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Regulation of the Human Thymidine Kinase Gene Promoter

by SV40 Large T Antigen

presented by

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has been accepted towards fulfillment
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Major professor

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REGULATION OF THE HUMAN THYMIDINE KINASE GENE PROMOTER BY SV40 LARGE T ANTIGEN

By

Michelle Marie Anderson

A DISSERTATION

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ABSTRACT

REGULATION OF THE HUMAN THYMIDINE KINASE GENE PROMOTER BY SV40 LARGE T ANTIGEN

By

Michelle Marie Anderson

The activity of human thymidine kinase (TK) is regulated during the course of the eukaryotic cell cycle, being highly induced at the onset of S phase. In quiescent cells infected with the DNA tumor virus Simian Virus 40 (SV40), this increase in TK activity is dependent upon the viral large T Antigen (T-Ag) protein and is accompanied by increases in both TK mRNA levels and the transcription rate of the gene. The study of SV40-mediated TK transcriptional induction is a potent model system for elucidating normal cell cycle control mechanisms, since regulatory pathways which have been bypassed to stimulate quiescent cells to re-enter the cell cycle and progress to S phase can be uncovered. In this study, both TK promoter elements and T-Ag protein domains required for activation of the TK promoter are identified. TK promoter-luciferase hybrid genes containing various wild-type and mutant TK promoter fragments were constructed and stably transfected into Rat-1 cells. Cells were then infected with recombinant adenoviruses expressing wild-type or mutant forms of T-Ag, and luciferase expression was determined as an indicator of TK promoter activity. Results show that a wild-type promoter fragment containing sequences to 135 bp upstream of the transcriptional start site is stimulated to high levels by viruses expressing either wild-type T-Ag or T-Ag proteins mutated within the p53-binding, ATPase, and helicase domains. The promoter, however, is not activated by a T-Ag protein defective in its ability to bind pRb and p107. In addition, wild-type T-Ag is incapable of activating a promoter fragment in which sequences resembling binding sites for the transcription factor E2F have been deleted.

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This implicates pRb (and/or p107) and an E2F family member as regulators of TK promoter activity and suggests that T-Ag activates the human TK promoter by disrupting associations between these proteins. T-Ag is also unable to stimulate expression of hybrid genes containing just the upstream (-135 to -67 bp, including the E2F-like sites) or downstream (-67 to +30 bp, including the TATA box) promoter halves, indicating that, in this system, both regions are required for high levels of activation.

For m

For my husband, Dr. Timothy R. Weichert, for his love and support, encouragement and understanding, and, most of all, for keeping me sane.

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Richard

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Literature Review

Proliferation of eukaryotic cells is a process entailing the accurate duplication of cell chromosomal content followed by division into two identical daughter cells. The ordered sequence of events culminating with cell division is collectively known as the "eukaryotic cell cycle", a complex and intricate process integrating aspects of cell nutrition, metabolism, and growth. The cell cycle is stringently controlled, being replete with numerous checkpoints and control mechanisms. For example, it is imperative that the DNA content of the cell is replicated once, and only once, during the course of any given cycle. Furthermore, DNA replication must be completed before cell division begins. The importance of the regulatory mechanisms controlling these events is illustrated by the various forms of cancer, diseases that result from the uncontrolled growth and proliferation of various cell populations. Therefore, knowledge of cell cycle regulatory events is essential to allow mechanistic insight into the cancer process. This chapter will provide an overview of cell cycle events and discuss general mechanisms of cell cycle control, with emphasis on particular proteins involved, as well as specific examples of several cell cycle-regulated genes.

1. Phases of the Cell Cycle

Cell cycle events are divided into four distinct phases (see Figure 1). S and M denote periods of DNA synthesis and mitosis, respectively. G1 and G2 are gap phases which effectively separate DNA synthesis from mitosis and during which cells synthesize or activate the required proteins for entry into and completion of the next phases of the cycle. In addition, there is an alternate phase removed from the cell cycle, a period of quiescence denoted G0.

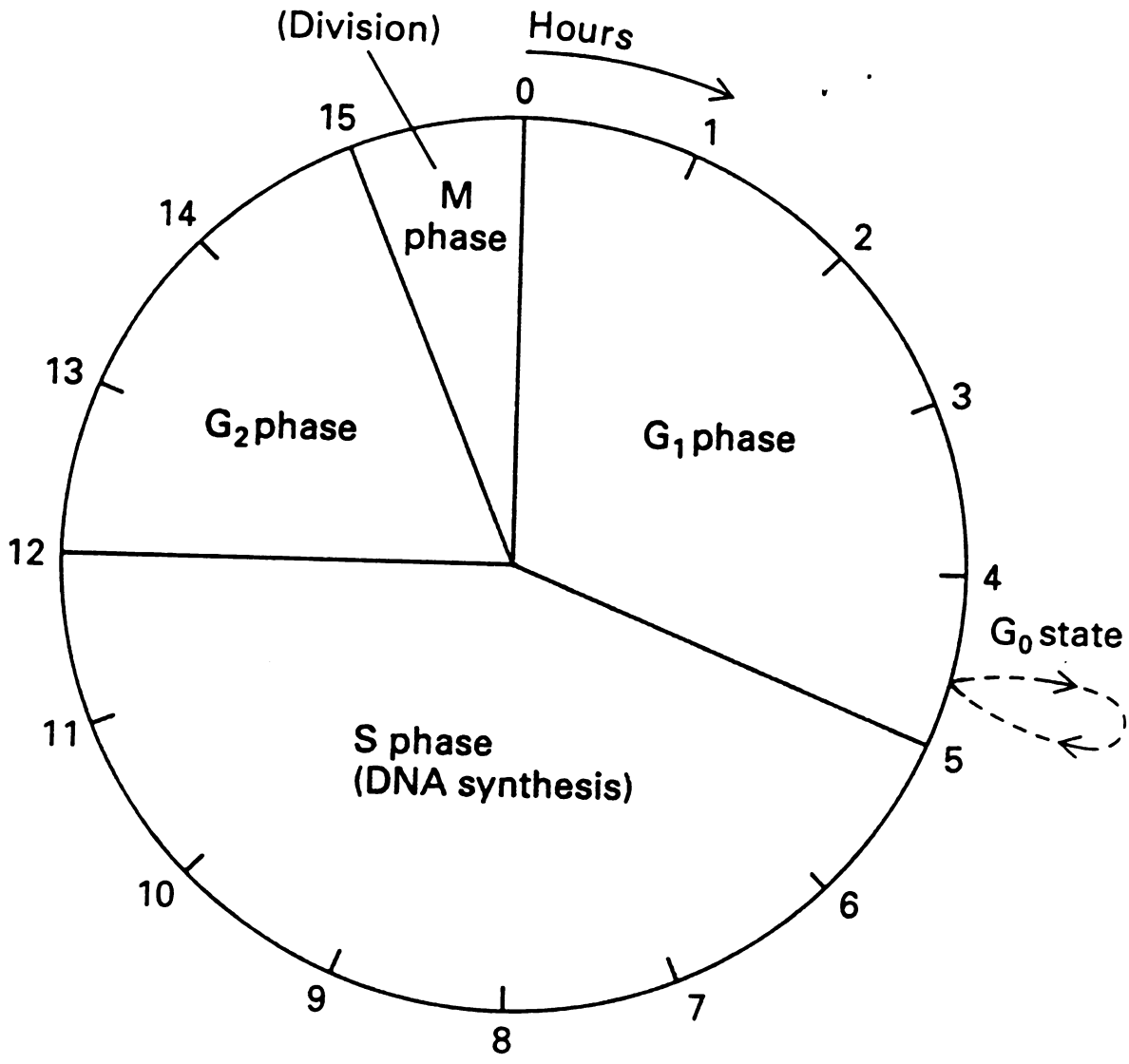

Figure 1. The eukaryotic cell cycle (64).

1.1 G1

In both mammalian cells and the budding yeast *Saccharomyces cerevisiae*, G1 is the interval during which major elements of cell cycle control are exerted (266, 288, 289). It is during this interval that the decision to progress through the cell cycle is made. Alternatives to cell cycle progression include initiation of cell differentiation and entry into quiescence, both of which occur during G1 (289). In yeast, the decision to pass through START, the point in G1 at which the cell is committed to traversing the remainder of the cycle (266), is based upon assessment of both cell size and nutritional status (266). A critical cell size, the attainment of which requires available nutrients, is required to pass START. In addition, the presence of mating pheromones arrest yeast in G1 and prevent cell cycle progression (266). In mammalian cells, a critical cell size is also required to enter S (266), and passage through G1 depends on cell size, nutritional status, cell density, and the concentrations of mitogens, or growth factors (266). The length of the G1 period is highly variable (266, 288, 289). Overall, smaller cells spend more time in G1 than larger cells, and embryonic cells, which divide quite rapidly, have practically non-existent G1 periods. G1 length is thus cell type and condition-dependent.

In mammalian cells, G1 biochemical events can be divided into four subphases based upon the effects of limiting growth factors and inhibitors and the subsequent time needed to reach S after the block is removed (289). The first subphase, competence, is characterized by changes in chromatin structure, increased transport of nutrients through the cell membrane, and expression of immediate early genes, i.e. genes that do not require *de novo* protein synthesis for expression. Platelet-Derived Growth factor (PDGF) has been termed a competence factor since quiescent cells must be treated with PDGF before they are able to begin passage through G1. The next subphase, entry, is characterized by protein and nucleic acid turnover to allow for some new macromolecular syntheses. In this stage, increases in the number of both polysomes and glycolytic enzymes occur. Progression, the third subphase, requires rapid net protein synthesis (in contrast to entry)

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as shown by the requirements for amino acids and the extreme sensitivity of cells to protein synthesis inhibitors. Enzymes required for DNA synthesis are made here, as is a critical G1 regulatory protein, the "Restriction" or "R" protein, to be discussed below. Finally, assembly is characterized by movement of the DNA synthesizing enzymes into the nucleus, and one theory postulates that enzymes are organized into a "replitase" complex for catalysis of DNA synthesis (289, 316). No serum or rapid protein synthesis is required for the final two hours of G1 or for the remainder of the cell cycle.

The major G1 control point in mammalian cells is the "Restriction" or "R" point, which is thought to occur somewhere within the final two hours of G1, prior to entry into S (266, 287, 288). The R control point is homologous to START (266) in yeast in that passage past R irreversibly commits the cell to traversing the remainder of the cycle. When the protein synthesis inhibitor cycloheximide is added to cells at various times after growth stimulation, R is defined as the point at which the drug no longer prevents entry into S (288). Beyond R, cells no longer require mitogens and are less sensitive to protein synthesis inhibitors. The concept of an R protein, which regulates passage past the Restriction point, is based upon kinetic experiments with cells treated at various times in G1 with cycloheximide (287). These experiments have suggested that passage past the Restriction point requires the synthesis of a labile protein, with a half-life of approximately two hours, in early G1. Furthermore, accumulation of this "R" protein to a critical concentration is required to initiate cell cycle progression (266, 328). Transformed cells, which are defective in Restriction point control, appear to have a stabilized R protein, since these cells are less sensitive to protein synthesis inhibitors, presumably due to lower levels of R protein degradation (41). In addition, transformed cells no longer require serum factors for transit past R (41, 328). Possible candidates for the R protein include cyclins A and E (84).

1.2 S Phase

Unlike prokaryotes, eukaryotes replicate DNA only once between divisions (205, 288), and the process of DNA replication occupies the S phase of the cell cycle. The length of S varies between species and between different developmental stages of the same species (205). However, within any particular cell type, S is constant in length, averaging in duration from 6 to 8 hours (205, 288). DNA replication occurs bidirectionally, initiating at multiple sites along the chromosomes, forming clusters of replication units otherwise known as "replicons" (205, 288). Individual genes replicate at defined periods during S. In addition, as chromosome structure must also be duplicated, histone synthesis occurs and is coupled to DNA synthesis (205, 288). Within ten minutes of the start of replication, DNA is packaged into nucleosomes and the chromosomal scaffold is reconstructed (205).

1.3 G2

G2 is the gap phase immediately preceding mitosis (288). During this period, cells prepare for entry into the mitotic phase of the cycle. As protein synthesis is required for entry into M phase, G2 is occupied with the synthesis and activation of proteins such as tubulin, a component of the mitotic apparatus, and important mitotic regulators like cyclins and the $p34^{cdc2}$ protein kinase, to be discussed below. In addition, cells round up and actin filaments reorganize.

The G2/M interface is another major control point within the cell cycle. In mammalian cells and budding yeast, the G2/M control point is weak compared to its G1/S counterpart. However, genes such as RCC1 (Regulator of Chromosomal Condensation) supposedly function during the S/G2/M interval of the cycle to link completion of DNA replication to the onset of mitosis (65). Other evidence for the exertion of G2 control derives from the fact that some anticancer drugs irreversibly arrest cells in G2 (288). In contrast, the G2/M interface is the major cell cycle control point for the fission yeast

Schizosaccharomyces pombe (266). It is at this point in the cycle that these cells monitor cell size and nutrient availability to decide upon further cell cycle progression.

1.4 M Phase

As previously stated, M phase is the period during which both nuclear division (karyokinesis) and cell division (cytokinesis) occur (241). The processes of cell division are subdivided into four stages. During prophase, the microtubules and microfilaments of the cytoskeleton depolymerize and rearrange, duplicated chromosomes condense to a compact state, RNA synthesis stops, and protein synthesis slows to 25% of its normal rate. During metaphase, the nuclear envelope breaks down, and the condensed chromosomes move to the cell midplane to form the "metaphase plate". In anaphase, identical daughter chromosomes segregate and move to opposite ends of the cell, and reformation of the nuclei and decondensation of the chromosomes occur in telophase. These events are then followed by cytokinesis which entails the formation of an actin- and myosin-dependent contractile ring to divide the cytoplasm in half.

1.5 G0

Cells decide whether or not to proliferate at the Restriction point in G1. In the presence of certain drugs, or if growth conditions are suboptimal (288), such as when cells attain a high cell density or are limited for serum/growth factors or amino acids, cells may withdraw from the cell cycle into a state of quiescence, termed G0. The G0 state is both kinetically and biochemically distinct from G1 (266, 288, 289). Macromolecular synthesis occurs at one-third the normal rate, there is low enzyme and transmembrane transport activities, and ribosomes exist as monosomes rather than polysomes. While some novel RNAs and proteins do appear, cell size is reduced. In fact, the small cell size and need for extra metabolism results in a longer time needed to traverse G1 and enter S upon restimulation (as compared to continually proliferating cells). While G0 is an alternate and transient state for most cell types, terminally-differentiated cells, such as hepatocytes and neurons, actually exist for long periods of time in G0 (288).

2. Signal Transduction and Activation of Gene Expression

In mammalian cells, entry and/or re-entry into the cell cycle, with subsequent progression through the early stages of G1, is mediated by mitogens. As previously stated, G1 events occurring prior to R are completely dependent upon the presence of growth factors and ongoing protein synthesis, while events occurring past R are mainly growth factor-independent. Thus, a brief discussion of the genes and proteins involved in mitogen-induced signal transduction pathways is warranted for better understanding of early cell cycle events.

2.1 Immediate Early Genes

Transmittal of a mitogenic signal results in the transient and rapid induction of a group of "immediate early genes". These genes are induced in the absence of protein synthesis and encode mainly for transcription factors of the AP-1, NFκB, and Myc families. The AP-1 transcription factor selectively binds promoter enhancer regions (297), predominantly within genes encoding enzymes required for proliferation. AP-1 itself exists as either a Jun-Jun homodimer or a Jun-Fos heterodimer (194, 297). The Jun protein contains a transcriptional activation domain at its N-terminus and dimerization and DNA-binding domains at its C-terminus. Transcription and translation of the Fos protein has been shown to be necessary for entrance of quiescent cells into the cell cycle (195, 275, 297, 319). While the Fos protein contains no DNA-binding domain, heterodimerization of Fos with Jun increases the DNA binding affinities of Jun, with complexes composed of different Fos/Jun family members having different affinities (194, 297). Fos and Jun are considered classic immediate early genes since the mRNAs of the various family members increase upon mitogenic stimulation.

The NFκB transcription factor (266) regulates genes involved in the inflammatory response, and thus plays a potential role in generation of the host defense response. Regulation of the NFκB protein is interesting in that, in unstimulated cells, it is tethered to

an inhibitor (IκB) in the cytoplasm. Upon mitogenic stimulation, NFκB is released from the inhibitor and translocates to the nucleus.

The Myc protein (231) is induced within several hours of mitogenic stimulation (in contrast to the induction of Fos and Jun, which occurs within minutes). Myc forms a heterodimer with a protein termed Max, and together Myc/Max form a transcription factor which appears to be required for the initiation of DNA synthesis. While no naturally-occurring promoter has, as yet, been demonstrated to be the target for regulation by Myc/Max, the heterodimer can activate transcription from a test promoter when overexpressed. Some studies also suggest that Myc can act as a competence factor, being able to substitute for PDGF in the G0 to S transitions.

2.2 The MAP Kinase Cascade

Mitogenic signals, culminating in the activation of nuclear transcription factors and genes are transduced by way of a protein kinase cascade. For example, Jun is activated by phosphorylation on serines in the N-terminal activation domain with concomitant dephosphorylation of residues within the C-terminal DNA-binding domain (32, 312). Likewise, activation of cytoplasmic phospholipase A_2 occurs via phosphorylation (330) and leads to the production of arachadonic acid, an important second messenger. Finally, transcriptional activation by Myc is induced by phosphorylation (237).

These phosphorylation events are mediated by proteins termed MAP Kinases or ERKs (Mitogen-Activated Protein Kinases or Extracellular Signal-Related Kinases), and the cascade, a series of sequentially-activated MAPKs, is appropriately termed the MAPK or MAP Kinase cascade (237, 294, 330). MAPKs are serine-threonine protein kinases (330) which are activated themselves by phosphorylation on tyrosine and threonine residues. Activation of the MAP Kinases occurs via MAP Kinase Kinases, otherwise known as MAPKKs or MEK. In the tradition of a kinase cascade, the MAPKKs are activated, by phosphorylation on their serine and threonine residues, by a MAP Kinase Kinase Kinase, or MAPKKK.

To date, several isoforms of MAPKs have been isolated. While cytoplasmically-located in unstimulated cells, immunolocalization experiments reveal translocation of the MAPKs to the nucleus following mitogenic stimulation (127, 212, 412). MAPKs have been shown to be important for G1 progression, since inhibition of MAPK activity via expression of antisense RNAs or dominant-negative mutants prevents cells from reaching S (330). Along these lines, targets for activation by MAPKs include proteins with putative roles in the release from cell cycle arrest, such as some of the immediate-early genes mentioned above (237, 294, 330).

2.3 The Ras-Raf Signal Transduction Pathway

There are many routes to the activation of the MAP Kinase cascade. In recent years, much study has been given to tracking the signals transduced via the product of the proto-oncogene ras (237), a protein found to be abnormally activated in a number of human cancers such as colon cancer. The Ras protein is G-protein-like in that it is active when bound to GTP and inactive when bound to GDP. Isoprenylation of Ras allows localization to the inner face of the plasma membrane, where it serves as an important relay molecule in signal transduction.

Attempts to trace the sequence of events connecting mitogenic stimuli at the cell surface with activation of gene expression in the nucleus have recently revealed a direct interaction between Ras and the product of another proto-oncogene: Raf (257, 330). The Raf protein is itself a serine/threonine protein kinase and is thought to be a MAPKKK since it is able to phosphorylate MAPKK *in vitro* (73, 203). Very recent evidence indicates that Raf is activated upon translocation to the plasma membrane and that, once membrane-bound, Raf activities are independent of Ras (360). The role of Ras in this signal transduction pathway may be merely to recruit Raf to the plasma membrane.

Convergent work from many laboratories has established a detailed signal transduction route from cell surface to nucleus (see Figure 2) (237). In the course of a mitogenic stimulus, a growth factor binds to its cognate receptor, most likely a tyrosine

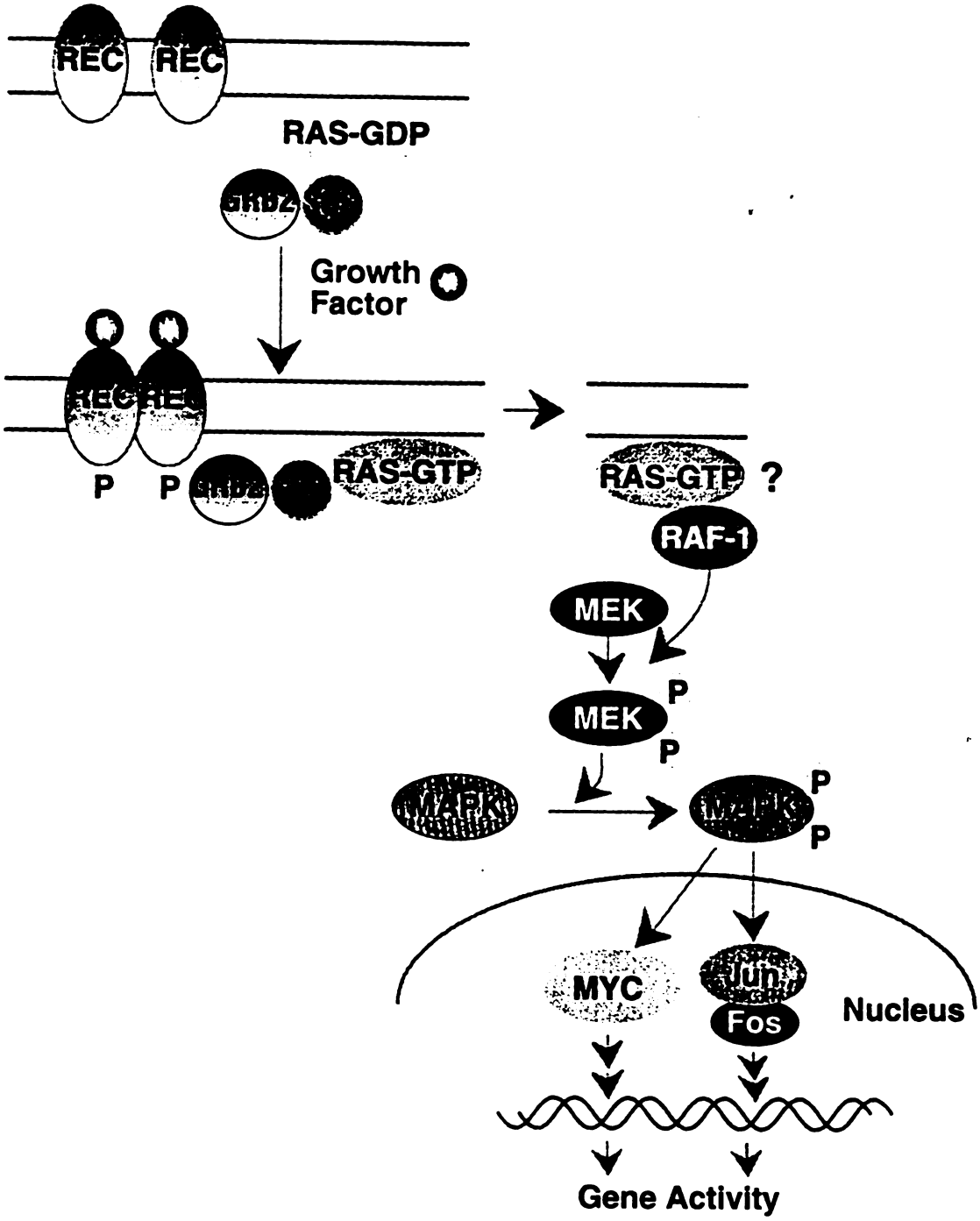


Figure 2. A signal transduction pathway involving Ras, Raf, and the MAP Kinase cascade (237).

kinase, in the cell membrane, resulting in activation and autophosphorylation of the receptor. The receptor signal is then intercepted by membrane-bound Ras via intermediary proteins that link Ras to the activated receptor. In mammalian cells, one such intermediary protein is GRB2 (Growth factor Binding protein), which binds the phosphorylated cell surface receptor. GRB2, in turn, is in complex with MSOS (Mammalian Son Of Sevenless) which binds and activates Ras by catalyzing the GDP to GTP exchange. Activated Ras then binds Raf and targets it to the plasma membrane whereupon the activated Raf phosphorylates MEK (MAPKK), initiating the MAPK cascade that concludes with activation of gene expression in the nucleus and subsequent cell cycle re-entry and/or progression.

3. A Universal Mechanism of Cell Cycle Control: Cyclins and CDKs

Regulation of mitogen-independent events, beginning in late G1 and continuing throughout the remainder of the cell cycle, relies upon the workings of an intrinsic cell cycle clock. In the past decade, three seemingly diverse lines of research have converged to identify what appears to be a universal mechanism of cell cycle control (reviewed in 278).

3.1 MPF

First, studies of the embryonic cell cycles in the frog *Xenopus laevis* identified a factor present in the cytoplasm of the mature oocyte/egg required for initiation of the onset of mitosis. Microinjection of this factor, termed MPF for Maturation or M-phase Promoting Factor, into G2-arrested oocytes induced entry into mitosis, initiating disassembly of the nuclear envelope, construction of the mitotic spindle, and maturation of the oocyte into an egg (201, 239, 269, 301). Furthermore, microinjection of MPF bypassed the usual need for protein synthesis for entry into mitosis (251, 269, 301). MPF has since been found to be highly conserved, being detected in the cytoplasms of both meiotic and mitotic eukaryotic cells (10, 208). In addition, it displays an M-phase-specific

histone H1 kinase activity (10, 199) and is comprised of two components, a 34 kD serine/threonine protein kinase, since identified as $p34^{cdc2/CDC28}$ (89, 245, 406), and a 45 kD protein now known to be a cyclin (119, 245, 278, 406).

3.2 The cdc2 Protein Kinase

A second line of research involving studies of cell division cycle (cdc) mutants in yeast led to the discovery of the $p34^{cdc2/CDC28}$ protein kinase mentioned above. In the fission yeast *Schizosaccharomyces pombe*, cdc mutants displayed a delay in mitotic onset, with division occurring at an increased cell size (276, 278). The genetic defect was localized to the cdc2 gene, which is homologous to the CDC28 gene in the budding yeast *Saccharomyces cerevisiae* (24, 140, 278). This gene encodes a 32-34 kD protein kinase that is required for progression past START as well as entry into mitosis in yeast (277, 300, 317). The $p34^{cdc2/CDC28}$ kinase is expressed constitutively throughout the yeast cell cycle, with phase-specific activity being regulated both by association with a cyclin protein, discussed below, and post-translational modifications involving the phosphorylation and dephosphorylation of various tyrosine and threonine residues (354).

Active cdc2 kinase phosphorylates substrates to drive cells into mitosis. Such substrates include the nuclear lamins A, B, and C, phosphorylation of which leads to lamina disassembly (142, 296, 386). In addition, caldesmon (406), an actin and calmodulin-binding protein, and the intermediate filament protein vimentin (54), are phosphorylated by the cdc2 kinase, leading to potential alterations of the organization of the microfilament cytoskeleton upon the onset of mitosis.

The universality of the $p34^{cdc2/CDC28}$ protein kinase was proven by the identification of human homologs, which were found in attempts to complement yeast cdc mutants with human cDNAs (208, 278). While yeast contain only one cdc2 protein kinase, higher eukaryotes contain multiple cdc2-related proteins (240, 249, 376) which can be grouped into families of cyclin-dependent kinases (cdks). As with the yeast cdc2 kinase, the mammalian cdks play roles in both the G1 and G2 phases of the cell cycle, with

different family members displaying distinct functions. For example, the prototypic cdc2 kinase, also known as cdk1, is required for entry into M in the mammalian cell cycle (29, 102, 320, 368, 377) while both cdk2 and cdk3 have been shown to play a role in G1 (102, 377). To date, as many as 6 mammalian cdks have been identified (249, 377).

3.3 Cyclins

Studies with marine invertebrate oocytes (sea urchins and surf clams) identified a set of proteins, termed cyclins, which were expressed periodically during the cell cycle (97, 327, 365). Specifically, cyclins accumulated during S and G2 and were degraded at the end of mitosis. Degradation of the cyclin proteins is required for exit from M phase (87, 270), is initiated by the cdc2 protein kinase (108), and occurs via the ubiquitin pathway (126).

There are now known to be multiple cyclins present in different phases of the cell cycle (159, 263, 347). In yeast (62, 77, 134, 266, 321, 395), the CLN 1, 2, and 3 genes encode the G1 cyclins. CLNs 1 & 2 are structurally similar, with CLN 3 being more divergent. Expression of CLNs 1 and 2 fluctuates periodically during the cell cycle, peaking in late G1 while CLN3 expression remains relatively constant throughout the cycle. Yeast G2 cyclins, CLBs 1-5, peak prior to the onset of M phase.

In higher eukaryotes, the G1 cyclins consist of cyclins C, D1, D2, and D3 (16, 100, 159, 178, 230, 263, 347, 363). As the cyclin C protein has not yet been identified, very little is known about it. However, similar to yeast CLN 3, cyclin C mRNA is present throughout the cell cycle, being slightly elevated in early G1. With the D-type cyclins, timing of expression varies among different cell types. D-type cyclins can be stimulated in G1 by growth factors, suggesting they may play a role as growth factor sensors. In addition, cyclin D-cdk complexes have been implicated in the phosphorylation and subsequent inactivation of the tumor suppressor proteins pRb and p107 (discussed below) and thus appear to be important for cell cycle progression. D-type cyclins interact predominantly with cdk2, cdk4, and cdk5. The expression of cyclin E (88, 192, 193, 263,

347) occurs later than that of cyclin C or the D-type cyclins, positioning it as a G1/S cyclin. Cyclin E, which associates mainly with the cdk2 protein kinase, potentially plays a role in the control of genes required for the transition from G1 to S since overexpression of cyclin E leads to a decrease in cell volume and an accumulation of cells in S and G2. Cyclin A (40, 124, 159, 262, 285, 330, 347, 382) represents an S/G2 cyclin, playing roles in both the progression through S phase and the onset of mitosis. Cyclin A also associates with multiple cdks: association of cyclin A with cdk2 regulates the onset of DNA replication while a cyclin A/cdc2 complex is involved with a regulatory checkpoint, making sure mitosis does not begin until DNA replication is complete. Finally, the classic mammalian mitotic cyclins are represented by the B-type cyclins, B1 and B2 (159, 263). These cyclins are expressed later than cyclin A and, in contrast to A, associate only with the cdc2 protein kinase. The B-type cyclins accumulate in the cytoplasm at the start of G2 and move to the nucleus at the G2/M interface, where association with cdc2 catalyzes nuclear lamina breakdown and other mitotic events. The cyclin proteins display a high degree of sequence conservation, especially within the "cyclin box" (263), a sequence of 100-150 amino acids which may be responsible for the interaction of the cyclins with the cdks.

The importance of cyclins in cell cycle control has been emphasized by the identification of cyclin overexpression and rearrangements in a variety of human cancers. For example, overexpression of cyclin D1 has been found in both parathyroid adenomas (159, 262, 263) and a variety of B-cell lymphomas. Overexpression of cyclin D1 in transgenic mice has been found to result in abnormal mammary cell proliferation and the development of mammary carcinomas (384), suggesting that cyclin D1 is actually the product of a proto-oncogene. The cyclin A gene has been found to be the site of integration for the Hepatitis B viral genome in the generation of one type of liver cancer (40, 159, 263, 383). The cyclin A protein also associates with the adenovirus transforming protein E1A in adenovirus-infected cells (123, 159).

In overview, yeast provide a simplified model of cell cycle control via cyclin/cdc2 complexes. In this system, one protein kinase, $p34^{cdc2/CDC28}$, associates with two types of cyclins to regulate cell cycle progression: CLN/cdc2 complexes regulate progression through START while CLB/cdc2 complexes initiate entry into and exit from M. The situation in higher eukaryotes is more complex, with multiple cyclin proteins associating with multiple cdks, generating combinatorial complexity and variation to effect passage through the various phases of the cell cycle.

4. Other Cell Cycle Control Genes

In addition to the proteins discussed in the previous sections, eukaryotic cells possess a number of other genes with critical roles in the regulation of cellular proliferation. A complete discussion of the multitude of proteins which cooperate to control cell cycle progression is infeasible. However, the following sections discuss a few of the more prominent and well known regulatory proteins. Many of these proteins constitute a family of tumor suppressor proteins, or proteins involved in the suppression of cell growth, while another is an example of a ubiquitous transcription factor involved in the phase-specific activation of essential replicative genes.

4.1 Rb

Retinoblastoma is a rare childhood tumor of the eye characterized by failure of the immature retinal cells to differentiate into photoreceptor cells (61). The genetic deficiency associated with this disorder involves the mutational inactivation (via deletions, insertions, or point mutations) of both of the retinoblastoma (Rb) alleles. The RB-1 gene encodes a 105-120 kD nuclear phosphoprotein. Cloning of the gene (149) revealed an N-terminal dimerization domain and a regulatory domain containing numerous serine and threonine residues as potential phosphorylation sites. Within the C-terminus is located a non-sequence-specific DNA-binding domain and a "pocket" region for protein-protein

interactions. The "pocket" region is utilized for the binding of viral oncoproteins as well as interactions with cellular proteins, to be discussed below.

In addition to retinoblastoma, Rb mutations have been associated with the generation of other tumor types, including those of the lung, bladder, breast, prostate, and bone (152), suggesting a growth suppressive function for the wild type Rb gene. Several lines of evidence directly implicate Rb as a tumor suppressor gene. First, reintroduction of the Rb gene into Rb $^{(-)}$ tumor cells partially suppresses tumorigenic potential. This is indicated by a decrease in the growth rate of the cells, inhibition of cell growth in soft agar, and decreased tumorigenic potential in nude mice (156, 362). In addition, the Rb protein (pRb) has been shown to be important for cell transformation and tumor formation by the DNA tumor viruses Adenovirus (Ad), Simian Virus 40 (SV40), Polyomavirus (Py), and Human Papilloma Virus (HPV). These viruses encode oncoproteins which effectively stimulate cell cycle progression in quiescent cells to allow for viral DNA replication. Many of these oncoproteins, such as the large T Antigens of SV40 and Py, the adenovirus E1A protein, and HPV E7, physically bind the Rb protein, and mutations which interfere with pRb binding also prevent viral transformation (67, 391, 392). The binding of pRb by viral oncoproteins mimics the loss of the Rb gene seen in naturally-occurring tumors. This is supported by the fact that the region in pRb required for interaction with SV40 large T-antigen and Ad E1A, for example, is the region most frequently mutated in tumors, suggesting that the oncoproteins bind pRb to inactivate/alleviate Rb-mediated growth suppression, thus allowing cell cycle progression.

The Rb protein is important for G1 progression, suppressing cell growth at a specific point in G1. Evidence for this is found in studies in which purified wild type Rb protein was microinjected at various times into synchronized populations of the Rb$^{(-)}$ Saos-2 cell line (128). pRb injection in early G1 prevented cells from entering S, while injection of pRb in late G1 (approximately 6-10 hours before the onset of S) had no effect on subsequent cell cycle progression. This same study provided direct evidence for the

inactivation of pRb function by SV40 large T antigen (T-Ag), since Saos-2 cells coinjected with pRb and an SV40 T-Ag peptide did not growth arrest. In addition, simian CV-1 cells injected with pRb underwent G1 growth arrest while pRb-injected simian COS7 cells (SV40-transformed CV-1 cells constitutively expressing T-Ag) did not.

Regulation of pRb activity occurs via post-translational modifications rather than by increases in either transcription or translation rates. Both pRb mRNA and protein are expressed constitutively throughout the cell cycle (68). However, the phosphorylation state of the protein varies in a cell cycle-dependent manner. pRb exists in an underphosphorylated form in quiescent, G0 cells or cells in early G1, and becomes hyperphosphorylated at the G1/S boundary, obtaining maximal phosphorylation in S phase (68). Further phosphorylation events may also occur upon entry into M, with extensive dephosphorylation occurring at the end of M phase. DeCaprio et al. (69) reports three distinct pRb phosphorylation events in mitogen-stimulated T cells, in early G1, the onset of S, and G2/M. The stepwise phosphorylation and dephosphorylation of pRb may reflect the involvement of pRb in numerous control points of the cell cycle. Similarly, the multiple steps may reflect methods for modulating pRb activity as opposed to simply turning activity on or off.

The fact that viral oncoproteins such as the SV40 T-Ag associate exclusively with the G0/G1 form of pRb (68) suggests that the underphosphorylated form of the protein is actually the growth-suppressive form. As mentioned previously, within the Rb protein, the binding sites for viral oncoproteins overlap the sites for binding cellular proteins. If pRb functions by binding, inhibiting, or activating various cellular effectors, the binding of viral oncoproteins to pRb would release these cellular proteins from pRb. Also, phosphorylation of pRb may alter pRb conformation and prevent binding to these cellular effectors, thereby acting as a molecular switch for cell cycle progression (68).

The cell cycle-dependent phosphorylation of pRb suggests that it may be a target of a cdk, and serves to link pRb to the cell cycle machinery. Tryptic phosphopeptide

mapping of pRb has identified multiple phosphorylated serine and threonine residues (155, 210), with many of the sites corresponding to cdc2 consensus phosphorylation sites (210). A physical association between pRb and the cdc2 kinase has been demonstrated (155, 210), but since the first pRb phosphorylation event occurs at G1/S, prior to activation of the major form of the mitotic cdc2 kinase, it is likely related kinases catalyze the G1 and S phase pRb phosphorylation events. Alternatively, a specific subset of cdc2 may be activated to phosphorylate pRb at G1/S. In support of this theory, a cyclin A requirement has been identified for pRb phosphorylation (155) and Williams et al. (394) have reported physical complexing between pRb and proline-directed protein kinase (PDPK), a cytoplasmic cdc2/cyclin A kinase complex structurally similar to, but functionally and biochemically distinct from, the nuclear histone H1 kinase.

Additional research is being done to understand the role of cyclins in regulation of pRb phosphorylation. A recent study by Hinds et al. (146) showed that constitutive expression of cyclins A and E, but not cyclins B1, B2, or D1, resulted in the generation of hyperphosphorylated pRb, yielding increased extractability of pRb from the nucleus and overriding an Rb-induced cell cycle block. Similar studies have shown that ectopic expression of cyclin D2 also induces pRb hyperphosphorylation (100) and that cyclin D-cdk4 complexes bind pRb *in vitro* (86, 100, 149). The results of these studies are intriguing in light of the timing of cyclin expression: cyclin A is not normally expressed until pRb is already in the hyperphosphorylated state while cyclin E and the D-type cyclins arise in G1 when pRb is undergoing phosphorylation.

The phosphorylation status of pRb during the cell cycle represents an equilibrium between regulating kinases and phosphatases. Candidates for regulating phosphatases (3) include both phosphoprotein phosphatase types 1 (PP1) and 2A (PP2A). Both phosphatases are able to dephosphorylate pRb *in vitro*. In addition, Alberts et al. (3) exploited the ability of hypophosphorylated pRb to bind tightly to a "nuclear anchor" (253) to show that microinjection of PP1 into either the cytoplasm or nucleus of G1 cells

increased the affinity of pRb for the nucleus and inhibited progression into S phase. Similar results were obtained with the microinjection of the catalytic subunit of PP2A directly into the nucleus. Finally, inhibitors of PP1 blocked pRb dephosphorylation.

The major focus for understanding the tumor suppressive action of pRb is determining the mechanism by which pRb exerts its growth repressive effects. Most evidence suggests that pRb acts by regulating transcription, directly or indirectly. pRb could regulate proliferation either by repressing growth stimulatory genes or activating growth inhibitory and differentiation-inducing genes. Evidence exists for both manners of regulation. A number of growth-stimulatory genes have been found to be transcriptionally repressed by pRb, one of which is the proto-oncogene c-fos. Transient cotransfection experiments showed that pRb inhibited the expression of a fos-CAT promoter-reporter gene construct following serum stimulation of quiescent NIH 3T3 cells (323). Progressive 5' deletions in the c-fos promoter identified a 31 base pair (bp) GC-rich region, termed the Retinoblastoma Control Element (RCE) which could confer Rb responsiveness to a heterologous promoter (323). Additionally, Pietenpol et al. (299) have shown that c-myc is also transcriptionally repressed by pRb. Transient cotransfections of skin keratinocytes with a c-myc-CAT reporter construct and a pRb expression plasmid showed direct transcriptional inhibition of the c-myc promoter by pRb.

The interaction of pRb with the TGFβs provides a good example of growth suppression via activation of growth-inhibitory genes. The TGFβs (β1, β2, and β3) comprise a family of homodimeric polypeptides which inhibit the growth of many cell types (i.e. epithelial, endothelial, fibroblast, lymphoid, and hematopoietic), blocking events in late G1 (200). The mechanism of inhibition by the TGFβs is unknown; however, Laiho et al. (200) have shown that TGFβ1 prevents the phosphorylation of pRb in late G1, and maintenance of pRb in the underphosphorylated state may then contribute to a block in cell cycle progression. The role of pRb on TGFβ1 transcription has been studied by Kim et al. (185). Using TGFβ1-Cat fusions and a pRb expression plasmid, they showed that

pRb increased the transcription of TGFβ1 fifteen- to twentyfold in mink lung epithelial cells, suggesting that TGFβ1 and Rb may be part of a common growth inhibitory pathway. In addition, examination of the TGFβ1 promoter revealed two RCE-like sequences (185). Additional studies showed that pRb also activated transcription of the TGFβ2 promoter. An interesting aspect to these studies is the discovery that the effect of pRb on gene transcription is cell type-dependent (185). For example, TGFβ1 transcription is activated by pRb in mink lung epithelial cells (which show a strong inhibitory response to TGFβ) but repressed by pRb in NIH 3T3 cells. Also, transcription from the c-myc and c-fos promoters is repressed by pRb in keratinocytes and NIH 3T3 cells, respectively, but activated by pRb in mink lung epithelial cells.

pRb may also influence gene transcription indirectly via interaction with and/or modulation of other cellular transcription factors. To date, the most highly studied interaction is that of pRb with the transcription factor E2F. E2F is a sequence-specific DNA binding protein and cellular transcription factor thought to regulate genes utilized in S phase (207, 274). Studies have shown that the underphosphorylated form of pRb physically binds E2F in G1 (17, 49, 410), thereby repressing transcriptional activity of E2F (63, 137, 144). As free, uncomplexed E2F accumulates at G1/S (273, 340), the hypothesis followed that phosphorylation of pRb at G1/S results in dissociation of pRb/E2F complexes, with subsequent activation of E2F-regulated genes (273). However, recent evidence now indicates that E2F/pRb complexes actually persist into and throughout S phase (340). As these complexes most likely represent a small fraction of underphosphorylated pRb remaining in the cell, phosphorylation of the remainder of pRb at G1/S serves to prevent further E2F/pRb interactions, thereby preserving the pool of free E2F. E2F/pRb complexes can also be dissociated upon the binding of viral oncoproteins to underphosphorylated pRb (15, 274). Therefore, one mechanism of Rb-mediated transcriptional repression is by the sequestration of cellular transcription factors such as E2F.

pRb may also utilize DNA binding proteins such as E2F as shuttle guides to promoters to directly bind promoters and repress transcription. For example, studies have shown that E2F/pRb complexes bind with high affinity to E2F-like sites (389), and binding of these complexes prevents gene transcription by interfering with the binding and/or activity of transcription factors at adjacent sites in the promoter. Thus, in this scenario, the E2F/pRb complexes are not transcriptionally inert; pRb effectively converts E2F from a positive to a negative regulator of transcription (see Figure 3). An interesting note is that E2F-like sites are found within the Rb promoter itself, suggesting a negative feedback loop controlling E2F activity and Rb transcription (389).

The effect of pRb on transcription has also been found to be mediated via the transcription factor Sp1, a factor which elevates transcription by binding to specific GC-rich sequences. Sp1 has been found to bind the RCE sequences within some promoters activated by pRb (186). In transient cotransfection studies, Sp1 enhanced pRb-mediated activation of a test promoter, and inactivation of the Sp1 binding sites within the RCE abolished transcriptional induction by pRb (186). Although no direct association between pRb and Sp1 has yet been detected, and not all Sp1-dependent promoters are stimulated by pRb, it is interesting to speculate upon the mechanism of action between pRb and Sp1; perhaps pRb mediates release of Sp1 from an inhibitory complex or facilitates the interaction of Sp1 with the transcriptional machinery, thus acting as a coactivator for transcription (186). A final note to the Rb story is that several other, as yet unidentified, cellular proteins have been found that bind to the Rb protein (70, 157, 172). It will be exciting to find the role these proteins play in growth suppression by pRb.

4.2 p107

The study of cellular proteins complexed with viral oncoproteins such as Ad E1A and SV40 T-Ag led to the discovery of p107 (92, 98, 139, 392, 408, 416), another nuclear phosphoprotein with a seemingly important role in the control of cellular proliferation. There is much evidence to suggest that p107 and pRb are related proteins.

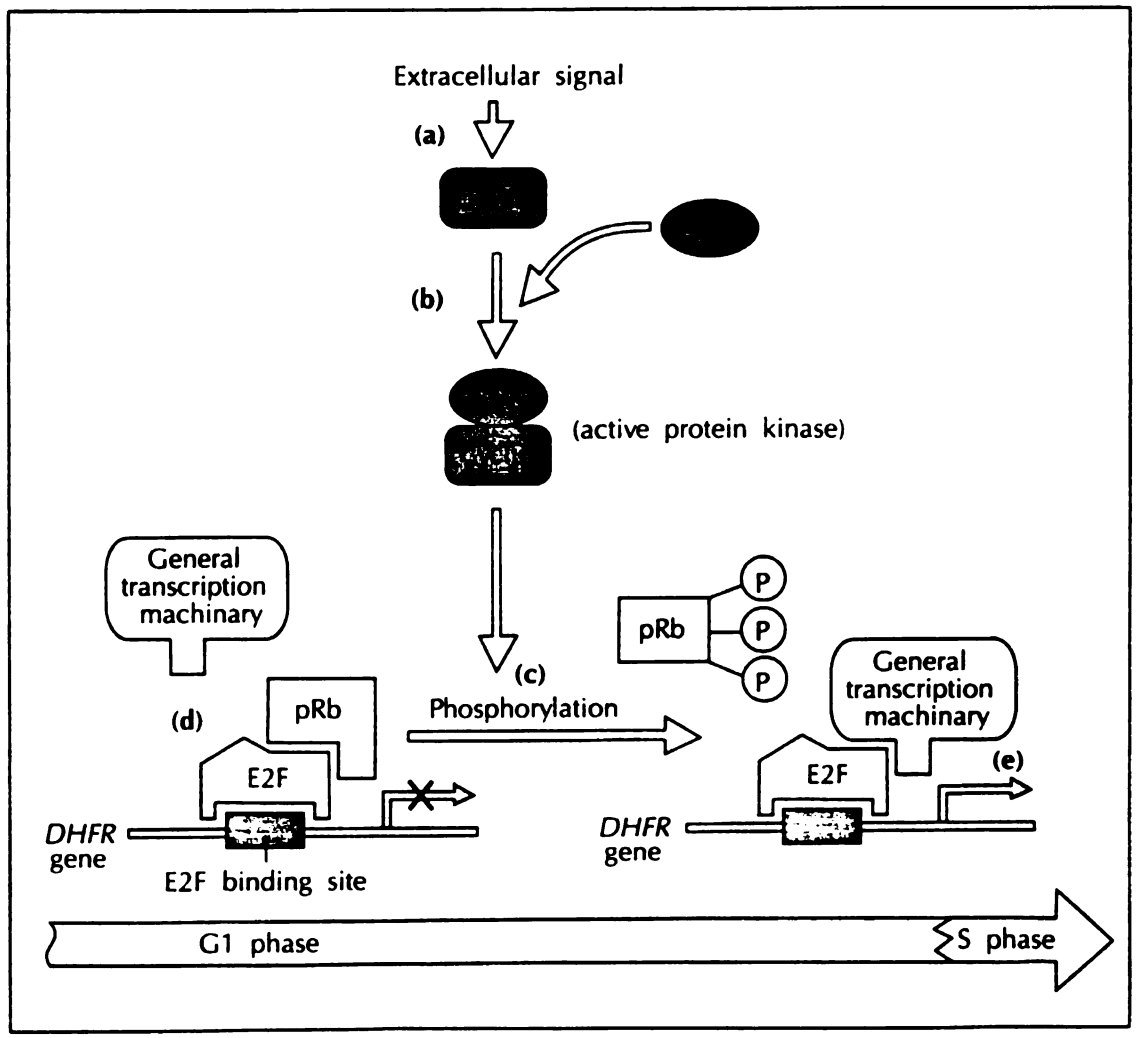


Figure 3. A model for pRb-mediated regulation of cellular gene transcription (149). a) G1 cyclins are synthesized or activated in response to environmental stimuli. b) Cyclins combine with a cdk partner to yield active protein kinases. c) The activated kinases phosphorylate and inactivate pRb. d) Transcription factors that had been repressed by pRb, such as E2F, e) can then promote transcription. Note that this figure illustrates transcriptional repression by DNA-binding of a pRb/E2F complex.

First, it was discovered that the same regions of E1A and T-Ag were required to bind both p107 and pRb (68, 93, 171, 387, 392). In addition, there are amino acid sequence homologies between p107 and pRb, particularly within the C-terminal two-thirds of the proteins, or "pocket domains" (416). Recall that the pocket domain is the region required for binding both cellular proteins and oncoproteins such as Ad E1A and SV40 T-Ag. For both p107 and pRb, this region is composed of two segments separated by a spacer region. For each, the spacer sequence is nonessential for binding T-Ag or E1A, as foreign sequences substituted for the spacer do not affect the ability to bind the viral oncoproteins. However, the spacer sequence does represent a major point of difference between p107 and pRb. First, the spacer sequences are nonhomologous between the two proteins and, second, the p107 spacer sequence binds cyclin A (99, 101), an association not seen with pRb.

The association of cyclin A with p107 was detected using GST-p107 fusion proteins and immunoprecipitations with α-cyclin A antibodies. Involvement of the spacer region was suggested when T-Ag peptides did not compete, and pocket mutations did not interfere, with cyclin A association, and confirmed when substitution of the p107 spacer for the pRb spacer conferred the ability to bind cyclin A upon pRb. Theories (99, 101) regarding the function of the p107-cyclin A association include 1) p107 as a target for regulation by a cyclin A-kinase complex, 2) other p107 pocket-bound proteins as targets for a cyclin A-kinase complex, and 3) p107 as a transducer for signals between pocket-bound proteins and a cyclin A-kinase complex bound at the pocket surface.

Another similarity between p107 and pRb is that the pocket regions of both proteins bind the same set of cellular proteins (99). Of special note is the fact the both proteins bind the transcription factor E2F, although in independent complexes (17, 42, 49, 74, 265, 348, 416). As mentioned in the previous section, pRb forms a complex with E2F during the G1 phase of the cell cycle. A G1-specific complex between p107 and E2F also exists. As demonstrated by Schwarz et al. (340), addition of GST-p107 to affinity-

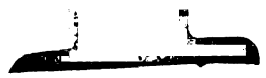
purified E2F produced a complex in a gel shift assay with mobility identical to a previously described E2F G1 complex; α-p107 antibodies successfully supershifted this complex, confirming p107 as a component. Similar experiments have since shown that cyclin E and the $p33^{cdk2}$ kinase are also components of this G1 complex (209, 416). While the pRb/E2F complex persists from G1 into S, the p107/E2F/cyclin E/$p33^{cdk2}$ G1 complex dissociates at G1/S, and an S-phase complex containing p107, E2F, cyclin A, and $p33^{cdk2}$ forms (74, 348, 416). While the exact roles these complexes play in regulating G1- and S-phase-specific cell cycle events are unknown, present theories focus upon the regulation of E2F activity, a topic which will be discussed in detail in a following section. In brief, E2F is transcriptionally inactive during G1 and S, the times at which it is found complexed with pRb and p107. Dissociation of the G1-specific p107/E2F complex at G1/S most likely contributes to the accumulation of transcriptionally-active "free" E2F at this point in the cell cycle. In addition, with both pRb and p107, the binding of oncoproteins such as Ad E1A or SV40 T-Ag dissociates complexes with cellular proteins, resulting in the release of free E2F.

Like pRb, p107 functions as a suppressor of cell growth. This was demonstrated by Zhu et al. (416) with a colony formation assay; stable transfection of a p107 expression plasmid into Saos-2 cells, an $Rb^{(-)}$ osteosarcoma cell line, resulted in a decrease in the number of colonies which arose. Flow cytometry analysis of the transfected cells revealed a decrease in the number of S phase cells with a corresponding increase in the number of G1-phase cells, suggesting a p107-mediated cell cycle arrest. However, while both pRb and p107 function as growth suppressors, the mechanisms of suppression appear to differ. For example, Dyson et al. (94) showed that the presence of p107 in tumor cells that have lost pRb is unable to compensate for the lack of pRb function. Additionally, Zhu et al. (416) demonstrated, by a number of criteria, that the mechanisms of p107- and pRb-mediated growth suppression were different. First, E1A was much more efficient at rescuing Saos-2 cells from a p107-mediated G1 block than a pRb-mediated G1 block.

Second, coexpression of cyclins A and E rescued the pRb-mediated growth arrest of Saos-2 cells (presumably via phosphorylation and inactivation of pRb) but not the p107-mediated growth arrest. Third, E2F-1, a recently cloned cDNA encoding E2F transcription factor activity, also rescued pRb-mediated but not p107-mediated growth suppression. As no previous *in vivo* association of p107 and E2F-1 has been detected (313, 416), and pRb and p107 associate with E2F in independent complexes, it is likely that a family of E2F proteins exist and that p107 and pRb associate with different family members. Finally, the ability to bind Ad E1A and SV40 T-Ag is required for growth suppression by pRb but not p107, since two p107 mutants unable to bind E1A were still capable of suppressing cell growth.

Like pRb, p107 functions as a transcriptional repressor and down-regulates E2F transcriptional activity (340, 416). Transient cotransfection assays with a p107 expression vector and an E2-Cat reporter plasmid show as much as a tenfold decrease in E2 promoter activity in the presence of p107, a repression comparable to that mediated by pRb. p107 also represses transcriptional activation by Myc (132). An *in vivo* association between p107 and Myc was detected via immunoprecipitation and immunoblotting with antibodies specific to p107 and Myc, respectively. To demonstrate p107-mediated repression of the transcriptional activity of Myc, the N-terminal trans-activation domain of Myc was linked to the Gal4 DNA binding domain and cotransfected with both a p107 expression plasmid and a CAT reporter gene linked to Gal4 binding sites. Repression required the pocket domain of p107. Thus, it is intriguing to speculate that one mechanism of p107-mediated growth suppression is via inhibition of the activities of cell proliferative transcription factors such as E2F and Myc/Max.

An obvious question arising from the p107 studies is whether or not this protein functions as a tumor suppressor. The evidence presented above suggests the role of p107 and pRb to be related, yet distinct. There are, however, no known naturally-occurring mutations within the p107 gene to suggest it is a tumor suppressor. Several hypotheses



may be presented to counteract this dilemma (416). For example, p107 mutations may exist yet be too subtle to detect. p107 mutations may also be lethal and not tolerated by the cell. Finally, perhaps no growth advantage is conferred upon cells with p107 mutations, rendering the mutations unnecessary. At this point in time, classification of p107 as a tumor suppressor awaits further studies to clarify the role of this protein in cell cycle regulation.

4.3 p53

Much research has been directed towards elucidating the functions and properties of one of the most well-known tumor suppressor genes: p53. Until the recent discovery of p16 (to be discussed in the next section), p53 had the distinction of being the most highly mutated gene in human cancers (79, 202, 295). This gene has been found to be mutated in 50% of cancers tested, with the most common aberration being complete loss of one allele with a missense mutation in the other ("Loss of Heterozygosity" or LOH, an occurrence particular to tumor suppressor loci) (79, 202, 311, 380). Despite the high mutation rate, however, the p53 gene has been found to be nonessential for normal cell proliferation and development (79, 380). For example, many tumor cells contain or express no functional p53, indicating that cell proliferation, per se, is not dependent upon p53 function. In addition, p53 null mice (harboring a homozygous deletion of the p53 gene) develop normally. These mice are highly susceptible, however, to the development of early tumors, suggesting that p53 may play a role in protection from tumors.

Initial evidence for the role of p53 as a tumor suppressor derives from studies with Friend virus-induced erythroleukemia in mice (79, 246). In these studies, leukemic clones contained inactivating rearrangements, deletions, or proviral insertions in the p53 gene which prevented p53 expression. This contrasts with the majority of murine retrovirus-induced leukemias in which oncogenes are activated by proviral insertion next to the proto-oncogene. In this case, the loss, rather than the activation, of p53 expression appeared important for tumor formation.

Studies with transformed cells provided yet another line of evidence for the tumor suppressor function of p53. In particular, Chen et al. (51) infected Saos-2 cells, an osteosarcoma cell line lacking endogenous p53 activity, with recombinant retroviruses encoding wild-type (WT) or mutant p53. Cells infected with WT p53-containing viruses became larger and flatter, had longer doubling times and reduced colony formation in soft agar, and were nontumorigenic when injected into mice, indicating that WT p53 had suppressed the neoplastic phenotype in these cells.

Further studies showed a role for p53 in cell immortalization. Transfection of tumor-derived mutant p53 into primary REF and chondrocytes resulted in immortalization of these cells (79). In addition, spontaneously immortalized mouse embryo fibroblasts often contain missing or mutated p53 alleles (79) and the ability of SV40 T-Ag to immortalize cells correlates with the ability of T-Ag to bind p53 (79, 223, 413). Thus, cell immortalization appears to be yet another outcome to the inactivation of p53 function.

Actual arrest of cell growth by WT p53 was demonstrated in a number of ways. First, a temperature-sensitive p53 mutant, p53Val135, transformed cells in cooperation with Ras at 37.5°C but arrested cells in G1 at 32.5°C (79, 246). Second, overexpression of WT p53 in mammalian and yeast cells either arrested cells in G1 (295) or led to apoptosis (79, 295, 311, 409). Finally, when cells were transfected with cDNAs encoding human WT p53 under the control of an inducible promoter (246), induction of p53 expression inhibited cell cycle progression in growing cells and inhibited the G1-to-S transition in serum-stimulated quiescent cells. No antiproliferative effect was seen with mutant p53 in the above induction system. The fact that *de novo* protein synthesis is required for cells to enter S phase after release from a p53-mediated block indicates that p53 arrests cells prior to or near the Restriction point in late G1 (222).

p53 expression correlates with the proliferative state of the cell. p53 mRNA and protein levels are low in serum-starved, quiescent cells (246, 295) but increase following mitogenic stimulation (79, 246, 295). Expression is highest in rapidly proliferating,

undifferentiated cells and decreases as cells are induced to differentiate (79). In addition, p53 levels increase upon exposure of cells to ultraviolet (UV) or γ-irradiation (177, 295), an aspect that will be discussed in greater detail below.

WT p53 is a 53 kD nuclear phosphoprotein which functions, in part, as a sequence-specific DNA-binding protein (22, 183, 184, 246, 311, 411). While no p53-related genes have yet been identified in invertebrates, the human p53 gene can act as a transcription factor and negative growth regulator in yeast (79). The N-terminal region of the p53 protein is acidic and harbors the trans-activation activity demonstrated in both yeast and mammals (79, 109). The basic C-terminal region contains both a DNA-binding domain and a nuclear localization signal, while the midsection of the protein is hydrophobic and plays a very important conformational role (79). Most p53 mutations, in fact, occur within this middle region of the protein (79). The WT p53 protein is relatively unstable, with a half-life of 15 minutes to 2 hours. Thus, in normal cells, levels of p53 protein are very low and barely detectable (311).

In contrast, the mutant p53 protein is quite stable, having a much longer half-life. This is illustrated by the fact that, in transformed cells and tumor cells, levels of p53 protein are much higher and easier to detect (79, 311). Furthermore, the mutant p53 protein is cytoplasmic (79, 246), is not able to bind DNA in a sequence-specific manner (184), and is transcriptionally inactive (104, 184, 315). Studies with the temperature-sensitive p53 gene, p53Val135, and conformation-specific monoclonal antibodies have revealed that a major difference between WT and mutant p53 is the actual conformation of the protein (136). In these studies, the proteins expressed from p53Val135 at 32.5°C versus 37.5°C were recognized by different monoclonal antibodies, indicating that conversion from the WT, nuclear, growth suppressive form of p53 to the mutant, cytoplasmic, growth promoting form involves adoption of a new protein conformation.

An additional property of both WT and mutant p53 proteins is the ability to oligomerize, forming dimers, tetramers, and higher order structures (384). In this respect,

an important activity of mutant p53 is dominant-negative inactivation of WT p53 function via the formation of mutant/WT oligomers. In support of this, transgenic mice with oncogenic p53 transgenes had a high level of tumor incidence, even in the presence of WT endogenous p53 (79) suggesting that mutant p53 overexpression overrode WT p53 expression, presumably via dominant-negative inactivation due to complex formation.

As with pRb, one mechanism for achieving suppression of cell growth is via transcriptional activation of growth-repressive genes. As mentioned previously, WT p53 is a transcriptional activator, a property which has been characterized and studied in a number of different laboratories. Cotransfection experiments have directly demonstrated WT p53-activated expression of genes adjacent to p53 binding sites. Fusion of the N-terminal p53 transcriptional activation domain to the Gal4 DNA binding domain resulted in transcriptional increase from a Gal4 promoter in yeast (109, 315). Similarly, WT p53 activated a reporter gene under the control of a Polyoma hybrid promoter linked to p53 binding sites (104, 184). The level of activation correlated with DNA binding by p53.

As the ability of p53 to suppress transformation correlates with its ability to activate transcription, identifying genes which are transcriptionally regulated by p53 is one method to elucidating the mechanism of p53-mediated growth suppression. One gene shown to be upregulated by p53 is muscle-specific creatine kinase (MCK) (79, 295). Cotransfection studies with a p53 expression plasmid and MCK-CAT revealed a ten- to eightyfold increase in activity with WT but not mutant p53 (388). In addition, p53 was found bound to a 50 bp region of the MCK upstream regulatory domain, and this region conferred p53 responsiveness to other reporter gene constructs (411). These experiments thus identified the "p53 response element" containing the core recognition sequence "TGCCT". Again, upregulation of MCK was via direct binding of p53 to this sequence, as mutant p53 was unable to bind.

Another gene stimulated by p53 is GADD45, a DNA damage response gene (177, 311). GADD45 synthesis increases when cell are exposed to DNA damaging agents, and

the GADD45 promoter contains a p53 binding site. Kastan et al. (177) have shown that WT but not mutant p53 binds to GADD45, and induction of GADD45 in response to ionizing radiation (IR) depends upon a WT p53 phenotype. This links p53 to a checkpoint mechanism for controlling cell proliferation in the event of DNA damage, a point to be discussed in greater detail below.

Other genes activated by p53 include WAF1 (96, 399) and p53 itself (71). WAF1, also known as $p21^{CIP1/WAF1}$ is an inhibitor of G1 cyclin-dependent kinases. Specifically, $p21^{CIP1/WAF1}$ appears to be involved in inhibition of cyclin A/cdk2 and cyclin E/cdk2 activities following cell exposure to IR, an event dependent upon the accumulation of WT p53. A key player in cell cycle arrest, the $p21^{CIP1/WAF1}$ gene itself has potential tumor suppressor activity. Upregulation of $p21^{CIP1}$ thus links p53 directly to the central cell cycle control mechanisms. While a direct interaction of p53 with its own promoter has not yet been demonstrated, the promoter does contain a consensus p53 binding site (71). Cotransfection of the p53 promoter with WT p53 resulted in a ten- to twentyfold increase in transcription (71). Thus, p53 appears to autoregulate its own synthesis, possibly via direct interaction with or stimulation of other proteins which recognize and bind the p53 response element.

In addition to transcriptional activation of genes involved in negative proliferation, growth suppression may be achieved via transcriptional repression of growth-stimulatory genes. To this end, p53 has been found to decrease the endogenous mRNA levels for B-myb, proliferating cell nuclear antigen (PCNA), and DNA polymerase α (223). B-myb is implicated in cell cycle progression of non-hematopoietic cells and may directly or indirectly activate PCNA and DNA polymerase α, both of which are involved in DNA synthesis. Additionally, cotransfection of p53 with a PCNA-CAT construct reduced transcription from the PCNA promoter six- to sevenfold (79).

p53 may also repress cell growth by inhibiting DNA replication. In this respect, WT p53 has been shown to block SV40 DNA replication both *in vitro* and *in vivo* (22,

115, 116). Bargonetti et al. (22) have shown that p53 binds to sequences adjacent to the late border of the SV40 replication origin. By competing with DNA polymerase α for binding to T-Ag (79, 246), p53 inhibits T-Ag's origin-binding and helicase activities. Likewise, plasmids containing the polyomavirus replication origin and p53 binding sites are defective as templates for the synthesis of DNA progeny in the presence of WT p53 and Py large T-Ag (183, 184, 311). Additionally, p53 has been found to interact directly with components of the DNA replication machinery (79); in HSV-1-infected cells, p53 colocalized with DNA polymerase α, PCNA, DNA ligase, Single-Stranded DNA Binding Protein (SSDBP), and Infected Cell Protein 8 (ICP8). This suggests that p53 is affecting the assembly and/or function of the DNA replication initiation complexes (115, 116, 246), an idea that potentially contradicts the hypothesis that p53 acts in G1, prior to "R".

Finally, p53 has been shown to play a role in apoptosis, or programmed cell death. In murine erythroleukemia cells (79), myeloid leukemia cells (409), and human colon tumor cells (344), to name a few, induction of exogenously-added p53 resulted in cell apoptosis. Thus, overexpression of WT p53 appears to result in either G1 arrest or apoptosis (79, 295, 311, 409).

There are multiple mechanisms for inactivating WT p53 function. As mentioned above, mutations which alter the conformation of the protein are common. p53 can also be inactivated via association with viral oncoproteins. For example, p53 has been found to form large oligomers with SV40 T-Ag (79), an event which stabilizes the half-life of the p53 protein; in SV40-infected cells, p53 is 100-fold more stable than in normal cells. Functional inactivation of p53 by T-Ag was demonstrated with transient cotransfection assays: WT p53 trans-activated a test gene construct in the absence of T-Ag, but in the presence of T-Ag, p53-specific trans-activation decreased greater than 90% (250). This effect was not seen in the presence of a T-Ag point mutant unable to bind p53. In addition, the ability to bind p53 is linked to the immortalization activity of T-Ag (223, 413). T-Ag mutations which abolish the ability to bind p53 result in decreased

immortalization efficiency. This indicates that removal of p53 activity by the formation of T-Ag/p53 complexes could allow cells to enter S phase for subsequent viral DNA replication.

p53 is also bound by both Ad E1B and HPV E6 (79). Ad E1B contacts the N-terminal trans-activation domain of p53 to inhibit p53-mediated transcriptional trans-activation and promote transformation of rodent fibroblasts in conjunction with E1A. *In vitro* binding assays demonstrated the interaction between the E6 proteins of the high risk HPV's (HPV-16 and HPV-18) and p53 (79). Interestingly, the E6 proteins from low risk HPVs (i.e. types 6 and 11, which cause benign warts) have either no or low affinity for p53. In contrast to Ad and SV40 oncoproteins, association of p53 with the HPV E6 proteins does not functionally inactivate p53 but, instead, targets p53 for degradation via ubiquitin-dependent proteolysis (79). Thus, in $HPV^{(+)}$ cervical carcinomas, p53 is WT but degraded while, in $HPV^{(-)}$ cervical carcinomas, p53 is mutated and functionally inactivated.

Recent studies have shown that p53 can also be inactivated by association with the cellular proto-oncogene mdm-2. Immunoprecipitation experiments revealed the presence of a 90 kD cellular protein complexed to p53 (147), with increased amounts of p90 found complexed to p53 during p53-mediated growth arrest. The gene encoding this protein has since been found to be identical to the "murine double minute 2" (mdm-2) gene (256), identified as an amplicon on the double minute chromosome of tumorigenic mouse cells (295). Overexpression of mdm-2 immortalizes primary rat cells (110), and cotransfection of Ras plus mdm-2 transforms cells. While mdm-2 is thought to encode a transcription factor, no mdm-2-responsive genes have yet been identified (21, 402). In terms of p53, overexpression of mdm-2 has been shown to inhibit p53-mediated transcriptional trans-activation (256, 295). This functional inactivation by mdm-2 is similar to the action of the viral oncoproteins suggesting that the oncogenic properties of the mdm-2 protein are due to interaction with p53 (19, 20, 110, 256, 282).

Of final note, inactivation of p53 can be achieved via cytoplasmic localization (295). Precedence for this form of inactivation derives from some breast cancers in which WT p53 is found in the cytoplasm rather than the nucleus. WT p53 relegated to the cytoplasm is inactive as a transcription factor and growth suppressor.

A role for p53 in the normal cell was not clear until it was discovered that p53 protein levels increase in response to DNA-damaging agents (i.e. IR, UV, actinomycin D, mitomycin C etc.) (177, 202). Accordingly, it was demonstrated that fibroblasts lacking p53 failed to arrest after IR treatment but that transfection of $p53^{(-)}$ cells with a WT p53 construct restored the ability to G1-arrest after IR exposure (177). Connected to this was the discovery that cells from patients with ataxia telangiectasia (AT, a human autosomal recessive disorder yielding high sensitivity to UV and predisposition to cancer) failed to accumulate p53 after UV exposure (177). Similar results were seen in cells exposed to PALA (N-(phosphonacetyl)-L-aspartate), a uridine biosynthesis inhibitor (202). Cell resistance to PALA results from amplification of the CAD gene which encodes the trienzyme complex carbamoyl phosphate synthase-aspartate carbamoyltransferase-dihydroorotase. It should be noted that gene amplification is a marker of genetic instability and a precedent to neoplasia. In experiments with PALA, exposed cells containing WT p53 arrested in G1 with no development of $drug^R$ colonies while cells harboring mutant p53 did not arrest in G1 and had a high frequency of $drug^R$ colonies.

A role for p53 as a component of a cell cycle checkpoint, as a "molecular guardian for DNA integrity" (79), can now be proposed. p53 could function in maintaining genomic stability, preventing genomic rearrangements, and preventing the growth of cells with genomic rearrangements. A model can be proposed in which the p53 protein is induced and activated following DNA damage, resulting in alteration of the transcriptional pattern in the damaged cell and inhibition of DNA replication. Cells arrest until DNA damage can be repaired. In the case of irreparable damage, apoptosis results. Damaged cells harboring mutant p53 would fail to arrest. In the absence of a monitoring

mechanism, cells would become genetically unstable in that they would continue to cycle and replicate damaged DNA. Chromosomal mutations and rearrangements would then accumulate, the end result being neoplasia and malignancy.

4.4 p16

Recently, another putative tumor suppressor gene has been identified which appears to be mutated more highly than p53 in human cancers. The p16 gene, which encodes a suppressor of cdk4 kinase activity, was discovered in 1993 in the lab of David Beach at Cold Spring Harbor (24). Beach and colleagues employed the yeast two-hybrid protein interaction screen, which involves reconstituting a functional Gal4 transcriptional activator from separate fusion proteins containing the Gal4 activation ($Gal4^{AD}$) and DNA-binding domains ($Gal4^{DB}$), to identify proteins which associate with cdk4. Cotransformation of yeast with $Gal4^{DB}$-p16 and $Gal4^{AD}$-cdk4 fusion proteins allowed growth on $His^{(-)}$ plates, verifying an interaction between the two proteins. In addition, GST-p16 bound only cdk4 from a mixture of *in vitro*-translated ^{35}S-labeled cdks, and monoclonal antibodies to p16 immunoprecipitated cdk4, and vice versa, from insect cells infected with baculoviruses encoding p16 and cdk4. Thus, the association is present *in vivo* as well.

Inhibition of cdk4 kinase activity by p16 was demonstrated by reconstituting an active cdk4/cyclin D complex that phosphorylated GST-Rb. Addition of extracts containing p16 abolished the phosphorylation of both GST-Rb and p107 by cdk4/cyclin D. It therefore appears that the role of p16 in the normal cell is to down-regulate cdk4 activity, possibly after pRb has been phosphorylated and inactivated.

The idea of p16 as a tumor suppressor derives from the studies of Kamb et al. (175). This group identified a chromosomal locus, termed MTS1 for Multiple Tumor Suppressor 1, on the short arm of chromosome 9. This region of this chromosome contains many chromosomal inversions, translocations, and heterozygous and homozygous deletions in glioma cell lines, lung cell cancers, leukemias, and melanomas,

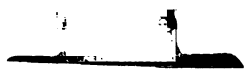
suggesting that it encodes a tumor suppressor. The MTS1 locus, in fact, encodes p16. Since its discovery, Kamb and colleagues have found deletions and mutations in the p16 locus in 75% of melanoma cells examined and in tumors from the lung, breast, brain, bone, skin, bladder, kidney, ovary, and lymphocytes. p16 therefore rivals p53 as the most mutated gene in human cancer.

4.5 E2F

As mentioned in the preceding sections, E2F is a ubiquitous cellular transcription factor implicated in the activation of a number of G1/S-phase genes. Its association with growth suppressive proteins such as pRb and p107 suggest it is an integral part of the cell cycle control machinery. The protein DRTF1 (Differentiation-Regulated Transcription Factor 1) was isolated from F9 embryonal carcinoma cells as a factor whose activity was down-regulated during cellular differentiation. DRTF1 appears to be related, and possibly identical, to E2F, as it recognizes the same DNA sequences and binds the same set of proteins (207).

E2F itself was discovered in 1986, during studies on E1A-mediated transcriptional control in adenovirus-infected cells (273), as a cellular DNA-binding protein that bound to elements within the Ad E2 promoter and stimulated transcription of the E2 gene. E2F recognized tandem, duplicate sites, containing the consensus sequence TTTCGCGCG within the Ad E2 promoter (273). Both the amount of E2F and the ability of E2F to bind sites within the E2 promoter increased following adenovirus infection (14, 196, 274). It was soon discovered that this increase in E2F protein and activity was attributable to 1) the action of the Ad E1A oncoprotein and 2) association with the adenovirus E4 protein.

In adenovirus-infected cells, E2F was found complexed to the 19 kD product of the Ad E4 gene, an association reported and subsequently studied by a number of laboratories (236, 272, 279, 318). While interaction of the E2F protein itself with the Ad E2 promoter was sufficient to stimulate transcription, promoter activity was greatly increased upon stable binding of an E2F/E4 complex. Apparently, in the absence of E4,



E2F bound weakly to the promoter sites, while association with E4 allowed cooperative and stable binding of E2F to the two adjacent sites in the promoter. This association was subsequently found to require the action of the E1A protein (14). As mentioned previously, E1A dissociates cellular complexes containing E2F, generating a pool of free E2F which, in the context of adenovirus-infected cells and adenoviral replication, is able to associate with E4 to stimulate transcription of adenovirus genes.

A pivotal link between E2F and eukaryotic cell cycle control was made with the discovery of an association between E2F and pRb. Since the ability of E1A to dissociate E2F complexes depends upon E1A sequences required for interactions with cellular proteins, researchers looked to see if any cellular E1A-binding proteins also interacted with or bound E2F (139, 408). An Rb-containing protein complex was found to bind DNA in a sequence-specific manner, recognizing sequences similar to the Ad E2 motif, thereby suggesting that E2F was a component (53). Gel shift assays in the presence of specific monoclonal antibodies, immunoprecipitations with α-E1A monoclonal antibodies, and Western blot analysis of proteins bound to affinity-purified E2F all confirmed an association between pRb and E2F (49) or DRTF1 (17). Thus, E2F is a cellular target for pRb. Complexes were dissociable by E1A, SV40 T-Ag, and HPV E7, and similar sequences (to those required for binding pRb) were required (50), indicating a common mechanism for the stimulation of cell proliferation by the viral oncoproteins.

A functional importance to the interaction between E2F and pRb is further suggested in that only the underphosphorylated, or growth-suppressive form of pRb is involved (49). In addition, E2F/pRb complexes are absent in human cervical carcinoma cell lines expressing HPV E7 or containing pRb mutations, suggesting that the loss of E2F/pRb is a contributing factor to cervical carcinogenesis (50). Deletion studies show that it is the C-terminal 135 amino acids of pRb that are required for interaction with E2F (145), the same sequences required for pRb-mediated growth suppression and repression of promoters containing E2F binding sites.

The activity of E2F is regulated, in part, via association with pRb. Hiebert et al. (144) demonstrated inhibition of E2F transcriptional activity by pRb via transient cotransfection assays in C33A cervical carcinoma cells. As indicated above, these cells contain a mutation in the RB1 gene and therefore contain free E2F as opposed to E2F/pRb complexes. Cotransfection of an E2 promoter-CAT reporter construct with a pRb expression plasmid resulted in a four- to fivefold decrease in expression from the E2 promoter. No effect was seen on a promoter in which the E2F sites were deleted or with cotransfection of mutant pRb. Additionally, E2F inhibition was relieved by coexpression of either HPV E7 or Ad E1A.

Recently, three independent laboratories have cloned a gene, termed E2F-1, encoding a protein with the properties of the transcription factor E2F (143, 173, 342). Analysis of the E2F-1 protein reveals several functional domains. The C-terminal region of the protein is acidic and contains overlapping trans-activation and pRb-binding domains. The overlapping nature of the trans-activation and pRb binding domains suggests a mechanism whereby binding of pRb masks the activation domain, thereby interfering with the ability of E2F to mediate transcriptional activation. The E2F DNA-binding domain was localized to within the N-terminal 284 amino acids of the protein. This region is basic in nature, contains an amphipathic helix-loop-helix motif thought to be important for DNA binding and/or protein dimerization, and is followed by a leucine zipper motif potentially involved in protein-protein interactions. Also of note is the presence of two potential cdk phosphorylation sites in the protein sequence (107).

Since the cloning of E2F-1, data has accumulated to indicate that the transcription factor "E2F" is actually a family of heterodimeric protein complexes. Initial evidence for dimerization derived from two sources. First, the structural analysis of the protein, mentioned above, revealed the helix-loop-helix and leucine zipper motifs common to dimerization domains and protein-protein interactions. Second, SDS-PAGE analysis of E2F protein purified from Hela cell lysates revealed multiple protein bands in the 50-60

kD range (158). Individual proteins, when electroeluted and refolded, displayed weak E2F DNA-binding activity. However, mixture of a 50 kD protein with a 60 kD protein regenerated the high-affinity DNA- and pRb-binding characteristic of E2F.

While one component of this complex is, of course, the 60 kD E2F-1 protein, there is, in fact, much evidence to suggest that E2F-1 is only one of a number of related, yet distinct, E2F proteins. A clue to the existence of multiple E2Fs first came when two of the three laboratories involved in the cloning of E2F-1 reported regulation of E2F-1 mRNA with respect to the growth state of the cell (173, 342); E2F-1 mRNA levels are low or undetectable in G0 quiescent cells but induced following mitogen stimulation. That no E2F-1 mRNA is detected during much of G1, a period during which the DNA-binding activity of E2F is clearly detectable, suggests the presence of additional E2F proteins.

It was then reported that, in tissue culture cells, E2F-1 binds pRb but not p107 (143). In correlation with this, Dyson et al. (94) demonstrated that p107 binds an E2F-1-like protein, but not E2F-1 itself. This conclusion was based upon the fact that various monoclonal antibodies specific to E2F-1 immunoprecipitated pRb but not p107, and that different protease digestion patterns were found for pRb-bound and p107-bound ^{32}P-labeled E2F polypeptides. Additionally, Chittenden et al. (53), following a study of E2F complexes formed in primary T cells, reported the finding of a number of novel forms of unbound and bound E2F. These forms had cell cycle kinetics, gel mobilities, and protein compositions distinct from any previously-described complexes, prompting this group to hypothesize the existence of different species of free E2F for different biological functions.

Concrete evidence for a family of E2F proteins came when Lees et al. (211) cloned the cDNAs for E2F-2 and E2F-3. Using low stringency hybridization conditions, they screened plaques from a human cDNA library with a ^{32}P-labeled cDNA encoding the DNA-binding domain of E2F-1, and uncovered two clones with amino acid homology to E2F-1. These proteins both bound a WT E2F recognition motif, bound pRb, and trans-activated genes in an E2F site-dependent manner. Different chromosomal locations,

however, confirmed their independence from E2F-1. It should be mentioned that E2F-1 homologs have recently been identified in mouse (220), *Xenopus* (207), *Drosophila* (281), and yeast (232), suggesting that "E2F" activity is evolutionarily conserved and functionally important.

The second component of the "E2F" heterodimer, a 46 kD protein termed DP-1 (DRTF1 Protein 1) was recently discovered as an E2F site-specific DNA-binding protein in F9 embryonal carcinoma (EC) cells (125). DP-1 is similar to E2F-1 in terms of its size, sequence, and location of the DNA-binding domain (207). It, too, contains amphipathic alpha helices for dimerization.

A number of studies verify that DP-1 is part of the E2F transcription factor (125, 197). First, immunoblot analysis of purified E2F with antibodies specific to DP-1 detected the 46 kD polypeptide. Second, α-DP-1 antibodies supershifted or abolished E2F-binding complexes on gel shifts, including complexes containing pRb or p107. Third, a GST-DP-1 fusion protein specifically bound a WT E2F motif. In addition to immunoprecipitation experiments (197), direct, physical interaction between DP-1 and E2F-1 *in vivo* was confirmed via the yeast two-hybrid system (18).

The functional significance of the E2F-1/DP-1 association appears to be a synergistic interaction to achieve high affinity DNA binding and transcriptional activation of E2F-dependent promoters (18, 197). As alluded to earlier, E2F-1 and DP-1 individually bind weakly to DNAs containing E2F motifs. DNA binding, however, is greatly increased upon association of the two proteins. In transcriptional activation studies, DP-1 does not activate, and E2F-1 poorly trans-activates, reporter gene constructs linked to E2F binding-site motifs. Trans-activation is significant, though, in the presence of the heterodimer.

Overall, DP-1 (or a DP-1-like protein) appears to be a frequent and widespread component of the transcription factor "E2F" (207). Whereas E2F-1 is a component of only a fraction of the "E2F" complexes, DP-1 has been found to be common to all E2F

complexes detected in Hela and NIH 3T3 cells, including all complexes formed with pRb and p107. Consistent with this is the fact that DP-1 mRNA is constitutively expressed. As with E2F-1, the recent identification of DP-1-related proteins indicates that DP-1 itself is a member of a family of proteins. Thus, it can be concluded that transcription factor "E2F" is comprised of an E2F family member and a DP-1 family member. This association presents the possibility of different E2F/DP-1 heterodimers forming at different times in the cell cycle to target different genes, thereby fine-tuning the regulation of "E2F" activity and cell cycle progression.

While the previous discussions have mentioned the transcriptional activation activity of "E2F", the function or role of the "E2F" transcription factor (hereafter referred to simply as E2F; individual components will be specifically designated, i.e. E2F-1) has not yet been discussed. As alluded to, E2F plays a role in the stimulation of cell growth. For example, serum starvation of cells transfected with an E2F-1 expression plasmid revealed that overexpression of E2F-1 could activate DNA synthesis in cells that would otherwise growth-arrest (166). In addition, microinjection of E2F-1 cDNA into already quiescent cells induced DNA synthesis, while expression of E2F-1 antisense RNA prolonged S phase (333), suggesting that E2F-1 plays a role in regulating S-phase entry.

Recent literature has indicated that E2F stimulates cell growth by activating genes required for proliferation. Specifically, E2F binding sites have been found in the promoters of many genes involved in the G1/S transition and activation of DNA synthesis, including dihydrofolate reductase (DHFR), thymidine kinase (TK), DNA polymerase α, c-myc, and cyclin D1. In addition, the level of E2F DNA-binding activity increases at the time when these genes are activated. As a more detailed discussion of the regulation of some of these genes follows in the next section, the following paragraphs present a brief synopsis of the role of E2F in the activation of G1 and S phase-specific genes.

DHFR is involved in purine biosynthesis and is required for cell growth, and TK catalyzes the conversion of thymidine to TMP (thymidine monophosphate), a compound

subsequently incorporated into DNA (107). mRNA levels for both of these genes increase at the G1/S interface. For DHFR, in particular, E2F has been demonstrated to be both necessary and sufficient for the observed transcriptional increase. When a minimal promoter containing only the DHFR initiator element and E2F sites is cloned upstream of a reporter gene, a thirty-fivefold increase in promoter activity is achieved during S phase; decreasing the number of E2F sites reduces the level of trans-activation (107). The role of E2F in the activation of TK, however, is less clear. Proteins from S phase extracts bind E2F motifs in both the human and mouse TK promoters. While specific E2F-containing complexes have been detected on the mouse TK promoter, similar associations have not been found with the human TK promoter (107). A few studies, however, report trans-activation of the murine TK promoter dependent upon E2F sites (85, 280) and stimulation of the human TK promoter by E2F-1 in transient transfection assays (166, 280).

DNA polymerase α, which catalyzes lagging strand DNA synthesis at the replication fork, shows an increase in mRNA as cells enter S phase. The polymerase α promoter contains one consensus E2F site and multiple similar sites (107). As with DHFR, promoter deletions which delete the E2F sites reduce the level of transcriptional increase, and transient transfection assays show trans-activation of a polymerase α-CAT reporter gene in the presence of E2F-1 (107, 333).

In contrast to the S phase-specific induction of DNA polymerase α, c-myc is rapidly and transiently induced in early G1 following mitogenic stimulation. Recall that Myc has been proposed to play a role in the G0 to G1 transition and entry into S phase. In terms of E2F, the major promoter of c-myc, P2, contains a sequence motif which is a) required for trans-activation by E1A and b) subsequently identified as a binding site for E2F (107). Transient transfection assays revealed that the c-myc promoter was activated by E2F-1 (283). Finally, E2F-1 bound the G1-specific cyclin D1 promoter in gel shift assays and activated cyclin D1 transcription in transient transfection assays (333).

In addition to being a stimulator of cell growth and regulator of entry into S phase, data indicate that E2F-1 may be proto-oncogenic and/or a mediator of apoptosis. Evidence for proto-oncogene status comes from studies of REF (rat embryo fibroblast) cells stably transfected with an E2F-1 expression construct (350). Stable transfectants expressing high levels of the E2F-1 transgene displayed morphological characteristics of transformation, including rounded foci, growth in soft agar, reduced requirement for serum growth factors, and an accelerated rate of proliferation. In addition, the transformed cells were tumorigenic when introduced into athymic mice. In contrast, coexpression of E2F-1 with p53 in mouse cells resulted in rescue of a p53-induced cell block followed by apoptosis, presumably due to the presence of conflicting signals in the cell regarding G1 arrest versus S phase entry (403).

The transcription factor E2F appears to play multiple roles in the regulation of cell cycle progression. Regulation of E2F activity is thus essential to cell viability and proliferation. In this regard, E2F activity is regulated by a variety of mechanisms, the most important being complex formation with cellular proteins. As mentioned previously, E2F associates with cellular proteins in a cell cycle-dependent manner. G1 finds E2F in complex with the underphosphorylated form of pRb (265) and, independently, with cyclin E, $p33^{cdk2}$, and p107 (74, 348, 370). During S phase, on the other hand, E2F is associated with cyclin A, $p33^{cdk2}$, and p107 (42, 285). As E2F transcriptional activity is repressed during these periods (112, 135), it is thought that association with these cellular proteins directly modulates E2F activity. Consistent with this, again, is the fact that these complexes are dissociated by the action of the Ad E1A, SV40 and Py T-Ags, and HPV E7, resulting in an accumulation of free, transcriptionally-active E2F.

The association of E2F with pRb warrants further discussion. Several groups have reported repression of E2F activity by pRb. For example, in transient cotransfection assays, E2F-mediated trans-activation was suppressed by WT but not mutant pRb (112). Suppression was inhibited by WT SV40-T-Ag, but not a T-Ag mutant unable to bind pRb.

In addition, E2F-1 mutants unable to bind pRb were unaffected. Since the E2F trans-activation and pRb-binding domains overlap, pRb binding masks the activation domain, thereby directly repressing E2F transcriptional activity. In addition, pRb binding may prevent the interaction of E2F with other proteins required for activation. In this respect, Hagemeier et al. (135) have shown that E2F binds TATA-binding protein (TBP), a protein essential to the formation of the transcription initiation complex; experiments utilizing GST-TBP and *in vitro*-translated E2F have successfully detected a direct interaction between the two proteins. Also significant is the discovery, via E2F deletion analysis, that the E2F sequences binding TBP are the same sequences utilized in pRb binding. These results directly link E2F to the transcriptional machinery, and postulate a mechanism for E2F-mediated activation via contact with the transcriptional apparatus.

A model for the functional interaction between E2F and pRb can now be proposed. The association of E2F with the underphosphorylated form of pRb in G1 suggests that one role for pRb is the sequestration of E2F in a transcriptionally-inactive form. Upon pRb phosphorylation at G1/S, transcriptionally-active E2F would be released, promoting the transcription of G1/S phase-regulated genes. While this model is consistent with the detected increase in free E2F at G1/S (274), it is inconsistent with the observation that E2F/pRb complexes actually persist into S (348). Therefore, the function of the E2F/pRb complex is unclear. It is possible that E2F remains associated with only a small percent of underphosphorylated pRb present in the cell at G1/S. Phosphorylation of the majority of pRb molecules would thereby serve to prevent further E2F interactions, preserving the pool of free E2F. The accumulation of free E2F could then be due to both newly synthesized E2F and release of E2F from other cellular complexes (i.e. p107-containing complexes).

As E2F is transcriptionally inactive while in association with pRb, it may be assumed that this complex is inert. Weintraub et al. (389), however, postulate that this complex is actually functionally active as a transcriptional repressor (diagrammed earlier in

Figure 3). In this model, the E2F site would alternate between a positive and a negative regulatory element, dependent upon the phosphorylation state of pRb in the cycle. Evidence to support this model comes from a number of experiments. First, in transient transfection assays designed to investigate the role of E2F in activation of the adenovirus E1A promoter, a competitor plasmid containing E2F sites was cotransfected with an E1A-CAT reporter. Presence of the competitor, which should bind up all endogenous E2F in the cell, resulted in activation of the E1A promoter, suggesting that E2F normally acts as an inhibitor of the E1A promoter. Additional experiments utilizing either an SV40 early promoter/enhancer-CAT construct or a minimal promoter(TATA-ATF)-CAT construct revealed that these promoters were inhibited in $Rb^{(+)}$ cell lines but activated in both $Rb^{(-)}$ cell lines and 293 cells, which exhibit stable expression of the E1A oncoprotein. Likewise, cotransfection of the reporter constructs with a pRb expression plasmid into Rb(-) cells resulted in E2F site-dependent promoter inhibition. Thus, these results indicate that free E2F is a transcriptional activator while pRb-complexed E2F is a transcriptional repressor and that E2F sites are negative elements in the presence of pRb and positive elements in the absence of pRb. Important implications also arise as to the function of pRb. In other words, pRb may do more than just sequester E2F and repress its activity; association with E2F imparts to pRb the ability to inhibit or activate cell cycle-regulated genes in a DNA site-dependent manner.

Additional cellular complexes also contribute to the regulation of E2F activity. Most notable is the association of p107 with E2F during both G1 and S. Presently, the roles of the E2F/p107 complexes are unclear. As with pRb, evidence exists for direct repression of E2F transcriptional activity by p107 (91, 370, 416). Thus, p107 may function simply to sequester and inactivate E2F at various points during the cell cycle. However, p107 may also play a role in targeting cyclin-dependent protein kinases to E2F complexes, either to phosphorylate and thereby additionally regulate E2F itself, to

phosphorylate other proteins contained in the complex, or to phosphorylate other promoter-bound proteins using E2F as a DNA site-specific chaperone.

Indeed, phosphorylation of E2F itself results in loss of the ability to both bind DNA and mediate trans-activation (91). E2F activity is inhibited during its association with the cyclin complexes. The notion of pocket proteins as targets for the kinases follows from work with pRb which suggests that phosphorylation of the pocket proteins by associated kinase complexes would release free E2F. Finally, the model of E2F as a chaperone (206, 274) is attractive. As indicated, association of E2F with cyclin-kinase complexes could generate an active kinase with sequence-specific DNA binding activity, thus targeting the kinase to promoters with E2F sites and allowing phosphorylation and modulation of other transcription factors bound nearby.

Finally, E2F associates with one other known p107- and pRb-related protein: p130 (57). p130 is a prominent E1A-associated cellular protein and is structurally similar to pRb and p107. As E1A mutants unable to bind p130, but able to bind p107 and pRb, are defective for the transformation of primary cells in cooperation with Ras, a role for p130 in cell proliferation and suppression of tumor formation is suggested. p130 is also found to be the major E2F-associated protein in G0 A31 fibroblasts and G1 fibroblasts released from serum starvation, as only small amounts of p107 and pRb are associated with E2F in these cells and under these conditions. Thus, either cell type-specific differences in E2F partners exist, or p130 predominates in G0. In any case, E2F/p130 complexes are also found in association with $p33^{cdk2}$, cyclin E, and cyclin A, suggesting that the previous discussions are relevant to interactions with this protein, as well.

Besides association with cellular factors, other mechanisms of regulating E2F exist. For example, E2F-1 is regulated, in part, by abundance, which is determined by the growth state of the cell. As mentioned, the E2F-1 mRNA is cell cycle regulated (173, 342), being repressed in senescent or quiescent cells and increasing just prior to S phase. Also mentioned is the role for heterodimerization with DP-1 in achieving high affinity

DNA binding and transcriptional activation, and the role phosphorylation plays in the modulation of E2F activity. In any case, regulation of and activation by E2F are complex issues, and the potential for E2F as an integral part of the cell cycle regulatory mechanism is only beginning to be realized.

The last several sections have striven to provide an overview of the various molecules and pathways involved in mediating cell cycle progression. Interactions are complex, and pathways intertwine to provide a highly tuned and highly sensitive system of regulation. The remaining sections involve exploring the regulation of a particular set of genes, those activated at the G1/S border and utilized for the onset of S phase. Particular attention will be paid to the regulation of thymidine kinase, the gene to which this dissertation is devoted.

5. S Phase-Regulated Genes

One approach to elucidating mechanisms of cell cycle control is to study the regulation of genes activated at specific phases of the cell cycle in hopes that the proteins involved in activation of the "model" gene are part of common cell cycle control mechanisms. In this respect, G1/S phase-regulated genes, which characteristically display a sharp induction at the G1/S interface, offer themselves as good model systems for the study of mechanisms controlling the progression of cells from G1 into S phase, one of the more highly regulated cell cycle transition points. The family of G1/S phase-regulated genes is comprised of many of the genes involved with the process of DNA synthesis, including dihydrofolate reductase, thymidylate synthase, thymidine kinase, DNA polymerase α, proliferating cell nuclear antigen, and replication-dependent histones. As the activation of these genes is temporally coupled to the process of DNA replication, study of the regulatory mechanisms governing their expression should provide insight into the mechanism behind this control point. For many of these genes, in both continuously cycling cells and quiescent cells mitogenically stimulated to re-enter the cell cycle, enzyme

activity is low in G0 and G1 and increases sharply at the onset to S phase, with maximal expression throughout S and G2. This section reviews briefly what is known about the regulation of each of these genes, with the exception of thymidine kinase; regulation of thymidine kinase will be discussed in detail in the following section.

5.1 Dihydrofolate Reductase

Dihydrofolate reductase (DHFR) catalyzes the conversion of dihydrofolate to tetrahydrofolate and is therefore required for the synthesis of purines, thymidylate, and glycine (107). Studies have shown that the level of DHFR is low in resting cells but increases upon cell stimulation (105, 167, 400). While the increase in DHFR expression is concomitant with entry of cells into S phase, the inhibition of DNA synthesis does not inhibit DHFR expression, indicating that the two processes are not tightly coupled.

The regulation of DHFR expression at the onset of S phase is complex and occurs at multiple levels. DHFR regulatory studies are most often done in methotrexate-resistant (MTX^R) cell lines, which express elevated levels of DHFR due to amplification of the DHFR genes. Studies are conducted with the amplified cell lines since DHFR expression in the single copy parental cells is too low for analysis. While DHFR genes may be amplified as much as 50-500 times, the amplified cell lines have been verified to have the same pattern of DHFR cell cycle regulation as the parental single copy cells (235).

Using MTX^R cell systems, numerous studies have provided evidence for the post-transcriptional regulation of DHFR. For example, studies in continuously cycling cells have demonstrated parallel increases in both DHFR enzyme activity and protein levels, suggesting that the increase of DHFR activity at S phase is due to the new synthesis of DHFR molecules as cells commit to DNA synthesis (235). Others have reported regulation at the level of mRNA stability and/or processing (216).

Numerous studies conducted in both continuously cycling cells and mitogenically-stimulated cells report a transcriptional element to the control of DHFR expression. Farnham and Schimke (105) determined the rate of DHFR gene transcription in MTX^R

mouse 3T6 cells synchronized by mitotic shakeoff. They determined that the rate of DHFR transcription is low, but detectable, in G1, increases sevenfold at the start of S phase, then drops almost immediately back to G1 levels, remaining low throughout the remainder of S and into G2. This mRNA increase at G1/S has been found to occur in hamster and human cells as well (107). Furthermore, two studies conducted with serum-stimulated mouse 3T6 cells report that the increase in the rate of DHFR synthesis following stimulation is due to an increase in the level of DHFR mRNA which, in turn, is due to an increase in the rate of production of mRNA (335, 400). Looking at the rate of DHFR hnRNA production, it was further illustrated that the rate of hnRNA production paralleled the rate of DHFR mRNA production, suggesting that the increase in DHFR gene expression was ultimately controlled at the level of transcription rather than processing of hnRNA to mRNA.

Analysis of the DHFR promoter region reveals that it does not have a TATA box (106). While TATA boxes are common in eukaryotic promoters to position the site of transcription initiation, they are lacking in many housekeeping genes. While no CCAAT boxes are found within the 5' untranslated region, two inverted CCAAT elements are located at the exon 1/intron 1 boundary (244), and the region directly upstream from the transcription initiation site contains four copies of a 48 bp repeat, each containing a GC box which binds Sp1 (90). Fractions of HeLa cell nuclear extracts enriched in Sp1 bind the consensus Sp1 sites in the mouse DHFR promoter, and HeLa cell extracts depleted of Sp1-containing fractions are incapable of efficient *in vitro* transcription from the DHFR promoter.

In the absence of a TATA box, Farnham and Means (106) identified an initiator element within the murine DHFR promoter and determined that positioning for accurate transcriptional initiation was determined by the binding of a protein termed HIP1 (for Housekeeping Initiator Protein 1) to the initiator element. Using DHFR promoter fragments with 5' and 3' deletions, and *in vitro* transcription reactions with HeLa cell

nuclear extracts, they defined a minimal promoter region to lie between -65 and +15 bp, relative to the transcriptional start site. In addition to a binding site for the transcription factor Sp1, this region contained the transcription initiation site, and DNAse protection assays revealed that the initiation site was bound by protein. It was further determined that both the Sp1 binding sites and the initiator element are required for accurate initiation, since the removal of either one abolishes transcriptional activity.

Besides selection of the transcriptional start site, a role for the HIP1 protein in the increase of DHFR transcription at G1/S has also been proposed. Means et al. (244) found that DHFR sequences between -270 and +20 bp, containing four Sp1 consensus elements and the HIP1 binding site, were sufficient to confer regulation to a heterologous luciferase reporter gene, as evidenced by a twentyfold increase in luciferase activity at G1/S. Furthermore, mutation of the HIP1 site abolished G1/S transcriptional induction. A HIP1 DNA affinity column was then used to isolate the 180 kD HIP1 protein, and the exact consensus binding sequence (TTCGCGCCA) was determined by gel shift analysis. It should be noted that the HIP1 binding sequence is similar to the consensus sequence for E2F, although it is unlikely that HIP1 itself is E2F since its size (180 kD) is approximately three times that of E2F (60 kD).

Recently, it has become known that E2F plays a prominent role in G1/S-phase transcriptional regulation of DHFR. A connection between E2F and DHFR was first established by Blake and Azizkhan (28) in studies with the hamster DHFR gene. DNAse footprinting of a segment of the DHFR promoter identified a protein-bound region containing two potential overlapping E2F binding sites. This arrangement was unique in that the "E2F" sites were found to be 3' to the major transcriptional start site. These E2F sites appear to be conserved in the human, mouse, and hamster DHFR promoters. Verification of an interaction between E2F and the DHFR promoter was achieved via gel shift analyses in which partially purified E2F protein and HeLa cell nuclear extracts formed comigrating complexes with DHFR promoter fragments. Furthermore, promoter

fragments mutated at the E2F sites were ineffective as gel shift competitors while, in *in vitro* transcription reactions, transcription from the mutated templates was deceased two- to fivefold in comparison with wild type promoter fragments. Finally, in transient transfection assays using wild type or mutated promoter fragments fused to CAT, activity from the mutant promoter was decreased fivefold. Thus, E2F binds the DHFR promoter and is required for efficient promoter expression.

Following previous studies which demonstrated binding of the purified, cloned E2F-1 protein to DHFR E2F sites (173), Slansky et al. (352) continued to examine the role of E2F in DHFR transcriptional regulation. They cloned murine DHFR promoter sequences between -20 and +9 bp, containing only the E2F sites and initiator sequence, upstream of a luciferase reporter gene and measured luciferase activity in cells which had been serum starved then serum stimulated. Luciferase activity increased to thirty-fivefold in mid S phase, indicating that E2F is sufficient to mediate the G1/S transcriptional increase in the absence of other protein binding sites. Furthermore, transient cotransfection of DHFR-Luciferase with CMV-E2F-1, a plasmid constitutively expressing E2F-1, followed by serum starvation, revealed that luciferase activity increased twenty-twofold relative to cells lacking the E2F-1 cDNA and that no luciferase induction was seen in cells transfected with constructs carrying DHFR promoter mutations in the E2F site. Thus, these studies show that the DHFR E2F element is both necessary and sufficient for G1/S regulation, providing one of the first pieces of strong evidence tying E2F to the activation of S phase-regulated genes.

5.2 Thymidylate Synthase

Thymidylate synthase (TS) catalyzes the reductive methylation of dUMP (163, 164) as the final step in the *de novo* synthesis of TMP, a precursor to DNA synthesis. In this regard, TS is an essential enzyme in proliferating cells and is actually an important target enzyme in cancer chemotherapy (13). In terms of expression, TS protein and

enzyme levels are high in cells undergoing DNA replication but low in other phases of the cell cycle and in non-dividing or arrested cells (163). As with DHFR, TS studies are often performed in cell lines harboring amplified TS genes since TS enzyme and mRNA levels are overproduced fiftyfold in 5-fluorodeoxyuridine-resistant ($FdUrd^R$) cells (164). Using these cells, TS protein levels have been found to increase tenfold 10 to 25 hours following serum addition to quiescent 3T6 mouse fibroblasts (298), a phenomenon found paralleled by a ninefold increase in the rate of TS protein synthesis as the serum-stimulated cells traversed S (164).

Like DHFR, regulation of the thymidylate synthase gene appears to occur at both the transcriptional and post-transcriptional level. A multitude of evidence exists for post-transcriptional regulation of the TS gene. For example, in one study (159), TS mRNA levels were measured in amplified mouse cell lines synchronized by centrifugal elutriation. While mRNA levels remained constant throughout the cell cycle, enzyme activity increased and was maximal during S phase. In a study by Ayusawa et al. (13), TS mRNA levels reportedly increased fourteenfold following serum stimulation of serum-starved human diploid fibroblasts, but the transcription rate remained the same in both resting and growing cells, suggesting post-transcriptional regulation. Likewise, nuclear run-on analysis in cells transfected with a human TS minigene revealed no difference in TS transcription rate between resting and growing cells (365). Finally, several studies by Chu and colleagues (55, 56) describe a potential negative autoregulatory mechanism mediated by the TS protein. These experiments, which focused on the study of TS translation in rabbit reticulocyte lysates, revealed that addition of purified recombinant TS protein inhibited the translation of TS mRNA by binding to the TS mRNA at the translation initiation site.

Evidence also exists for transcriptional regulation. Northern blot analysis of TS mRNA levels in serum-stimulated $FdUrd^R$ mouse 3T6 cells demonstrated a twenty- to fortyfold increase in TS mRNA levels 5 to 25 hours following serum addition, as cell

progressed from G0 into S (163). Subsequent pulse-labeling studies detected no change in mRNA stability in cycling versus noncycling cells, suggesting that the increase in mRNA levels may be due to an increase in transcription rate. By measuring the rate of incorporation of ^{3}H-UTP into TS hnRNA, the rate of transcription was estimated to increase three- to fourfold as the cells entered S. However, as the transcription rate increases only three- to fourfold, while mRNA levels increase twenty- to fortyfold, some of the above-mentioned post-transcriptional regulatory mechanisms must also contribute to induction of TS activity at G1/S. TS mRNA content is also cell cycle-regulated in continuously cycling cells (163) since in these same FdUrd^{R} mouse 3T6 fibroblasts synchronized by mitotic shakeoff, Northern blot analysis detected a five- to eightfold increase in TS mRNA as cells passed from G1 into mid S phase.

Features of both the human (176) and mouse (72) thymidylate synthase promoters include the absence of both TATA and CCAAT boxes. Due to the lack of an initiator element, transcription initiates at multiple sites (72, 75). Both promoters do, however, contain a G+C-rich region (120, 176). While the human TS gene does not contain Sp1 sites within the promoter region itself, the G+C-rich region extends into intron 1 where three Sp1 sites are located. DNAse footprint assays with purified Sp1 has detected two Sp1 binding sites within the mouse TS promoter (169). Mutation of either Sp1 site results in a threefold decrease in TS enzyme expression. The mouse TS promoter also contains a binding site for a protein termed CIII (169), a possible member of the Fos family of transcription factors. While the exact identity of the CIII protein is unknown, mutation of the CIII binding domain reduces TS transcription approximately twelvefold.

An interesting aspect to regulation of both human (365) and mouse (12, 219) TS genes is that both sequences upstream of essential basal promoter elements and intron sequences are required. In studies utilizing TS minigene constructs, it has been found that, while both regions are required, neither are sufficient, as omission of either region results

in constitutive expression of both mRNA and enzyme. The requirement for introns suggests that splicing may play a role in the regulation of thymidylate synthase (12).

Finally, a putative E2F site has been identified within the TS promoter (107). In gel shift assays, this site binds a cellular protein that is effectively competed away by a consensus E2F motif. No further information, however, is available on the role, if any, of E2F in thymidylate synthase promoter regulation.

5.3 DNA Polymerase α

DNA polymerase α (Pol α) is the principal polymerase in eukaryotic DNA replication where it is responsible for catalyzing the synthesis of lagging strand DNA. Pol α is similar in sequence to other prokaryotic and eukaryotic replicative polymerases (396). The activity of DNA polymerase α, but not polymerases β or δ, increases markedly during the S phase of the cell cycle (369). This increase is suppressed in the presence of cycloheximide and Actinomycin D, suggesting that the phase-specific increase in enzyme activity is due to *de novo* synthesis of Pol α and/or synthesis of an activator protein. In monkey and rat cells, pulse-chase studies followed by immunoprecipitation with a Pol α-specific antibody indicated that the increase in Pol α activity at S is indeed due to an increase in the rate of Pol α synthesis. Thus, in higher eukaryotes, Pol α enzyme activity and *de novo* protein synthesis positively correlates with the stage of cellular proliferation, coupled to the onset of DNA replication.

Studies with serum-starved and stimulated human lung fibroblasts (396) provide a correlation between levels of Pol α transcription, protein synthesis, and enzyme activity, suggesting regulation at a transcriptional level in quiescent cells stimulated to proliferate. In these experiments, Northern blot assays detected a greater than twentyfold increase in Pol α mRNA 18 hours following serum stimulation. This mRNA increase was paralleled by a corresponding tenfold increase in Pol α enzyme activity. These results were confirmed by Wahl et al. (381) studying human Pol α regulation in serum-stimulated quiescent 293 and K562 cells. However, different results were obtained by this group

with continuously cycling cells synchronized by either mitotic shakeoff or centrifugal elutriation. Under these conditions, Pol α mRNA levels, translation rates, and enzyme activity were constitutively expressed, with only a slight increase detected prior to S and a slight decrease noted in G2. Thus, regulatory mechanisms appear to differ in continuously cycling cells and quiescent cells stimulated to proliferate.

In 1991, Pearson et al. (292) isolated a Pol α genomic clone and identified the essential promoter region by analyzing the activity of Pol α deletion mutants, linked to a luciferase reporter gene, in transiently transfected NIH3T3 cells. Results indicated that sequences between -248 and +45 bp relative to the transcriptional start site directed Pol α expression in cycling cells, while elements between -1515 and +45 bp conferred a late serum response to the reporter gene. In addition, the kinetics of luciferase RNA expression from the Pol α promoter paralleled that of the endogenous gene.

Analysis of the first 248 bp of promoter sequence revealed the absence of a TATA box but the presence of a putative initiator sequence similar to that found in DHFR. Also present is an inverted CCAAT element and sequences similar to the consensus binding sites for the transcription factors Sp1, AP1, AP2, and E2F. DNAse protection assays with partially-purified nuclear proteins from HeLa extracts verified protein binding to these regions. In particular, purified E2F was found to bind to synthetic oligomers containing one of the sequences from the Pol α promoter. Another study (333) utilized gel mobility shift assays to demonstrate binding of E2F-1 to the Pol α promoter.

A definitive role for E2F in Pol α regulation has not been proven (107). Deletion studies have not implicated any specific sequence element in the mediation of the late serum response. While successive 5' deletions reduce the magnitude of gene expression in both cycling and mitogenically-stimulated quiescent cells, S phase-specific induction of Pol α is not abolished by the deletion of any specific sequence element. It appears, then, that multiple sequence elements may be coordinately involved in conferring the S phase-specific response. As there are multiple putative E2F sites throughout the promoter,

including one element downstream of the transcriptional start site, it is also possible that multiple E2F factors work to confer the response.

5.4 PCNA

Proliferating cell nuclear antigen, or PCNA, was first identified via immunoflourescence as a protein whose appearance correlated with the proliferative state of the cell (374). It was also originally described as a nuclear antigen, restricted to proliferating cells, that reacted with sera from patients with lupus erythrematosus (254, 260). It was later discovered that this protein was identical to a protein previously known as "cyclin", a protein often confused with, but completely different from, the regulatory cyclins described earlier.

PCNA is a 36 kD, highly conserved, nuclear phosphoprotein (37, 38, 308). Synthesis of PCNA is both cell cycle and growth state regulated (374) in that high levels are detected in rapidly dividing normal cells, transformed cells, and tumor cells, while levels are low or barely detectable in quiescent cells. Synthesis also increases six to sevenfold after serum stimulation, shortly before the onset of DNA replication, and decrease after passage through S. In cells treated with hydroxyurea, the increase in synthesis at S phase is not affected, indicating synthesis is not triggered by DNA replication. However, hydroxyurea does prevent a decrease in PCNA synthesis after S, suggesting that this phase of regulation is responsive to S phase events.

The intranuclear location of PCNA has been found to vary during S phase, with the location within the nucleus corresponding to sites of ongoing DNA synthesis (308). Since the pattern of nuclear distribution appeared to depend upon the occurrence of DNA synthesis, or events triggered by DNA synthesis (38), a role for PCNA in DNA replication was suggested.

Verification of a role for PCNA in DNA replication came with its isolation from fractionated human 293 extracts and the demonstration that it is required for the *in vitro* replication of SV40 DNA (307, 308). PCNA was subsequently found to be identical to a

previously discovered DNA Polymerase δ (Pol δ) auxiliary protein (39, 307) which stimulated Pol δ activity by increasing enzyme processivity and was required for leading strand DNA synthesis at a replication fork (308). In addition, PCNA has been found to be necessary for cell cycle progression since both DNA synthesis and cell cycle progression are inhibited upon exposure of BALB/c 3T3 cells to antisense PCNA oligonucleotides (162).

The PCNA gene has been cloned, its DNA sequence determined, and a promoter region identified (374). Promoter identification was achieved by linking various fragments of the PCNA gene to a TK cDNA and transfecting $TK^{(-)}$ts13 cells to $TK^{(+)}$. As with the previously described S phase-regulated genes, the PCNA promoter lacks a TATA box. It does, however, contain an inverted CCAAT element, multiple GC boxes representing putative Sp1 binding sites, and a number of AP2 consensus motifs.

It is generally believed that regulation of the PCNA gene occurs mainly at the post-transcriptional level (46). In particular, in the full-length PCNA gene (containing both introns and exons) nuclear run-on analyses show similar transcription rates in both G0 and serum-stimulated cells. Determination of hnRNA levels by RT-PCR (reverse transcription polymerase chain reaction) shows an increase in hnRNA levels in cells stimulated to proliferate, and a corresponding increase in mRNA stability as cells progress from G0 to S. As the gene is transcribed at the same rate throughout the cycle, increased mRNA levels are most likely the result of increased hnRNA and mRNA stability.

5.5 Replication-Dependent Histones

Histones (238, 369) are basic proteins which associate with DNA to assemble chromatin. There are a number of different classes of histones. For example, the replication-independent or replacement variants accumulate in non-dividing and terminally-differentiated tissues. In contrast, expression of the replication-dependent variants is tightly linked to the S phase of the cell cycle. Replication-dependent histone proteins include the octamer core components, H2A, H2B, H3, and H4, as well as the

linker protein, H1. The proteins are expressed from five to twenty well-conserved genes, clustered on multiple chromosomes. These genes are unique in that there are no introns and the 3' end contains a conserved palindromic sequence rather than a poly(A) tail.

Synthesis of the replication-dependent variants is cell cycle-regulated (234), being restricted to S phase in continuously cycling cells and in cells stimulated to proliferate. Inhibition of DNA replication with drugs such as hydroxyurea or cytosine arabinoside rapidly inhibits histone synthesis. *In vitro* translation and nucleic acid hybridization studies reveal that histone mRNAs are only present in the nucleus and cytoplasm during S phase. While the efficiency of expression differs for the various histone genes, the relative ratios of transcripts from the genes remains constant throughout the cell cycle (238, 369), implying that the individual genes are regulated in a parallel manner. In fact, most histone proteins are synthesized coordinately with the onset of DNA replication.

Fluctuations in histone protein levels are also paralleled by changes in histone mRNA levels (238, 369). While mRNA levels fluctuate as much as thirty- to fiftyfold during the cell cycle, the transcription rate of the histone genes is only two to fivefold higher in S than in G1 or G2. So, again, both transcriptional and post-transcriptional modes of regulation are involved, with changes in transcription rate, efficiency of mRNA processing (i.e. 3' end formation, as there are no introns), transport to the cytoplasm, and mRNA half-life potentially contributing to the thirty- to fiftyfold changes in mRNA levels (238).

Studies on histone mRNA half-life provide compelling evidence for the post-transcriptional regulation of the histone genes (238, 351, 369). mRNA half-life is 45 to 60 minutes in S phase, decreasing to 10 to 15 minutes when DNA synthesis is inhibited or cells reach the end of S. The 3' stem loop appears to be the feature identifying the mRNA species as histone mRNA. The stem loop region is both necessary and sufficient for histone mRNA degradation and is capable of conferring regulation of mRNA half-life to heterologous mRNAs. In addition, mRNA degradation is prevented by the inhibition of

protein synthesis. Together, these results indicate that histone mRNA stability is sensitive to changes in DNA synthesis, and suggest that a short-lived protein is possibly involved in mRNA degradation (369).

An additional component to post-transcriptional regulation is the processing of the 3' end of the mRNA (148). Processing is mediated by the U7 snRNP particles, where the 5' ends of U7 snRNP contact regions of purine-rich sequence downstream from the conserved stem loop structure in the histone pre-mRNA. G1 extracts are deficient in 3' end processing, concurring with the theory that U7 snRNP 5' ends are unavailable for contact in G0/G1 (due possibly to conformational inaccessibility and/or binding to other proteins) but are exposed in S phase.

A third component to post-transcriptional regulation is transport of RNA species from the nucleus to the cytoplasm. Schümperli and colleagues (369) demonstrated that incorporation of ^{3}H-uridine into cytoplasmic H3 mRNA increased at G1/S then decreased in G2. These changes could be due to increased export of newly synthesized hnRNA from the nucleus to the cytoplasm at G1/S, rather than an increase in mRNA stability.

In terms of transcriptional regulation, the transcription rate of the histone genes has been found to increase an average of three to fivefold upon entry into S phase (238). In addition, transcription rate decreases upon inhibition of DNA synthesis, onset of differentiation, or quiescence (369, 378). Artishevsky et al. (10, 11) showed that 1 kb of 5' flanking hamster H3 sequences conferred cell cycle regulation to a linked Neo reporter gene; Neo mRNA levels increased three to fivefold at G1/S, consistent with the change in endogenous histone transcription. Further deletion analysis localized the cell cycle control signal to a 150 bp region containing a TATA box, 2 CAAT elements, 4 Sp1 consensus binding sites (GGGCGGG) and multiple G+C-rich inverted repeats important for efficient *in vitro* transcription. Gel shift analyses verified that cellular factors bind to the H3 promoter in a cell cycle-specific manner, binding specifically at G1/S.

Similar studies were also conducted with the histone H4 gene. H4 was found to transcribed three to tenfold more efficiently in HeLa S phase nuclear extracts than in non S-phase extracts (369). Using *in vitro* run-off transcription assays with nuclear extracts, maximal transcription was found to require the TATA box, cap site, and elements 70 to 110 nucleotides upstream of the transcription initiation site (138). Promoter elements were recognized preferentially in extracts from synchronized S phase HeLa cells, with the cell cycle specificity suggesting the involvement of a specific transcription factor interacting with H4 promoter elements.

Continuing studies with the H4 proximal promoter have revealed the presence of two *in vivo* protein binding domains, termed site I and site II (150). Three proteins (HiNF-A, C, and E) have been found to bind independently to site I in both actively proliferating and differentiating cells. Deletion of site I results in a four to sixfold decrease in H4 *in vitro* transcription. The protein HiNF-D binds site II, and deletion of site II abolishes H4 *in vivo* expression. In addition, the HiNF-D-site II interaction is proliferation-specific in that S phase extracts contain measurable HiNF-D binding activity, while activity is barely detectable in G1 extracts. Tumor-derived or transformed cells, however, exhibit constitutively-elevated HiNF-D binding throughout the cell cycle.

Furthermore, transcriptional down-regulation of the H4 gene at cessation of proliferation or onset of differentiation or quiescence is associated with the loss of binding of the nuclear HiNF-D protein to the H4 proximal promoter (150). To investigate a possible transcriptional component to HiNF-D activity, H4-CAT transgenes were constructed. Transgene expression was found to parallel endogenous histone mRNA expression, and the tissue representation of reporter gene expression paralleled that of HiNF-D activity. Thus, transcriptional regulation is suggested, and HiNF-D is important for proliferation-specific H4 expression.

Finally, the coordinate control of the various histone genes was investigated by comparing, via gel shift analyses, the H3 and H1 promoter DNA-binding activities with

the H4-HiNF-D interaction (379). Like H4-HiNF-D, both the H3 and H1 promoter interacted specifically with proteins in S phase extracts, but displayed low binding activity in G1 extracts of normal diploid cells. Binding activities were also constitutively high in transformed and tumor-derived cells, and were down-regulated at the onset of quiescence and differentiation. Thus, key oscillatory cell cycle and growth control mechanisms operate to modulate histone promoter-DNA interactions from three independent histone genes.

6. Thymidine Kinase

In comparison with the S phase-regulated genes discussed in the previous section, thymidine kinase (TK) is one of the most highly induced cell cycle-regulated enzymes. In addition to high levels of induction, the fact that it is a salvage enzyme and nonessential for cellular survival, allows for a multitude of molecular manipulations. TK has become one of the most widely studied cell cycle-regulated genes, and is an excellent model system for the study of mechanisms governing the G1/S transition. As this dissertation is devoted to understanding the mechanisms underlying S phase-specific TK promoter activation, the following section provides a summary of the current knowledge of TK properties and regulation.

6.1 Biochemical and Physical Properties of TK

TK is a ubiquitous enzyme which plays a key role in the pyrimidine salvage pathway, where it catalyzes the phosphorylation of deoxythymidine (dT) to deoxythymidine 5' monophosphate (dTMP), a precursor to DNA synthesis (see Figure 4) (329). As such, TK plays an integral role in the regulation of intracellular thymidine pools. The *de novo* pathway for dTMP synthesis proceeds via the reductive methylation of deoxyuridylic acid (329), and requires both the thymidylate synthase and dihydrofolate reductase proteins.

Mammalian cells contain two TK isozymes. The minor form is the mitochondrial form of the enzyme (26) and does not fluctuate in activity throughout the cell cycle. In contrast, the major, or cytosolic (2), form of the enzyme attains high levels in cycling cells. As this dissertation is concerned only with cytosolic TK, the remaining information pertains only to this form of the enzyme.

HeLa cytosolic TK has been purified via ion exchange and affinity (thymidine sepharose) chromatography (345). The enzyme has a native molecular weight of 96 kD. Denaturing SDS-PAGE analysis reveals a subunit molecular weight of 24,000, suggesting the active enzyme is actually a tetramer.

Genomic and cDNA TK clones have been isolated from a variety of organisms, including human (35), mouse (224), hamster (214), chicken (248), and Fowlpox virus (31). Upon transformation of the cloned eukaryotic genes into mouse cells, scoring for conversion of a $TK^{(-)}$ phenotype to $TK^{(+)}$, it was found that these genes were expressed like the normal mouse gene, being regulated in a growth-phase-dependent manner (see below) (338). The human TK gene has been sequenced in its entirety (111), occupying 12.9 kbp of DNA and encoding a 1500 bp mRNA (36).

6.2 TK Gene Expression

As mentioned, TK gene expression is governed by both the growth state and cell cycle position of the cell. Terminally-differentiating and nonproliferating cells express low levels of TK (129), while actively proliferating cells display high levels of expression. In addition, in serum-starved, G0-arrested cells stimulated to re-enter the cell cycle by the addition of serum (168), replating at a lower density (338), or infection with a virus such as Simian Virus 40 (SV40) (190, 305, 361), TK activity is low throughout G0 and G1, increases sharply at G1/S, and remains high throughout S, G2, and into M before declining prior to the start of the following G1 phase. Likewise, in continuously cycling cells (25, 338), enzyme activity increases at least ten- to twentyfold in S phase, concomitant with the onset of DNA replication.

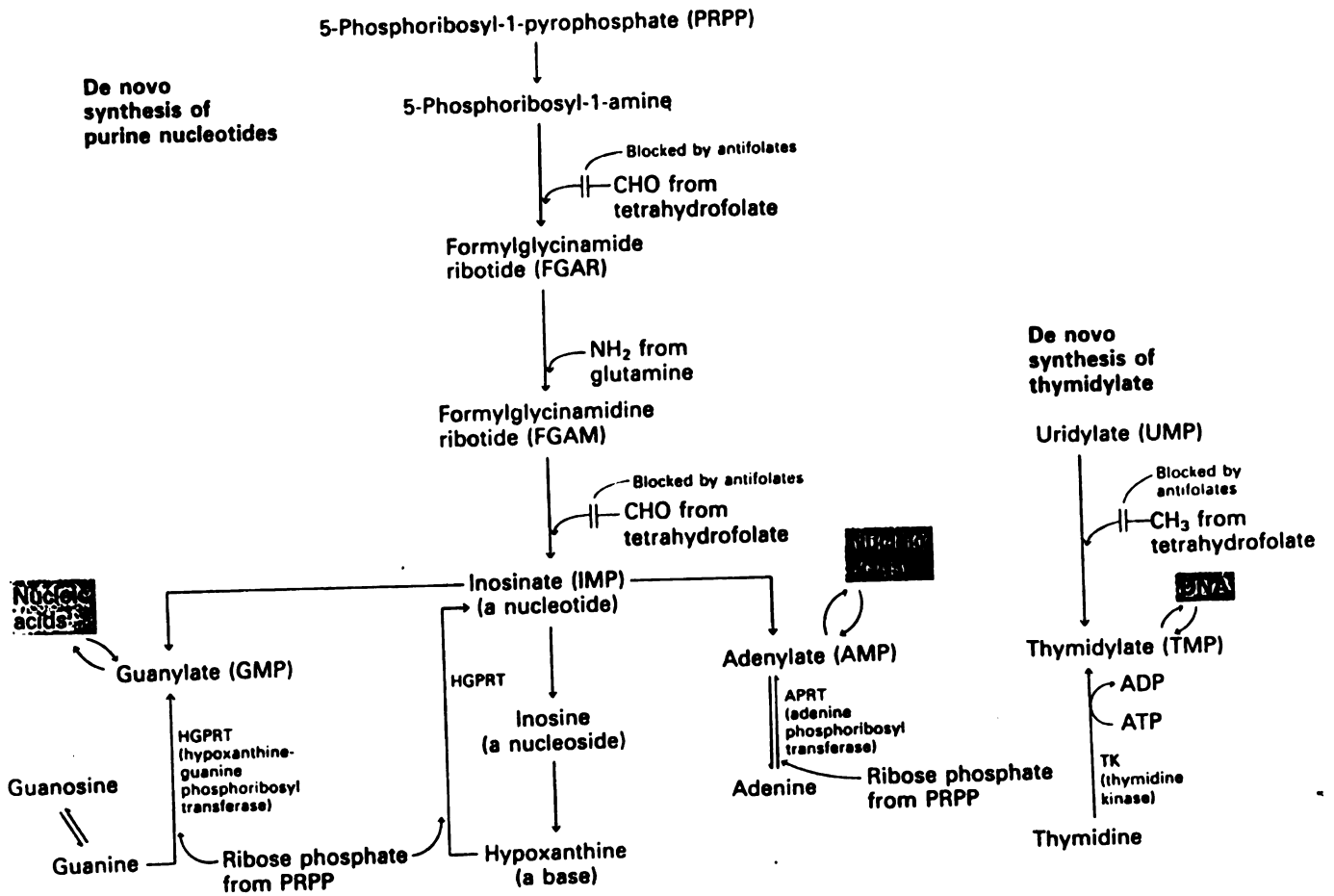


Figure 4. ***De novo*** **and salvage pathways to obtain purine nucleotides and thymidylate (64).**

Studies in mitogenically-stimulated cells have indicated that the increase in TK activity at the start of the DNA replicative phase is not inhibited by DNA synthesis inhibitors such as bromodeoxyuridine (BrdU) (190), 1-β-D-arabinofuranosyl cytosine (Ara C) (190), hydroxyurea (168), and aphidicolin (59). As with DHFR and TS, these studies suggest that induction of TK enzyme activity and the onset of DNA replication are not tightly linked processes. The decrease in TK activity following S phase is sensitive to DNA synthesis inhibitors (25), however, suggesting some involvement of S phase events in TK expression.

Of interest is the fact that the increase in TK activity at S is inhibited with low levels of the RNA synthesis inhibitors actinomycin D (59, 168) and 5,6-dichloro-β-D-ribofuranosyl benzimidazole (59) and the protein synthesis inhibitor cycloheximide (59, 60, 168, 190). TK induction is delayed if the drugs are added prior to, but not later than, the R point. These data suggest that the S phase-dependent induction in TK expression is dependent upon both RNA and protein synthesis, either of TK itself or an activator protein. It is particularly intriguing to think of the required activator protein as the labile Restriction point protein postulated by the"R" hypothesis for G1/S regulation.

6.3 Regulation of TK

Regulation of TK is complex, occurring at both the transcriptional and post-transcriptional levels. While post-transcriptional regulatory mechanisms may predominate in continuously cycling cells, transcriptional regulation appears to play a significant role in quiescent cells that have been stimulated to re-enter the cell cycle.

Regulation of TK is complex and somewhat confusing, with different modes of regulation being reported in different systems. Sherley and Kelly (346) examined TK regulation in continuously cycling HeLa cells synchronized via centrifugal elutriation or mitotic selection. Western blot analyses revealed that both enzyme activity and protein levels increased more than fifteenfold at G1/S. In contrast, Northern blot analyses revealed a less than a threefold increase in mRNA levels, an increase insufficient to

account for the large induction in enzyme activity and protein levels. Pulse-labeling and pulse-chase studies revealed a tenfold increase in the rate of TK protein synthesis in S relative to G1, suggesting that an increase in the efficiency of translation of TK mRNA occurs as cells begin DNA replication. The stability of the TK protein was also found to decrease upon cell division, with the half-life decreasing from 40 hours to less than one hour, resulting in no new TK protein in new G1 cells.

In 1991, Kauffman and Kelly (179) reported that the sequences responsible for degradation of TK were located within the C terminus of the protein. They found that both deletion of the C-terminal 40 amino acids, or fusion of sequences from the bacterial β-galactosidase gene to the TK C-terminus resulted in constitutive high levels of TK expression throughout the cell cycle. It was later found that these same C-terminal mutations allowed for expression of TK protein and enzyme in quiescent, G0 cells when the cDNA was expressed from a heterologous promoter (180).

Much evidence for post-transcriptional regulation also derives from studies with chimeric TK genes, i.e. TK cDNA recombinant clones expressed from heterologous promoters. TK cDNA sequences were sufficient to confer G1/S-phase regulation in a variety of genes [human (359), hamster (215), chicken (129, 248), and mouse (221)].

In our lab, Ito and Conrad (161) showed that, while the pattern of TK mRNA expression is determined by the promoter used, the pattern of TK enzyme expression and protein levels is independent of RNA levels. Their studies utilized a set of human TK cDNAs expressed from either the human TK or SV40 early promoters. With the human TK promoter, mRNA levels were low throughout G1 and increased at S. In contrast, with the SV40 early promoter, the mRNA peaked 2 to 4 hours following the addition of serum, then remained high throughout the remainder of G1 and into S. In both cases the enzyme levels and activity were low until 10 to 12 hours following stimulation, then increased as much as forty- to fiftyfold. Deletion of the TK 5' untranslated region (UTR), the AUG, or the first sixteen amino acids had no effect, suggesting that the regulation was not

occurring at the level of translation initiation. Deletions of the poly(A) site and 430 bp of the 3' UTR also had no effect.

Carozza and Conrad (43) continued this analysis by transfecting TK^- Rat-3 cells with a construct linking the TK cDNA to a hybrid SV40-human TK promoter fragment and measuring TK mRNA, protein, and enzyme levels in serum-starved, serum-stimulated cells. Results confirmed that mRNA levels were fairly constitutive throughout G1 and S, but that protein and enzyme levels were low during G1 and increased at S. Pulse-labeling studies to measure the rate of TK synthesis indicated no change in TK translation efficiency. In contrast, pulse-chase studies to measure TK protein half-life identified an increase in half-life from 1.5 hours in G1 to 20 hours in S.

Finally, there is some evidence to suggest that regulation occurs via post-translational processing, by changes in phosphorylation status, for example. TK is a nuclear phosphoprotein. A study of TK regulation in serum-starved and stimulated human HL-60 cells (48), a promyelocytic leukemia cell line, revealed that TK enzyme activity, but not TK mRNA or protein levels, increased following stimulation, and that this increase was paralleled by an increase in TK phosphorylation. Treatment with cycloheximide inhibited both TK activity and extent of phosphorylation, but not the level of the TK protein. Furthermore, phosphoamino acid analysis revealed that TK is phosphorylated on serine, and the addition of a serine/threonine phosphatase inhibitor to crude cell extracts increased the level of TK activity. Phosphorylation of TK at G1/S may therefore contribute to the increased stability of the protein in S phase.

TK is also regulated at the level of transcription. An interesting study by Gudas et al. (133) demonstrates the uncoupling of transcriptional and post-transcriptional regulatory mechanisms in mitogenically-stimulated cells arrested under a variety of conditions. In these studies, Chinese hamster fibroblasts were arrested by serum deprivation, which arrests cells in G0, or by isoleucine starvation or lovastatin inhibition, both of which arrest cell in G1. Under all arrest conditions, the endogenous TK mRNA

remained low or undetectable in arrested cells but increased at G1/S following stimulation. Nuclear run-on analyses, however, detected an increase in transcription rate (three to fourfold following stimulation) in the serum-deprived cells only, suggesting the transcriptional component of regulation was unique to the G0-to-S transition. This was confirmed in cells transfected with a hybrid gene composed of the TK promoter and a β–globin reporter gene; increases in β-globin mRNA at G1/S were detected only in serum-starved and stimulated cells, with mRNA levels expressed constitutively in cells arrested by the other methods.

In cells mitogenically-stimulated to re-enter the cell cycle from a quiescent, G0 state, the ten to twentyfold increase in TK protein levels at G1/S has been found to be paralleled by a corresponding increase in TK mRNA. This was demonstrated in a number of studies. First, Stuart et al. (361) detected an increase in endogenous TK mRNA in both serum-stimulated and SV40-infected simian CV1 cells, and reported that this induction coincided with the onset of DNA replication. Second, Lieberman et al. (221) detected an increase in cytoplasmic TK mRNA expressed from TK minigenes stably transfected into mouse cells. Finally, Coppock and Pardee (60) noted a similar increase in mRNA for the endogenous BALB/c 3T3 gene. Thus, it seems that, in mitogenically-stimulated cells, TK mRNA levels often determine the level of TK protein.

An increase in the transcription rate of the TK gene has also been detected at the G1/S interface in mitogenically-stimulated cells. Studies report a six- to sevenfold, or a two- to fourfold, increase in the transcription rate of the endogenous TK gene in serum stimulated simian CV1 (359) or mouse BALB/c 3T3 (60) cells, respectively. Also, an elevenfold increase has been measured in mouse L929 cells transfected with a TK minigene (221), and a three to fourfold increase was determined in SV40-infected CV1 cells (326).

In accordance with a role for the promoter in TK gene regulation, a number of labs have demonstrated conferral of G1/S-phase regulation to a heterologous reporter gene by

fragments of the TK promoter. Travali et al. (373) demonstrated that a CAT gene under the control of a 451 bp TK promoter fragment is maximally expressed in S phase in serum-stimulated BALB/c 3T3 cells. Other labs have confirmed this result, showing that TK promoter fragments can confer S phase regulation to reporter genes such as Neo (187, 325, 326), CAT (7, 113), and β-globin (113, 133), as well as to heterologous, non-cell cycle-regulated promoters such as Herpes Simplex Virus TK promoter (187). Thus, both coding and noncoding sequences contribute to TK regulation, and different levels of regulation predominate in different cell types and under different growth conditions.

6.4 The Human TK Promoter

Localization of the human TK promoter region has been accomplished by a combination of primer extension and S1 nuclease mapping studies, followed by analysis of promoter mutants linked to genes in transfection studies (111, 198). The human TK promoter sequence from -135 to +30 bp, relative to the transcriptional start site (198) is shown in Figure 5. Promoter elements and putative regulatory sequences are indicated. Among other elements to be discussed below, the human TK promoter contains two mRNA start sites, differing in position by 7 bp, a noncanonical TATA box (TTTAAA) located approximately 25 bp upstream from the major CAP site (-25), two inverted CCAAT elements, located approximately at -40 and -70 bp, and a number of G+C-rich regions which include the sequence GGGCGG known to bind the transcription factor Sp1 (122). In comparison, the chicken TK promoter contains three copies of a closely-related inverted CCAAT element, and the hamster TK promoter contains only one CCAAT box at -40 bp but multiple GC elements. In contrast, the mouse TK promoter does not contain a TATA box or a CCAAT element, but has many GC boxes that bind Sp1 and many transcriptional start sites.

In studies on TK promoter strength, Kriedberg and Kelly (198) localized a minimal promoter fragment to just 83 bp upstream of the cap site. This fragment linked to the TK

Figure 5. The human TK promoter (326). (A) Sequence of the human TK promoter from -135 to +30 bp relative to the major transcriptional start site. (B) Locations and sequences for a number of putative transcription factor-binding sites. Nucleotides which do not match the consensus sequence are given in lowercase. Rev indicates that the consensus sequence is found on the strand opposite the one shown.

A

$^{-135}$TCCTCCCACG $^{-125}$AGGGGGCGGG $^{-115}$CTGCGGCCAA $^{-105}$ATCTCCCGCC $^{-95}$AGGTCAGCGG

$^{-85}$CCGGGCGCTG $^{-75}$ATTGGCCCCA $^{-65}$TGGCGGCGGG $^{-55}$GCCGGCTCGT $^{-45}$GATTGGCCAG

$^{-35}$CACGCCGTGG $^{-25}$TTTAAAGCGG $^{-15}$TCGGCGCGGG $^{-5}$AACCA $^{-1}$GGGGC $^{+5}$TTACTGCGGG

$^{+15}$ACGGCCTTGG $^{+25}$AGAGT

B

Transcription Factor	TK Promoter Sequence	Sequence Location
SP1 ($^{G}/_{T}{}^{G}/_{A}$GGC$^{G}/_{T}{}^{G}/_{A}{}^{G}/_{A}{}^{G}/_{T}$)	GGGGCGGG	-123 to -116
	GGGCTGcG	-118 to -111
	CCGCC (Rev)	-100 to -96
	GGGCGc	-83 to -78
	tGGCGG	-65 to -60
	cGGCGGGG	-62 to -55
	GGGGCcGGc	-58 to -50
	TcGGCGcGG	-15 to -7
E2F ($TTT^{G}/_{C}{}^{G}/_{C}CG^{G}/_{C}$)	CgGCCAAA (Rev)	-112 to -105
	TCCCGC	-102 to -97
	aTTGGCcC	-75 to -68
	CGCGGgAA (Rev)	-11 to -4
AP2 ($CCC^{A}/_{C}N^{G}/_{C}{}^{G}/_{C}{}^{G}/_{C}$)	GGCGGGc (Rev)	-121 to -115
	CCCATGGC	-69 to -62
	GGCGGGGc (Rev)	-61 to -54
Yi (CCCNCNNNNCT)	CCCGCCAGGtc	-101 to -91
	AGGTCAGCGGc (Rev)	-95 to -85
CCAAT	ATTGG (Rev)	-44 to -40
	ATTGG (Rev)	-75 to -71
TATA	TTTAAA	-25 to -20
p300 (GGGAGTG)	CTCCC (Rev)	-133 to -129
	CTCCC (Rev)	-103 to -99
	CACgCC (Rev)	-35 to -30
	GGGcGcTG	-83 to -76
	GGGAcgG	+12 to +18

Figure 5.

cDNA transfected $TK^{(-)}$ cells to $TK^{(+)}$ with an efficiency equivalent to a longer (430 bp), wild-type promoter piece. No promoter activity was detected with a 53 bp promoter fragment, which transformed with an efficiency 250-fold below that of wild-type.

Arcot et al. (7) undertook a more extensive analysis of elements involved in promoter strength. Using TK promoter fragments linked to CAT, they assayed promoter strength by measuring CAT activity after transfection of the constructs into mouse L cells. Their results indicated that promoter strength decreased with increasing N-terminal promoter truncations, with a 139 bp fragment, containing both inverted CCAAT elements and one GC box, still retaining 64% of the total promoter activity (as compared to activity expressed from a 444 bp fragment). Deletion to 88 bp upstream of the cap site, with both CCAAT elements still remaining, reduced activity to 28% of wild-type activity.

These results indicated that the Sp1 binding sites are important for overall promoter strength. DNAse footprinting studies with crude nuclear HeLa extracts and purified Sp1 protein have confirmed that Sp1 binds GC boxes throughout the human TK promoter (8). Similarly, studies with the mouse TK promoter indicate that Sp1 is critical for promoter function (113, 114) in that mutation of an Sp1 site eliminates murine TK promoter function (82, 114).

Initial studies on the role of the CCAAT boxes in the human TK promoter suggested that these two elements are functionally equivalent (7), since they both were found to bind the same set of cellular proteins in competitive gel shift assays. These studies also showed that only one of the two CCAAT boxes was required for promoter activity. In addition, the presence of two CCAAT boxes did not result in twice as much promoter strength. DNAse footprinting studies revealed more protection around the proximal (-40) CCAAT box, prompting the hypothesis that binding of proteins to the proximal CCAAT element precludes binding to the distal CCAAT element (7). To test this, TK promoter mutants were designed in which the spacing between the two CCAAT boxes was increased (8). Removing steric hindrance thereby allowed equal protein

binding to both CCAAT elements, and TK promoter strength was increased by a magnitude of nearly threefold.

The equivalency of the CCAAT boxes has since been contested by Lipson and colleagues (225). Looking in the context of the full-length 460 bp TK promoter fragment, they linked promoter fragments containing either or both of the CCAAT boxes to a luciferase reporter gene and found that deletion of the proximal CCAAT box had no effect on promoter expression but deletion of the distal (-70) CCAAT box reduced luciferase expression to 55% of the parental promoter fragment containing both CCAAT boxes. Thus, in this case, the two CCAAT elements do not appear to be functionally equivalent, as the distal CCAAT box is able to substitute for the proximal CCAAT box, but not vice versa. Mutation of either CCAAT box was also found to reduce luciferase expression to 40 to 45% of wild-type levels, indicating that both CCAAT elements are actually needed for full promoter strength. The quantitative differences in results obtained with CCAAT box excision versus mutation may be due to the ability of other protein-binding sites to functionally substitute for missing elements when the CCAAT boxes are removed. Furthermore, competitive gel shift analyses utilizing oligonucleotide probes encompassing either or both CCAAT elements identified a protein which binds to the distal, but not the proximal, CCAAT box. This protein complex only forms, and is only competed away, with the probe containing the distal CCAAT element.

The roles of the CCAAT elements have not been clarified in terms of promoter regulation. Point mutations generated within each of the CCAAT elements were ineffective at altering the S-phase transcriptional regulation of a transfected TK-Neo hybrid gene in serum-stimulated Rat-3 cells (6), suggesting the CCAAT elements are not involved in promoter regulation. In one gel shift study, though, a G1/S-specific change was detected in the pattern of proteins bound to the CCAAT elements within the human TK promoter (191), suggesting the cell cycle-specific release of a protein or protein complex. This study, however, has not been reproducible in our lab (325) or others (188)

where gel shift analyses have detected only constitutive patterns of protein binding throughout the cell cycle. A role for the CCAAT box-binding proteins in the regulation of TK during cellular senescence has been proposed due to a study which reported the appearance of protein complexes bound to CCAAT boxes 12 to 24 hours following serum stimulation of young, but not old, IMR-90 human diploid fibroblasts (285). In addition, human tumor cells, which express TK at elevated levels, have been found to exhibit constitutively elevated levels of serum-independent protein binding to CCAAT boxes (47). This is in contrast to nontumor cells, in which protein binding was dependent upon the presence of serum. Thus, it appears that the elevated levels of TK expression in tumor cells may possibly be due to the increased ability of these cells to activate the CCAAT elements. In summary, while the CCAAT boxes appear to be important for overall promoter activity, their role in regulation is unclear.

6.5 Studies on TK Promoter Regulation

Promoter regions containing regulatory elements have been localized by assaying the activity of reporter genes linked to fragments of the TK promoter. Roehl and Conrad (324) linked promoter fragments containing successive N-terminal deletions to Neo and measured Neo mRNA levels in stably-transfected serum-starved and serum-stimulated Rat-3 cells. Northern blot analyses detected a four- to sevenfold increase in Neo mRNA levels at G1/S from all promoter fragments except one deleted to just 67 bp upstream of the transcriptional start site. This 67 bp promoter fragment yielded constitutively low mRNA levels in serum stimulated Rat-3 cells, indicating that serum-regulatory elements lie between -135 and -67 bp within the TK promoter.

Likewise, Roehl et. al. (326) continued these studies in CV1 cells stably transfected with the TK-Neo hybrid genes. Cells were serum-starved, then stimulated by the addition of serum or infection with SV40, and Neo mRNA levels were measured by the Northern blot procedure. In both serum-stimulated and SV40-infected cells, Neo mRNA expressed from 444 and 135 bp promoter fragments increased at G1/S. In

contrast, the 67 bp promoter fragment constitutively expressed Neo in serum stimulated cells but showed a regulated pattern of expression (i.e. a G1/S increase in Neo mRNA levels) in SV40-infected cells. This data confirmed the location of serum-regulatory elements between -135 and -67 bp in the human TK promoter. It also suggested that additional viral-specific regulatory elements lie downstream of -67 bp and that the two mitogens, serum and SV40, may operate via independent pathways. Thus, the promoter could be functionally subdivided into two domains: 1) a downstream domain, containing sequences between -67 and +30 bp, sufficient for viral-mediated TK promoter induction and 2) an upstream domain, containing sequences between -135 and -67 bp, required for serum-mediated promoter regulation.

Kim and Lee (188) have further demonstrated that this upstream domain is sufficient for serum-mediated promoter regulation. They formed a series of triple DNA constructs composed of 5' truncations of the human TK promoter, linked to a small fragment of the HSV TK promoter containing the TATA element and 50 bp of sequence upstream of the transcriptional start site, linked to Neo. Constructs were transfected into hamster K12 cells, a temperature-sensitive (ts) cell line, and Neo mRNA levels were analyzed in serum-stimulated pools and colonies. Results showed no regulation with a 63 bp human TK promoter fragment, but a fragment consisting solely of the 70 bp upstream region (-135 to -67 bp) conferred cell cycle dependence to the linked heterologous promoter and reporter gene.

To further define the region required for serum-mediated regulation, DNAse footprinting analyses of this 70 bp region were conducted, revealing the presence of four protein binding sites (189). Sequences between -88 and -113 bp were then mutated, to eliminate two adjacent DNA binding domains, and the constructs were stably transfected into K12 cells for analysis of Neo mRNA levels in serum-stimulated cells. The mutation abolished G1/S phase regulation and resulted in constitutively high levels of expression of Neo message. This localized the serum-regulatory sequences to a 25 bp segment between

-88 and -113 bp. This is particularly interesting since this region contains a number of putative E2F-binding sites. In addition, sequences between -67 and -88 bp were thought to serve as enhancers for basal transcription, since deletion of these sequences retained regulation of the promoter, but at a lower level of expression.

Referring to Figure 5, the downstream TK promoter domain (-67 to + 30 bp), which is sufficient for regulation in SV40-infected CV1 cells, essentially contains the TTTAAA sequence and one inverted CCAAT box. The upstream promoter domain (-135 to -67 bp) contains the distal inverted CCAAT box and encompasses the 25 bp serum-regulatory element (-88 to -113 bp). As mentioned above, this region contains several sequences with homology to the consensus binding site for the transcription factor E2F. Also included are a pair of sequences resembling the Yi consensus site (82); Yi has been identified as a protein complex in mouse cells that exhibits G1/S-specific binding to the murine TK promoter. Scattered throughout the entire 135 bp promoter fragment containing both domains are multiple binding sites for Sp1 as well as sequences resembling binding sites for p300 (358), a cellular phosphoprotein required for adenovirus E1A-mediated induction of cell DNA synthesis. This protein has also been postulated to interact, directly or indirectly, with SV40 large T Antigen (404).

To identify cellular proteins potentially involved in TK promoter regulation, gel shift analyses have been performed using whole cell extracts and the 70 bp (-135 to -67) region as a probe (188). In this study, two major complexes were found with constitutive binding profiles throughout G1, S, and G2. Refinement of the probe to the recently defined 25 bp serum-regulatory element (containing the sequences resembling E2F-binding sites), along with the use of nuclear extracts, revealed the formation of four complexes in gel shift assays (218). While three of these complexes bound constitutively throughout the cycle, the binding activity of the remaining complex (complex III) increased fivefold at the G1/S border and persisted throughout S phase. Using a variety of specific antisera, it was found that the formation of the cell cycle-dependent complex III was eliminated by

preincubation with monoclonal antibodies specific for p107 and cyclin A, and one of the constitutive complexes was eliminated with an antibody against $p33^{cdk2}$. In contrast, antibodies directed against pRb and $p34^{cdc2}$ had no effect. These results indicate that the S phase-dependent protein complex is dependent upon both cyclin A and p107, and that a cell cycle-dependent protein kinase is linked to activation of an S phase-dependent cellular promoter. The authors postulate that cyclin A and $p33^{cdk2}$ associate to serve as transcriptional coactivators to maintain human TK transcription throughout S phase. Furthermore, substrates for the cyclin A/cdk2 kinase activity may include factors bind to the adjacent CCAAT boxes and the TTTAAA element. As discussed below, the role of E2F in human TK promoter regulation is still unclear.

These studies are remniscient of ones performed with the murine TK promoter. As previously mentioned, the murine TK promoter differs markedly from the human TK promoter in that it contains no CCAAT or TATA boxes. Sequences located between -174 and +159 bp, relative to the translation initiation site, are required to confer S phase-specific expression to a linked reporter gene (113). Thus, some of the translated sequences are required, a situation similar to that with the DHFR and TS genes. DNAse footprint and gel mobility shift assays with the 170 bp murine TK promoter fragment in serum-starved and stimulated nontransformed mouse A31 cells (82, 83) identified a constitutive protein complex due to the binding of Sp1, a G1-specific complex termed Yi_1, and an S phase-specific complex termed Yi_2. Yi_2 binding activity is low or undetectable in G0 and G1, and increases markedly at G1/S. Apparently, Yi_1 is replaced by Yi_2 at G1/S, and this conversion is phosphorylation-dependent, since it is inhibited by alkaline phosphatase (83). Of particular interest is that the Yi consensus binding sequence (CCCNCNNNCT) is somewhat similar to the E2F consensus sequence. Under conditions of either serum starvation or inhibition of protein synthesis, Yi_2 binding activity is found to be unstable in nontransformed A31 cells but stable in transformed BPA31 (benzo[a]pyrene-transformed BALB/c 3T3) cells. This result, in particular, satisfies the

criteria for a Restriction point protein, i.e. a protein which is labile in untransformed cells yet stable in transformed cells (35).

In gel shift analyses using monoclonal antibodies specific for pRb and cdc2 (83), it was found that αpRb eliminated both Yi complexes, suggesting the presence of pRb or a pRb-like protein. Yi binding activity also cofractionates with a protein of MW 110, the size of pRb. αcdc2 selectively inhibited the formation of Yi_1, and H1 kinase activity cofractionates with both Yi complexes. So, the Yi_1 complex appears to be composed of a murine form of pRb and a cdc2 kinase. A model has been proposed involving the phosphorylation of pRb by cdc2 kinase at G1/S, releasing transcriptional inhibitions by Yi_1. This would be followed by formation of a transcriptionally-active Yi_2 complex and subsequent cell cycle progression.

Further analysis of protein-bound regions of the mouse TK promoter identified one region (MT2) containing sequences similar to E2F binding sites, suggesting the involvement of an E2F-like protein (82). In gel shift assays, GST-E2F bound a labeled oligonucleotide containing the MT2 site. Using extracts from serum-starved and serum-stimulated cells, gel shift assays revealed four cell cycle-regulated bands: 1) an E2F-G0/G1 complex which was detected in G0 and G1 but disappeared before G1/S, 2) E2F-S, which appeared near G1/S and increased dramatically in S 3) free E2F, which was low prior to S then increased dramatically, and 4) Yi_2. These complexes are similar to previously-reported E2F-containing complexes in other systems (17, 42, 49, 74, 209, 265, 285, 348). Deoxycholate abolished the bound G0/G1 and S phase murine TK complexes, increasing the abundance of the free E2F complex and thereby implicating the association of E2F with other proteins to form phase-specific complexes. The presence of a murine form of E2F was verified by Western blot analysis of proteins eluted from excised, shifted bands.

Gel shift analyses utilizing monoclonal antibodies to inhibit or supershift bands found that the E2F-G0/G1 complex also contains p107, and that the E2F-S complex also contains p107, cdk2, cyclin E and cyclin A (85). In addition, mutation of the MT2 (E2F)

binding region resulted in constitutive high levels of expression of a reporter gene driven by the mouse TK promoter (114), suggesting that the E2F-G0/G1 complex is a transcriptional repressor. Thus, E2F is involved in the regulation of the murine TK promoter. Furthermore, Ogris et al. (280) demonstrated that the murine MT2 E2F-binding site is a target for trans-activation by polyomavirus large T-Ag. They used TK-CAT hybrid genes transiently transfected into mouse 3T3-LT cells, a cell line which conditionally expresses a dexamethasone-induced polyomavirus large T-Ag, to show that T-Ag trans-activated CAT expression from promoters with wild-type, but not mutated, MT2 E2F sites. When nuclear extracts prepared before and after induction of large T-Ag were used in gel shift assays with a synthetic murine E2F TK binding site, it was found that higher order structures formed prior to large T-Ag induction and were dissociated upon large T-Ag induction, with a concomitant induction in free E2F levels and a corresponding increase in TK mRNA levels in serum-starved cells. Mutations introduced into the pRb-binding domain of large T-Ag revealed that mutant large T-Ag was reduced in its ability to trans-activate the murine TK promoter in transient transfection assays and that mutant 3T3-LT cells were unable to induce the synthesis of endogenous TK mRNA. A recent study also shows that the endogenous murine TK gene is induced by WT polyomavirus large T-Ag but not by a mutant T-Ag unable to bind pRb (264). Taken together, these studies show that critical cell cycle regulatory proteins, i.e. pRb, p107, cdc kinases, cyclins A and E, and E2F, are involved in the regulation of the murine TK promoter and, to some extent, the human TK promoter.

Unlike murine TK, a role for E2F in the regulation of human TK is still unclear and remains to be demonstrated. Regulation involving E2F is implied, since deletion of the E2F-like sites abolishes G1/S phase regulation. However, direct interaction of E2F with the human TK promoter has not been detected, since an oligonucleotide containing a consensus E2F binding site was ineffective at competing away human TK protein complexes, and a high concentration of E2F antiserum was required to affect formation of

the G1/S-specific complex, complex III (218). Also, pRb was not detected in any of the gel shift complexes formed with the human TK promoter, as indicated above. In contrast, Johnson et al. (166) have demonstrated the activation of a human TK promoter-CAT construct by an E2F expression plasmid. So, a consensus remains to be reached as far as E2F involvement in human TK promoter activation .

While much work has focused on understanding the mechanisms of TK gene regulation in serum-stimulated cells, regulation in SV40-infected cells is a relatively unexplored domain. Papovaviruses require cell cycle machinery for the replication of viral DNA, the transcription of viral RNA, and the translation of viral proteins. In terms of viral DNA replication, the viruses require that cells to be in S phase of the cell cycle so that the appropriate cellular replication factors are available. They therefore act as potent mitogens, inducing quiescent cells to re-enter the cell cycle, and stimulating cell cycle progression and DNA synthesis.

In 1964, Kit et al. (190) showed that TK enzyme activity is induced after the infection of primary African green monkey kidney cells or established simian CV1 cells with SV40. Postel and Levine (305) showed, in 1976, that this TK induction was dependent upon expression of T-Ag, since tsA mutants (temperature-sensitive mutations in the T-Ag gene) were able to stimulate TK activity at the permissive, but not the nonpermissive, temperature.

As mentioned previously, SV40 efficiently induces TK mRNA. Roehl et al. (326) have recently demonstrated a three- to fourfold increase in the transcription rate of the endogenous TK gene in SV40-infected CV1 cells. As mentioned previously, using CV1 cells stably transfected with TK-Neo hybrid genes, they showed that transcription from a severely truncated 67 bp promoter fragment, containing the TATA box and one inverted CCAAT element, was still induced at the G1/S interface in serum-starved, SV40-infected cells. Thus, T-Ag is capable of trans-activating the TK promoter via regulatory elements potentially different from those utilized in serum-stimulated cells. Since many of the

effects of T-Ag are due to interactions with cellular proteins (such as pRb, p53, and TBP, discussed in greater detail below), studies such as these emphasize the role of SV40 as a valuable tool, for identifying both TK promoter elements and potential cellular proteins involved in viral-mediated TK transcriptional induction. It is hoped that elucidating the mechanism for the T-Ag-mediated induction of TK will provide insight into general mechanisms for cell cycle control.

7. SV40 and Large T Antigen

7.1 SV40 Overview

Simian virus 40 (SV40) was discovered in 1960 as a contaminant of poliovirus vaccines prepared from rhesus monkey kidney cells (371). Its ability to cause tumors when injected into newborn hamsters earned it a classification as a tumor virus.

SV40 is a nonenveloped virus. Its capsid is icosahedral in shape and is composed of three different viral proteins (95). The SV40 genome is double-stranded, covalently-closed, circular, DNA, 5243 bp in size, and encodes two early gene products and three major late gene products. The early gene proteins are the small and large tumor antigens (t-Ag and T-Ag, respectively). These proteins are produced via differential splicing of a common mRNA species, and share a common N-terminus while harboring unique C-termini. The late gene products are the viral capsid proteins VP1, VP2, and VP3, with VP1 being the major capsid protein. Viral DNA in the virion is associated with four cellular histones, namely H2A, H2B, H3, and H4. In this respect, the viral DNA shares the same nucleosome structure and histone composition as cellular DNA.

SV40 maintains a narrow host range (95). The permissive host is monkey epithelial cells, and in these cells, a productive infection, resulting in the production of viral progeny and concomitant lysis of the host cell, occurs. SV40 is also known as a vacuolating virus, since a characteristic of the lytic infection in a permissive host cell is the production of multiple vacuoles within the cell. In nonpermissive cells, such as that of

mouse or rat, a nonproductive infection occurs. This is characterized by interruption of the viral life cycle, with no production of viral progeny, and may result in immortalization of primary cells and/or neoplastic transformation of tissue culture cells. In this respect, no tumors are formed in infected host animals, due to occurrence of a lytic infection, but tumors do arise when the virus is introduced into animals of other species, such as the newborn hamsters mentioned above.

Viral infection consists of two stages (95). The early stage occurs prior to the onset of DNA replication and involves modulation of various aspects of host cell metabolism. In particular, cellular gene expression is activated and cellular DNA synthesis is induced to prepare cells for the S phase of the cell cycle. This is necessary, as viral DNA replication is dependent upon host cell proteins and replicative machinery available only in S. This requirement for an S-phase environment undoubtedly accounts for the potent mitogenicity of SV40. The late stage of infection follows viral DNA replication, and is characterized by synthesis of the virion proteins and assembly of viral progeny. A complete infection cycle requires approximately 48 to 72 hours.

Upon infection, the first step in viral entry into a cell is adsorption to cell surface receptors. Virus binding is then followed by internalization into the cell, transport to the nucleus, and uncoating of viral DNA for subsequent transcription and replication of the viral genome. Transcription of early viral mRNAs begins after uncoating. Transcription is carried out by the cellular RNA Polymerase II, as evidenced by sensitivity of the enzyme to α-amanitin. The SV40 early promoter contains a number of transcriptional regulatory elements. A TATA box is located approximately 30 bp upstream from the early RNA initiation site. In addition, there are a number of G+C-rich regions, including three 21-bp repeats, located 40 to 103 bp upstream from the RNA initiation site, which bind the transcription factor Sp1 to activate transcription from the early promoter. An enhancer element is also located upstream of the 21-bp repeats and is composed of two 72-bp repeats.

Viral transcription is regulated by the early gene product and viral replicative protein, large T Antigen (T-Ag). As will be discussed in greater detail below, multiple binding sites for the T-Ag protein exist within SV40 DNA. The binding of T-Ag to sites within the viral DNA near the early viral promoter effectively represses early transcription by blocking the binding of RNA Polymerase II. In this respect, T-Ag autoregulates its own synthesis. Repression of early transcription then triggers the onset of viral replication. T-Ag trans-activates the viral late promoter and late transcription follows. Late transcription requires both the synthesis of viral early proteins and viral DNA replication. The SV40 late promoter is located upstream of the enhancer element, oriented in the direction opposite that of the early promoter, and viral late genes are transcribed from the strand opposite that used for early gene transcription.

In contrast to cellular DNA, viral DNA replicates many times per cell cycle. It does, however, replicate as a minichromosome, identical in structure to the cellular chromatin. The SV40 origin of replication (95, 182) consists of a 64 bp core sequence composed of three distinct domains. The center domain contains four copies of a pentanucleotide sequence (GAGGC) organized as an inverted repeat, and serves as recognition sites for the binding of T-Ag. On the late gene side of this domain is a 17-bp A+T-rich segment which serves as the site of initial unwinding of viral DNA. Finally, the early gene side of the central domain contains a 15-bp imperfect repeat in which DNA melting occurs.

Briefly, the steps involved in replication of SV40 DNA are as follows: 1) T-Ag binds to the pentanucleotide repeats within the replication origin and assembles as a nucleoprotein structure. 2) T-Ag contains ATPase and helicase activities and thereby catalyzes the unwinding of the DNA strands with formation of a replication bubble containing two diverging replication forks. 3) DNA polymerases α and δ then catalyze DNA synthesis, with polymerase α-primase complex synthesizing lagging strand synthesis and polymerase δ/PCNA catalyzing leading strand synthesis. Synthesis is bidirectional and

discontinuous, and is complete when the two diverging replication forks meet. Finally, cellular topoisomerases relieve supercoiling tensions and segregate the two multiply-intertwined daughter molecules that result.

As mentioned, SV40 is dependent upon host cell proteins for the processes of viral transcription, translation, and DNA replication. This means that viral mechanisms are, in fact, very similar to cellular processes. SV40 thereby becomes a valuable tool for the study of cellular processes such as transcription, and DNA replication. It also offers insight into mechanisms that regulate cell cycle transitions, such as that from G1 into S, and pathways to tumorigenicity and neoplasia. It is this aspect of SV40 which is employed in the studies described in this dissertation.

7.2 t-Ag

t-Ag is encoded within a 2500 nucleotide segment of the SV40 early region (27). The protein is 174 amino acids, with the N-terminal 82 amino acids being common to T-Ag and the remaining 92 C-terminal amino acids being unique. The exact role of t-Ag in the viral life cycle is unknown. Studies with viral mutants that delete or alter t-Ag indicate that it is not directly required for viral DNA replication, but, as this protein is encoded by all Papovaviruses, it most likely has some role (309), and evidence exists to suggest that it does, in fact, play an overall helper role in activities mediated by the T-Ag protein.

For example, T-Ag is able to transform many cell types on its own, while t-Ag is incapable of transforming cells alone. However, viruses carrying mutations in T-Ag which concomitantly abrogate the production of functional t-Ag are deficient in the transformation of resting cells, suggesting t-Ag plays a helper role in T-Ag-mediated transformation (27). Likewise, viral mutants which do not produce t-Ag induce tumors in hamsters after a longer latency than wild-type SV40, and, in lytic infections in monkey kidney CV1 cells, produce lower yields of infective progeny (118). In many cases, the

defect imposed by the missing t-Ag protein can be restored by the addition of serum or phorbol ester tumor promoters, suggesting that t-Ag substitutes for growth factors (356).

Recently, a role for t-Ag in cell proliferation and the establishment and maintenance of transformation has been proposed based upon a discovered association between t-Ag and protein phosphatase 2A (PP2A) (337, 356, 407). PP2A is a major cellular serine/threonine phosphatase, and is comprised of three subunits: a 37 kD catalytic subunit (C) and two regulatory subunits (A and B). The protein core, comprised of the A+C subunits, associates with multiple forms of the B regulatory subunits, with binding of both C and B subunits occurring on A. Through a variety of methods involving the mixture of t-Ag protein with various subunits of PP2A, it was discovered that t-Ag binds the A regulatory subunit of PP2A, and that this interaction is blocked by binding of the B regulatory subunit to A (356, 407), suggesting that B and t-Ag bind identical or mutually exclusive sites on the PP2A enzyme and thereby compete with each other for binding. As there are various forms of the B regulatory subunit, some investigators have even detected an binding exchange between B and t-Ag on PP2A subunit A (356).

Binding of t-Ag to PP2A has been found to inhibit the catalytic activity of the enzyme, as assayed by the effect of t-Ag on exogenous substrates of PP2A. For example, Yang et al. (407) looked at the ability of myosin light chain kinase and ERK1 to phosphorylate their respective substrates in the presence of t-Ag and PP2A. As these kinases are normally dephosphorylated and inactive in the presence of PP2A, substrate phosphorylation would be indicative of inactivation of PP2A activity. A similar study was conducted by Sontag et al. (356), in this case, looking at the activity of MEK1 and ERK1, both components of the MAP Kinase cascade, in the presence of t-Ag and PP2A. In both cases, the kinases were active, indicating PP2A was inactive in the presence of t-Ag. Furthermore, transient transfection of t-Ag into CV1 cells resulted in increases in both ERK1 and MEK1 activities, with a corresponding increase in phosphorylation of the kinases, presumably via the inactivation of PP2A (356).

In this respect, then, t-Ag has been found to stimulate cell proliferation. Transient transfection of t-Ag into serum-starved CV1 cells resulted in stimulation of cell growth almost as well as that induced by the addition of serum (407). The stimulation of cell growth presumably arises by blockage of PP2A activity and deregulation of the MAP Kinase cascade, as evidenced above. Additionally, t-Ag inhibits the dephosphorylation of T-Ag protein and p53 (337). p53 growth suppressor activity is modulated by phosphorylation, and highly phosphorylated T-Ag binds pRb. Therefore, one possible outcome of this scenario is cell cycle progression due to the titration and sequestration of pRb coupled with the inactivation of p53 (103). Furthermore, an increase in the phosphorylation and subsequent inactivation of pRb could also be postulated. Together, this data suggests that t-Ag may actually play a subtle but important role in the increase in cell proliferation and cell cycle progression upon SV40 infection.

7.3 T-Ag

As mentioned earlier, T-Ag is the major replicative and transforming protein of SV40. Synthesized during the early phase of infection, it accumulates in the nucleus where it effectively alters the pattern of cellular gene expression and stimulates quiescent cells to enter S phase (103). During the transition to the late phase of infection, T-Ag represses early transcription, initiates viral DNA replication, and stimulates late gene expression and virion production.

T-Ag is a 708 amino acid, 90 kD phosphoprotein that shares its N-terminal 82 amino acids with t-Ag (27). Synthesized in the cytoplasm, the majority of the protein localizes to the nucleus while a small percent (<5%) is located to the plasma membrane (95). The T-Ag protein is highly modified (95); modifications include N-terminal acetylation, phosphorylation, poly ADP-ribosylation, glycosylation, and acylation.

T-Ag also possesses multiple functions which segregate to multiple functional protein domains. The domain structure of the T-Ag protein is shown in Figure 6. Note that many of the domains contain activities pertinent to the protein's replicative functions,

including those for DNA-binding (viral and non-specific cellular), ATPase activity and ATP-binding, helicase activity (both ATP-dependent DNA and RNA helicases), and the binding and stimulation of the cellular DNA polymerase α. Many domains are also pertain to the mitogenic and promoter trans-activation functions of T-Ag, and involve the binding of cellular proteins, including pRb/p107 and p53. In addition, T-Ag has also been shown to interact with TATA Binding Protein (TBP) (131) and may possibly associate with p300 (404), a cellular protein which interacts with and aids adenovirus E1A in inducing cell DNA synthesis (358).

In addition, T-Ag contains a zinc-finger motif which may actually play a role in the oligomerization of T-Ag, a requirement for the performance of many viral regulatory functions (303). Alternatively, the zinc-finger domain may function to maintain the overall conformation of the T-Ag protein and mediate interactions between the DNA-binding domain and the ATPase/ATP-binding domain (414). The nuclear localization signal (NLS) is responsible for translocation of the protein into the nucleus while the C-terminal host-range (hf/hr) domain is required late in a productive viral infection, most likely for mediation of virus assembly (303). This domain is fully functional in an infected cell, even when expressed as a separate polypeptide (103), and allows human adenovirus to grow in nonpermissive monkey cells (303).

As will be discussed below, the transformation and trans-activation activities of T-Ag localize predominantly to the N-terminal domains of the protein. Of interest, as well, is the fact that several of the T-Ag regions show considerable homology to adenovirus E1A (67). For example, amino acids 9-19 of T-Ag encodes a transformation function analogous to the p300-binding function of E1A conserved region 1 (CR1) while amino acids 102-107 of T-Ag and the E1A conserved region 2 (CR2) (258) both constitute pRb/p107 binding sites. The individual activities of T-Ag will be discussed in more detail below.

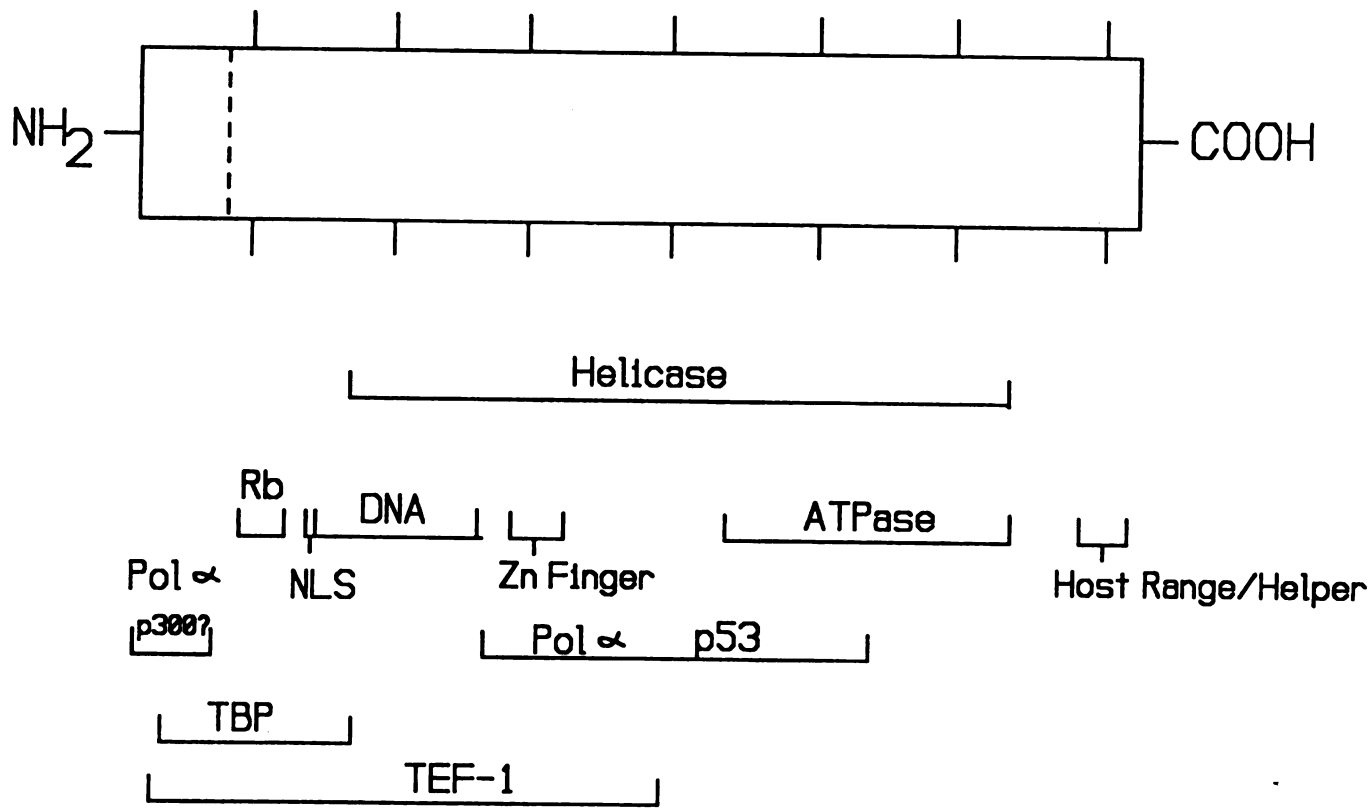


Figure 6. SV40 large T antigen protein domains. Vertical marks on the map represent every 100 amino acids. The exonI-exonII boundary at amino acids 82-83 is indicated by a vertical dashed line. Brackets below the map indicate domains of known function.

7.3a Interactions with Cellular Proteins

T-Ag is involved in many activities besides the initiation of viral DNA replication and regulation of viral transcription. Specifically, T-Ag exerts a powerful mitogenic effect on infected cells, inducing quiescent cells to re-enter the cell cycle and undergo DNA synthesis. T-Ag is also a potent activator of both viral and cellular promoters, and functions to immortalize and/or transform nonpermissive cells in tissue culture. As shown in Figure 6 above, T-Ag interacts with a variety of key cellular proteins, many of them regulatory in nature, and it is widely believed that these interactions are fundamental to the ability of T-Ag to alter the metabolism of host cells.

One important interaction is the association between T-Ag and the tumor suppressor protein pRb. Complex formation between these two proteins was first discovered by DeCaprio et al. (67) in immunoprecipitates from lysed monkey cells expressing T-Ag. Monoclonal antibodies specific to both T-Ag and pRb coprecipitated both proteins, suggesting complex formation, and studies with T-Ag mutants indicated that complex formation was dependent upon amino acids 105-114 in the T-Ag protein.

Ludlow et al. (229) further showed that it was the underphosphorylated species of pRb (which predominates during G1) that associates with T-Ag. Moreover, all detectable unphosphorylated pRb was found bound to T-Ag in G1, with dissociation of the complex in S and reformation early in the next G1. Assuming the un- or underphosphorylated G1 form of pRb to be the growth-suppressive form, this indicated that pRb activity could be suppressed by either phosphorylation or association with T-Ag.

It is now known that pRb binds cellular proteins such as the transcription factor E2F, that E2F transcriptional activity is repressed when complexed to pRb, and that T-Ag effectively dissociates these Rb-containing complexes to release free and active E2F (49), which is then able to activate genes required for cell cycle progression. An important pathway to mitogenic stimulation by T-Ag is hereby implicated. As will be discussed

below, there is also some correlation between the ability to bind pRb and the transformation activities of T-Ag.

Similarly, T-Ag also binds the tumor suppressor protein p107. p107 was, in fact, first identified through its associations with T-Ag and adenovirus E1A (92, 98, 139, 392, 408). The region of T-Ag required for binding p107 is also the same region needed for binding pRb (74, 93, 171, 387, 392). Recall that p107 also complexes with cellular proteins, associating with cdk2, cyclin E, and E2F in G1 (209) and cdk2, cyclin A, and E2F in S phase (74, 348). Again, binding of T-Ag to p107 dissociates these complexes, releasing the various factors (17, 49, 50, 265). Interestingly, besides the effect of T-Ag on p107 activities, p107 itself is able to inhibit the initiation of viral DNA replication by T-Ag (5), a point that will be discussed in greater detail in a following section.

A third tumor suppressor protein bound to T-Ag is p53. Amino acids 325-625 of T-Ag are required for this interaction. There is a good correlation between the ability of T-Ag to transform cells and the ability of T-Ag to bind p53. As with p107, p53 also inhibits the ability of T-Ag to initiate and replicate SV40 DNA. Mechanisms most likely involve blocking the binding of DNA polymerase α to T-Ag. Friedman et al. (115) demonstrated that p53 strongly inhibited T-Ag helicase activity and that, while p53 did not inhibit the ATP-dependent conversion of T-Ag monomers to hexamers that occurs prior to initiation of viral DNA replication, it did inhibit the ability of the hexamers to assemble on DNA containing the viral origin of replication, thereby indicating that T-Ag hexamers are the target of p53 inhibition.

Conversely, T-Ag inhibits p53-mediated transcriptional activity, as shown in transient cotransfection assays (165). Filter binding and gel shift assays further indicated that T-Ag blocks the binding of p53 to DNA (23), and p53 transcriptional activity is dependent upon the binding of p53 to consensus sites within promoters.

T-Ag has also been found to interact with DNA polymerase α (80). In particular, T-Ag binds the large catalytic subunit of polymerase α, an interaction that requires the N-

terminal 83 amino acids of T-Ag. T-Ag also displays a higher affinity for human polymerase α than calf polymerase α, suggesting that polymerase α is at least partly responsible for the primate-specific replication of SV40 DNA *in vivo* and *in vitro*. Indeed, there is further evidence to suggest that polymerase α is a cell specificity factor. Purified polymerase α from permissive human or monkey cells can restore T-Ag-dependent replication to non-permissive mouse cells that are normally inactive for SV40 DNA replication (58).

In association with polymerase α, T-Ag also binds RP-A (Replication Protein A or human single-stranded DNA binding protein) (81). Via ELISAs and modified immunoblot procedures, it has been determined that RP-A itself associates with DNA polymerase α. Thus, protein-protein interactions between RP-A, DNA polymerase α-primase, and T-Ag may play a role in the initiation of SV40 DNA replication. In this respect, a correlation has been shown between T-Ag complex formation and viral DNA synthesis (303). Furthermore, T-Ag has been found to stimulate the activity of DNA polymerase α-primase (58).

Of interest is the fact that pRb inhibits the stimulation of polymerase α by T-Ag (336). In an *in vitro* replication system using purified calf thymus or human polymerase α and primed single-stranded DNA, T-Ag stimulated both primase and polymerase α activity several-fold. Stimulation was, however, abolished by preincubation of T-Ag with pRb suggesting that competition between pRb and polymerase α for binding to T-Ag is a potential regulatory step in the initiation of viral DNA replication.

In accordance with its transcriptional trans-activation functions, T-Ag has also been shown to associate with TATA Binding Protein (TBP), an integral component of transcription pre-initiation complexes, and Transcription Enhancing Factor I (TEF-I) (130). These associations were detected in assays involving incubation of purified TBP with GST fusion constructs containing segments of the T-Ag protein. Results revealed that TBP interacted with amino acids 5-172 of the T-Ag protein while TEF-I binding

required amino acids 5-383. Furthermore, both of these fragments were able to activate transcription from both the SV40 late promoter and a simple promoter composed of a binding site for the transcription factor TEF-1 and a TATA box. This supports previous work which showed that TEF-1 binding sites within the SV40 late promoter were sufficient to confer T-Ag inducibility to a normally uninducible HSV TK promoter. Mutation of the TEF-1 sites within the SV40 late promoter abolished T-Ag-mediated activation. This work suggests that T-Ag may transcriptionally activate via interactions with components of the transcriptional apparatus. Precedence for this comes from studies with adenovirus E1A showing that E1A mediates some of its effects by interacting with proteins bound to TATA boxes (150, 153, 349, 401).

Finally, a potential association exists between T-Ag and p300. p300 is a ubiquitous, cellular, nuclear phosphoprotein found in both proliferating and resting cells (405). In addition, p300 is a sequence-specific DNA binding protein with an affinity for enhancer-like sequences (322). While p300 protein levels remain constant throughout the cell cycle, activity may be modified by cell cycle phase-specific phosphorylation. Recently, p300 was found to coimmunoprecipitate with a TBP-specific antibody, indicating that it is a component of TBP-containing transcription complexes (1).

p300 is significant in that it binds to the N-terminus of the adenovirus E1A protein, and is the only protein known to bind E1A in this region (358). Association with p300 is required for the ability of E1A to transform cells (358), inhibit the activity of transcriptional enhancer elements (358), and induce cellular DNA synthesis (154). The association between p300 and T-Ag is suggested because T-Ag is able to functionally complement E1A N-terminal mutants unable to bind p300 (404). This complementation involves the N-terminus of T-Ag, specifically amino acids 17-27, and does not involve the T-Ag pRb- or p53-binding domains. Thus, the N-terminal region of the T-Ag protein may be involved directly or indirectly with p300, an association which may further contribute to the mitogenic and transformation activities of T-Ag.

7.3b Mitogenic Activities

SV40 T-Ag, along with other viral oncoproteins such as adenovirus type 5 E1A and HPV type 16 E7, is a potent mitogen (259). Studies in a number of laboratories have shown that infection of quiescent cells with SV40 virus results in the induction of cellular RNA and protein synthesis (118), induction of cellular and viral DNA synthesis (118), and cell division (353). These activities occur in the absence of t-Ag, indicating that T-Ag itself is sufficient (118).

The mitogenic activity of T-Ag has been found to involve at least four independent functions of the protein (76, 78). Evidence for this comes from studies analyzing the ability of various T-Ag mutants to induce cell cycle progression. Specifically, T-Ag proteins defective in the ability to bind pRb, p107, p130, or p53, ones containing mutations in the DNA-binding domain, and ones containing mutations in the N-terminus which might affect the association of T-Ag with cellular proteins such as TBP, TFIIB, TEF-1, and p300, were analyzed. Mutant proteins were microinjected into serum-starved, quiescent cells, and the uptake of BrdU was measured as an indicator of DNA synthesis. Results showed that all T-Ag proteins carrying individual mutations were still capable of inducing DNA synthesis, although with a short (approximately 3 hour) delay relative to the wild-type protein. Furthermore, T-Ag double mutants were even slower at promoting the G0 to S-phase transition, indicating that the individual mutations were each independent in their actions. Finally, complementation studies with various combinations of mutants resulted in restoration of DNA induction at wild-type kinetics.

Using this data, then, a model has been proposed where each T-Ag function mutated represents an interaction with a different cellular protein (i.e. pRb, TBP, TEF-1 etc.). Each of these proteins may, in turn, be part of separate signal transduction cascades that feed into a common measuring device. The measuring device may involve the accumulation of cyclins (D, A, and E) and activation of cyclin/cdk complexes. The rate of

progression from G1 into S would then be proportional to the intensity of the mitogenic functions of T: the more active functions there are, the faster progression occurs.

7.3c Transformation and Immortalization

The fate of a cell infected by SV40 varies, depending upon the species and cell type (372). In cells permissive for the replication of viral DNA (i.e. primate), a productive infection ensues, resulting in the generation of viral progeny and lysis of the cell. In contrast, in nonpermissive cells (i.e. rodent), viral DNA integrates into the genome. No viral DNA replication occurs, no viral progeny are produced, and the cell may, in fact, survive. Surviving cells may then undergo the processes of immortalization, and abortive and/or stable transformation.

Immortalization is the ability of cells to proliferate indefinitely. Characteristic phenotypes of a transformed cell (372) include loss of contact inhibition with growth to high saturation densities, a decreased requirement for growth factors, loss of anchorage-dependent growth, growth on monolayers of normal cells, and tumor formation upon injection of cells into susceptible animals.

Transformation involves multiple stages (372). Following infection, nonpermissive cells will first undergo abortive transformation, in which cells express the characteristics of a transformed phenotype for several generations before reverting back to the normal, untransformed phenotype. This occurs in a large number (as many as a few percent) of infected cells. A small fraction of the abortively-transformed cells will then become stably transformed. In this case, the viral DNA becomes successfully integrated into the host chromosome, and the transformed phenotype is expressed repeatedly throughout generations.

In addition to the stimulation of cell cycle progression, SV40 T-Ag, Ad E1A, and HPV (types 16 and 18) E6 and E7 share the ability to both immortalize and transform cells (323, 259). With SV40, large T-Ag alone has been found capable of immortalizing

primary cell cultures and transforming established rodent cell lines (reviewed in 334), although t-Ag may assist, especially under conditions of limiting amounts of T-Ag.

Much work has been done to try to identify the protein domains of T-Ag required for mediating transformation and immortalization. With respect to the immortalization functions of T-Ag, it has been demonstrated that primary mouse embryo fibroblasts (MEF) can be immortalized following transfection of the gene encoding T-Ag (413), and that the N-terminal 626 amino acids of the T-Ag protein are sufficient (367). In addition, the specific and non-specific DNA-binding activities, DNA helicase, pRb- and p107-binding, and the adenovirus helper-host range function of T-Ag are not essential for the immortalization of primary MEFs (413). In contrast, the N-terminal 135 amino acids appear sufficient for the immortalization of primary rat embryo fibroblasts (REF) (413). Thus, T-Ag protein regions required for immortalization appear to vary with cell type.

Further studies have narrowed the region containing immortalization activity for MEFs to amino acids 347 to 626 (413), essentially the C-terminal region of the T-Ag protein. Surprisingly, the pRb-binding domain does not appear to be required for this process. This result actually contradicts that of LaRose et al. (204) who studied polyoma large T-Ag mutants and found that proteins defective for pRb-binding were unable to immortalize primary REFs. Again, this emphasizes what seems to be a very prominent cell type-specific difference in the regions of T-Ag required for immortalization. Furthermore, Zhu et al. (413) reported cosegregation between the ability of SV40 T-Ag to bind p53 and immortalization of primary MEFs; all T-Ag mutants unable to bind p53 were unable to immortalize. Thus, the ability to complex with and inactivate p53 may be one requirement, at least in some cell types, for preparing cells for immortalization and possibly subsequent transformation.

In studies on transformation by T-Ag, it has been shown that the N-terminal 121 amino acids of T-Ag are sufficient to transform established mouse 10T1/2 cells (415). However, transformation of REF-52 cells, an established rat cell line, requires a

considerably larger portion of T-Ag, namely the first 600 amino acids (293). Zhu et al. (415) explored further the ability of T-Ag point, linker, insertion, and deletion mutants to transform both REF-52 and 10T1/2 cells. Also examined was the requirement for the binding of pRb. Using a monolayer overgrowth assay, in which transformation was indicated by the formation of dense foci on top of a monolayer of untransformed cells following transfection with T-Ag DNA, they found that mutations within the first exon of T-Ag (amino acids 1-82) decreased transformation of both cell lines. Mutations in the pRb- and p53-binding domains had only a slight effect on transformation of 10T1/2 cells, but there was a tight correlation between the ability to bind p53 and pRb and the ability to transform REF-52 cells. The requirement for p53-binding was further confirmed by Lin and Simmons (223) who found that T-Ag proteins defective for binding p53 had a reduced ability to induce transformed cell foci in two normal diploid human cell lines (D.551 skin fibroblasts and WI-38 lung fibroblasts) while mutants that bound p53 induced foci at wild-type levels. Thus, transformation of human and REF-52 cells requires the binding of p53, and p53 and pRb, respectively, and multiple regions of T-Ag (i.e. the first exon, and p53- and pRb-binding domains) are required for full transformation by SV40.

Other studies have reported that the N-terminal 147 amino acids of T-Ag are sufficient to transform secondary REF cells (355) and produce chromosomal aberrations in human fibroblasts transfected with T-Ag (397). Genome instability is a first step in transformation. Thus, the mechanism for transformation by T-Ag is complex and appears to vary depending upon cell type (i.e. rat, human, mouse) and stage of establishment of cells in culture (i.e. primary versus secondary cell lines).

7.3d DNA-Binding and Replicative Activities

As was mentioned previously, T-Ag binds specifically to multiple sites within the SV40 genome to mediate the processes of transcriptional regulation and initiation of viral DNA replication. Via DMS protection and interference experiments, the consensus binding sequence has been determined to be the pentanucleotide 5'-GAGGC-3' (170, 331,

366, 370, 398). Multiple copies of this pentanucleotide sequence comprise three main binding sites for T-Ag within and around the SV40 origin of replication.

Binding Site I (331) is located on the early side of the SV40 core origin of replication, downstream from the early transcriptional start site. Of the three binding sites, T-Ag binds strongest to Site I, and binding of T-Ag both to Sites I and II results in repression of early transcription at high concentrations of T-Ag. Site I contains three copies of the pentanucleotide sequence, but a 17 bp core sequence, consisting of two pentanucleotides separated by a 7 bp spacer, is sufficient to mediate high affinity binding by T-Ag.

Binding Site II (292) is located within the core origin of replication. This site comprises the central core domain mentioned earlier, and binding of T-Ag to Site II is required for the initiation of viral DNA replication. Site II contains four pentanucleotide sequences arranged as inverted repeats. The pentanucleotides initiate the binding of T-Ag in proper orientation for the assembly of T-Ag hexamers over each half of the origin, a requirement in the initiation of viral DNA replication. The inverted orientation of the repeats serves to orient the T-Ag hexamers in opposite directions for eventual bidirectional helicase activity during DNA replication.

Binding Site III is located on the late side of Site II. T-Ag binds weakest to Site III, and little is known about its function. In addition, T-Ag is able to bind, non-specifically, to double-stranded cellular DNA, as evidenced by its retention on double-stranded DNA cellulose columns (44, 121, 122, 291, 309). The consequences of non-specific DNA-binding by T-Ag are unknown.

The DNA-binding domain within the T-Ag protein has been determined by a variety of methods. McVey et al. (242) used a nitrocellulose filter-binding assay to analyze the ability of progressively smaller N-terminal proteins to bind DNA, while Paucha et al. (291) used an immunoprecipitation assay to monitor the effect of T-Ag mutants on DNA binding. Results showed that the N-terminal 272 amino acids of T-Ag were

sufficient for the binding to both cellular and viral DNA, and amino acids 139-220 contained the ability for specific origin-binding. It is unknown if this smaller region also confers binding to cellular DNA. Furthermore, binding appears to be regulated by post-translational modifications since binding activity differed for T-Ag fragments produced in mammalian versus bacterial cells (242). Finally, no helix-turn-helix motifs, common to many DNA-binding proteins, are found within this region of T-Ag (242), although there is a stretch of basic amino acids (Lys-Lys-Lys-Arg-Lys) which could potentially function as a site of interaction with DNA (174).

During the initiation of DNA replication, T-Ag monomers assemble, in an ATP-dependent reaction, as hexamers on the core origin, with one hexamer covering each half (292). The hexamer bound to the early half of the origin forms first and cooperatively promotes the assembly of the hexamer covering the late half, and the inverted pentanucleotide sequences orient the direction of the hexamers. Mutational analysis indicates that spacing is critical, suggesting that the T-Ag double hexamer has rigid steric constraints on its interaction with the core origin.

The development of an *in vitro* SV40 DNA replication system (217) has provided most of the information on the steps involved in replication. Basically, after binding of the T-Ag double hexamers to the core origin, T-Ag induces structural changes in the DNA (292), melting the DNA in the core inverted repeat (IR) domain and untwisting the DNA in the A+T-rich (AT) domain (66). Then, bidirectional unwinding via T-Ag helicase activity ensues (390).

The next steps of replication require ATP hydrolysis, HSSB (Human Single-Stranded DNA Binding protein), and topoisomerase activity to relieve supercoiling tension. It has long been thought that cellular topoisomerases fulfilled this role; however, a T-Ag-associated topoisomerase activity has recently been discovered (233). This activity immunoprecipitated with T-Ag-specific monoclonal antibodies and cosedimented with the T-Ag hexamer.

T-Ag then associates with the DNA polymerase α-primase complex (268), presumably guiding the polymerase α-primase complex to the template DNA (233) and positioning it on the lagging strand (268). The polymerase α-primase complex repeatedly associates and dissociates from the replication fork to synthesize and extend RNA primers; in doing so, the rate of movement of the T-Ag helicase is reduced (268). DNA polymerase δ holoenzyme, containing PCNA and the activator protein RF-C (66), then positions itself on the leading strand. Polymerase δ holoenzyme is processive and can synthesize DNA at a rate of 400-600 nt/minute. However, in the SV40 system, the rate of synthesis is only 200 nt/minute, equivalent to the rate of T-Ag unwinding. Thus, the rate of leading strand synthesis is limited by the unwinding by T-Ag. Formation of full-length covalently-closed circular products then requires a 3' to 5' exonuclease activity, RNAse H, and DNA ligase (66).

T-Ag-dependent replication can be inhibited by association with the p107 tumor suppressor protein (5). Apparently, the p107 pocket region inhibits the assembly of T-Ag hexamers on the SV40 origin of replication. If hexameric complexes have preformed on viral DNA, p107 binds the complex and interferes with the interaction between T-Ag and DNA polymerase α. In this way, viral DNA replication may be regulated in that p107 effectively sequesters T-Ag monomers in G1, preventing their assembly at the core ori and/or inhibiting the initiation of viral DNA replication. At the G1/S interface, then, the phosphorylation of p107 would result in the release of T-Ag monomers, allowing for assembly and initiation in S phase.

Regulation of T-Ag replicative functions most likely occurs via phosphorylation (reviewed in 103 and 310). This is suggested by reports of differential phosphorylation of T-Ag in infected monkey cells, varying phosphate turnover rates at different T-Ag sites, and decreased binding affinity for SV40 DNA by highly phosphorylated T-Ag. Indeed, there are two major clusters of phosphorylation sites in the T-Ag protein, one at both the N- and C-terminus, lying outside of the DNA-binding and helicase domains. Highly

phosphorylated sites include serines (Ser) 106 and 112, within the pRb-binding domain, and serines 120 and 123 and threonine (Thr) 124, adjacent to the NLS and origin DNA-binding domain.

Negative regulation of T-Ag activities by phosphorylation is evidenced by the fact that dephosphorylation of T-Ag serine residues by alkaline phosphatase resulted in enhanced binding of T-Ag to SV40 DNA and initiation of *in vitro* DNA replication. Thus, highly phosphorylated T-Ag appears unable to bind cooperatively to SV40 ori, form the double hexamer nucleoprotein complex, and unwind ori DNA. Furthermore, G1 extracts have been found to be inefficient at supporting *in vitro* SV40 DNA replication unless supplemented with PP2A (103); PP2A activity is low in G1 and increases at S.

Positive regulation by phosphorylation is also suggested in connection with the Thr124 residue. Replacement of Thr124 with alanine, a nonphosphorylated amino acid, resulted in a T-Ag that was unable to replicate SV40 DNA in monkey cells, bound poorly to the Site II ori sequence, and was defective in *in vitro* SV40 DNA replication. Moarefi et al. (255) further showed that the Thr124 mutant was actually able to bind DNA but was defective in origin unwinding, perhaps due to an inability to form the proper double hexamer complex. Furthermore, bacterially-produced T-Ag was only active in SV40 ori binding and *in vitro* SV40 DNA replication after incubation with a cdc2 kinase, which phosphorylates Thr but not Ser residues. Taken together, this data indicates that phosphorylation of Thr124 is required for the replicative activities of T-Ag.

In summary, it appears that T-Ag replicative activities are at a maximum when the protein is phosphorylated on Thr124 but dephosphorylated on serines. Models (103, 310) for regulation can therefore be proposed involving fluctuating cell cycle-specific levels of phosphorylated (inactive) and unphosphorylated (active) T-Ag. Perhaps highly phosphorylated T-Ag binds the tumor suppressor proteins in G1, thereby promoting cell cycle progression. Activation of a phosphatase, such as PP2A, and possibly a cdc2-like kinase at S would then result in production of active T-Ag.

7.3e Trans-activation

As mentioned, T-Ag is intimately involved in transcriptional regulation, repressing viral early transcription by binding to sites on the early side of the SV40 origin of replication and activating transcription from the SV40 late promoter (33, 34, 45, 130, 181, 414). T-Ag is also a promiscuous trans-activator, activating transcription from a variety of other cellular and viral promoters (4, 130, 227, 264, 267, 375, 414). As this dissertation is concerned with trans-activation of the human TK promoter by T-Ag, this property of T-Ag is of particular interest.

Zhu et al. (414) explored extensively the regions of the T-Ag protein involved in trans-activation. Particularly, they looked at the ability of various mutant T-Ags to activate the expression of CAT hybrid genes containing either the SV40 late promoter or the long terminal repeat from the Rous Sarcoma Virus (RSV LTR) in transiently transfected CV-1 cells. They found that a 138 amino acid N-terminal fragment of T-Ag retained trans-activation activity, albeit at a reduced level. Furthermore, mutations within the N-terminal 85 amino acids also resulted in reduced abilities to trans-activate both promoters. There was, however, no effect on trans-activation by mutations in the pRb-binding domain, NLS, or the C-terminal ATPase, hr/hf (host range and adenovirus helper function), p53-binding, and helicase regions. The conclusion, then, was that the N-terminus of T-Ag (i.e. the first 85-138 amino acids) contains the major trans-activation activity. This is particularly interesting since it is the N-terminus of T-Ag which is involved in interactions with cellular proteins such as TBP, TEF-1, and possibly p300, in addition to the known associations with pRb and p107.

Zhu and colleagues (414) also determined that the overall conformation of the T-Ag protein was critical to trans-activation activity, since a number of mutations in other regions of the protein, namely the DNA-binding and zinc-finger domains, abolished or severely reduced trans-activation. In particular, two mutations which abrogated the ability of T-Ag to bind DNA had no detectable levels of trans-activation. DNA-binding is

unlikely to be a requirement for transcriptional activation, however, since the truncated 138 amino acid fragment, which lacks the DNA-binding domain, retained trans-activating activity. Mutations within the zinc finger region also severely diminished activation. As this region plays mainly a structural role, it is proposed that all these mutations abolish trans-activation by altering the overall conformation of the T-Ag protein.

There are a number of possible mechanisms by which T-Ag could mediate transcriptional activation. First, it may do so directly by binding to sites within promoter DNAs. Second, it may do so indirectly via associations with cellular factors. In this respect, T-Ag could indirectly modulate the activity of pre-existing factors or induce the synthesis of new factors. Alternatively, it may function by making direct contact with cellular proteins.

As alluded to above, it is unlikely that the mechanism of activation requires direct DNA-binding by T-Ag, although Keller and Alwine (181) did see a correlation between the ability of T-Ag to bind DNA and its ability to activate transcription from the SV40 late promoter. Specifically, full activation from the late promoter was only stimulated by T-Ags able to bind DNA. This does not prove, however, that actual DNA-binding was involved. Nevertheless, it is possible that the SV40 late promoter and RSV LTR represent a class of promoters for which DNA-binding by T-Ag is a requirement for trans-activation. Other promoters may not have this requirement. There may be multiple mechanisms of trans-activation by T-Ag, with different mechanisms operating to stimulate different promoters.

In support of the claim that DNA-binding is not required for transcriptional activation by T-Ag, it is noteworthy that the majority of promoters known to be activated by T-Ag do not contain the pentanucleotide consensus binding sites (414). Furthermore, in another study, T-Ag mutants lacking the DNA-binding domain could still activate the Ad E2 and SV40 late promoters (357), and an NLS mutant, which resulted in strictly

cytoplasmic T-Ag still activated the SV40 late promoter (393). Thus, it is unlikely that the mechanism for activation involves direct DNA-binding by T-Ag.

As mentioned above, T-Ag may also activate promoters by altering the activity of cellular factors. For example, T-Ag has been shown to modulate the activity of, and induce the synthesis, of cellular factors. In particular, Saffer et al. (332) reported that Sp1 mRNA and protein levels increased nearly 100-fold in SV40-infected cells. Thus, some promoters may be stimulated simply due to the increased amounts of Sp1.

In another example, Gallo et al. (117) studied a set of proteins known as Band A factors, unknown simian cellular factors that bind sequences within the SV40 late promoter that are required for trans-activation by T-Ag. In particular, the Band A factors appear to be modified and/or induced following infection with SV40. The proteins were found to bind more stably to their target sites, as indicated by DNAse footprint analysis. Furthermore, they were found to be in higher molecular weight forms after infection, produced heterogeneic gel shift patterns with infected versus non-infected extracts, suggesting the induction of new family members after infection, and had altered chromatographic properties on ion exchange columns, suggesting the modification of pre-existing forms and/or synthesis of new forms of the proteins.

Further studies (130) revealed that deletion of the nucleotides containing the Band A factor recognition sites resulted in loss of T-Ag-mediated activation. This region was found to contain two octamer protein binding sites, one of which overlapped with two binding sites for the TEF-1 factor. This region was additionally found to be capable of conferring T-Ag inducibility to the normally unresponsive HSV TK promoter. Finally, transient cotransfections with a hybrid gene, composed of a CAT reporter and the SV40 late promoter, a T-Ag expression plasmid, and an expression plasmid encoding Octamer Binding Protein 1 (Oct) revealed that Oct protein expression actually repressed promoter activity and that T-Ag relieved the inhibition.

Casaz et al. (45) confirmed these results by showing that mutation of the TEF-1 sites abolished detectable activation by T-Ag. By DNAse footprinting mutant promoters, they verified that inducibility by T-Ag was correlated with the ability of TEF-1 to bind DNA. Finally, a direct interaction between SV40 T-Ag and TEF-1 was demonstrated (131). Thus, at least for the SV40 late promoter, a mechanism for promoter activation is emerging involving T-Ag-mediated displacement of Oct protein from promoter sites with subsequent stimulation of binding of TEF-1.

Direct interactions have been demonstrated between T-Ag and other cellular proteins as well. Mentioned earlier was the association between T-Ag and TBP (131), suggesting that T-Ag may trans-activate by making direct contacts with components of the transcription complex. T-Ag has also been shown to associate with the transcription factor AP-2 (252), inhibiting both the sequence-specific binding of AP-2 to cognate promoters and AP-2-dependent transcriptional activation. The significance of this association is not yet clear.

A question arises as to the role of pRb in T-Ag-mediated transcriptional activation. Knowing the associations between T-Ag, pRb, and the transcription factor E2F, it seems plausible that inactivation of pRb activity would be a major mechanism for trans-activation. Evidence for such a mechanism is, however, conflicting. For example, the studies of Zhu et al. (414) report that T-Ags defective for binding either pRb or p53 trans-activated the SV40 late promoter and RSV LTR at wild-type levels in transient transfection assays, suggesting that interactions with these proteins were not required. In support of this, Trifillis et al. (375) looked at the ability of T-Ag to trans-activate the SV40 late and RSV LTR promoters in both $pRb^{(-)}$ and $pRb^{(+)}$ human osteosarcoma cell lines; trans-activation occurred efficiently in both cell lines, with eight- to tenfold increases in CAT activity and seven- to eightfold increases in CAT mRNA occurring from transfected hybrid gene constructs after introduction of T-Ag. Thus, an interaction

between T-Ag and pRb appears not to be required for trans-activation of the SV40 late and RSV LTR promoters.

Trans-activation of other promoters (especially those induced during viral infection), however, may require an interaction with pRb. Along these lines, Mudrak et al. (264) looked at the transcriptional activation of murine thymidine kinase (TK), thymidylate synthase (TS), dihydrofolate reductase (DHFR), PCNA, and DNA polymerase α genes following induction of polyomavirus large T-Ag expression. In each case, mRNA levels were induced with wild-type T-Ag but not a mutant T-Ag unable to bind pRb. Likewise, in transient transfections with a TK-CAT hybrid gene, CAT activity was detected only after expression of the wild-type T-Ag protein; no expression was detected in the presence of the pRb-binding-defective T-Ag. So, for these genes, at least, trans-activation seems to require inactivation of pRb by T-Ag.

7.4 SV40 T-Ag and Thymidine Kinase

The remainder of this dissertation is devoted to elucidating a mechanism for the trans-activation of human thymidine kinase gene promoter by SV40 T-Ag. As indicated earlier, TK is an excellent model system for understanding events which regulate the G1/S transition in the eukaryotic cell cycle. SV40 T-Ag is a powerful tool to study these events since the viral life cycle depends upon cellular factors. Thus, by studying viral regulation of cellular genes induced upon infection, cellular factors normally involved in the regulation of that gene may be identified and knowledge of by-passed control mechanisms, with a corresponding understanding of normal cell cycle control pathways, is gained.

Sufficient evidence has already been presented attesting to the fact that SV40 T-Ag activates TK. Namely, TK enzyme activity, protein levels, mRNA levels, and transcription rate all increase in an S phase-specific manner following infection of quiescent cells with SV40. Furthermore, these increases can be attributed to the activities of T-Ag. The chapters that follow examine in depth both the TK promoter elements and

T-Ag protein domains involved in the process of T-Ag-mediated activation of TK in an attempt to solve a piece of the G1/S-phase regulatory puzzle.

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Chapter 2

Trans-activation of the Human Thymidine Kinase Promoter by SV40 Large T Antigen Involves Both theT Antigen pRb/p107-Binding Domain and Promoter Sequences Resembling E2F Binding Sites

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Abstract

Infection of quiescent cells with the DNA tumor virus SV40 induces expression of the cellular thymidine kinase gene at least ten- to twentyfold. This activity of the virus depends upon the viral replicative protein, large T Antigen (T-Ag). We have previously shown that the rate of TK gene transcription increases three- to fourfold at G1/S in SV40-infected CV1 cells, suggesting that one mechanism by which SV40 induces TK is via direct or indirect interactions between T-Ag and the thymidine kinase promoter. Here, experiments designed to define both TK promoter elements and T-Ag functional domains required for TK transcriptional induction are presented. A system has been established in which stable Rat-1 transfectants harboring TK-Luc hybrid genes are infected with a recombinant adenovirus expressing wild-type or mutant forms of T-Ag, and luciferase expression is measured as an indicator of promoter activity. The results of these studies show that 1) a 135 bp TK promoter fragment is activated by viral infection, in this system 2) the final induction levels are a result of both T-Ag-dependent and independent mechanisms 3) the T-Ag pRb/p107-binding domain, but not the p53-binding, helicase, or ATPase domains, is involved in trans-activation of the TK promoter and 4) T-Ag-mediated induction of TK-Luc hybrid genes is severely diminished with a TK promoter fragment in which E2F-like binding sites, located between -98 and -113 bp, have been removed. This data therefore suggests the involvement of E2F or a related factor, and pRb (and/or p107) in activation of the human TK promoter by SV40 T-Ag.

Introduction

The processes of DNA replication and cell division are fundamental components of the eukaryotic cell cycle, a complex network of integrated, highly regulated pathways which cooperate to facilitate cell proliferation. While the cycle is replete with control mechanisms, entries into both S and M, the DNA synthesis and mitotic phases of the cycle, respectively, are particularly highly regulated. With regard to the G1/S transition, a major control point termed the Restriction Point, or R, occurs in late G1 and regulates entry into S. Once cells have passed R, they are committed to traverse the remainder of the cell cycle.

In order to understand the nature of the regulatory mechanisms governing passage from G1 into S, much research has been devoted to studying the regulation of genes that are activated at the G1/S border. Many of the genes involved with DNA synthesis, including dihydrofolate reductase (DHFR) (27), thymidylate synthase (TS) (48), and thymidine kinase (TK) (28) display this G1/S phase-specific activation and thus serve as model systems for the elucidation of what is hoped to be common cell cycle control mechanisms. Of these, TK is one of the most highly induced genes and therefore one of the most widely-used model systems.

TK catalyzes the conversion of dT to dTMP, a precursor to DNA synthesis. TK enzyme activity is highly regulated by the growth state of the cell in that activity is high in rapidly proliferating cells but low in terminally-differentiated or nonproliferating cells (42, 45). TK activity is also highly regulated by cell cycle position; in both continuously cycling cells and quiescent cells which have been mitogenically stimulated to re-enter the cycle, TK enzyme activity is low in G0 and G1 but increases sharply (ten- to twentyfold, or more) at the G1/S boundary, remaining high throughout G2 and into M (28). As with many of the G1/S phase-regulated genes, regulation is complex, occurring at both the transcriptional and post-transcriptional levels. A variety of factors may contribute to the

existence of multiple levels of regulation, including differences in cell type (i.e transformed versus nontransformed cells) and the growth state of the cell (i.e. continuously cycling versus quiescent, mitogenically-stimulated cells). For example, a number of studies with continuously cycling cells, including the transformed HeLa cell line, have indicated regulation to be post-transcriptional, since the increase in enzyme activity is paralleled by a corresponding increase in protein levels while mRNA levels remain fairly constant (55). In contrast, transcriptional regulation has been implicated in the case of quiescent, nontransformed cells mitogenically stimulated to re-enter the cell cycle. In serum stimulated cells, increases in endogenous TK enzyme activity and protein levels have been found to be paralled by a corresponding increase in mRNA levels (61) and a smaller, but significant, increase in gene transcription rate (60). Furthermore, studies in our lab and others have shown that a fragment of the TK promoter containing 444 bp upstream and 30 bp downstream of the transcriptional start site is sufficient to confer G1/S-phase regulation to a linked heterologous reporter gene (2, 51).

The cis-acting elements within the human TK promoter required for G1/S transcriptional induction in serum-stimulated cells have been further mapped via 5' deletion studies and mutational analyses. In this lab, we linked fragments of the TK promoter, successively truncated from the 5' end, to the bacterial neomycin resistance (*Neo)* gene and assayed mRNA levels in stably transfected cells by Northern blot (RNA) hybridization assays (51, 52). Results showed that a promoter fragment containing sequences 135 bp upstream plus 30 bp downstream of the TK transcriptional start site retained serum-mediated G1/S transcriptional induction; however, truncation to only 67 bp of upstream sequence abolished regulation, indicating that promoter elements between -135 and -67 bp are required for regulation in serum-stimulated cells. This result was confirmed by Kim and Lee (32) who also demonstrated that sequences between -135 and -67 bp are sufficient to confer G1/S-phase transcriptional regulation to a linked *Neo* gene.

Analysis of the promoter region between -135 and +30 bp reveals several putative regulatory elements (see Figure 5 of Chapter 1, page 69). Included within the -135 to -67 bp region is one inverted CCAAT box, several elements resembling binding sites for the transcription factor Sp1, and sequences similar to the consensus recognition sites for the transcription factors E2F and Yi (14). Yi is a protein identified in mouse A31 cells that forms G1/S-specific complexes with the mouse TK promoter. An additional inverted CCAAT box and a noncanonical TATA box (TTTAAA) are located downstream of -67.

DNAse footprint analyses have confirmed protein binding to each of these sites (3, 33), but the role of most of these elements, if any, in serum-mediated regulation is unclear. Mutational studies with the CCAAT box sequences determined that at least one CCAAT box is required for maximal promoter expression in continuously cycling cells (2), but the effect of these mutations on cell cycle-specific regulation was not tested. In addition, one gel shift mobility assay study, reporting G1/S-specific changes in nuclear protein complex formation on a CCAAT box element (35), has not been reproducible. Likewise, a role for the Sp1 elements in S phase-specific TK promoter induction has not been tested.

Recently, however, it was shown that replacement of sequences between -88 and -113 bp with nonhomologous sequences abolished G1/S phase-specific regulation of the TK promoter (33). Promoter fragments mutated in either half of this region (i.e. containing replacements of sequences between -88 and -101 bp or between -102 and -113 bp) were ineffective for competing for nuclear protein complex formations in gel mobility shift assays and displayed a loss in S phase-regulated transcription *in vivo*. The sequences contained within this 25 bp segment include the potential E2F binding sites, implicating E2F in serum-mediated G1/S regulation. This is intriguing since E2F sites are found in the promoters of many G1/S phase-regulated genes. In particular, E2F has been found to be both necessary and sufficient for G1/S induction of the DHFR promoter (56). While E2F has been shown to activate human TK gene expression in transient transfection assays (26), direct interaction of E2F with the human TK promoter has not yet been

demonstrated. Gel shift mobility studies with the 25 bp fragment mentioned above identified a constitutive nuclear protein complex containing the $p33^{cdk2}$ kinase and the G1/S-specific formation of a complex containing both cyclin A and p107 (39), proteins which have all been shown to interact with E2F (6, 47). However, oligomers containing a single consensus E2F site were ineffective at competing with the human TK promoter fragment for nuclear protein complex formation, and E2F antisera was inefficient at affecting complex formation (39), thereby questioning the role of E2F in TK promoter regulation.

As noted above, the -88 to -113 bp TK promoter segment also contains sequences similar to the mouse Yi consensus binding sites. While the Yi protein has not yet been identified, there is speculation that Yi may be similar to E2F, as the cell stage-specific Yi complexes have been found to contain the $p34^{cdc2}$ kinase and pRb (15), and pRb is a known E2F-associated protein (8). Ogris et al. (49) have also demonstrated a role for E2F in activation of the mouse TK promoter and have shown that the E2F site within this promoter is a target for trans-activation by Polyoma virus large T Antigen. In contrast to the mouse TK promoter, however, pRb has not been found associated with any of the human TK promoter complexes (39). Thus, important similarities and differences may exist between regulation of the human and mouse TK genes, and much work remains to be done to clarify the cellular proteins and promoter elements involved in transcriptional regulation of the human gene.

In addition, infection of serum-starved quiescent cells with the small DNA tumor virus Simian Virus 40 (SV40) effectively induces cells to re-enter the cell cycle and progress to S phase (34, 57), indicating that SV40 acts as a potent mitogen. As this virus requires the use of host cell replicative enzymes and proteins for the synthesis of progeny viral DNA, stimulation of quiescent or nonproliferating cells to a proliferating state is essential to virus survival. Therefore, the study of cellular gene expression in virus-

infected cells provides clues to the normal cell cycle control mechanisms which have been bypassed to coerce normally quiescent cells to proliferate.

Infection of cells with SV40 results in induction of both cellular DNA synthesis (57) and TK gene expression (34), and these inductions depend upon the viral replicative protein, large T-Antigen (T-Ag) (50). T- Ag is a multifunctional protein actively involved in the initiation of viral DNA replication, regulation of viral transcription, and trans-activation of many cellular and viral genes. The biochemical activities of the protein include those devoted to replicative functions, such as ATPase and helicase activities (10, 63), as well as those potentially involved in its mitogenic activities, such as the ability to bind DNA (7, 44, 64) and the ability to bind a variety of cellular proteins, including DNA polymerase α (12, 13), pRb (11, 41), and p53 (38). .

While much work has been done to elucidate TK promoter elements utilized in serum-mediated regulation, little work has been done to examine TK gene regulation in SV40-infected cells. The TK promoter is a good model system since it is the first cellular promoter naturally activated by T-Ag during viral infection to be studied, and SV40 therefore offers itself as a potent tool to uncovering clues to TK regulation. Previous studies in our lab have shown that, in SV40-infected CV1 cells, the mRNA levels increase at G1/S, concomitant with the increase in enzyme activity and protein levels, and this increase exceeds that detected in serum-stimulated CV1 cells (61). Nuclear run-on transcription assays in SV40-infected CV1 cells have also detected a modest (approximately fourfold) increase in the rate of TK gene transcription (52) indicating that some of the regulation is occurring at the level of transcription.

We have initiated a study of the TK promoter elements and SV40 T-Ag protein domains required for SV40-mediated TK transcriptional regulation. It is hoped that elucidation of the T-Ag domains required will yield clues as to the cellular proteins involved in promoter activation. We have designed a system which involves the infection of Rat-1 cell lines, stably transfected with a TK promoter-Luciferase hybrid gene (TK-

Luc), with recombinant adenoviruses expressing either wild-type or mutant forms of SV40 T-Ag. Our results indicate that the C-terminal p53-binding, ATPase and helicase activities of T-Ag are not involved in TK regulation. However, mutations within the pRb/p107-binding domain of T-Ag reduced TK promoter induction to a background level, suggesting the involvement of pRb and/or p107 in TK promoter regulation.

Based on this, and considering the known interaction of pRb with E2F (8) and the ability of T-Ag to disrupt this association (9), we examined the role of the TK promoter E2F-like sites in regulation by substituting sequences encompassing these sites with nonhomologous sequences, a mutation identical to that used by Kim and Lee (33) to study the role of this region in serum-mediated regulation. Luciferase induction by T-Ag-expressing adenoviruses was severely impaired in cell lines transfected with the mutant TK-Luc construct. These results indicate that the putative E2F binding sites are important for T-Ag-mediated TK promoter induction, invoking a potential activation mechanism involving associations between T-Ag, pRb/p107, and an E2F-related protein.

Materials and Methods

Cell culture. Rat-1 cells were cultured at 37°C in Dulbecco's Modified Eagle's medium (DME) supplemented with 10% calf serum (HyClone Laboratories, Logan, Utah). Stably-transfected cells containing hygromycin-resistance (*Hgm*) genes were maintained in medium containing 50 μg/ml hygromycin B (CalBiochem-Novabiochem Corporation, La Jolla, CA). Cells were grown in medium without hygromycin B for all virus infection experiments.

Viruses. Recombinant adenovirus vectors expressing either wild-type or mutant forms of SV40 large T Antigen (Ad T/dl309) were a kind gift from Dr. Charles Cole and Jun Chen at Dartmouth University. Briefly, these viruses contain T-Ag coding sequences, under control of the SV40 promoter, inserted in place of the E1A and E1B genes in Adenovirus dl309 (29). Small 6 bp deletions within the SV40 origin of replication abrogated SV40-directed replication to render the viruses completely replication-defective in the absence of the adenovirus E1A protein. Mutations within the T-Ag coding sequence include point mutations and small insertions or deletions and are described elsewhere (30, 67).

Viral stocks were prepared by infecting confluent monolayers of human 293 cells, a cell line transformed with adenovirus sequences and constitutively expressing E1A (21), with single-plaque-derived virus resuspended in 5 ml 1X DME + 2 % calf serum. An MOI of 1 gave the highest viral titers. CPE (cytopathic effect) was observed two to three days following infection, as indicated by the rounding up and detachment of cells. At this point, cells were scraped from the plate, and both cells and cell supernatant were transferred to a sterile centrifuge tube. Cells were lysed by two rounds of freezing and thawing then centifuged at 2000 RPM for 30 min at 4°C to pellet cell debris. Supernatant was filtered through a 0.45μm filter, aliquoted, and stored at -70°C. Viral titers were determined by plaque assays on 293 cells and generally ranged between $5x10^8$ PFU/ml and $1x10^9$

PFU/ml. The absence of wild-type recombinant virus was verified by plaquing viral stocks on HeLa cells. Mock virus is a 293 cell lysate prepared by a procedure identical to that used for generating viral stocks except that confluent monolayers of 293 cells received 5 ml of 1X DME + 2% calf serum medium without virus.

To prepare UV-irradiated virus stocks, a minimal volume of virus was sterilely transferred to an appropriately-sized tissue culture plate (i.e. 2.5 ml virus per 60 mm diameter dish) then placed uncovered in a UV Stratalinker 1800 cross-linker (Stratagene, La Jolla, CA). Viruses received 6 hits of 1200 μJoules ultraviolet light in 2 min intervals, and viral solutions were mixed by swirling plates between each hit. Virus was irradiated just prior to use, then placed on ice. To minimize light exposure and prevent potential light-induced repair mechanisms, irradiated stocks were kept covered with aluminum foil, and infections were performed in the absence of tissue culture hood fluorescent lights.

Ad T/dl309 infections. To synchronize cells in G0, cells were plated at a density of $1x10^5$ cells per 60 mm diameter tissue culture plate, grown to confluence (approximately 5 days), then serum starved for 24 h in medium containing 0.5% calf serum. For infection, the low serum medium was removed at time zero and saved. Medium from each cell line was kept separate. Virus was diluted in 1X DME + 0.5% calf serum, and cells were infected at a multiplicity of infection (MOI) of 200 in a total volume of 0.6 ml low serum medium. Infections were done for 1.5 h at 37°C. Following infection, the low serum medium that had been removed at time zero was replaced, and plates were returned to the 37°C incubator until harvest.

Plasmid and promoter mutation constructions. PGL2-Basic, a promoterless luciferase vector, was obtained from Promega Corporation (Madison, WI). pY3 (4) is a plasmid conferring Hgm^R. 135-Luc consists of TK promoter sequences between -135 and +30 bp, relative to the transcriptional start site, subcloned upstream of the firefly luciferase gene

(*Luc*) in PGL2-Basic, and was generated by digestion of plasmid pH10-TK, which contains the human TK cDNA under control of this 135 bp TK promoter fragment, with *Sca*I followed by isolation of the resulting 779 bp fragment containing the promoter piece. *Hin*dIII linkers were then added and the desired 165 bp fragment was liberated by digestion with *Hin*dIII + *Sal*I. The fragment was subcloned into PGL2 Basic at the *Xho*I and *Hin*dIII sites within the polylinker. HTK-Luc contains 131 bp of the Herpes Simplex Virus (HSV) TK promoter (-75 to +56 bp) (43) linked to *Luc* and was constructed by digestion of pTK2, a plasmid placing the human TK cDNA under control of the HSV TK promoter, with *Eco*R1. A 1 kb piece was subsequently isolated, ligated with *Sac*I linkers, and further digested with *Sac*I + *Bgl*II. The resulting fragment was subcloned into PGL2 Basic at the polylinker *Sac*I and *Bgl*II sites. 135(E2F)-Luc contains TK promoter sequences between -135 and +30 bp, with a substitution of nonhomologous sequences for promoter sequences between -98 and -113 bp, linked to *Luc*. Sequences between -98 and -113 bp comprise the distal portion of the 25 bp serum regulatory region described by Kim and Lee (33) and contain two adjacent protein binding domains and two of three putative E2F recognition sites. The mutation was generated via oligonucleotide-mediated mutagenesis in M13, as described by Kunkel (37). A 40 bp oligonucleotide, with 12 bp matches on either side of the sequence to be mutated, was used in the mutagenesis. The presence of the desired mutation was confirmed by dideoxy DNA sequencing and the resultant mutant promoter fragment was excised from M13 with appropriate restriction endonucleases and subcloned upstream of *Luc* in the PGL2-Basic vector.

DNA transfections. Stable transfections into Rat-1 cells were performed using *Lipofectin* Reagent (BRL Life Technologies, Gathersburg, MD) according to the procedure supplied by the manufacturer. Briefly, Rat-1 cells were plated at a density of $5x10^5$ cells per 60 mm diameter tissue culture dish 24 h prior to transfection. All DNA preparations used were either banded in CsCl or prepared with Qiagen Maxiprep columns (Qiagen

Corporation, Chatsworth, CA). For each transfection to be done, 8 μg TK-Luc and 2 μg pY3 were coprecipitated, resuspended in 10 μl of sterile ddH_2O, then mixed with 90μl of 1X DME minus serum and penicillin/streptomycin. In separate sterile tubes, one tube for each transfection to be done, 20 μl *Lipofectin* reagent was mixed with 80 μl 1X DME minus serum and penicillin/streptomycin. The DNA and lipofectin solutions were then combined, mixed gently, and allowed to stand at room temperature for 15 min, after which 1.8 ml 1X DME minus serum and penicillin/streptomycin was added. Cells were rinsed twice with several mls of 1X DME minus serum and penicillin/streptomycin, overlayed with the DNA/*Lipofectin* solution and incubated at 37°C in an atmosphere of 5% CO_2 for 10 hours, after which time the DNA/*Lipofectin* solution was removed and replaced with 5 mls 1X DME + 10 % calf serum media. 48 h after removal of the *Lipofectin* solution, cells were split into medium containing 200 μg/ml hygromycin B. When resistant colonies emerged, 10-50 colonies were pooled and several colonies were picked and propagated as clonal cell lines. After initial selection, transfected cells were maintained in medium containing 50 μg/ml hygromycin B.

Preparation of whole cell extracts and luciferase assays. 1X Luciferase Cell Culture Lysis Buffer was prepared by dilution of 5X Luciferase Cell Culture Lysis Buffer [125 mM Tris, pH 7.8 with H_3PO_4, 10 mM CDTA (1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid), 10 mM DTT, 50% glycerol, and 5% Trition X-100] obtained from Promega Corporation (Madison, WI). D-Luciferin (sodium salt), acetyl coenzyme A, $MgCO_3$, and Tricine were obtained from Sigma (ST. Louis, MO) and used to prepare the luciferase substrate solution according to the recipe (270 μM coenzyme A, 470 μM Luciferin, and 530 μM ATP) supplied by Promega Corporation with its Luciferase Assay System. After preparation, substrate solution was aliquoted and stored at -70°C until use. To prepare whole cell extracts for analysis of luciferase activity, cells were removed from the incubator 72 h following infection. Medium was removed, and cells were rinsed with 2 ml

cold PBS, then stored on a bed of ice during harvest. To harvest, cells were scraped from the plates with a rubber policeman in 1.5 ml cold PBS into a microfuge tube on ice. Cells were pelleted and the PBS was removed by aspiration. Cell pellets were then resuspended in 100 µl 1X Cell Culture Lysis Buffer and allowed to lyse for 15 min at room temperature. Extracts were then centrifuged for 1 min at room temperature to pellet cell debris and DNA. Supernatants were transferred to fresh microfuge tubes and stored at -70°C until assayed.

The luciferase assay procedure was done according to the protocol supplied by Promega Corporation. Briefly, extracts and substrate were thawed and allowed to equilibrate to room temperature. 20 µl extract was placed in an 8x50 mm polypropylene tube (Turner Designs, Sunnyvale, CA) in a Turner TD-20E Luminometer (Turner Designs). 100 µl of substrate solution was then injected and luciferase expression was measured during a 1 min reaction. Luciferase activity in each sample (expressed as light units [LU]) was normalized to total protein content to calculate the specific activity of the sample (LU/µg protein). For each infection, fold inductions in luciferase expression were determined by dividing the specific activity at 72 h following infection by the corresponding activity at time zero.

TK enzyme assays. Assays for endogenous TK enzyme activity were performed using the whole cell extracts prepared in the 1X Luciferase Cell Culture Lysis Buffer. Assays were performed as previously described (61). TK activities (cpm) were normalized to total protein content, and endogenous TK enzyme inductions were calculated by dividing TK specific activity (cpm/µg protein) at 72 h after infection by specific activity at time zero.

Protein quantitation, sodium dodecyl sulfate-polyacrylamide gel electophoresis, and Western blotting. The amount of protein in the whole cell extracts was determined using the Bio Rad Protein Assay system (Bio Rad, Richmond, CA) based upon the method of

Bradford (5). To verify the presence of SV40 large T Antigen, 25-30 μg protein from whole cell extracts were mixed with SDS Gel Loading Buffer (0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 2% [w/v] SDS, 0.72 M 2-mercaptoethanol, and 0.00125% bromphenol blue), boiled, and electrophoresed on 8% SDS-Page Bio-Rad minigels in a buffer composed of 0.124 M Tris, 0.959 M glycine, and 0.5% (w/v) SDS at 150 V. After electrophoresis, proteins were transferred to polyvinylidene difluoride polyscreen membrane (Dupont-NEN, Boston, MA) at 30 V for 1.5 h in Western transfer buffer (20 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) using the Novex miniprotein transfer system (Novex Corp., San Diego, CA). Membranes were blocked overnight in 5% nonfat dried milk in 1X PBS + 0.1% Tween 20 (PBS/Tween) at 4°C. After a brief rinse in PBS/Tween, membranes were incubated with the T-Ag-specific monoclonal antibodies L19 (23), which recognizes N-terminal T-Ag determinants or PAB416 (kindly supplied by the lab of Dr. Ed Harlow, Massachusetts General Hospital Cancer Center), which recognizes C-terminal T-Ag determinants, for 1 h at room temperature with gentle agitation. Antibodies were diluted 1:20 and 1:500, for L19 and PAB416, respectively, in PBS/Tween + 5% nonfat dried milk. Removal of the 1° antibody was followed by four 15-min washes in PBS/Tween, then incubation with 2° antibody, a goat anti-mouse IgG-HRP conjugate (Bio Rad, Richmond, CA) diluted 1:5000 in PBS/Tween + 5% nonfat dried milk, for 1 h at room temperature with gentle agitation. Removal of the 2° antibody solution was again followed by four 15-min washes with PBS/Tween, after which the membranes were reacted with the *Lumiglow* chemiluminescent reagents (Kirkegaard and Perry, Gaithersburg, MD) as per manufacturer's protocol. Blots were covered with plastic wrap and immediately used to expose x-ray film (Amersham Hyperfilm MP, Amersham Corporation, Arlington Heights, IL).

Results

Generation of stable TK-Luc Rat-1 cell lines and use of T-Ag-encoding viruses. The goal of this study was to identify both TK promoter elements and T-Ag protein domains required for viral-mediated trans-activation. To facilitate the analysis of various combinations of wild-type and mutant TK promoters and T-Ag proteins, an assay system measuring TK promoter-directed luciferase expression was utilized. Luciferase was chosen as the reporter gene because its expression can be measured very sensitively and accurately. To begin, a variety of TK promoter-luciferase hybrid genes (TK-Luc) were constructed, as detailed in Materials and Methods. The TK-Luc constructs used in these studies are diagrammed in Figure 1. 135-Luc represents the wild-type promoter fragment and consists of TK sequences extending from 135 bp upstream (-135) to 30 bp downstream (+30) of the major transcriptional start site (36). Putative regulatory elements located within this region are listed in Figure 5 of Chapter 1 (page 69) and include a number of sequences resembling binding sites for the transcription factor E2F. 135(E2F)-Luc contains a mutant promoter fragment with nonhomologous sequences substituted from -98 to -113 bp, effectively replacing two of three adjacent putative E2F sites. HTK-Luc is a control plasmid that contains HSV TK promoter sequences extending from 75 bp upstream to 56 bp downstream of the HSV transcriptional start site. Elements contained within this fragment include the HSV TATA box and one of the upstream control regions, DSI, both required for maximal promoter expression. Since HSV TK is a non-cell cycle-regulated promoter, it should not be responsive to T-Ag in this system.

The TK-Luc hybrid genes described above were stably cotransfected, along with a plasmid conferring *Hgm*^{R}, into Rat-1 cells. Rat cells were chosen for these experiments because they form good monolayers, are fast-growing, easily transfected, and respond well to serum starvation. In previous experiments, we have shown that transfected human TK minigenes are highly regulated in these cells (51). Stable, rather than transient,

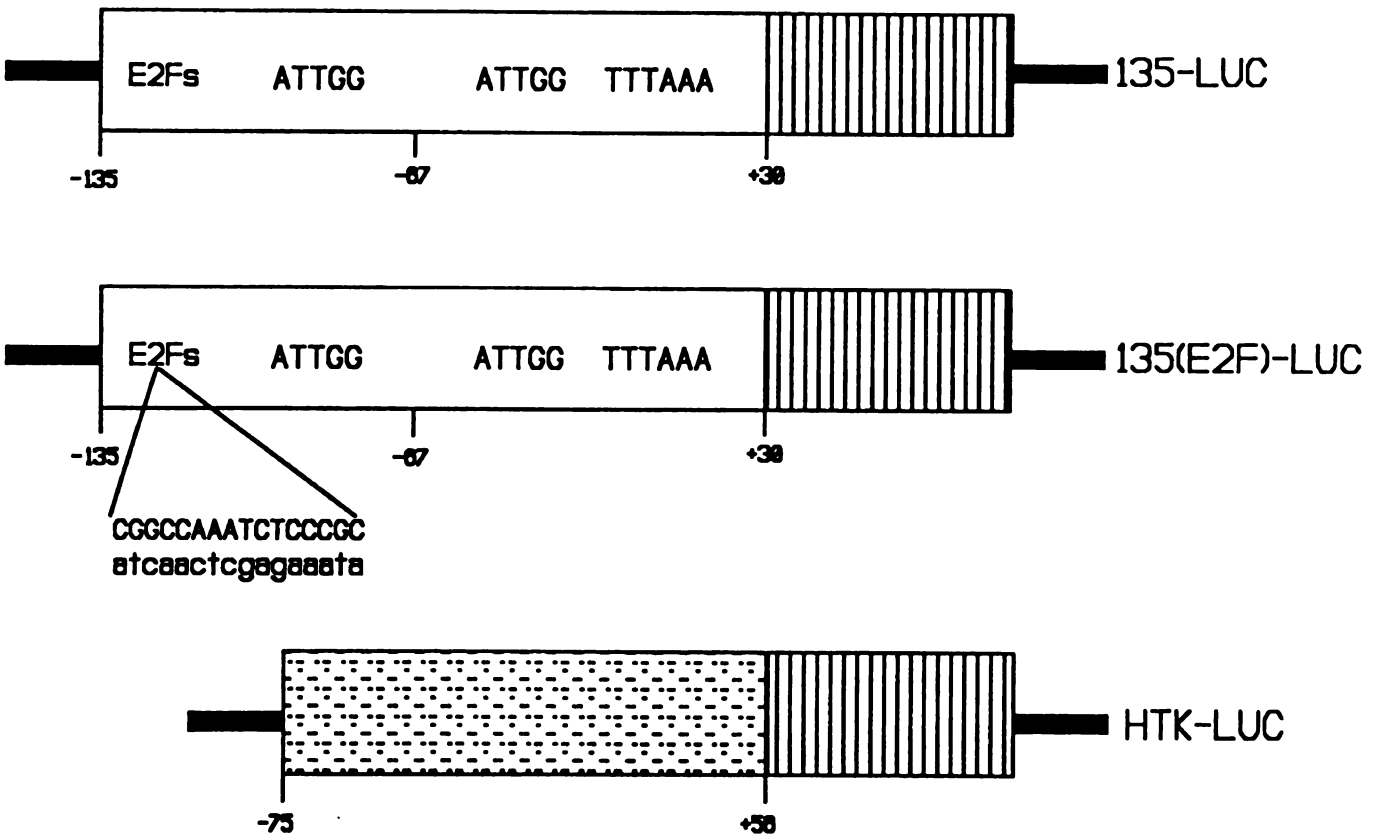


Figure 1. Structure of human TK promoter-Luciferase hybrid genes. Open bar: human TK promoter region. Stippled bar: HSV TK promoter sequences. Vertical striped bar: luciferase reporter gene. Solid bars: prokaryotic vector sequences. Putative regulatory elements within the human TK promoter region are indicated. Numbers refer to sequence locations within the respective TK promoter. Sequences mutated in 135(E2F)-Luc are indicated: the wild-type promoter sequence is shown in upper case and the nonhomologous substitution sequence is shown below it in lower case.

transfections were chosen for a number of reasons. First, T-Ag is a promiscuous transcriptional activator (1, 69), having a tendency to stimulate a multitude of genes when they are presented as plasmid templates. In fact, initial analyses of the TK-Luc hybrid genes were attempted via transient cotransfections into simian CV1 cells with plasmids expressing either wild-type or mutant forms of SV40 large T-Ag. In those experiments, the magnitude of induction of luciferase expression from negative control plasmids, such as the promoterless luciferase vector PGL2-Basic, was as great or greater than that of the TK-Luc constructs, making the delineation of specific T-Ag/TK promoter interactions impossible. In addition, controlling for variable parameters, such as transfection efficiencies, in transient transfections involving T-Ag is difficult; due to the promiscuity of T-Ag trans-activation, almost all of the reporter plasmids normally used for this type of control are activated. In contrast, only a select population of chromosomal genes are activated following SV40 infection. This is illustrated, for example, by the RSV LTR, which has been shown to be trans-activated by T-Ag in an episomal state in transient transfection assays (69), but is not activated upon SV40 infection when it is stably integrated into the chromosome (52). We therefore felt that stable transfections would eliminate problems with variable transfection efficiencies, normalization, and non-specific trans-activation by T-Ag, and that events leading to activation of the integrated TK-Luc gene would most closely approximate the mechanisms utilized to induce cellular genes following infection with SV40.

A second reason for using stably transfected cells is that a number of technical problems exist with transient assays when looking at gene expression in cells that are to be serum starved then stimulated. Most notably, it is difficult, within the time constraints of the technique, to both effectively synchronize cells in G0 and efficiently introduce DNA, since cells must be actively dividing in order to take up DNA. For this reason, stable transfections were the most attractive method for our studies.

Following transfection, *Hgm*^{R} colonies were selected, and individual colonies were picked and expanded as clonal cell lines. In addition, pools of 10 to 50 colonies were generated. After expansion, cycling populations of each colony and pool were tested for luciferase expression, as described in Materials and Methods. Colonies expressing no luciferase activity were discarded. Likewise, colonies expressing abnormally high levels of activity (i.e. thousands of light units) were not chosen for analysis. Colonies chosen for analysis contained moderate levels of luciferase activity in cycling cells.

The system we have used to introduce T-Ag into cells is viral infection using an adenovirus vector. Infection, as opposed to transfection, was chosen as the best method for introducing T-Ag into cells for two reasons. First, infection is a much more efficient process; 100% of the cells can be infected. This is particularly important when dealing with a quiescent population of cells, where transfections are quite inefficient. Second, by using infection, our system would, again, most closely parallel the natural sequence of events occurring upon infection of a host cell by SV40.

Recombinant adenoviruses were used for the Rat-1 cell infections for two reasons, as well. First, it was discovered previously in our laboratory that SV40 is unable to either infect and/or express its early genes in rat cells (52). Either required cell surface receptors are missing, preventing virus binding, or cellular factors necessary for the transcription of viral genes are lacking. Since T-Ag appears to be efficiently expressed from plasmids in transient transfection assays (31, 69), and reporter genes expressed from the SV40 early promoter (i.e. pSV2Neo) are expressed in rodent cells (25, 68, 70), it seems likely that the problem lies with the viral receptor or mechanism of virus internalization. Second, several of the T-Ag mutants we want to study are replication defective and therefore cannot be grown as SV40 virus. In theory, the defective viruses could be grown in Cos7 cells, which are simian cells stably transformed with SV40 that constitutively express wild-type T-Ag, but many of the mutants are trans-dominant and therefore do not grow well, even in these cells. To alleviate these problems, the recombinant adenovirus vector Ad T/dl309,

which contains T-Ag coding sequences in place of the adenovirus E1A and E1B genes, was used as a mechanism for delivering T-Ag to the rat cells. This virus is completely replication defective in the absence of the E1A and E1B proteins, but can be propagated on 293 cells, which constitutively express E1A, and should effectively deliver T-Ag coding sequences to the cell nucleus where they can be transcribed and translated.

Establishing the luciferase assay system. Preliminary experiments were conducted to establish conditions for the efficient infection of rat cells with the recombinant adenoviruses. Stably transfected Rat-1 cells were infected at MOIs of 0, 10, 25, 50, 100, and 200, and cells were harvested for both luciferase and endogenous TK enzyme assays at 0, 12, 24, 36, 48, 60, and 72 h following infection. In addition, coverslips containing infected cells were processed for detection of T-Ag by immunofluorescence at each of the time points.

The number of cells expressing T-Ag increased with both increasing MOI and time of incubation, with 100% of the cells staining positive for T-Ag by 72 h post-infection at an MOI of 200. Likewise, expression of both the transfected luciferase gene and the endogenous TK gene increased gradually throughout the time course, attaining the highest levels of induction, again, by 72 h post-infection at an MOI of 200 (data not shown). All subsequent experiments were therefore carried out by infecting for 72 h at an MOI of 200.

To assay for T-Ag-mediated TK promoter induction, Rat-1 cell lines stably transfected with the TK-Luc hybrid genes were grown to confluence, serum starved for 24 h, then infected, as noted above, with a recombinant adenovirus expressing SV40 large T Antigen. Whole cell extracts were prepared at both the time of infection (T=0) and 72 h after infection (T=72) and assayed for luciferase activity as described in Materials and Methods. For most experiments, extracts were also assayed for endogenous TK enzyme activity, to verify that cells had efficiently arrested and restimulated, and subjected to SDS-PAGE analysis with subsequent immunoblotting for SV40 large T Antigen, to verify

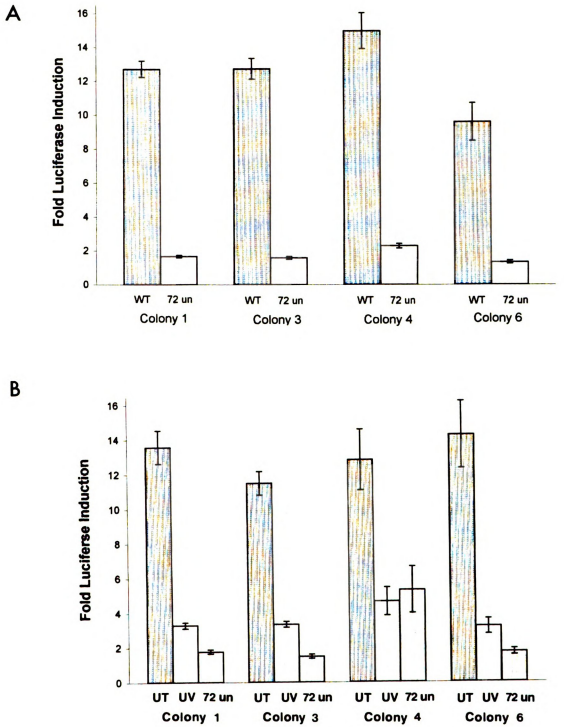
infection. Both luciferase and TK enzyme activities were normalized to total protein levels. Experiments were often performed with duplicate sets of plates and repeated several times with multiple batches of virus preparations. As we detected some interassay variability in the levels of T-Ag produced following infection, data was discarded for any experiment in which T-Ag expression and/or endogenous TK enzyme inductions were very low. Finally, expression of each of the individual TK-Luc constructs was measured in a set of multiple, independent clonal cell lines.

Measurement of luciferase expression in multiple clonal cell lines was used to circumvent potential integration site effects. While analysis of pools of colonies is a popular alternative to the use of multiple independent colonies, preliminary results with 135-Luc revealed that luciferase expression in colonies, but not pools, was significantly induced in the presence of T-Ag (data not shown). Since cell lines were generated by cotransfection, with subsequent selection for Hgm^R, the presence of the *Luc* gene was not directly selected. The pools tested, then, represent a heterogeneous population of colonies, many of which may harbor the *Hgm*, but not the *Luc*, gene.

Induction of TK-Luc hybrid gene constructs in cells infected with wild-type Ad T/dl309. Cells from Rat-1 clonal cell lines containing stably-integrated 135-Luc were synchronized in G0, then infected with Ad T/dl309 recombinant adenovirus, as described above and in Materials and Methods. Figure 2A shows the luciferase induction levels, relative to the T=0 time point, for each of four clonal cell lines infected with wild-type virus. The graph is a composite, with the results representing the average level of induction determined from multiple experiments. Induction levels range from nine- to fifteenfold, indicating that the 135 bp promoter fragment is activated in this system.

To verify that activation of 135-Luc is due specifically to viral gene expression, serum-starved cells were infected with UV-irradiated virus in parallel with the untreated Ad T/dl309. Luciferase induction levels, representing the average of multiple experiments,

Figure 2. Induction of luciferase in 135-Luc Rat-1 cells infected with untreated or UV-irradiated wild-type virus. Results are shown for four independent clonal cell lines. Confluent cells were serum-starved then infected with recombinant adenovirus expressing SV40 T-Ag (Ad T/dl309). Whole cell extracts were prepared for luciferase analysis at both the time of infection (0 h) and 72 h after infection. Results are grouped by colony, and each bar represents the average of multiple experiments. Fold luciferase induction indicates the increase in luciferase expression at 72 h relative to expression at 0 h and is calculated as described in Materials and Methods. Error bars reflect the standard error of the mean. **(A)** Luciferase induction following infection with untreated, wild-type (WT) virus. 72 un refers to uninfected cells maintained in low serum medium throughout the infection. For each sample, the number of experiments (n) comprising the average is as follows: Colony 1: WT, n = 27; 72 un, n =17. Colony 3: WT, n = 22; 72 un, n = 15. Colony 4: WT, n = 17; 72 un, n = 11. Colony 6: WT, n = 11; 72 un, n = 9. **(B)** Luciferase induction following infection with UV-irradiated (UV), wild-type virus. UV-irradiated virus stocks were prepared as described in Materials and Methods. Untreated (UT) virus was not subjected to UV-irradiation and is identical to the WT virus displayed in panel (A). 72 un is as described for panel (A). For each sample, the number of experiments comprising the average (n) is as follows: Colony 1: UT, n = 10; UV, n = 9; 72 un, n = 9. Colony 3: UT, n = 10; UV, n = 10; 72 un, n = 9. Colony 4: UT, n = 5; UV, n = 5; 72 un, n = 5. Colony 6: UT, n = 5; UV, n = 5; 72 un, n = 4.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Fold Luciferse Induction
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Colony 1
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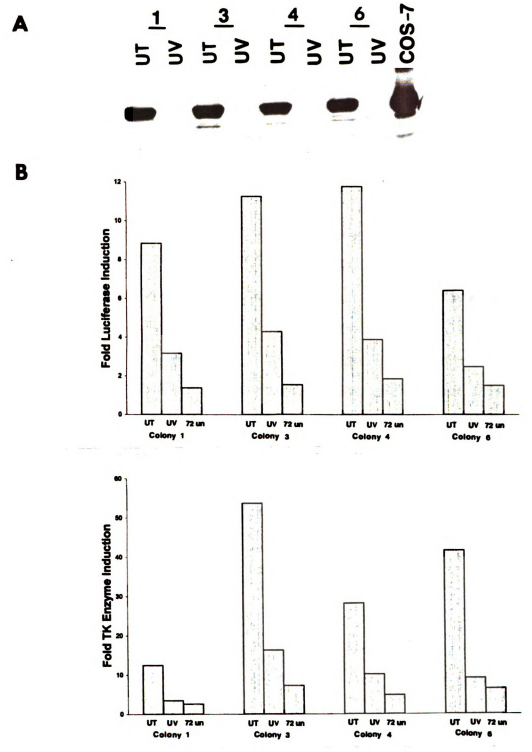
Colony 3
UT UV 72 un
Colony 4
UT UV 72 un
Colony 6

Figure 2.

are depicted in Figure 2B. For each colony, luciferase expression after viral infection is reduced approximately 70% by UV irradiation of the virus. Western blot analyses of whole cell extracts, using the T-Ag-specific monoclonal antibody PAB416, reproducibly showed no detectable T-Ag in extracts from cells infected with UV-irradiated virus, indicating that the UV-irradiation treatment effectively abolished the expression of T-Ag. A representative immunoblot from one experiment is shown in Figure 3A, and Figure 3B compares the patterns of luciferase and endogenous TK enzyme inductions in that same experiment. The TK pattern is representative of that seen in all experiments involving infection with UV-irradiated virus. In particular, note that endogenous TK enzyme induction levels are similarly reduced.

The ramifications of these results are twofold. First, activation of the TK promoter is largely dependent upon the expression of viral early genes, since, in the absence of detectable levels of T-Ag, expression from both the transfected TK-Luc hybrid gene and the endogenous TK gene is greatly reduced. Second, there exists a mechanism for T-Ag-independent, or background, activation, as UV-irradiated virus preparations are still capable of activating 135-Luc to 30% of wild-type levels. This background could derive from activation due to signal transduction events triggered by virus binding to cell surface receptors. Precedence for this is found in the work of Zullo et al. (71) who detected increases in c-myc and c-fos mRNA levels following exposure of cells to either polyomavirus empty virions or recombinant VP1 protein, suggesting that the interaction of polyomavirus with cell surface receptors was sufficient to trigger activation of these cellular genes. Alternatively, the background level of induction seen in our experiments may be due to residual, yet undetectable, T-Ag expression or to the presence of a 293-derived factor in the virus preparations. That the latter explanation is particularly unlikely is illustrated in Figure 10, which shows practically no induction of 135-Luc in cells mock-infected with a 293 cell lysate.

Figure 3. T-Ag protein, luciferase, and endogenous TK enzyme expression in 135-Luc Rat-1 cells infected with UV-irradiated virus. Results are shown for one experiment with four independent clonal cell lines. Confluent cells were serum-starved then infected with either UV-irradiated (UV) or untreated (UT) wild-type Ad T/dl309 virus at an MOI of 200. Whole cell extracts were prepared at both the time of infection (0 h) and 72 h after infection and analyzed by Western blot for T-Ag protein and by enzyme assay for luciferase and endogenous TK enzyme expression, as described in Materials and Methods. **A)** Immunoblot of T-Ag protein in infected cell extracts. For each colony, 25 μg protein from 72 h whole cell extracts was loaded on an 8% SDS-PAGE minigel and analyzed as described in Materials and Methods. T-Ag was detected using a monoclonal antibody specific for C-terminal determinants of the protein (PAB416). COS7: 10 μl of a whole cell extract prepared from continuously cycling COS7 cells (SV40-transformed simian CV1 cells which stably express T-Ag) was loaded as a marker for T-Ag. **(B)** Increase in luciferase expression at 72 h after infection, relative to expression at 0 h, for cells infected with UV-irradiated (UV) virus, unirradiated virus (UT) or uninfected cells (72 un). **(C)** Increase in endogenous TK enzyme expression at 72 h after infection, relative to expression at 0 h for cells infected as in (B). For both panels (B) and (C), values reflect normalization to total protein content (see Materials and Methods for calculations).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Colony 3
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Colony 6
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Colony 6

Figure 3.

To verify that the T-Ag-mediated activation described above is specific to the TK promoter, clonal cell lines containing HTK-Luc were infected with both untreated and UV-irradiated Ad T/dl309, and assayed for luciferase expression at 0 and 72 h following infection. As mentioned earlier, the HSV TK promoter is a constitutively expressed, non-cell cycle-regulated promoter (54) and should not be specifically activated in the presence of T-Ag. Figure 4A is a composite graph displaying average levels of luciferase induction for five independent colonies. Expression in four of the five colonies was induced only two- to threefold upon infection with the untreated Ad T/dl309 virus. This level of activation is consistent with the low level of T-Ag-independent induction detected above. The induction of HTK-Luc Colony 9 is more substantial, being approximately sixfold.

Average induction levels for HTK-Luc colonies infected with UV-irradiated virus are shown in Figure 4B. As before, no T-Ag was detected by Western blotting in extracts from cells infected with UV-irradiated virus, while cells infected with untreated virus contained significant amounts of T-Ag (data not shown). Luciferase induction levels attained following infection with the UV-irradiated virus are not much lower than those attained upon infection with untreated, wild-type virus, indicating that the two- to threefold induction of HTK-Luc (sixfold induction for Colony 9) is indeed the result of T-Ag-independent events.

In summary, these experiments have shown that the 135 bp TK promoter is induced nine- to fifteenfold by infection with a T-Ag-expressing virus. Approximately 70% of this induction is T-Ag-dependent and promoter-specific, while the remaining 30% induction arises from T-Ag-independent mechanisms and is not promoter-specific.

Induction of 135-Luc in cells infected with mutant Ad T/dl309 viruses. Having established that the -135 to +30 bp human TK promoter fragment is specifically activated by T-Ag, we next wished to determine the domains of the T-Ag protein required for this activation. The T-Ag protein, diagrammed in Figure 5, contains many functional domains,

Figure 4. Luciferase induction in HTK-Luc Rat-1 cells infected with untreated or UV-irradiated wild-type virus. Confluent cells were serum-starved then infected with recombinant adenovirus expressing SV40 T-Ag (Ad T/dl309). Whole cell extracts were prepared for luciferase analysis at both the time of infection (0 h) and 72 h after infection. The graphs are composites, with each bar representing the average of multiple experiments. Fold luciferase induction indicates the increase in luciferase expression at 72 h relative to expression at 0 h and is calculated as described in Materials and Methods. Error bars reflect the standard error of the mean. **(A)** Luciferase induction following infection with untreated, wild-type (WT) virus. Results are shown for four independent clonal cell lines. 72 un refers to uninfected cells maintained in low serum medium throughout the 72 h infection. For each sample, the number of experiments (n) comprising the average is as follows: Colony 1: WT, n = 4; 72 un, n = 2. Colony 8: WT, n = 5; 72 un, n = 4. Colony 9: WT, n = 7; 72 un, n = 6. Colony 10: WT, n = 5; 72 un, n = 6. **(B)** Luciferase induction in cells infected with UV-irradiated virus (UV) or unirradiated (untreated) virus, and in uninfected cells (72 un). Results are shown for three independent colonies. For all samples, n = 2.

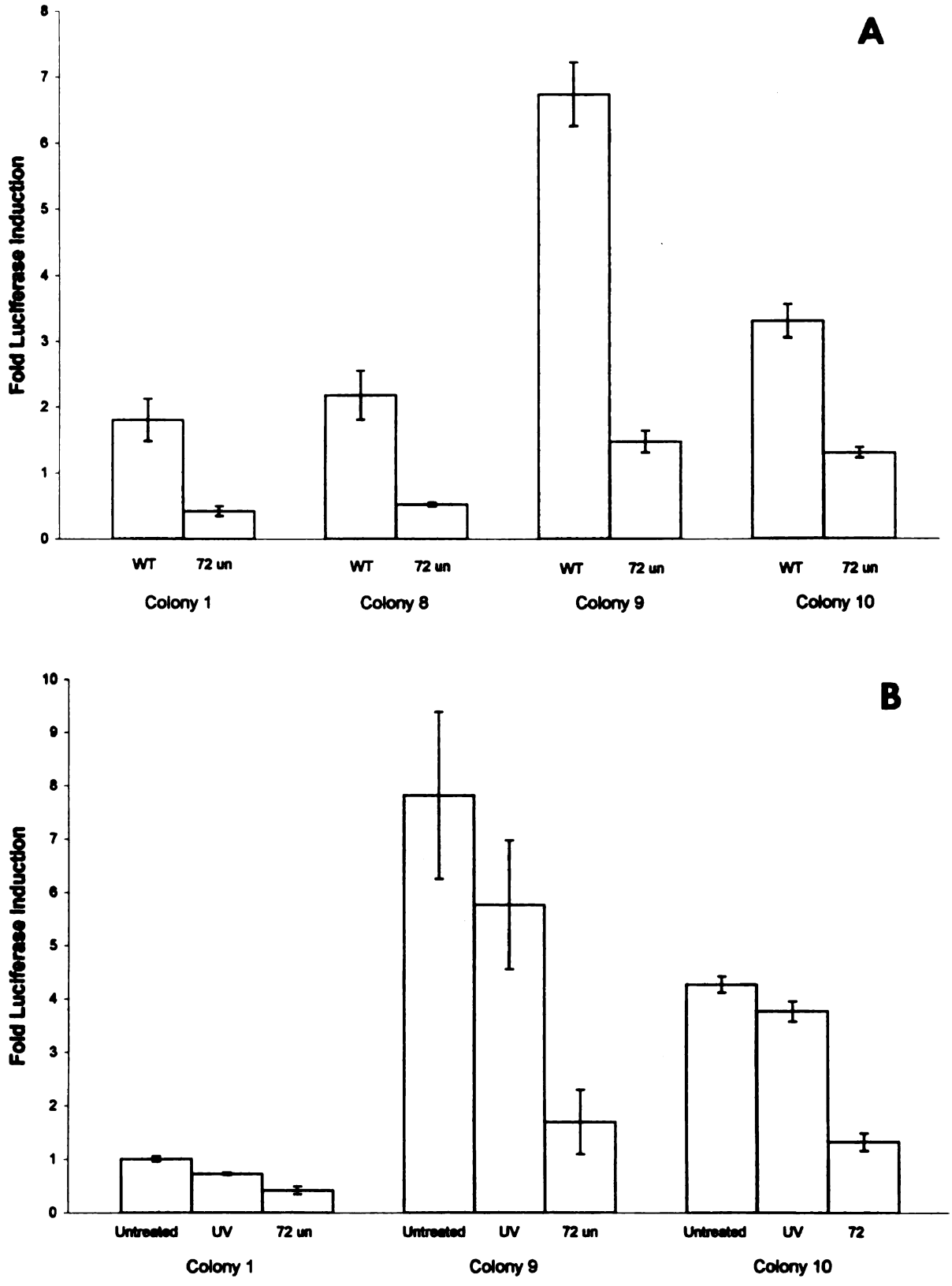
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Figure 4.

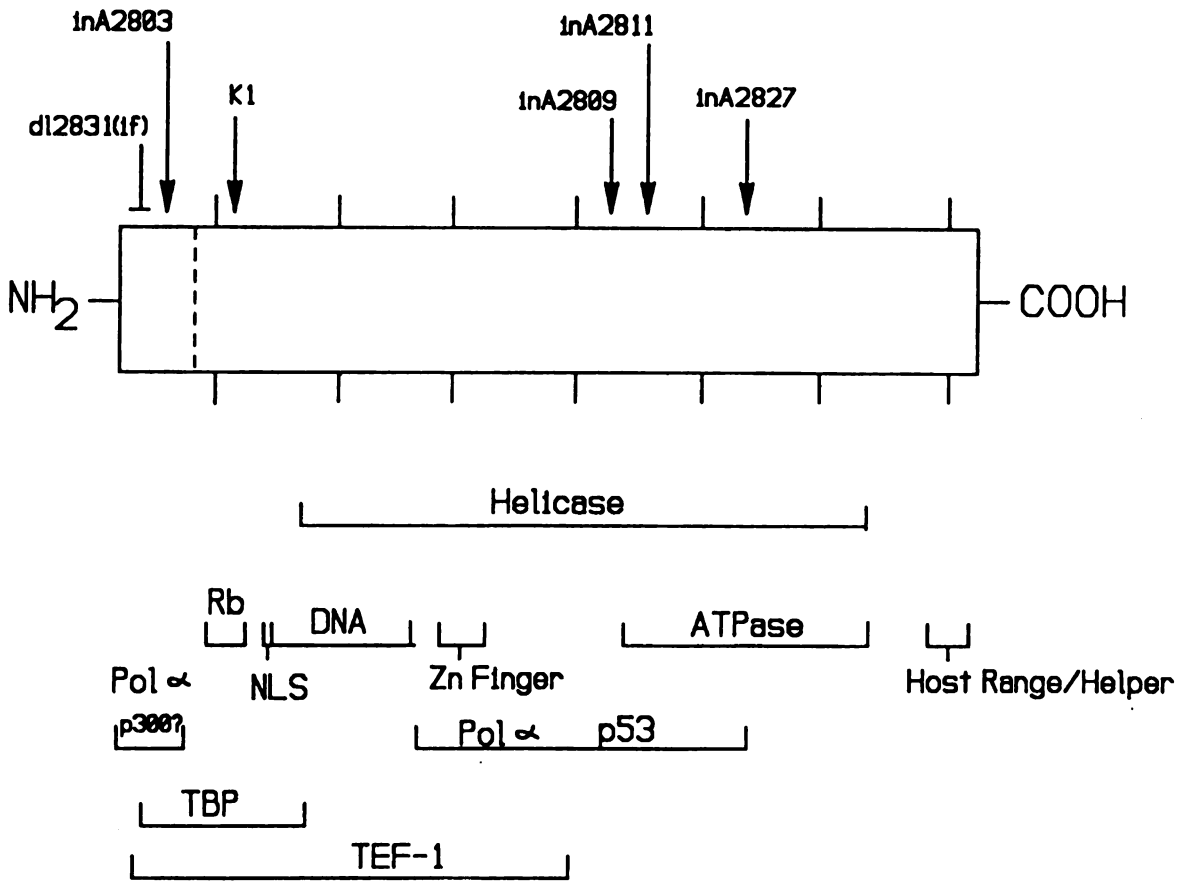


Figure 5. SV40 large T antigen protein domains and mutations. Vertical spikes on the map represent every 100 amino acids. The exonI-exonII boundary at amino acids 82-83 is indicated by a vertical dashed line. Labeled brackets below the map indicate domains of known function. T antigen mutations analyzed in this study are indicated above the diagram. "in" indicates an insertion and "dl" refers to a deletion. In-frame mutations are designated by (if).

some of which are involved in the binding to, or interaction with, cellular proteins such as the tumor suppressor gene products pRb (11, 41), p107 (17), and p53 (38), the TATA binding protein TBP (22), DNA polymerase α (12, 13), and p300 (65), a protein known to be required for the adenovirus E1A-mediated induction of cellular proliferation (59). It is hoped that the identification of T-Ag protein domains required for activation of the human TK promoter will provide additional clues as to the cellular proteins involved in TK regulation.

To assess the requirement for various T-Ag functional domains, a series of Ad T/dl309 viruses expressing mutant T-Ag proteins were obtained from Dr. Charles C. Cole and Jun Chen at Dartmouth. The mutant genes contain point mutations or small insertions or deletions within T-Ag coding sequences. As shown in Figure 5, three mutations (inA2809, inA2811, and inA2827) are located within the overlapping C-terminal ATPase, helicase, and p53-binding domains of the protein, while the remaining three mutations (inA2803, dlA2831, and K1) localize to N-terminal domains utilized for interaction with cellular factors. In particular, the K1 mutation renders T-Ag defective for binding both pRb and p107 (11, 19). T-Ags harboring the inA2809 and inA2811 mutations are defective in the ability to bind p53 while inA2827 T-Ag binds p53 with wild-type affinity (68). Previous studies with plasmids encoding these mutations in transient transfection assays (69) revealed that neither the C-terminal mutations nor the K1 pRb-binding mutant had an effect on trans-activation of either the SV40 late or RSV LTR promoter. The remaining two N-terminal mutations (inA2803 and dlA2831), however, had reduced abilities to trans-activate the RSV LTR, but not the SV40 late, promoter. The previous studies also indicated the the N-terminal 138 amino acids of T-Ag, which encompass sequences up to and including the nuclear localization signal, are necessary and sufficient for the trans-activation activities of T-Ag. Based upon these results, then, it was predicted that the inA2803 and inA2831 T-Ag N-terminal mutants would be most likely to show an inability, or reduced ability, to trans-activate 135-Luc in this system. In addition, since the

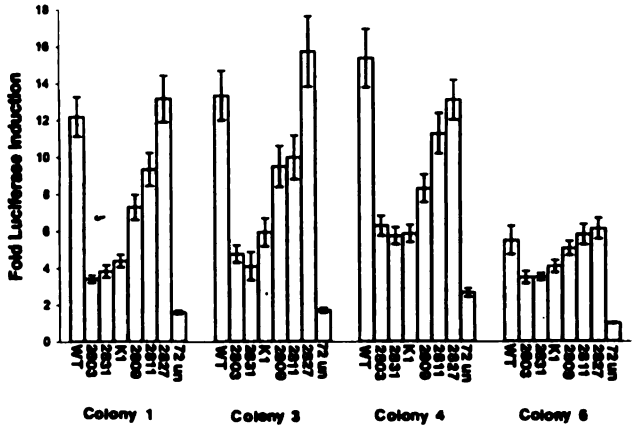
transcription factor E2F has been proposed to be involved with the activation of S phase-regulated genes, we were very interested in determining the effect of the pRb-binding T-Ag mutant on TK promoter activation.

Figure 6A shows a composite bar graph depicting average levels of induction in luciferase expression for four clonal 135-Luc cell lines infected with either wild-type or mutant recombinant adenoviruses. With the exception of Colony 6, induction levels with the wild-type virus ranged from twelve- to fifteenfold. Luciferase and endogenous TK enzyme inductions from one experiment with one infected cell line are depicted in Figure 6B. Note that the pattern of endogenous TK enzyme expression parallels that of luciferase expression, indicating that the transfected TK-Luc hybrid gene and endogenous TK gene behave similarly in this system. This pattern of TK enzyme expression was reproduced in all 135-Luc colonies in each experiment involving infection with the mutant viruses.

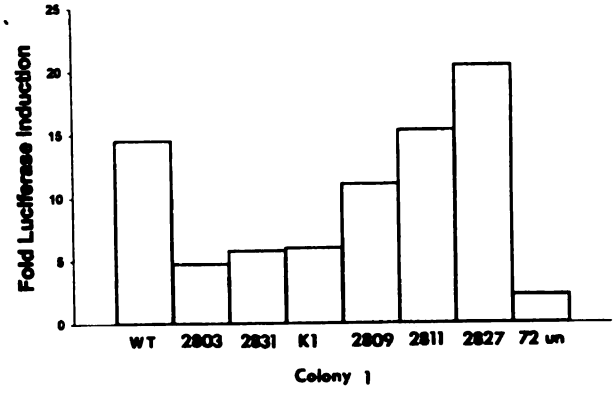
The T-Ags harboring C-terminal mutations (inA2809, inA2811, and inA2827) appeared to have little or no effect on the trans-activation of the -135 to +30 bp promoter fragment. Average induction levels with the inA2827 mutant virus were consistently at or above the levels attained with wild-type virus. Induction levels with the p53-binding mutants inA2809 and inA2811, however, appear slightly reduced relative to the wild-type virus in the composite graph. It would therefore appear that the T-Ag p53 binding activity contributes to TK trans-activation. It was noted, however, that induction with these two viruses was at or near wild-type levels in most of the experiments conducted, with reductions in activity present in only a small fraction of assays. Since some of these experiments were done early, before T-Ag levels in infected cell extracts were routinely checked, we did Western blots to measure the levels of T-Ag in all samples included in the composite graph above. Results showed that T-Ag protein was present at or near wild-type levels in the majority of the experiments conducted, but reduced in the same experiments exhibiting reduced luciferase induction. Thus, it appeared that the slightly decreased induction levels could be explained by decreased T-Ag protein levels in

Figure 6. Luciferase and endogenous TK enzyme inductions in Rat-1 cells infected with mutant Ad T/dl309 viruses. Results are shown for four independent colonies containing 135-Luc. Confluent cells were serum-starved then infected with recombinant adenoviruses expressing either wild-type or mutant forms of SV40 T-Ag. Whole cell extracts were prepared and analyzed for both luciferase and endogenous TK enzyme expression at 0 and 72 h after infection, as described in Materials and Methods. The identity of the virus used for infection is indicated below each bar. 72 un refers to uninfected cells maintained in low serum medium throughout the 72 h infection. Fold luciferase induction refers to the increase in luciferase expression at 72 h following infection relative to expression at 0 h. Values reflect normalization to total protein content. **(A)** Composite graph depicting average levels of luciferase induction for four independent clonal cell lines. Bars are grouped by colony. Each bar represents an average level of induction calculated from multiple experiments. The number of experiments (n) comprising each average are as follows: Colonies 1 and 4: WT, n = 8; 2803, 2809, 2811, 2827, 2831 and K1, n = 7; 72 un, n = 6. Colony 3: WT, n = 8; 2803, 2809, 2811, 2827, and K1, n = 7; 2831, n = 5; 72 un, n = 6. Colony 6: n = 5 for all. Error bars reflect the standard error of the mean. **(B)** A comparison of the patterns of luciferase and endogenous TK enzyme inductions in one experiment with one infected clonal cell line (135-Luc Colony 1). Each bar represents the level of induction attained in one experiment only. All data was taken from the same experiment.

A


Fold Luciferase Induction
WT
2803
2831
K1
2809
2811
2827
72 un
Colony 1
Colony 3
Colony 4
Colony 6

B


Fold Luciferase Induction
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K1
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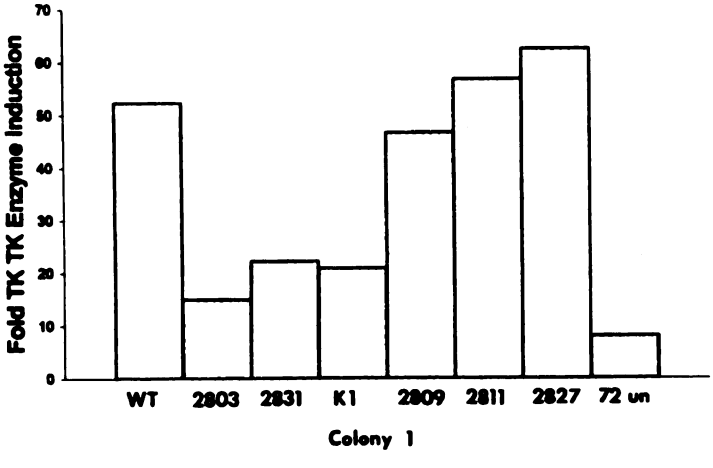

Fold TK TK Enzyme Induction
WT
2803
2831
K1
2809
2811
2827
72 un
Colony 1

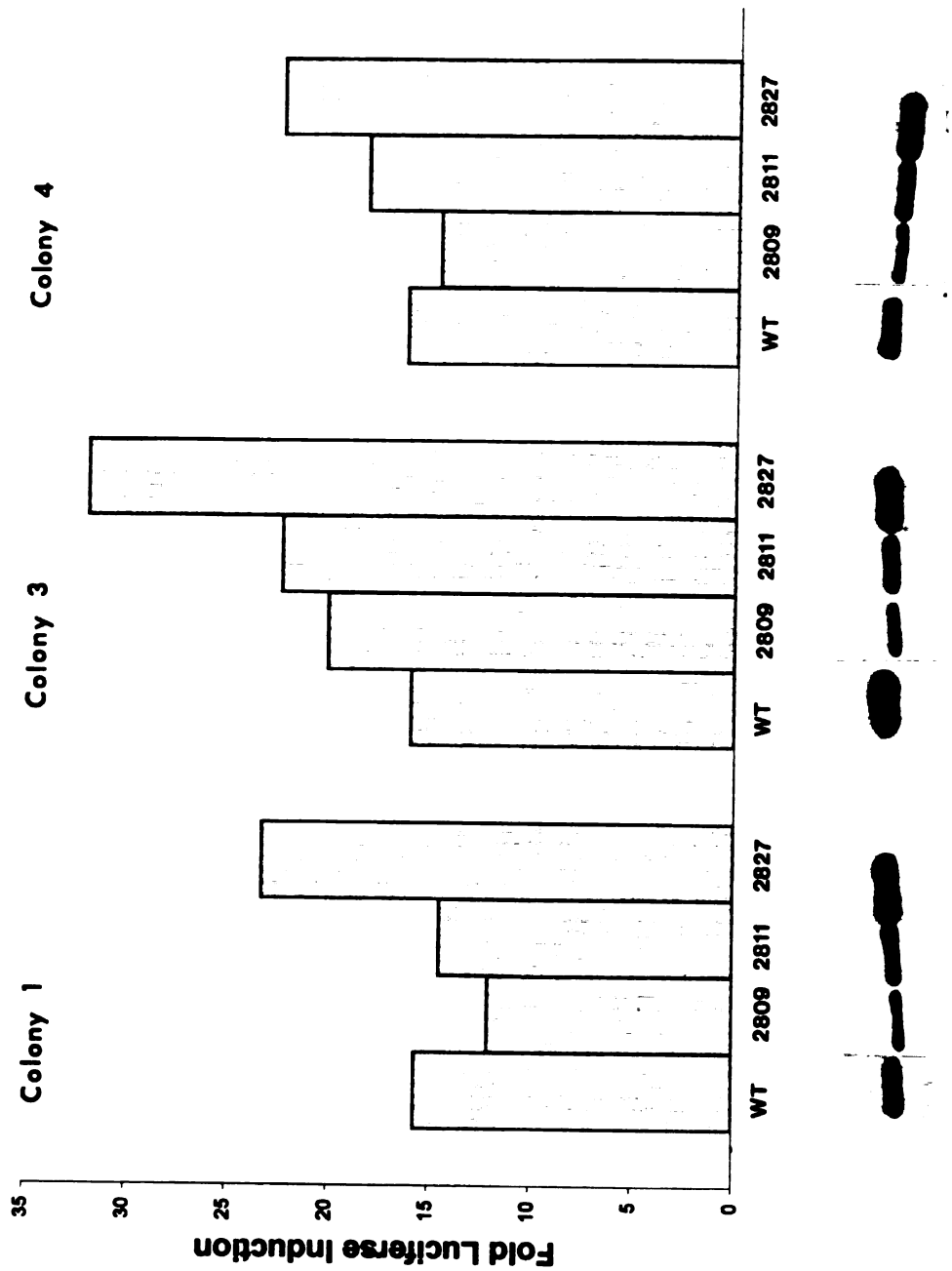
Figure 6.

individual experiments, rather than by a true trans-activation defect in these viruses. To verify this, Figure 7 shows Western blots performed with a monoclonal antibody specific for the N-terminal region of T-Ag, and corresponding luciferase induction levels for one representative experiment in which inA2809 and inA2811 T-Ag proteins were expressed near wild-type levels. Note that the luciferase inductions attained with these viruses are near wild-type levels as well. Results with the inA2827 virus are also included and show greater than wild-type levels of both T-Ag protein and luciferase expression. Therefore, the C-terminal T-Ag protein domain encoding the ATPase, helicase, and p53 binding activities are not required for T-Ag-mediated TK trans-activation.

In contrast to the results with the C-terminal mutants, and as shown in the composite graph in Figure 6A, luciferase induction levels were consistently decreased 50-70% (approximately two to threefold) in cells infected with viruses expressing the N-terminally mutated T-Ags (inA2803, inA2831, and K1). The residual induction was consistent with that of background, as discussed previously. Initially, these results were very exciting, since they supported the prediction that the N-terminal domains of T-Ag are involved with trans-activation of TK. Particularly, they indirectly implicated proteins such as pRb, p107, and, by extrapolation, E2F, as participants in TK promoter regulation.

To ascertain that the decrease in luciferase induction levels attained with these viruses was actually due to a defect in T-Ag trans-activation activity, rather than decreased levels of T-Ag protein, whole cell extracts were analyzed for T-Ag expression by Western blotting using the PAB416 monoclonal antibody, which specifically recognizes epitopes within the C-terminal region of T-Ag. The immunoblots for representative experiments are shown in Figure 8. Immunoblots of extracts from inA2803- and inA2831-infected cells consistently showed extremely low levels of expression. This was true for all colonies in all experiments. This was also true when Western blots were performed using other antibodies, including an N-terminal-specific T-Ag monoclonal and a polyclonal antibody. It therefore appears that these two mutant viruses encode unstable

Figure 7. T-Ag protein and luciferase induction levels in Rat-1 cells infected with viruses carrying C-terminal T-Ag mutations. Results are shown for three independent colonies containing 135-Luc. Confluent cells were serum starved, then infected with with either wild-type (WT) or mutant (2809, 2811, 2827) Ad T/dl309 viruses. Luciferase expression was analyzed in whole cell extracts prepared at both the time of infection (0 h) and 72 h after infection. Results were normalized to total protein content and used to calculate the increase in luciferase expression (fold luciferase induction) at 72 h after infection, relative to 0 h. 72 h-infected cell extracts were also analyzed for T-Ag protein expression by immunoblotting with a monoclonal antibody specific for the N-terminus of the T-Ag protein. For each colony, the data was taken from one experiment, and each bar represents the level of luciferase induction attained in that particular experiment. For each sample, the corresponding immunoblot result, depicting T-Ag protein expression at 72 h after infection, is shown below the graph.


Fold Luciferase Induction
0
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25
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35
Colony 1
Colony 3
Colony 4
WT
2809
2811
2827

Figure 7.

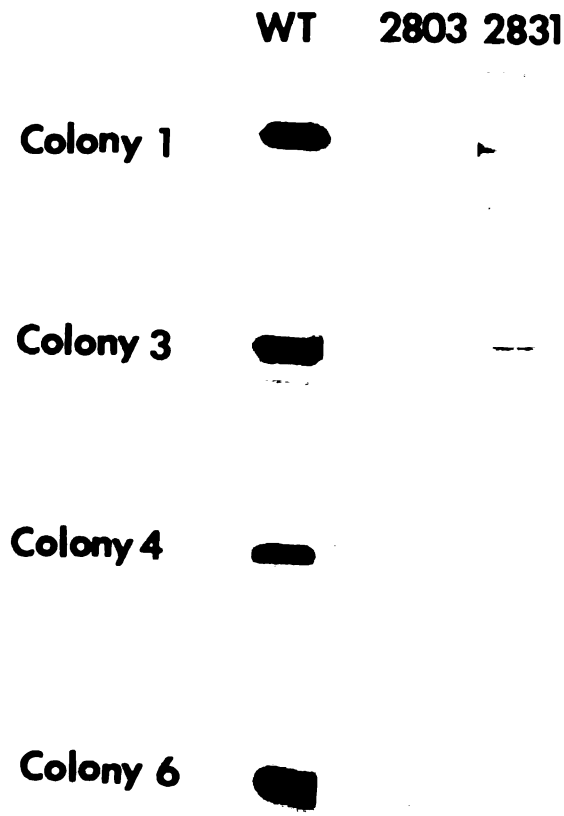


Figure 8. T-Ag protein levels in Rat-1 cells infected with the inA2803 and dl2831 mutant adenoviruses. Results are shown for four independent clonal cell lines carrying 135-Luc. Each colony represents one infection experiment. Cells were serum-starved then infected with either wild-type (WT) or mutant (2803, 2831) recombinant virus. Whole cell extracts were prepared 72 h following infection. T-Ag protein was detected by Western blot analysis, as described in Materials and Methods.

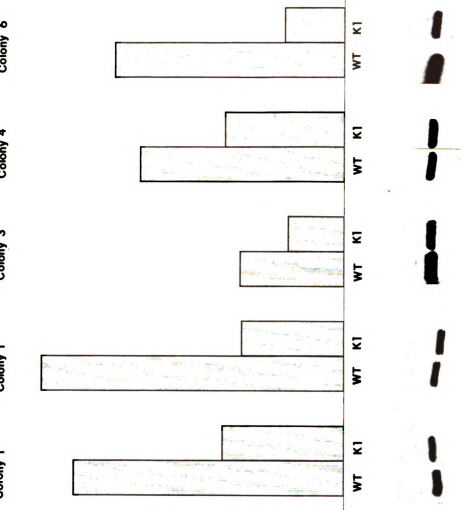
T-Ag proteins, and the severely diminished levels of luciferase induction are most likely due to the production of insufficient T-Ag rather than to a functional defect in T-Ag trans-activation activity. The role of these mutations in trans-activation cannot, therefore, be evaluated.

Results with the pRb-binding mutant, K1, are, however, different than those obtained with inA2803 and inA2831. Referring to Figure 9, K1 T-Ag protein levels are nearly equivalent to that attained with the wild-type virus, yet luciferase induction levels remain approximately 50% of wild-type. Thus, TK promoter induction is impaired. This strongly suggests that the ability to bind pRb or p107 is important to T-Ag's trans-activation activity.

In summary, these studies show that the p53-binding, ATPase, and helicase domains of T-Ag are not involved in viral-mediated TK transcriptional induction. Trans-activation of the TK promoter is, however, severely reduced in response to a T-Ag protein defective in its ability to bind the tumor suppressor proteins pRb and p107. Thus, these cellular proteins are implicated as cell cycle regulators of TK, a result which prompts further exploration into the role of E2F in mechanisms of TK promoter regulation. Furthermore, all of the mutant viruses, even those which make little T-Ag protein, show a low level of induction. We propose that this due to T-Ag-independent mechanisms of activation, as described in the previous section.

Regulation of 135(E2F)-Luc in cells infected with Ad T/dl309 viruses. One mechanism by which pRb and p107 exert their anti-proliferative effect is via the binding of E2F, resulting in repression of the transcriptional activity of this factor. This effect is abrogated in the presence of DNA tumor virus proteins such as SV40 T-Ag and Adenovirus E1A. While sequences resembling E2F binding sites are located within the human TK promoter, the binding of E2F itself has not yet been demonstrated, and the identity of the protein(s) recognizing these binding sites is unknown. Since the results

Figure 9. T-Ag protein and luciferase induction levels in Rat-1 cells infected with K1/dl309. Results are shown for four clonal cell lines containing 135-Luc. Confluent cells were serum-starved, then infected with either wild-type or K1 recombinant adenovirus. Luciferase expression was analyzed in whole cell extracts prepared at both the time of infection (0 h) and 72 h after infection. Results were normalized to total protein content and used to calculate the increase in luciferase expression (fold luciferase induction) at 72 h after infection, relative to 0 h. 72 h-infected cell extracts were also analyzed for T-Ag protein expression by immunoblotting with a monoclonal antibody specific for the C-terminus of the T-Ag protein (PAB416). For each colony indicated, the data was taken from one experiment, and each bar in the graph represents the level of luciferase induction attained in that particular experiment. For each sample, the corresponding immunoblot result, depicting T-Ag protein expression at 72 h after infection, is shown below the graph.


Fold Luciferase Induction
0
2
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6
8
10
12
14
16
Colony 1
Colony 1
Colony 3
Colony 4
Colony 6
WT
K1
WT
K1
WT
K1
WT
K1
WT
K1

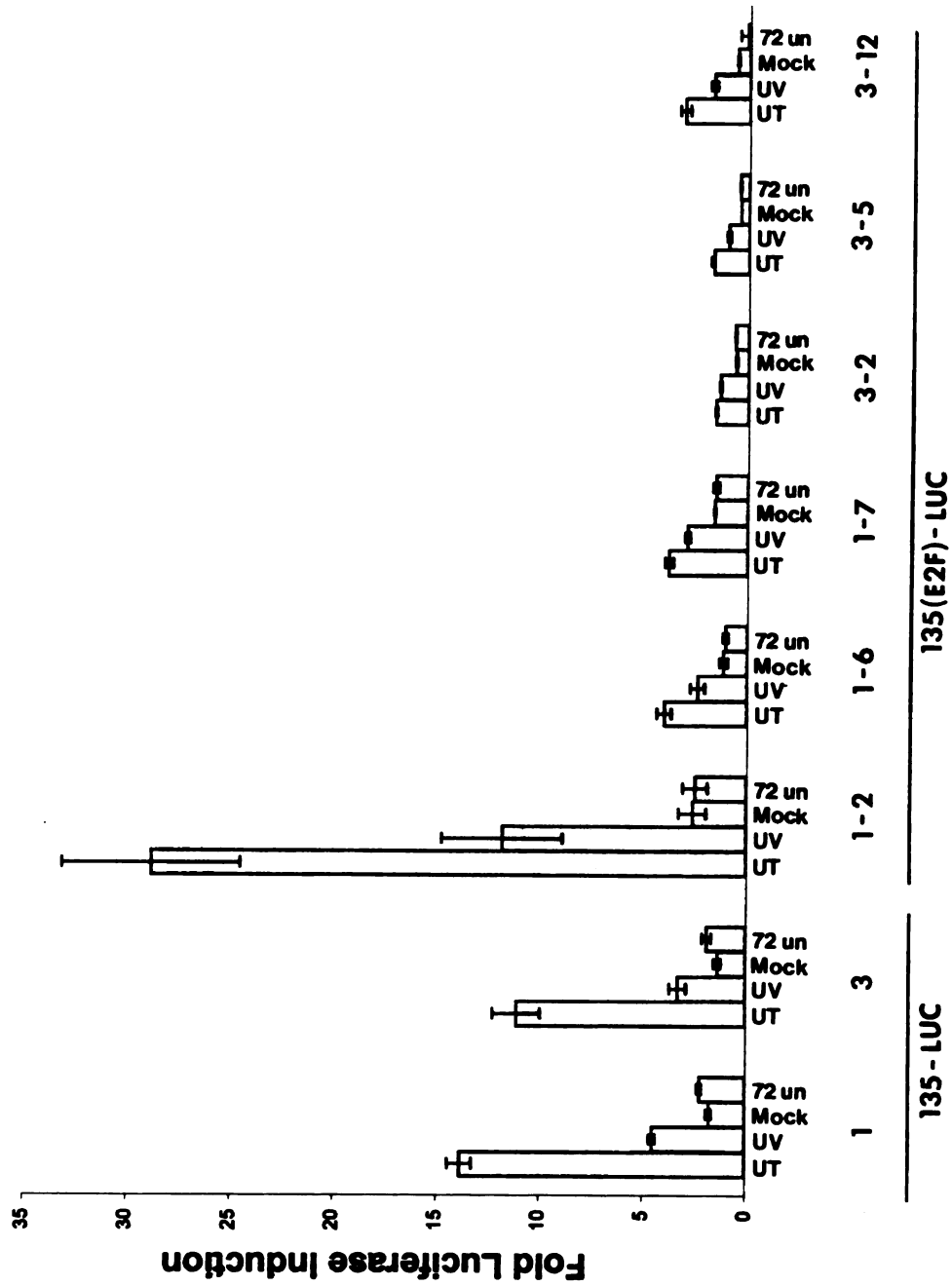
Figure 9.

discussed above implicate pRb and/or p107 as regulators of TK transcription, an E2F-like protein is implicated by association, as well. As a first step towards confirming this association, the E2F-like TK promoter sequences were analyzed for their contribution to TK transcriptional activation by T-Ag. To do this, the 135(E2F)-Luc hybrid gene was tested for its ability to be activated by T-Ag. Recall that 135(E2F)-Luc contains mutation substituting nonhomologous sequences for E2F binding sites located between -98 and -113 bp. If these binding sites do indeed constitute a T-Ag-mediated regulatory element, it is predicted that wild-type T-Ag will be impaired in its ability to activate expression from the mutant promoter.

Stably-transfected 135(E2F)-Luc cell lines were serum starved, then infected with wild-type Ad T/dl309 virus, as described previously. Six independent 135(E2F)-Luc clonal cell lines were tested. In addition, two 135-Luc colonies were infected to provide for a wild-type control in the same experiments. Cells were infected with either untreated or UV-irradiated wild-type Ad T/dl309 virus preps, or were treated for 72 hours with an equivalent volume of a 293 cell lysate ("mock" infection). The 293 cell lysate was used in an attempt to determine if the background levels of induction inherent to these assays is due to the presence of a trans-acting factor released from 293 cells during preparation of the viral stocks.

Luciferase induction levels are depicted in Figure 10. The graph is a composite, with the results representing the average level of induction determined from multiple experiments. Note that the parental, unmutated 135-Luc constructs exhibit characteristic inductions of eleven- to fourteenfold with the untreated, wild-type virus. Correspondingly, luciferase expression from cells infected with the UV-irradiated virus are decreased to approximately 30% of the untreated levels. Also, luciferase expression in mock-infected cells is decreased to only 40% of the level attained with the UV-irradiated virus and is no higher than that seen in untreated cells at 72 h. This suggests that the majority of the background induction seen is due not to the presence of a 293 cell-derived

Figure 10. Induction of luciferase by wild-type virus in Rat-1 cells stably transfected with 135(E2F)-Luc. Results are shown for two colonies carrying the wild-type 135-Luc hybrid gene and 6 colonies carrying the mutant 135(E2F)-Luc hybrid gene. Confluent cells were serum-starved, then infected with either untreated (UT) or UV-irradiated (UV) wild-type Ad T/dl309. In addition, some cells were left uninfected and maintained in either low serum medium (72 un) or treated with a lystate from 293 cells (mock). Whole cell extracts prepared at 0 and 72 h after infection were assayed for luciferase activity. Fold luciferase induction was calculated as described in Materials and Methods and represents the increase in luciferase expression at 72 h after infection, relative to 0 h. Viruses used are given directly below the graph, and the identities of the colonies tested are listed below the viruses. The graph is a composite, with each bar representing an average level of luciferase induction, as determined from multiple experiments. The number of experiments (n) comprising each average is as follows: 135-Luc (for both colonies): UT, n = 6; mock, n = 4; 72 un, n = 4; UV, (Colony 1) n = 3 and (Colony 3) n = 4. 135(E2F)-Luc: (for Colonies 1-2, 1-6, and 3-12) UT, n = 6; UV, n = 4; mock, n = 4; 72 un, n = 4. (for colonies 1-7, 3-2, and 3-5) UT, n = 4; UV, n = 2; mock, n = 2; 72 un, n = 2. Error bars reflect the standard error of the mean.


Fold Luciferase Induction
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UT
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Mock
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1
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1-7
3-2
3-5
3-12
135-LUC
135(E2F)-LUC

Figure 10.

trans-acting factor(s) but is attributable, instead, to viral-dependent, but not T-Ag-dependent, events.

Luciferase induction in five of the six 135(E2F)-Luc transfectants is severely diminished relative to the wild-type promoter. Specifically, induction levels with untreated wild-type virus range from 1.5-fold for Colony 3-2 to fourfold for Colony 1-6. Moreover, luciferase expression is not decreased much farther by UV-irradiating the virus, indicating that the mutant TK promoters in these transfectants are not specifically activated by T-Ag. This is the predicted result if the E2F binding sites are required for TK trans-activation by T-Ag. The pattern seen with the remaining colony (1-2) is more confusing. As shown, with the untreated Ad T/dl309 virus, luciferase expression in Colony 1-2 is induced to a level more than twice that attained with the parental 135-Luc constructs. Also, analogous to the parental controls, expression in cells infected with the UV-irradiated virus is approximately one-third that of the untreated level. Expression in mock-infected and 72 h uninfected extracts is negligible. Thus, except for being overinduced, Colony 1-2 exhibits a wild-type response to T-Ag. While we do not understand this result, it may be due to integration effects, such as integration next to a T-Ag-responsive enhancer element. In any case, the fact that five of the six mutant cell lines tested show severe defects in activation by T-Ag suggests that the E2F binding sites are indeed important for activation.

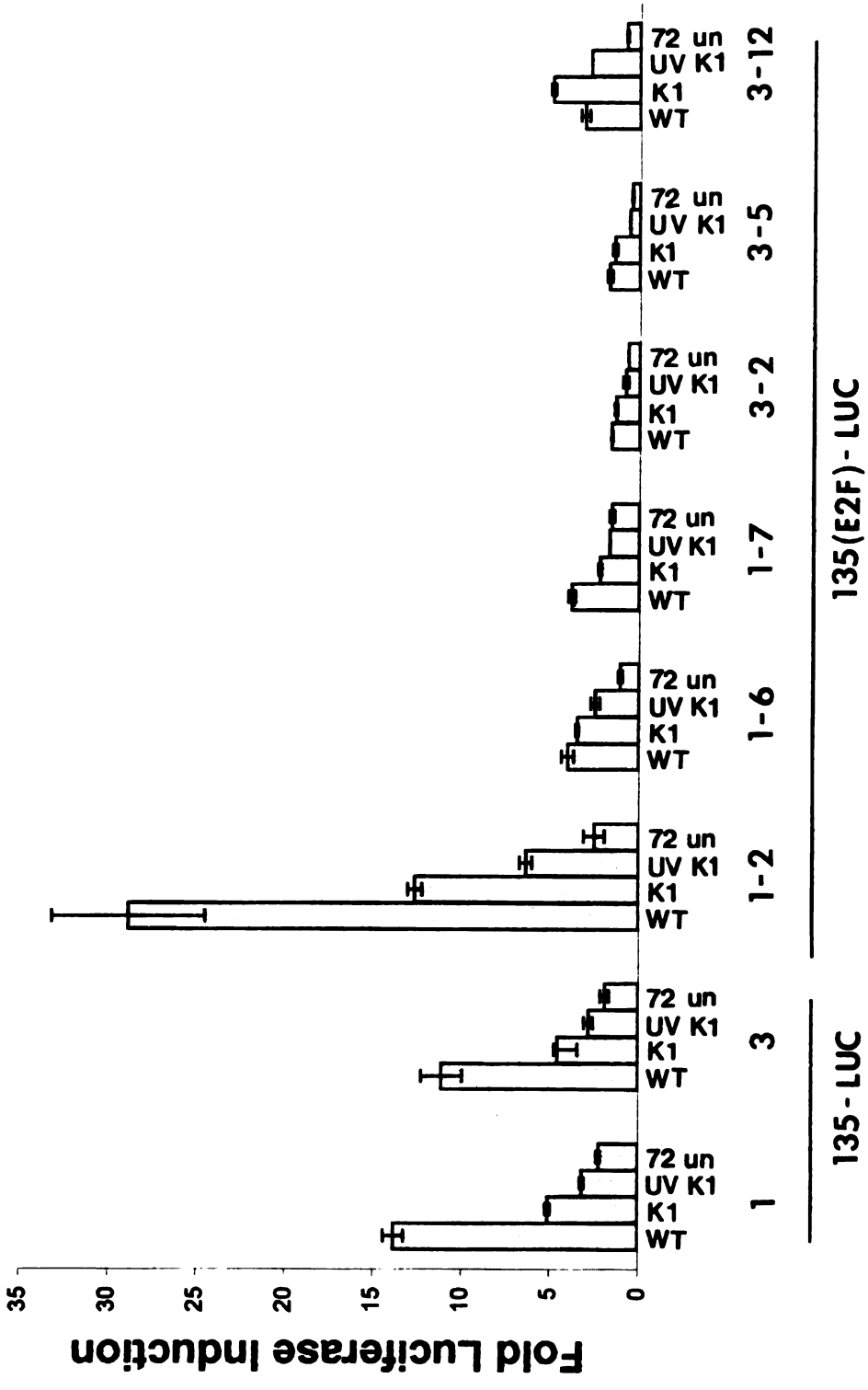
As with most experiments described in this chapter, Western blot analyses were performed to confirm the presence of T-Ag protein in infected cell extracts. For the data depicted in Figure 9, T-Ag was present at significant levels in extracts from cells infected with the untreated wild-type virus, and, as illustrated previously, T-Ag protein was undetectable in extracts from cells infected with the UV-irradiated virus.

Since both the T-Ag pRb/p107 binding site and the TK promoter E2F-like binding sites appear to be important for activation, a mechanism for the regulation of TK promoter activity, involving an interaction between pRb or p107 and an E2F-like protein, can be

envisioned. In a further attempt to establish the existence of such an interaction, the colonies tested above were infected with either untreated wild-type virus, untreated K1 mutant virus, or UV-irradiated K1 mutant virus. If activation is controlled by an interaction between pRb and/or p107 with a protein binding to the promoter E2F sites, then the K1 virus should not be any more defective than wild-type virus in its ability to activate the mutant promoter. If, however, the protein recognizing the E2F sites does not associate with pRb and/or p107, then the deficits represented in the promoter and viral mutations should be independent and additive, and infection of the 135(E2F)-Luc transfectants with the K1 mutant virus would be expected to result in levels of induction even lower than those observed upon infection of either wild-type promoter constructs with mutant virus or mutant promoter constructs with wild-type virus. UV-irradiated K1 virus is included for the purpose of assessing whether the T-Ag pRb-binding domain is the only T-Ag domain required for stimulation of the TK promoter; if the pRb-binding domain is the only region involved, luciferase induction levels should be equivalent with both untreated and irradiated K1 virus preparations. In contrast, if other domains of T-Ag are also involved, UV-irradiation of the K1 virus should result in even further decreases in luciferase expression.

Results are shown in Figure 11. For all colonies, the levels of T-Ag protein were equivalent in wild-type and K1-infected extracts (data not shown). Note that the pattern of luciferase induction produced is nearly identical to the pattern obtained in the preceeding experiment. Induction of parental 135-Luc colonies with the untreated K1 virus is reduced nearly 60% relative to the levels achieved with the untreated wild-type virus and is similar to that seen in the previous experiment with the UV-irradiated wild-type virus. Induction levels with UV-irradiated K1 virus are slightly farther reduced, but not much. As before, 135(E2F)-Luc Colony1-2 displays a response similar to the wild-type constructs.As shown, the remaining 135(E2F)-Luc colonies exhibit the response predicted if T-Ag is activating the TK promoter by disrupting an association between

Figure 11. Induction of luciferase by K1 virus in Rat-1 cells stably transfected with 135(E2F)-Luc. Results are shown for two colonies carrying the wild-type 135-Luc hybrid gene and 6 colonies carrying the mutant 135(E2F)-Luc hybrid gene. Confluent cells were serum-starved, then infected with either untreated, wild-type recombinant adenovirus (WT), untreated K1 virus (K1) or UV-irradiated K1 virus (UV K1). In addition, some cells were left uninfected and maintained in low serum medium (72 un). Whole cell extracts prepared at 0 and 72 h after infection were assayed for luciferase activity. Fold luciferase induction was calculated as described in Materials and Methods and represents the increase in luciferase expression at 72 h after infection, relative to 0 h. Viruses used are given directly below the graph, and the identities of the colonies tested are listed below the viruses. The graph is a composite, with each bar representing an average level of luciferase induction, as determined from multiple experiments. The number of experiments (n) comprising each average is as follows: 135-Luc (for both colonies): WT, n = 6; K1, n = 4, UV K1, n = 2; 72 un, n = 4. 135(E2F)-Luc: (for Colonies 1-2 and 1-6) WT, n = 6; K1, n = 4; UV K1, n = 2; 72 un, n = 4. (for Colony 1-7) WT, n = 4; K1, n = 3; UV K1, n = 2; 72 un, n = 2. (for Colony 3-2) WT, n = 4; K1, n = 4; UV K1, n = 2; 72 un, n = 2. (for Colony 3-5) WT, n = 4; K1, n = 2; UV K1, n = 2; 72 un, n = 2. (for Colony 3-12) WT, n = 6; K1, n = 4; UV K1, n = 1; 72 un, n = 4. Error bars represent the standard error of the mean.


Fold Luciferase Induction
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35
135-LUC
135(E2F)-LUC
1
3
1-2
1-6
1-7
3-2
3-5
3-12
WT
K1
UV K1
72 un

Figure 11.

pRb and E2F. Induction levels with the K1 virus are at or near levels produced with the untreated wild-type virus, being even higher than wild-type in Colony 3-12, and induction levels are not much lower with the UV-irradiated K1 virus. These results are consistent with SV40 activating the TK promoter via the E2F sites, with the T-Ag pRb-binding domain being primarily responsible for this effect.

Discussion

The experiments described here were designed to elucidate both TK promoter elements and T-Ag protein domains required for TK transcriptional regulation in SV40-infected cells. To facilitate promoter analysis, a series of TK-Luc hybrid genes was constructed and stably transfected into Rat-1 cells, and luciferase expression was measured, as an indicator of promoter activity, in serum-starved cells stimulated by infection with a recombinant adenovirus expressing SV40 large T Antigen. A 135 bp TK promoter, containing both CCAAT boxes, the TATA element, and a number of sequences resembling E2F consensus binding sites, was induced an average of ten- to fifteenfold in the presence of wild-type T-Ag protein. Infections with UV-irradiated virus established that the majority of this induction (approximately 70%) was dependent upon the expression of T-Ag, but that a certain level of T-Ag-independent but viral-dependent background induction existed (approximately 30% of the total induction with untreated wild-type virus). A likely source for background induction is activation resulting from the triggering of signal transduction events by virus binding to cell surface receptors. This phenomenon has been previously observed in studies with polyomavirus where it was determined that an interaction of the viral capsid protein VP1 with the cell surface was sufficient to induce early increases in the mRNA levels for some cellular genes (71).

Using a series of adenoviruses expressing mutant forms of T-Ag protein, it was determined that the p53-binding, helicase and ATPase domains of T-Ag are not important for TK trans-activation. This was evidenced by the fact that three C-terminally mutated viruses (inA2809, inA2811, and inA2827) induced expression of 135-Luc to near wild-type levels. The inA2809 and inA2811 mutations are located within the T-Ag helicase domain and render the protein defective for p53 binding. Previous analyses with these T-Ag mutants established that, while they are defective in the immortalization and transformation of rodent cells (68, 70), they trans-activate the viral SV40 late and RSV

LTR promoters to wild-type levels in transient transfection assays (69). The inA2827 mutation lies within the T-Ag ATPase domain. T-Ags carrying this mutation bind p53, immortalize and transform primary mouse cells (68, 70) and also trans-activate these viral promoters with wild-type efficiency (69). Thus, C-terminal sequences have been found to be dispensible for T-Ag-mediated trans-activation of certain viral promoters (69), and, while p53-binding activity positively correlates with the ability of T-Ag to transform and immortalize, it also appears to be dispensible for trans-activation. While the data presented here supports these conclusions, our results are especially unique and significant in that TK is a cellular promoter, intimately attuned to the growth state of the cell, that is naturally activated following infection with SV40. Thus, in contrast to studies with viral promoters, understanding the mechanisms by which T-Ag induces TK promoter activity has profound implications in terms of basic cell cycle control processes.

Studies on T-Ag-mediated trans-activation in transient transfection assays have also shown that the N-terminal 138 amino acids of T-Ag contain the trans-activation activity (69). It was therefore predicted that mutations within the N-terminal region of T-Ag would be most likely to show an effect in our assay sytem. Pursuant to this, we studied the expression of TK-Luc hybrid genes following infection with three viruses carrying N-terminal T-Ag mutations. Two of the mutations studied, inA2803 and dlA2831, are located within the first exon of the T-Ag protein and have been shown, in transient transfection assays, to result in reduced trans-activation of some viral promoters (69). Unfortunately, we have found that these mutant viruses produce unstable T-Ag proteins, and we were therefore unable to determine the effect of these mutations on the trans-activation of TK by T-Ag.

In contrast, the third N-terminal mutant, K1, did produce a stable protein, and its effect could therefore be studied. Luciferase induction levels in K1-infected cells were typically 50-60% below wild-type levels and just above the level of T-Ag-independent background expression, indicating that this mutant is defective in activation of the TK

promoter. Since the K1 virus is unable to bind both pRb and p107 (11, 19), tumor suppressor proteins with integral roles in cell cycle regulation, these results implicate pRb and/or p107 as regulators of TK promoter activity.

Our results with the K1 virus are unique in that they contradict many studies conducted on T-Ag-mediated trans-activation of viral promoters. We have shown the pRb-binding domain of T-Ag to be required for mediating activation of the human TK promoter. Others, however, have reported that the K1 virus trans-activates the SV40 late and RSV LTR promoters to wild-type levels in transient transfection assays (69), indicating that this domain of T-Ag is not required for stimulation of these viral promoters. Our results also indicate that pRb and/or p107 are involved in the activation of TK transcription. Trifillis et al. (62), however, reported the efficient trans-activation of both the SV40 late and RSV LTR promoters by T-Ag in both pRb^+ and pRb^- human osteosarcoma cell lines, indicating that pRb was not involved in regulating the expression of these viral promoters. Thus, it appears there may be fundamental differences in regulation between cellular and viral promoters; while pRb may not be required for "general trans-activation, it does appear to be necessary for the specific activation of the human TK promoter. In support of this, it should be noted that Mudrak et al. (46) have reported the transcriptional activation of a variety of murine cellular genes, including TK, in response to wild-type polyomavirus large T-Ag but not to a pRb-binding T-Ag mutant. This concurs with our results, and establishes pRb (and/or p107) as a fundamental component in the regulation of some growth-related cellular genes.

Since pRb and p107 bind and inactivate the transcription factor E2F (20, 24), and tumor proteins like T-Ag dissociate these complexes, resulting in the release of transcriptionally-active, free E2F (9), E2F itself may be a regulator of TK promoter activity. To date, E2F appears to be important for the regulation of the murine TK promoter (16, 46). While sequences resembling E2F binding sites are present in the human TK promoter, its role in the regulation of this gene is still unclear.

To test for the possible involvement of E2F, or an E2F-related factor, in T-Ag-mediated TK promoter activation, we studied the expression of a hybrid gene construct in which the E2F-like binding sites were replaced with nonhomologous sequences. Results show that these binding sites are important for activation and are consistent with the protein(s) binding these sites being a transcriptional activator. Luciferase induction levels for five of six 135(E2F)-Luc stably-transfected colonies were severely decreased, relative to unmutated parental constructs, upon infection with the wild-type T-Ag-expressing virus. Induction levels were, in fact, at or below the level of background defined by the previous UV-irradiated virus studies. The remaining 135(E2F)-Luc transfectant displayed a wild-type phenotype, expressing luciferase at levels greater than that of the parental constructs. As the integration sites have not been analyzed for these transfectants, it is possible that this hybrid gene integrated in the vicinity of a strong enhancer or regulatory element that responds to T-Ag.

Finally, regulation via an association between pRb and/or p107 and the protein(s) recognizing the E2F-like sites is suggested since the untreated wild-type and K1 viruses induced luciferase expression in 135(E2F)-Luc cell lines to relatively equivalent levels. This indicates that the promoter and viral mutations affect the same regulatory pathway, rather than being components of independent pathways.

A word should be said about SV40 small T antigen (t-Ag). Specifically, it should be noted that the wild-type recombinant adenovirus used in these studies also produces t-Ag. Thus, while specific activation of 135-Luc by the wild-type recombinant adenovirus is presumably due to the actions of T-Ag, the possibility exists that t-Ag contributes as well. t-Ag has been reported to trans-activate some promoters (adenovirus E2), but not others (the SV40 late promoter) (40). It has been postulated that t-Ag enhances the ability of limiting amounts of T-Ag to trans-activate (40), or enhances the activity of trans-activation-defective T-Ags produced at normal levels (69), but the role of t-Ag in trans-activation is still largely unknown. More importantly, t-Ag has been shown to stimulate

cell proliferation by inhibiting the actions of regulatory phosphatases such PP2A (53, 58, 66). It is possible, then, that a portion of the designated T-Ag-dependent induction seen in these assays is attributable to the mitogenic effects of t-Ag. Alternatively, t-Ag could be contributing to the level of T-Ag-independent background induction. This is unlikely, though, since UV-irradiation should also abolish expression of t-Ag.

In terms of the mutant viruses used in these studies, we would expect only the N-terminal inA2803 and inA2831 mutant viruses to produce mutant t-Ag proteins, sine these mutations lie within the region shared between the T-Ag and t-Ag proteins (the N-terminal 82 amino acids). These mutants were unstable, though, and not evaluated anyhow. All other mutant viruses, including K1, should produce wild-type t-Ag protein, since these mutations are C-terminal to the common region between the two T antigens. Since K1 was the only mutant which displayed a defect in trans-activation, and K1 makes wild-type t-Ag, our conclusions specifying T-Ag-mediated activation of the human TK promoter dependent upon the T-Ag pRb-binding domain are valid. However, the K1 virus did not always reduce luciferase expression completely to background level. This was evident particularly in the final experiment which showed a slight additional decrease in luciferase expression following infection with UV-irradiated K1 virus. Thus, the low level of residual induction may be due to the actions of t-Ag. Alternatively, additional T-Ag domains may be involved.

A model for TK promoter regulation can now be envisioned in which an activator protein, perhaps one of the many members of the E2F family, associates with, and is repressed by, pRb or p107 during the G0 and G1 phases of the cell cycle. Upon SV40 infection, T-Ag binds pRb or p107, leading to dissociation of complexes containing these proteins, and allowing the freed activator to bind the E2F-like consensus sites within the TK promoter and stimulate transcription.

Further development of this model would, of course be greatly facilitated by identification of the protein(s) recognizing the E2F-like binding sites. DNAse-footprinting

assays have revealed that a 25 bp promoter fragment containing these binding sites is indeed bound by protein (33). Furthermore, gel shift analyses with this segment of the promoter has detected constitutive binding of the $p33^{cdk2}$ protein kinase and G1/S specific binding of a complex dependent upon cyclin A and p107 (39). Interestingly, pRb was not detected as a component of any of the complexes formed. Likewise, both an E2F antiserum and an oligomer containing a single E2F consensus site were ineffective at abrogating or altering the formation of any of the detected complexes, suggesting that E2F-1, at least, is not the protein binding these sites. As indicated above, there are, however, multiple family members of the E2F transcription factor, including some which interact specifically with p107 rather than pRb (18). The protein involved in human TK promoter regulation may be one of these related forms. These results also suggest that it may be the ability of T-Ag to bind p107, rather than pRb, which is involved in TK trans-activation. If cyclin A associates with the promoter-bound activator protein at G1/S, transcription could be stimulated further by cyclin-A/cdk2-mediated phosphorylation of either the activator protein itself or other transcription factors bound to adjacent promoter sites. Together, these results indicate that key cell cycle regulatory proteins are involved with the S phase-specific mediation of TK transcription.

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Chapter 3

Thymidine Kinase Promoter Domains Utilized in SV40-Mediated Transcriptional Induction

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Abstract

We have examined the ability of simian virus 40 (SV40) large T antigen (T-Ag) to activate independent regions of the human thymidine kinase (TK) promoter. Previously, we studied the expression of a series of hybrid genes containing fragments of the human TK promoter linked to the bacterial neomycin resistance gene (TK-Neo), in serum-stimulated or SV40-infected stably transfected CV1 cells. Results showed that sequences between -135 and -67 bp relative to the transcriptional start site were required for regulation in serum-stimulated cells, while sequences between -67 and +30 bp were sufficient for regulation in SV40-infected cells. In the present study, we confirm this result by examining the expression of a TK-Neo hybrid gene containing a full-length promoter fragment with an internal deletion of sequences between -135 and -67 bp (dl-138/67-Neo); Northern blot results reveal a four- to sixfold induction in *Neo* mRNA levels in stably transfected SV40-infected, but not serum-stimulated, CV1 cells. Thus, the TK promoter can be subdivided into two functional domains: 1) an upstream domain containing sequences between -135 and -67 bp, both necessary and sufficient for regulation in serum-stimulated cells and 2) a downstream domain, containing sequences between -67 and +30 bp, sufficient for regulation in SV40-infected cells. To analyze the ability of the individual promoter domains to be induced by SV40 T-Ag, we linked fragments of the TK promoter containing upstream domain sequences (-135 to -67 bp), downstream domain sequences (-67 to +30 bp), or the promoter harboring the internal deletion (dl-138/67) to the firefly luciferase gene, and studied the expression of the resulting hybrid genes in stably transfected Rat-1 cells infected with a recombinant adenovirus expressing SV40 T-Ag. Results, however, show that T-Ag is unable to specifically activate the independent promoter regions in this system. Since previous results show stimulation of a 135 bp TK promoter by T-Ag, it appears both promoter regions are required for activation in Rat-1 cells.

Introduction

Thymidine kinase (TK) catalyzes the phosphorylation of dT to dTMP, a precursor to DNA synthesis (41). Although a salvage pathway enzyme, TK plays an integral role in controlling cellular thymidine pools. TK activity is regulated by both the growth state and cell cycle position of the cell. Activity is low in quiescent, G0 cells, differentiated cells, and cells traversing the G1 phase of the cell cycle (20, 32, 35). Activity then increases markedly (tenfold or more) at the onset of S phase, remaining high throughout S and G2 before declining at the end of M (20). Due to both the high level of G1/S induction, and the nonessentiality of the enzyme for cell proliferation, TK has become one of the most widely manipulated G1/S phase-regulated genes and one of the most popular model systems for understanding regulatory mechanisms governing the transition from G1 into S phase in the eukaryotic cell cycle.

Small DNA tumor viruses, such as Simian Virus 40 (SV40) and Polyoma virus, have also been utilized as tools to understanding cellular processes. Because of the small genome size (approximately 5000 bp), these viruses rely almost completely upon the infected host cell for the production of viral progeny, requiring host cell proteins and enzymes for transcription of viral RNA, replication of viral DNA, and translation of viral proteins. Thus, the mechanisms underlying these viral events should closely approximate and provide insight into the corresponding cellular processes. Since viral DNA replication requires S phase-specific enzymes and proteins, these viruses act as potent mitogens, altering the patterns of cell transcription upon infection to induce cells to enter S phase and begin DNA synthesis. In this respect, SV40 induces cell DNA synthesis in the absence of serum growth factors (44) and also activates expression of thymidine kinase (26). The mitogenic activity of SV40 depends upon the major viral replicative protein, large T Antigen (T-Ag) (37).

SV40 T-Ag is a 90 kDa, multifunctional, nuclear phosphoprotein. The protein can be divided into distinct functional domains comprising the various protein activities (14, 54). Activities include an ATPase and ATP-dependent helicase (7, 23, 48), specific and non-specific DNA binding (5, 34, 50), and the ability to bind a variety of cellular proteins, including the tumor suppressors p53 (29), pRb (8, 31), and p107 (12), as well as DNA polymerase α (9, 10), and TATA-Binding Protein (TBP) (17). The protein also contains a function analagous to the Adenovirus E1A p300-binding activity (52), suggesting T-Ag interacts directly or indirectly with p300, a cellular protein required for adenovirus E1A to induce cell DNA synthesis (45). Related to its pRb-binding activity, T-Ag is also capable of dissociating complexes containing the E2F transcription factor (6), allowing for the release of free, transcriptionally active E2F and the potential induction of a number of S phase-regulated genes. T-Ag is also a potent transcriptional activator (1, 54), capable of trans-activating a number of cellular and viral promoters. It is likely that T-Ag's ability to interact with a variety of cellular proteins is an integral part of its trans-activating and mitogenic properties.

Since SV40 large T Antigen is capable of inducing TK activity, we were interested in exploring the interactions of T-Ag with the TK promoter, effectively using T-Ag as a tool to uncovering the cellular proteins involved. The human TK gene is regulated, in part, at the level of transcription. In serum-starved cells stimulated by either the addition of serum or infection with SV40 virus, the increase in TK enzyme levels and activity at G1/S is paralleled by a corresponding increase in TK mRNA levels (47). In addition, there is a small, but significant, increase in the transcription rate of the gene, with the transcription rate increasing five- to sevenfold in serum-stimulated cells (46) and three- to fourfold in SV40-infected cells (40).

Most of the work on TK transcriptional regulation has been carried out in serum-stimulated cells. In particular, we and others have shown that sequences lying between 135 and 67 bp upstream of the transcriptional start site (27) are necessary and sufficient to

confer serum-mediated regulation to a heterologous reporter gene (38, 40) or heterologous promoter (24). Deletion analysis has narrowed the serum-regulatory region to a 25 bp segment containing a number of putative E2F binding sites (25), and gel shift analyses have detected interactions between this promoter region and major cell cycle regulatory proteins such as p107, $p33^{cdk2}$ kinase, and cyclin A (30), implicating these as regulators of serum-mediated TK transcriptional induction. A requirement for E2F, in this case, has not been proven but is implied, as deletion of the E2F sites results in constitutive TK mRNA expression (25).

Less is known about the regulation of TK in SV40-infected cells. As mentioned above, we have detected both an increase in TK mRNA levels and TK transcription rate following infection of simian CV1 cells with SV40. Recently (40), we constructed a series of hybrid genes, linking fragments of the TK promoter to the bacterial neomycin resistance (*Neo*) gene. Following stable transfection into CV1 cells, pools of resistant colonies were serum starved, then stimulated by either the addition of fresh serum or infection with SV40, and levels of Neo mRNA were examined. Results showed that an S phase-specific pattern of mRNA expression was retained by a promoter fragment containing 135 bp of upstream regulatory sequence in both SV40-infected and serum-stimulated cells. Deletion to only 67 bp of upstream sequence abolished regulation in serum-stimulated, but not SV40-infected, cells. While these results were consistent with a role for elements between -135 and -67 bp in serum-mediated regulation, they were also surprising. Taking into account the known interactions between SV40 T-Ag, p107/pRb, and E2F (6), deletion of the serum regulatory region containing the putative E2F sites was predicted to abolish SV40-mediated regulation as well. Instead, our results indicated that the TK promoter can be subdivided into two functional domains: an upstream domain, containing sequences between -135 and -67 bp, sufficient and required for regulation in serum-stimulated cells, and a downstream domain, containing sequences between -67 and +30 bp, sufficient for

regulation in SV40-infected cells. Furthermore, they suggested that SV40 and serum stimulate the TK promoter by different, although possibly overlapping, pathways.

The studies described in this chapter were directed at further elucidation of the TK promoter regions required for viral-mediated induction. First, a TK promoter fragment harboring an internal deletion of sequences between -135 and -67 bp was used to confirm the above results in stably transfected CV1 cells. Second, experiments are described in which the independent upstream and downstream TK promoter regions were analyzed for activation in the presence of SV40 T-Ag. Specifically, hybrid genes were constructed linking TK promoter fragments containing the upstream domain alone, the downstream domain alone, or both, to a luciferase reporter gene (TK-Luc). Luciferase expression was then analyzed in stably transfected Rat-1 cells infected with a recombinant adenovirus vector expressing SV40 T-Ag.

These experiments were designed to address a number of questions. First, we were interested in determining if T-Ag could independently activate sequences within the upstream promoter domain. While our previous results in CV1 cells indicated that these sequences are not required for regulation, results from the previous chapter, showed involvement of upstream E2F-like sites in viral-mediated induction. Thus, there may be more than one promoter domain that responds to T-Ag, and the upstream promoter sequences might, in fact, be sufficient for regulation. Second, we were interested in exploring further the sequences within the downstream domain required for viral-mediated regulation. Lying within the -67 to +30 bp promoter region is the TTTAAA sequence and an inverted CCAAT element. Studies with the adenovirus E1A protein have shown that promoter-specific trans-activation by E1A is mediated by proteins bound to the TATA box (19, 43, 51). As T-Ag has been shown to interact with TBP (17), we were interested in determining if the TTTAAA sequence was the element conferring regulation to the downstream promoter domain. The results obtained, however, indicate that neither the isolated upstream nor the downstream promoter region is significantly activated by wild-

type SV40 T-Ag in this system. These results also indicate that, in Rat-1 cells, both promoter domains are required for maximal induction by T-Ag, suggesting cooperativity between the two halves, destruction of an important protein binding site during promoter separation, and/or involvement of a cell type-specific factor in TK induction.

Materials and Methods

Cell culture. CV1 cells were cultured in Dulbecco's Modified Eagle's medium containing 5% fetal calf serum and 5% calf serum (HyClone Laboratories, Logan, Utah). Rat-1 cells were cultured in Dulbecco's Modified Eagle's medium (DME) supplemented with 10% calf serum. Both cell lines were grown at 37° C in an atmosphere containing 5% CO_2. Stably transfected CV-1 cells containing the bacterial neomycin resistance gene (*Neo*) were maintained in medium containing 100 μg/ml G418 (Gibco-BRL Life Technologies, Gaithersburg, MD) while stably-transfected Rat-1 cells containing hygromycin resistance genes (*Hgm*) were maintained in medium containing 50 μg/ml hygromycin B (CalBiochem-Novabiochem Corporation, La Jolla, CA). Cells were grown in medium without the respective drug during serum stimulation and virus infection experiments.

Viruses. The SV40 virus used was purified by equilibrium centrifugation in a cesium chloride gradient. Recombinant adenovirus vectors expressing either wild-type or mutant forms of SV40 large T-Antigen (Ad T/dl309) were a kind gift from Dr. Charles Cole and Jun Chen at Dartmouth University. These viruses contain T-Ag coding sequences, under control of the SV40 promoter, inserted in place of the E1A and E1B genes in the adenovirus dl309 (21) virus. Small 6 bp deletions within the SV40 origin of replication render the viruses completely replication-defective in the absence of E1A. Mutations within the T-Ag coding sequence include point mutations and small insertions or deletions, and are described elsewhere (22, 53).

Ad T/dl309 viral stocks were prepared by infecting confluent monolayers of human 293 cells, a cell line transformed with adenovirus sequences and constitutively expressing E1A (16), with single-plaque-derived virus resuspended in 5 ml 1X DME + 2 % calf serum. A multiplicity of infection (MOI) of 1 gave the highest viral titers. CPE (cytopathic effect) was observed two to three days following infection, as indicated by the

rounding up and detachment of cells. At this point, cells were scraped from the plate, and both cells and cell supernatent were transferred to a sterile centrifuge tube. Cells were lysed by two rounds of freezing and thawing then centifuged at 2000 RPM for 30 min at 4°C to pellet cell debris. Supernatant was filtered through a 0.45μm filter, aliquoted, and stored at -70°C. Viral concentrations were determined by plaque assays on 293 cells and generally ranged between 5 x 10^8 PFU/ml and 1 x 10^9 PFU/ml. The absence of wild-type recombinant virus was insured by plaquing viral stocks on HeLa cells.

To prepare UV-irradiated virus stocks, a minimal volume of virus was sterilely transferred to an appropriately-sized tissue culture plate (i.e. 2.5 ml virus per 60 mm diameter dish) then placed, uncovered, in a UV Stratalinker 1800 cross-linker (Stratagene, La Jolla, CA). Viruses received 6 hits of 1200 μJoules ultraviolet light in 2 min intervals, and viral solutions were mixed by swirling plates between each hit. Virus was irradiated just prior to use, then placed on ice. To minimze light exposure and prevent potential light-induced repair mechanisms, irradiated stocks were kept covered with aluminum foil and infections were performed in the absence of tissue culture hood fluorescent lights.

Viral infections (SV40 and Ad T/dl309) and serum stimulation. Synchronization of CV1 cells in G0 was achieved by plating cells on 100 mm diameter tissue culture plates followed by growth to confluence and serum starvation for 24 h in medium containing 0.1% fetal calf serum. To serum stimulate CV1 cells, low serum medium was removed at time zero and replaced with fresh medium containing 10% serum. For SV40 infection of CV1 cells, low serum medium was removed at time zero and saved. Cells were then infected with SV40 at an MOI of 15. Infections were done for 1 h at 37° C in 1 ml of low serum medium. At the end of the infection, the low serum medium that had been removed from the cells at time zero was added back to the plates.

Similarly, for infection of Rat-1 cells with Ad T/dl309, cells were plated at a density of 1x10^5 cells per 60 mm diameter tissue culture plate and synchronized in G0 by

growth to confluence (approximately 5 days) followed by serum starvation for 24 h in medium containing 0.5% calf serum. The infection procedure was analogous to that described for SV40 with the following modifications: cells were infected at an MOI, and infections were done for 1.5 h at 37° C in a total volume of 0.6 ml low serum medium. Low serum medium removed at time zero was kept separate for each cell line and replaced at the end of the infection.

Plasmid and promoter mutation constructions. PGL2-Basic, a promoterless luciferase vector, was obtained from Promega Corporation (Madison, WI). pY3 (3) is a plasmid conferring *Hgm*^{R}. 67-Luc contains TK promoter sequences between -67 and +30 bp, relative to the transcriptional start site, subcloned upstream of the firefly luciferase gene (*Luc*) in PGL2-Basic. This construct was generated by excising the 97 bp TK fragment from pH10-TK, a plasmid containing TK cDNA under control of a 135 bp TK promoter, by digestion with *Nco*I, addition of *Sal*I linkers, and subsequent digestion with *Hin*dIII + *Sal*I. The isolated fragment was cloned into the *Xho*I and *Hin*dIII sites within the PGL2-Basic polylinker. Hybrid-Luc consists of human TK promoter sequences between -135 and -67 bp linked to a fragment of the Herpes Simplex Virus (HSV) TK promoter subcloned upstream of *Luc* in PGL2-Basic. The HSV TK promoter sequences utilized are encompassed in an *Eco*RI-*Bgl*II (-75 to +56 bp) restriction fragment and include both the HSV TATA element and the DS1 control sequence (33). The composite human TK/HSV TK promoter is identical to that utilized by Kim and Lee (24). Construction of Hybrid-Luc was a multistep process. First, a 1500 bp HSV TK fragment was excised from pTK2, a plasmid containing the human TK cDNA under control of the HSV TK promoter, by digestion with *Eco*R1, followed by addition of *Nco*I linkers and subsequent digestion with *Nco*I. Second, this fragment was ligated to a 3 kb fragment containing human TK promoter sequences between -135 and -67 bp, generated by digestion of pH10-TK with *Nco*I. Resulting ligation products were screened for proper orientation via restriction

enzyme digestion. To excise the hybrid promoter, the desired construct was digested with *Sal*I, followed by the addition of *Sac*I linkers and digestion with *Sac*I + *Bgl*II. The 209 bp hybrid promoter fragment was then subcloned into the *Sac*I and *Bgl*II restriction sites within the PGL2-Basic polylinker.

Plasmid dl-138/67-Neo consists of TK promoter sequences between -444 and +30 bp, with an internal deletion of sequences between -135 and -67 bp, linked to *Neo*. Construction of this plasmid utilized the oligonucleotide-mediated mutagenesis procedure described by Kunkel (28). To generate the mutation, a 30-base oligonucleotide containing 15-bp matches on either side of the sequence to be mutated was used in the procedure. The presence of the desired deletion was confirmed by DNA sequencing, and the mutant promoter fragment was subsequently removed from M13 by digestion with *Hin*dIII + *Sal*I and subcloned into pBluescript upstream of a promoterless *Neo* gene. Finally, dl-138/67-Luc was constructed by digestion of dl-138/67-Neo with *Hin*dIII + *Sal*I and subcloning of the resulting promoter fragment into the *Xho*I and *Hin*dIII sites within the PGL2-Basic polylinker.

DNA transfections. The stable transfection of CV1 cells with dl-138/67-Neo was done via the $CaPO_4$ transfection procedure described by Wigler et al. (49). Stable transfectants were selected in medium containing 400 μg of G418 per ml. When resistant colonies were clearly visible, 10 to 50 colonies were pooled and expanded for analysis. After the initial selection, transfected cells were maintained in medium containing 100 μg of G418 per ml.

Stable transfections into Rat-1 cells were performed using *Lipofectin* Reagent (BRL Life Technologies, Gaithersburg, MD) according to the procedure supplied by the manufacturer. Briefly, Rat-1 cells were plated at a density of $5x10^5$ cells per 60 mm diameter tissue culture dish 24 h prior to transfection. All DNA preparations used were either CsCl-banded for purity or prepared with Qiagen Maxiprep columns (Qiagen Corporation, Chatsworth, CA). For each transfection to be done, 8 μg TK-Luc and 2 μg

pY3 were coprecipitated, resuspended in 10 µl of sterile ddH_2O, then mixed with 90µl of 1X DME minus serum and penicillin/streptomycin. In separate sterile tubes, one tube for each transfection to be done, 20 µl *Lipofectin* reagent was mixed with 80 µl 1X DME minus serum and penicillin/streptomycin. The DNA and lipofectin solutions were then combined, mixed gently, and allowed to stand at room temperature for 15 min, after which 1.8 ml 1X DME minus serum and penicillin/streptomycin was added. Cells were rinsed twice with several mls of 1X DME minus serum and penicillin/streptomycin, overlayed with the DNA/*Lipofectin* solution and incubated at 37°C in an atmosphere of 5% CO_2 for 10 h, after which time the DNA/*Lipofectin* solution was removed and replaced with 5 mls 1X DME + 10 % calf serum media. 48 h after removal of the *Lipofectin* solution, cells were split into medium containing 200 µg/ml hygromycin B. When resistant colonies emerged, 10-50 colonies were pooled and several colonies were picked and propagated as clonal cell lines. After initial selection, transfected cells were maintained in medium containing 50 µg/ml hygromycin B.

Preparation of total RNA. Total RNA was prepared from tissue culture cells by a modification of the method of Favaloro et al. (15). Briefly, cells were washed once with PBS without calcium and magnesium, then lysed with 1 ml of lysis buffer (100 mM Tris-HCl, pH 7.5, 12 mM EDTA, 150 mM NaCl, 1% SDS, 200 µg/ml proteinase K) per 10-cm-diameter plate. Cell lysates were scraped from the plate, and cellular DNA was sheared by several passages through a 22 gauge needle. The lysate was then incubated at 37°C for 45 to 60 min. Nucleic acids were extracted twice with phenol/chloroform then ethanol precipitated. The precipitate was resuspended in 400 µl TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) to which a mixture containing 10 mM $MgCl_2$, 1 mM DTT, 10 U of RNasin, and 10 U of RNase-free DNase was added. The samples were incubated at 37°C for an additional 45 to 60 min, after which they were extracted twice with phenol/chloroform, once with chloroform, then ethanol precipitated. The precipitated

RNAs were resuspended in 150 µl TE, and concentrations were determined by reading absorbances at 260 nm.

Northern blot analyses and nucleic acid hybridizations. Equal amounts of total RNA (20 µg) of each sample were separated on a 1.2% agarose-formaldehyde gel containing 500 ng/ml ethidium bromide. After electrophoresis, gels were photographed to ascertain that equal amounts of RNA were loaded in each lane. RNA was transferred to nitrocellulose filters by blotting overnight. Prehybridization, hybridization, and washes after hybridization were done as described previously (38). To determine the amounts of *Neo* mRNA on blots, several different film exposures were scanned on an Ambis Image Acquisition and Analysis system. To calculate induction values, the amount of mRNA at 24 h was divided by the amount at time zero.

A ^{32}P-radiolabeled probe was prepared with the Boehringer Mannheim Random Primed DNA Labeling kit in accordance with the manufacturer's specifications. The following DNA fragments were used to probe for expression of the relevant gene: neo, a 916-bp *Hin*dIII-*Nco*I fragment from within the *Neo* gene; β-2 microglobulin, a 600-bp *Pst*I fragment from the human cDNA; SV40 early transcripts, a 1,169-bp *Hin*dIII fragment from the early region of the viral genome.

Preparation of whole cell extracts and luciferase assays. 1X Luciferase Cell Culture Lysis Buffer was prepared by dilution of 5X Luciferase Cell Culture Lysis Buffer [125 mM Tris, pH 7.8 with H_3PO_4, 10 mM CDTA (1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid), 10 mM DTT, 50% glycerol, and 5% Trition X-100], obtained from Promega Corporation (Madison, WI). D-Luciferin (sodium salt), acetyl coenzyme A, $MgCO_3$, and Tricine were obtained from Sigma (ST. Louis, MO) and used to prepare luciferase substrate solution according to the recipe (270 µM coenzyme A, 470 µM Luciferin, and 530 µM ATP) supplied by Promega Corporation with its Luciferase Assay System. After preparation, substrate solution was aliquoted and stored at -70°C until use.

To prepare whole cell extracts for analysis of luciferase activity, cells were removed from the incubator 72 h following infection. Medium was removed, and cells were rinsed with 2 ml cold PBS, then stored on a bed of ice during harvest. To harvest, cells were scraped from the plates with a rubber policeman in 1.5 ml cold PBS into a microfuge tube on ice. Cells were pelleted and the PBS was removed by aspiration. Cell pellets were then resuspended in 100 µl 1X Cell Culture Lysis Buffer and allowed to lyse for 15 min at room temperature. Extracts were then centrifuged for 1 min to pellet cell debris and DNA. Supernatents were transferred to fresh microfuge tubes and stored at -70°C until assayed.

The luciferase assay procedure was done according to the protocol supplied by Promega Corporation. Briefly, extracts and substrate were thawed and allowed to equilibrate to room temperature. 20 µl extract was placed in an 8x50 mm polypropylene tube (Turner Designs, Sunnyvale, CA) in a Turner TD-20E Luminometer (Turner Designs). 100 µl of substrate solution was then injected and luciferase expression was measured during a 1 min reaction. For each sample, luciferase activity (expressed as light units [LU]) was normalized to total protein content to calculate the specific activity of the sample (LU/µg protein). Luciferase induction for each time point was then calculated by dividing the specific activity of the sample (in light units/µg protein) by the specific activity at time zero.

TK enzyme assays. Assays for endogenous TK enzyme activity were performed using the whole cell extracts prepared in the 1X Luciferase Cell Culture Lysis Buffer. Assays were performed as previously described (47). TK activities (cpm) were normalized to total protein content and endogenous TK enzyme inductions were calculated by dividing TK specific activity (cpm/µg protein) at 72 h after infection by the specific activity at time zero.

Protein quantitation, sodium dodecyl sulfate-polyacrylamide gel electophoresis, and Western blotting. The amount of protein in the whole cell extracts was determined using the Bio Rad Protein Assay system (Bio Rad Corp., Richmond,CA) based upon the method of Bradford (4). To verify the presence of SV40 large T Antigen, 25-30 μg protein from whole cell extracts were mixed with SDS Gel Loading Buffer (0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 2% [w/v] SDS, 0.72 M 2-mercaptoethanol, and 0.00125% bromphenol blue), boiled, and electrophoresed on 8% SDS-Page Bio-Rad minigels in a buffer composed of 0.124 M Tris, 0.959 M glycine, and 0.5% (w/v) SDS at 150 V. After electrophoresis, proteins were transferred to polyvinylidene difluoride polyscreen membrane (Dupont-NEN, Boston, MA) at 30 V for 1.5 h in Western transfer buffer (20 mM Tris-192 mM glycine-20% methanol, pH 8.3) using the Novex miniprotein transfer system (Novex Corp., San Diego, CA). Membranes were blocked overnight in 5% nonfat dried milk in 1X PBS + 0.1% Tween 20 (PBS/Tween) at 4°C. After a brief rinse in PBS/Tween, membranes were incubated with the T-Ag-specific monoclonal antibodies L19, which recognizes N-terminal T-Ag determinants (18) or PAB416 (kindly supplied by the lab of Dr. Ed Harlow, Massachusetts General Hospital Cancer Center), which recognizes C-terminal T-Ag determinants. Antibodies were diluted 1:20 or 1:500 for L19 and PAB416, respectively, in PBS/Tween + 5% nonfat dried milk, for 1 h at room temperature with gentle agitation. Removal of the 1^{o} antibody was followed by four 15-min washes in PBS/Tween then incubation with 2^{o} antibody, a goat anti-mouse IgG-HRP conjugate (Bio Rad Corp., Richmond, CA) diluted 1:5000 in PBS/Tween + 5% nonfat dried milk, for 1 h at room temperature with gentle agitation. Removal of the 2^{o} antibody solution was again followed by four 15-min washes with PBS/Tween, after which the membranes were reacted with the *Lumiglow* chemiluminescent reagents (Kirkegaard and Perry, Gaithersburg, MD), as per manufacturer's protocol. Blots were covered with plastic wrap and immediately used to expose to x-ray film (Amersham Hyperfilm MP, Amersham Corporation, Arlington Heights, IL).

Results

Regulation of dl-138/67-Neo in SV40-infected CV1 cells. In chapter 2 of this dissertation, we showed that both the pRb/p107-binding domain of SV40 T-Ag and TK promoter sequences resembling E2F binding sites are important for the T-Ag-mediated transcriptional activation of TK in Rat-1 cells. The mechanism of activation most likely relies upon the ability of T-Ag to bind and dissociate complexes formed between pRb (and/or p107) and an E2F-related factor. In a previous study (40), however, we had examined the expression of a series of TK-Neo hybrid genes which contain TK promoter fragments linked to the bacterial neomycin resistance gene (*Neo*), in SV40-infected CV1 cells, and found a G1/S-specific increase in *Neo* mRNA levels with a hybrid gene containing only a 67 bp TK promoter fragment (67-Neo). As 67-Neo contains promoter sequences to 67 bp upstream of the major transcriptional start site (27), it lacks the upstream putative E2F-binding sites, located between -68 and -113 bp. This data contradicts our results in Rat-1 cells by suggesting that the putative E2F-binding sites are not required for TK regulation in CV1 cells.

Since 67-Neo contains a highly truncated promoter, we reasoned it possible that the results obtained in CV1 cells were misleading. Therefore, to confirm this result, dl-138/67-Neo was generated. This construct is illustrated in Figure 1A and contains TK promoter sequences between -444 and +30 bp, with an internal deletion of sequences between -135 and -67 bp. It has previously been shown that sequences between -135 and -444 bp account for approximately 40% of promoter activity in transient transfection assays (2), but are dispensable for regulation (24, 38, 40). We therefore predicted that dl-138/67-Neo would exhibit higher basal levels of expression than 67-Neo.

dl-138/67-Neo was constructed as described in Materials and Methods. The plasmid was stably transfected into CV1 cells, and $G418^R$ colonies were selected. Pools of 10 to 50 colonies were generated for analysis. To analyze *Neo* mRNA levels in the

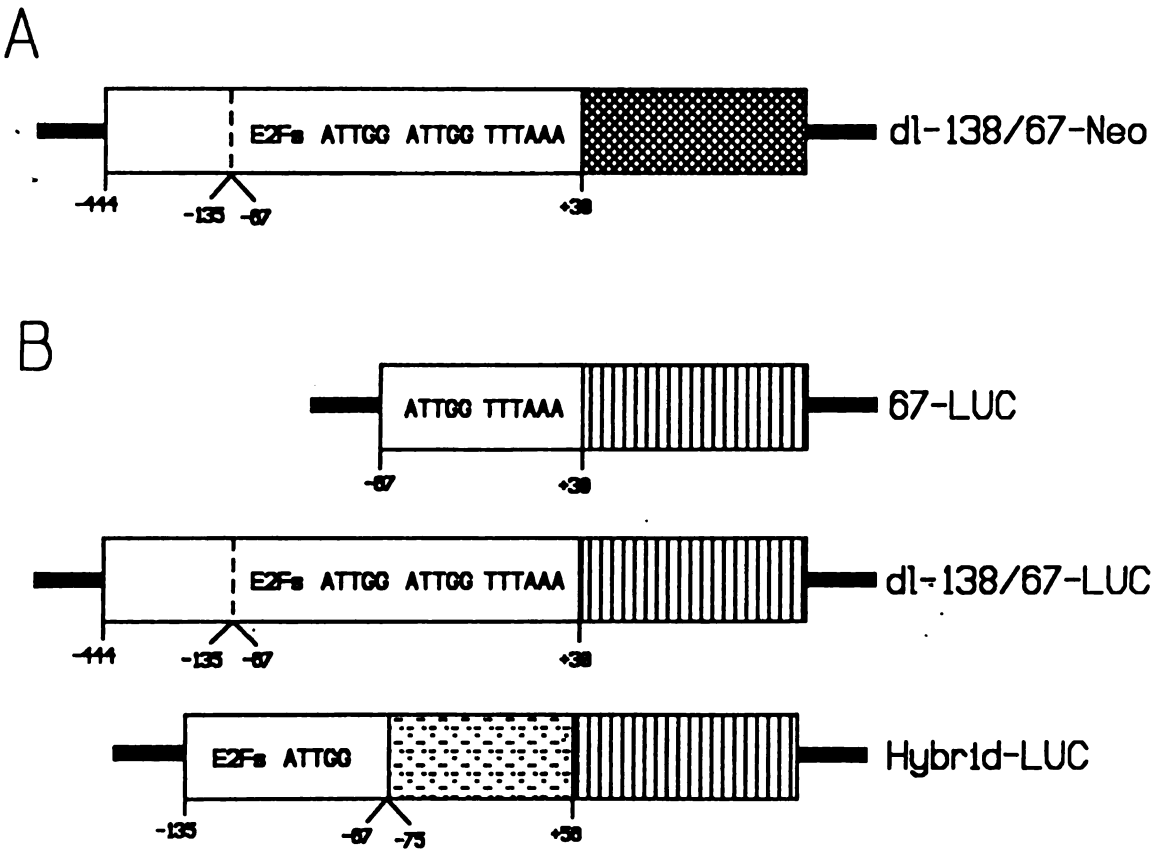


Figure 1. Diagram of human TK promoter-reporter gene constructs. A) TK-Neo hybrid gene B) TK-LUC hybrid genes Open bar: human TK promoter region. Stippled bar: HSV TK promoter sequences. Vertical striped bar: luciferase reporter gene. Hatched bar: neo reporter gene. Solid bars: prokaryotic vector sequences. Putative regulatory elements within the human TK promoter are indicated. Numbers refer to sequence end points within the various promoter regions.

transfected cells, cells were synchronized in G0 by growth to confluence and serum starvation for 24 h in 0.1% calf serum medium, then stimulated by either the addition of fresh serum or infection with SV40. Since the endogenous CV1 cell TK gene is induced between 8 and 10 h following serum stimulation and between 14 and 18 h following SV40 infection (47), total RNA was prepared at 0, 6, 12, 18, and 24 h after stimulation. Patterns of mRNA expression were then determined via Northern blot analysis with a *Neo* probe.

Results obtained with two independent pools of transfectants are shown in Figure 2. In serum-stimulated cells, expression from the dl-138/67 promoter increased slightly between 0 and 6 h, then remained constant between 6 and 12 h, as cells progressed from G1 into S. In contrast, expression was induced between 12 and 18 h following infection with SV40. The lengthened period of time required to reach G1/S in SV40-infected versus serum-stimulated cells reflects the additional time needed to transcribe and translate SV40 T-Ag. Previous studies in this lab have verified that T-Ag mRNA appears between 12 and 18 h following infection of CV1 cells (40). Quantitation of mRNA levels for dl-138/67-Neo revealed a six- to eightfold induction at G1/S, in correlation with previous results demonstrating a four- to sixfold induction in mRNA from a 444-Neo construct (40). This data therefore confirms our previous results, indicating that TK sequences between -135 and -67 bp are not required for G1/S transcriptional induction in SV40-infected CV1 cells.

Generation of stable TK-Luc cell lines. The above results, along with previously published work, suggest that the human TK promoter can be subdivided into two independent regulatory domains: an upstream domain containing sequences between -136 and -67 bp both necessary and sufficient for regulation in serum-stimulated cells, and a downstream domain containing sequences between -67 and +30 bp sufficient for regulation in SV40-infected CV1 cells. As mentioned previously, the upstream domain contains several E2F-like binding sites. Within the downstream domain, the TATA box

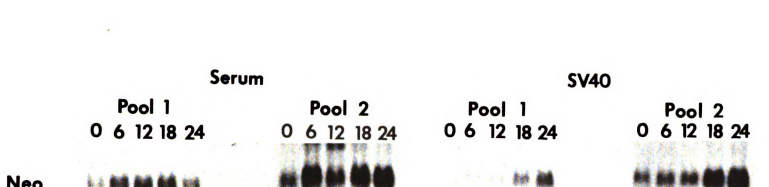


Figure 2. Northern blot analysis of dl-138/67-Neo hybrid gene constructs in stably transfected CV1 cells. Total RNA from two independent pools of cells was extracted at the indicated times (hours) after serum stimulation or SV40 infection and analyzed as described in Materials and Methods. In the top panel, the blot was hybridized with a *Neo* probe, and in the bottom panel, the same blot was rehybridized with a β-2 microglobulin probe.

(TTTAAA) is a putative regulatory element. Precedence for the role of a TATA box in promoter-specific activation is found in studies with adenovirus E1A. In particular, E1A was found to physically associate with proteins bound to TATA boxes (19) and mutation of the TATA box within the hsp70 promoter resulted in retainment of promoter function (i.e. induction in response to heat shock) but loss of E1A-mediated activation. As SV40 is also known to associate with TBP (17), this element may be involved in mediating induction of the downstream promoter domain by SV40..

We decided to investigate more closely the differences in activities between these two promoter regions, hoping to determine regulatory elements within each that are required for induction in SV40-infected cells. To facilitate an independent study of each region, it was decided to physically separate the two domains. In particular, we were interested in addressing a number of specific questions. First, is T-Ag capable of independently activating the upstream promoter domain? Our results have indicated that the putative E2F-binding sites within this region are required for viral-mediated induction in Rat-1, but not CV1 cells. The possibility exists that these sites are also sufficient for conferral of regulation in both cell types. Second, does the TATA box contribute to regulation of the downstream domain in SV40-infected cells? This analysis would ultimately involve site-directed mutagenesis of the TTTAAA sequence. Finally, what domains of the T-Ag protein are required for regulation? In the previous chapter, a panel of T-Ag mutant viruses was used to implicate pRb and/or p107 and an E2F-related protein as regulators of the 135 bp TK promoter fragment. This same panel of mutant viruses mighty be useful in determining required TK promoter domain-specific elements and associated proteins.

To analyze promoter activities in response to SV40 T-Ag, the luciferase assay system described in Chapter 2 of this dissertation was utilized. Rationale for use of this system, and establishment of assay parameters, is discussed in detail in Chapter 2 as well. Briefly, the system involves the generation of Rat-1 cell lines stably transfected with TK

promoter-luciferase gene constructs. Serum-starved cells are then infected with a recombinant adenovirus expressing SV40 T-Ag, and luciferase expression is measured at 0 and 72 h after infection as an indicator of promoter activation.

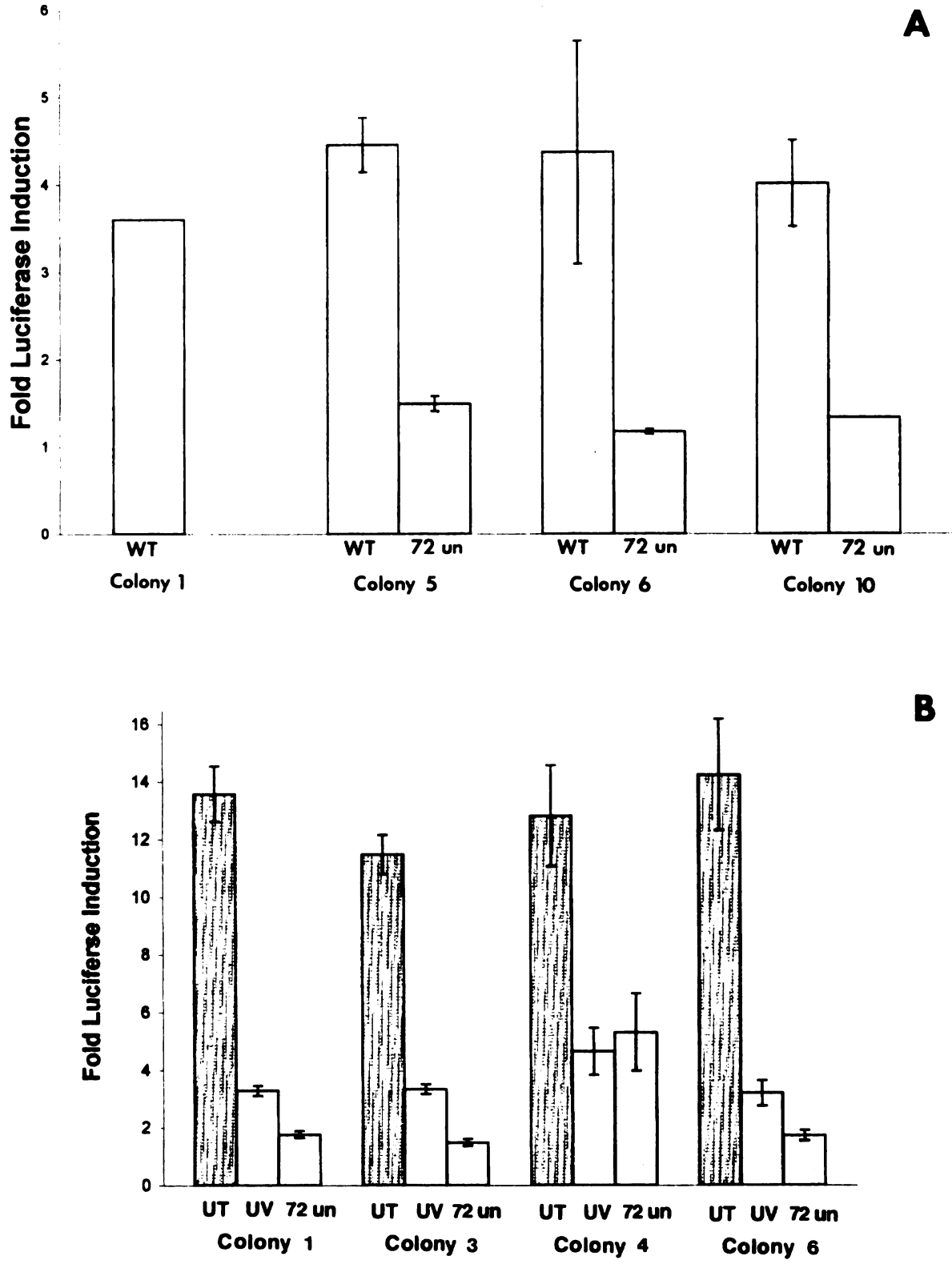
Generation of fragments containing the individual promoter domains was accomplished by digestion of a 165 bp TK fragment, containing sequences between -135 and +30 bp, with the restriction endonuclease NcoI, essentially splitting the fragment at bp -67. Sequences between -67 and +30 bp were subcloned directly upstream of the firefly luciferase gene, while sequences between -135 and -67 bp were linked first to a segment of the HSV TK promoter, then subcloned upsteam of *Luc*, as described in Materials and Methods. Figure 1B diagrams the various TK-Luc constructs generated for these experiments. Putative regulatory elements are indicated. 67-Luc contains the downstream promoter region (-67 to + 30 bp) and is analogous to 67-Neo, mentioned above. dl-138/67-Luc contains the TK promoter with the internal deletion of upstream domain sequences (from -135 to -67 bp) and is analogous to dl-138/67-Neo, discussed above. This construct was generated to confirm results obtained with the highly truncated 67 bp promoter. Hybrid-Luc contains the sequences comprising the independent upstream domain (-135 to -67 bp) linked to the segment of the HSV TK promoter for the purpose of providing a TATA box for transcription initiation.

The TK-Luc constructs were stably cotransfected, along with a plasmid conferring Hgm^R, into Rat-1 cells. Following transfection, Hgm^R colonies were selected, and individual colonies were picked and expanded into clonal cell lines. After expansion, cycling populations of each colony were tested for luciferase expression. Colonies expressing no luciferase activity were discarded. Likewise, colonies expressing abnormally high levels of activity (i.e. thousands of light units) were not selected for further analysis. Colonies chosen for analysis contained moderate levels of luciferase activity in cycling cells.

Induction of 67-Luc and dl-138/67-Luc in cells infected with Ad T/dl309. Stable transfectants containing 67-Luc were first tested for activation by wild-type T-Ag. As mentioned previously, to assay for promoter activity, cells were synchronized in G0, then infected with a recombinant adenovirus expressing wild-type SV40 T-Ag (Ad T/dl309). Cells were harvested, and whole cell extracts were prepared at both the time of infection (time zero) and 72 h following infection (t=72). Luciferase activity was determined as described in Materials and Methods. For each experiment, both Western blot analyses and assays for endogenous TK enzyme activity were performed to confirm that all cell lines had been infected and were expressing similar levels of T-Ag protein, and that cells had been effectively arrested and restimulated. Experiments exhibiting very low levels of T-Ag protein and/or endogenous TK enzyme inductions were discarded.

Induction levels for four independent 67-Luc colonies infected with wild-type Ad T/dl309 are shown in Figure 3A. The graph is a composite, with the results representing an average level of induction determined from multiple experiments. In addition, data from Chapter 2 is included, showing, for comparison, induction of the -135 to +30 bp TK promoter fragment (135-Luc) in response to both untreated and UV-irradiated wild-type virus (Figure 3B). Note that luciferase expression in each of the 67-Luc colonies is induced only three- to fourfold relative to activity at time zero while induction levels for 135-Luc range between ten- and fifteenfold. This suggests that 67-Luc is not significantly activated by T-Ag in this system. Furthermore, the three- to fourfold level of induction seen here correlates well with the level of background induction obtained upon infection of 135-Luc with UV-irradiated virus preparations (Chapter 2). Moreover, recall from Chapter 2 that HTK-Luc, which contains a small fragment of the non-cell cycle-regulated HSV TK promoter, exhibited inductions of two- to threefold in the presence of wild-type T-Ag. Taken together, this data suggests that the small magnitude of induction from the 67 bp promoter is not dependent upon specific activation by T-Ag, but is instead the result

Figure 3. Induction of luciferase in 67-Luc Rat-1 cells infected with wild-type Ad T/dl309. (A) Results are shown for four independent clonal cell lines. Confluent cells were serum-starved then infected with recombinant adenovirus expressing wild-type SV40 T-Ag. An MOI of 200 was used. Luciferase activity was assayed in whole cell extracts prepared at both the time of infection (0 h) and 72 h after infection. Infection and assay procedures are described in detail in Materials and Methods. Fold luciferase induction indicates the increase in luciferase activity at 72 h post-infection relative to activity at 0 h. WT indicates infection with wild-type virus, and 72 un refers to uninfected cells maintained in low serum medium throughout the infection time period. The graph is a composite, with each bar representing an average level of luciferase induction, as determined from multiple experiments. The number of experiments (n) comprising each average are as follows: Colony 1: WT, n = 2. Colony 5: WT, n = 5; 72 un, n = 4. Colony 6: WT, n = 5; 72 un, n = 3. Colony 10: WT, n = 4; 72 un, n = 1. Error bars represent the standard error of the mean. **(B)** For comparison, data from Chapter 2 of this dissertation (Chapter 2, Figure 2B, page 163) is given showing luciferase induction in 135-Luc Rat-1 colonies infected with either untreated (UT) or UV-irradiated (UV) recombinant adenovirus. Infection and cell harvest conditions are identical to those described above. See Chapter 2 for discussion and statistical details.


A
Fold Luciferase Induction
0
1
2
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4
5
6
WT
Colony 1
WT
72 un
Colony 5
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72 un
Colony 6
WT
72 un
Colony 10
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4
6
8
10
12
14
16
UT
UV
72 un
Colony 1
UT
UV
72 un
Colony 3
UT
UV
72 un
Colony 4
UT
UV
72 un
Colony 6

Figure 3.

of other mechanisms of activation, such as events triggered by virus binding to cell surface receptors.

To confirm that the 67 bp promoter is not specifically induced by T-Ag, two 67-Luc colonies were infected with UV-irradiated preparations of wild-type Ad T/dl309. Western blot analysis confirmed that no detectable T-Ag protein was produced in cells infected with irradiated virus, while T-Ag was easily detected in cell extracts infected with untreated virus (data not shown). Average luciferase inductions are shown in Figure 4. In this particular experiment, Colony 5 was activated nearly sixfold by the untreated wild-type virus, while Colony 6 was activated only 1.4-fold. These activations are still T-Ag-independent, however, since the luciferase induction levels are not decreased much farther in cells infected with the UV-irradiated virus preparation, relative to cells infected with the untreated virus preparation. As the T-Ag protein is not detected in extracts from irradiated virus-infected cells, this further indicates that 67-Luc is induced to background levels only.

As a final analysis, 67-Luc colonies were serum-starved, then infected with recombinant adenoviruses encoding either wild-type or mutant forms of T-Ag. The mutant viruses used here are the same as those described in detail in Chapter 2 and diagrammed in Figure 5 of that chapter. In brief, the mutant virus panel contained three viruses with mutations within the N-terminal region of T-Ag, and three viruses with mutations in the C-terminal region (i.e. ATPase, helicase, and p53-binding domains) of T-Ag. Of the three N-terminal mutations, K1 is defective in its ability to bind both pRb and p107 (8, 13) and was shown, by the experiments described in Chapter 2, to be deficient in its ability to activity the full-length 135-Luc promoter construct. The remaining two N-terminal mutations, inA2803 and dlA2831, encode unstable T-Ag proteins, as determined by Western blot analyses. Activation of 135-Luc with these two mutant viruses was always found to be at background levels due to production of insufficient T-Ag protein. In contrast, all three of the C-terminal mutants encode stable T-Ag proteins, and induction

Figure 4. Induction of luciferase in 67-Luc Rat-1 cells infected with UV-irradiated virus. Results are shown for two independent clonal cell lines. UV-irradiated virus was prepared as described in Materials and Methods. Confluent, serum-starved cells were infected with either untreated (UT) or UV-irradiated (UV) wild-type Ad T/dl309 at an MOI of 200. Luciferase activity was determined as described for Figure 3. Each bar in the graph represents an average level of induction, calculated from duplicate plates of cells in one experiment (n = 2). Error bars reflect the standard error of the mean.

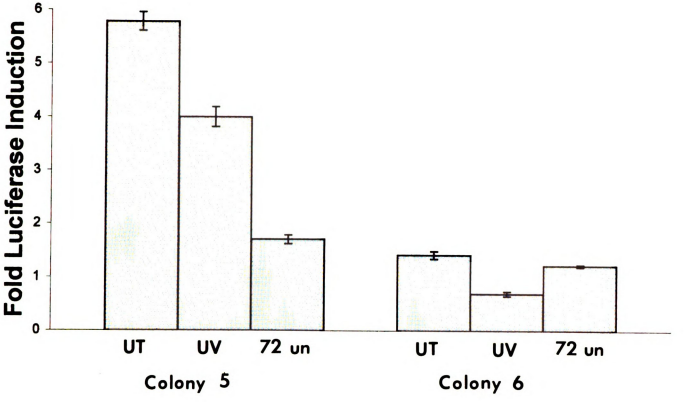

Fold Luciferase Induction
0
1
2
3
4
5
6
UT
UV
72 un
Colony 5
UT
UV
72 un
Colony 6

Figure 4.

of 135-Luc was always near the level of induction attained with the wild-type virus. In terms of 67-Luc, then, we predicted that if activation was specific to the action of T-Ag, differences in the levels of induction would be seen upon infection with the various mutant viruses. In particular, induction levels should be lower with viruses carrying the inA2803 and dlA2831 mutations, since these viruses produce barely detectable levels of T-Ag protein.

In Figure 5A, a composite graph depicting the average levels of luciferase induction in three 67-Luc colonies infected with the various wild-type and mutant T-Ag viruses is shown. Note that induction levels are essentially constant for all three colonies with all viruses, ranging in magnitude from two- to fourfold. In particular, cells infected with viruses carrying the inA2803 and dlA2831 mutations exhibited induction levels equivalent to those attained with wild-type virus and are similar to the level produced with the inA2827 mutant virus, which expresses T-Ag protein at levels greater than wild-type virus (see Chapter 2). Furthermore, extracts harvested from uninfected cells, retained in the serum-starved state throughout the course of infection (72 h uninfected), expressed luciferase to levels near 50% of wild-type, emphasizing that all inductions are within background range.

Induction of the endogenous Rat-1 TK gene in one 67-Luc colony infected with the panel of wild-type and mutant viruses is shown in Figure 5B. Corresponding luciferase inductions are shown for comparison. The results represent one experiment but are representative of the endogenous patterns detected in all experiments in which 67-Luc was infected with mutant viruses. Note that the pattern for TK enzyme expression follows the pattern predicted for a T-Ag-dependent response: induction levels are decreased with the inA2803 and dlA2831 mutant viruses, and are at or above the level of the wild-type virus with the inA2809, inA2811, and inA2827 mutant viruses. This indicates that lack of response with 67-Luc is specific to the transfected 67 bp TK promoter fragment and not

Figure 5. Luciferase and endogenous TK enzyme inductions in 67-Luc Rat-1 cells infected with mutant Ad T/dl309 viruses. Confluent, serum-starved cells were infected with recombinant adenoviruses expressing either wild-type (WT) or mutant (2803, 2831, 2809. 2811, 2827, K1) forms of SV40 T-Ag. Both luciferase and endogenous TK enzyme activities were determined in whole cell extracts prepared at 0 and 72 h after infection, as described in Materials and Methods, and fold induction at 72 h, relative to activity at 0 h, was calculated. 72 un = uninfected cells maintained in low serum medium throughout the 72 h infection period. **(A)** Average luciferase inductions are shown for three independent colonies. Bars are grouped by colony, and the identities of the viruses used and colonies tested are given below the graph. The graph is a composite, with each bar representing an average level of luciferase induction determined from multiple infections. The number of experiments (n) comprising each average is as follows: for Colonies 5 and 6, n = 2 for all; for Colony 10, n = 3 for all. Error bars reflect the standard error of the mean. **(B)** A comparison of the patterns of luciferase and endogenous TK enzyme inductions in one experiment with one infected clonal cell line (67-Luc Colony 5). Each bar represents the level of induction attained in that one experiment only. All data was taken from the same experiment.

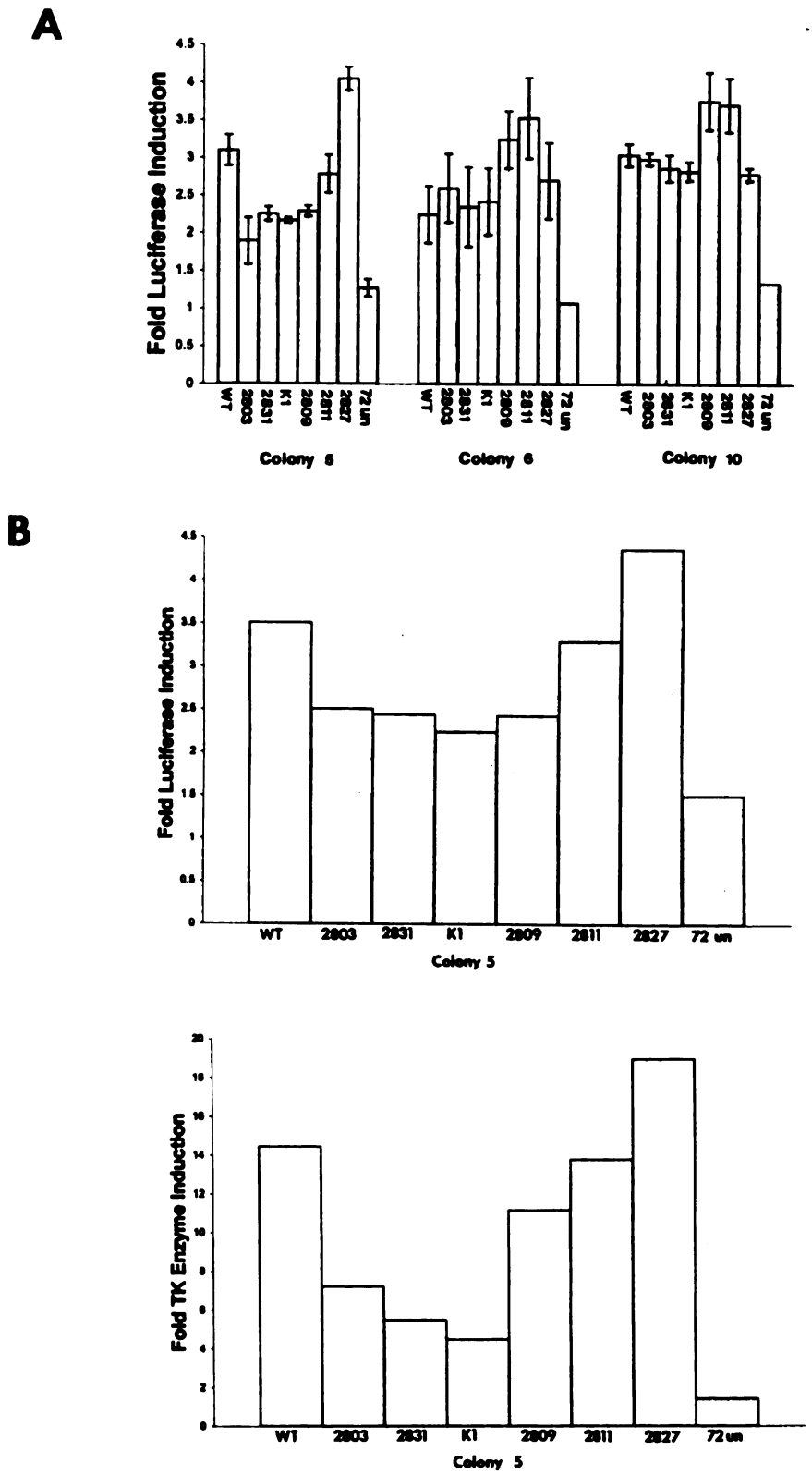

A
Fold Luciferase Induction
WT
2803
2831
K1
2809
2811
2827
72 um
Colony 6
Colony 6
Colony 10
B
Fold Luciferase Induction
WT
2803
2831
K1
2809
2811
2827
72 um
Colony 5
Fold TK Enzyme Induction
WT
2803
2831
K1
2809
2811
2827
72 um
Colony 5

Figure 5.

due to a problem with the growth state of the cell. Thus, the 67 bp TK promoter fragment is not activated by SV40 T-Ag in this system.

Again, the possibility remained that the results obtained with 67-Luc were misleading, due to the highly truncated nature of the promoter fragment. To test this, stable Rat-1 transfectants harboring dl-138/67-Luc were infected with wild-type virus. Four independent colonies were tested, and induction levels averaged only 4.5-fold (data not shown). Since this correlated with the levels obtained with 67-Luc, no further studies were conducted with these colonies.

The results obtained with 67-Luc, then, appear to be valid. These results were both unexpected and disappointing. Since induction of the 67 bp promoter fragment had been detected in SV40-infected CV1 cells, an analogous result was expected here. Also, activation of 67-Luc by Ad T/dl309 was a prerequisite to analyzing the effect of promoter mutations, such as substitutions within the TATA element, on TK regulation. Absence of specific activation of the wild-type 67-Luc parental construct precludes further mutational analysis.

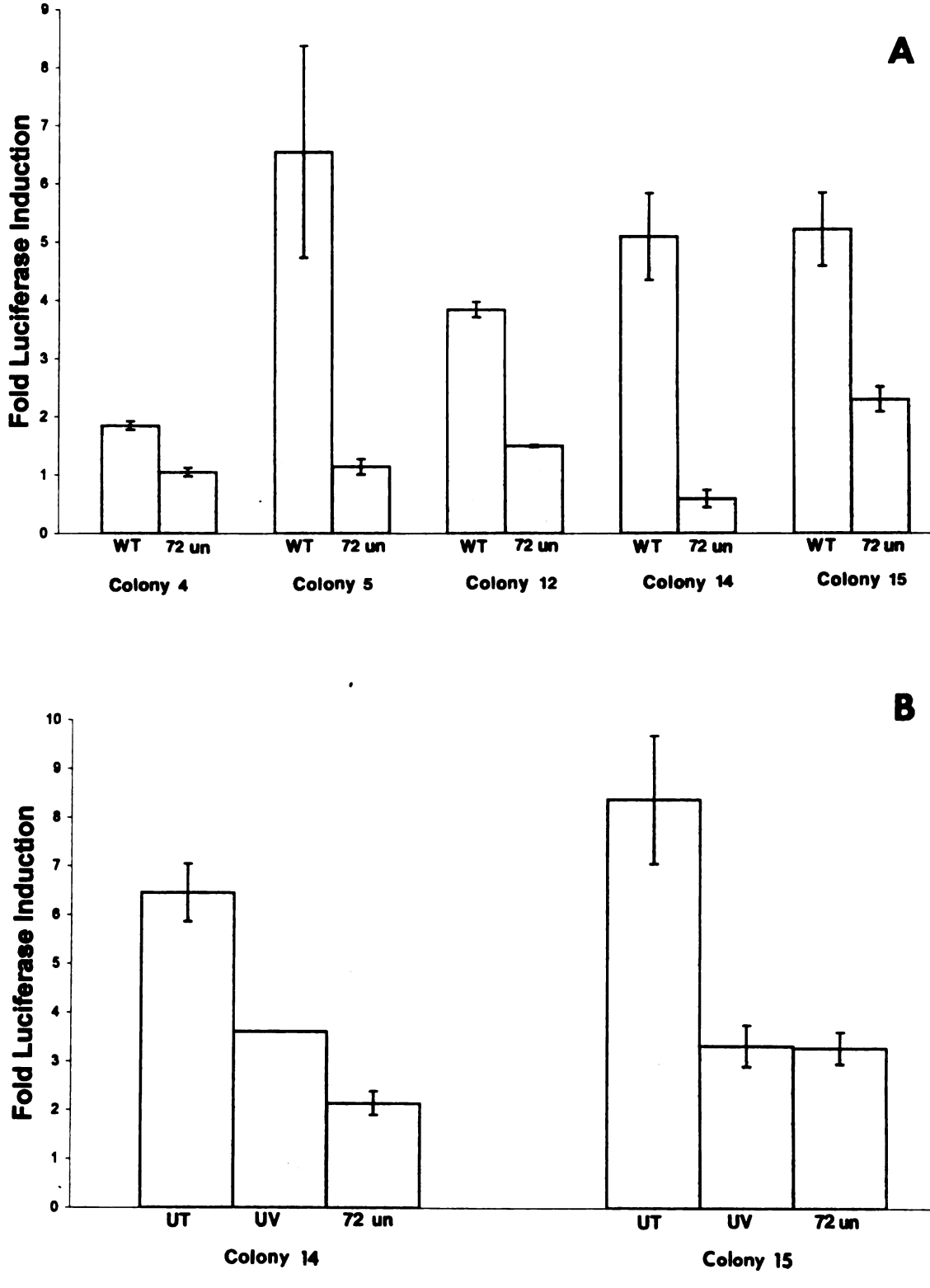
Induction of Hybrid-Luc in cells infected with Ad T/dl309. To determine whether T-Ag was capable of inducing the upstream promoter domain when separated from the downstream domain, the Hybrid-Luc construct was generated (see Figure 1B). As described earlier, this construct contains a hybrid promoter consisting of human TK promoter sequences between -135 and -67 bp connected to 131 bp of the HSV TK promoter containing the HSV TATA box and DSI control region. The HSV TK promoter sequences are included to provide a TATA box for transcription initiation and should not contribute to any T-Ag-mediated increase in luciferase expression. First, the HSV TK promoter is not cell cycle-regulated, as mRNAs expressed from this promoter have been shown to be constitutively expressed (42). Second, experiments described in Chapter 2 explored the ability of T-Ag to activate HTK-Luc, a construct in which

luciferase expression is driven by the same 131 bp of HSV TK promoter sequence; results showed that the HSV TK promoter sequences were not activated by T-Ag.

Figure 6A shows the average induction levels for five independent Hybrid-Luc colonies infected with wild-type Ad T/dl309. Three of five colonies induced to levels fivefold or higher, slightly above background level, while the remaining two colonies were induced an average of only two- to fourfold. The fact that three of the colonies expressed luciferase at levels slightly higher than background was encouraging, since it suggested the possibility of a small T-Ag-dependent effect. As this human TK promoter region contains the E2F-like binding sites, some stimulation of Hybrid-Luc was predicted. The levels of induction, however, are less than 50% of those attained with the 135-Luc colonies, indicating that, if these sequences are being utilized, other sequences are also required for maximal promoter induction. As the induction levels for these colonies are still very close to background levels, however, the evidence supporting a claim for T-Ag-dependent stimulation is unconvincing.

In an effort to further determine whether T-Ag is responsible for the Hybrid-Luc inductions, cells from two of the Hybrid-Luc colonies were serum-starved then infected with either UV-irradiated or untreated wild-type Ad T/dl309 viruses. Extracts were prepared at 0 and 72 h following infection, as per protocol. Results are depicted in Figure 6B. In this experiment, for both colonies, luciferase activity increased six- to eightfold in cells infected with the untreated wild-type virus. TK enzyme assays revealed, for both colonies, low levels of endogenous TK activity at time zero, with twenty- to thirtyfold increases in activity at 72 h post-infection, indicating that cells were effectively arrested and restimulated. Cells infected with the UV-irradiated virus expressed luciferase at only 40-60% of the levels attained in cells infected with untreated virus. While this decrease in induction is not as great as that seen following infection of 135-Luc cells with UV-irradiated virus (a 70% to 75% reduction), the difference is distinct. This suggests, again, that there may be some level of T-Ag-dependent activation occurring.

Figure 6. Luciferase induction in Hybrid-Luc Rat-1 cells infected with untreated or UV-irradiated wild-type virus. (A) Average luciferase induction levels in cells infected with untreated recombinant adenovirus expressing wild-type SV40 T-Ag (WT). Results are shown for five independent clonal cell lines. As described previously, confluent, serum-starved cells were infected at an MOI of 200, and luciferase activity was determined in whole cell extracts prepared 72 h after infection. Luciferase activity was also measured in uninfected cells (72 un). The increase in luciferase activity at 72 h post-infection, relative to activity at the time of infection (0 h) is plotted as fold luciferase induction. Each bar on the graph represents an average level of luciferase induction, calculated from many experiments. The number of experiments (n) comprising each average is as follows: Colony 4: n = 2 for both WT and 72 un. Colony 5: WT, n = 5; 72 un, n = 4. Colony 12: WT, n = 3; 72 un, n = 4. Colonies 14 and 15: n = 6 for both WT and 72 un. Error bars reflect the standard error of the mean. **(B)** Average levels of luciferase induction in cells infected with either untreated (UT) or UV-irradiated (UV) wild-type recombinant adenovirus. UV-irradiated virus was prepared as described in Materials and Methods. Infections and luciferase assay procedures are as described above. Uninfected cells (72 un) were also analyzed for luciferase expression. Results are shown for two independent colonies. Each bar depects the average level of luciferase induction determined from duplicate plates infected in one experiment. Error bars are as for paanel (A).


A
Fold Luciferase Induction
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1
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4
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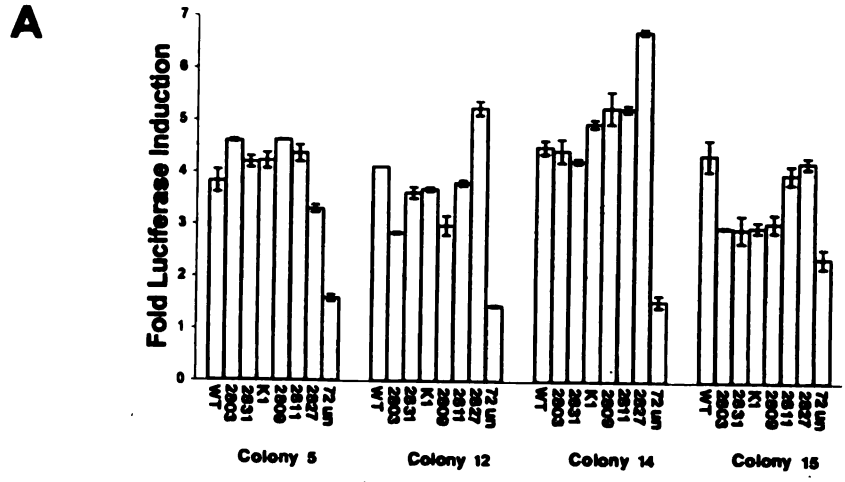
6
7
8
9
WT
72 un
Colony 4
Colony 5
Colony 12
Colony 14
Colony 15
B
Fold Luciferase Induction
0
1
2
3
4
5
6
7
8
9
10
UT
UV
72 un
Colony 14
Colony 15

Figure 6.

To this point, the data regarding induction of the upstream promoter domain has been inconclusive. As a final attempt to determine whether Hybrid-Luc is specifically activated by T-Ag, four colonies were infected with the panel of wild-type and mutant viruses described earlier. Induction results are shown in Figure 7A and represent the average of duplicate plates in one experiment. A T-Ag-dependent pattern of luciferase expression was not seen with any of the mutant viruses. For example, in several of the colonies, luciferase induction levels with the T-Ag-overexpressing mutant, inA2827, are decreased with respect to wild-type virus, while levels in cells infected with the viruses producing unstable T-Ag proteins (inA2803 and dlA2831) are greater than wild-type levels. Figure 7B shows the pattern of induction for endogenous TK enzyme activity from one set of plates for one colony in this experiment (corresponding luciferase inductions are again included for comparison). The remaining three colonies displayed identical patterns in endogenous TK enzyme expression. Note that, as with 67-Luc, this pattern exhibits the predicted variations with the mutant viruses. These results suggest, then, that Hybrid-Luc is not specifically activated by T-Ag in this system. Since 135-Luc was efficiently activated by T-Ag (see Figure 3B and Chapter 2), but Hybrid-Luc and 67-Luc were not, indicates that both promoter domains are required for T-Ag-mediated activation in Rat-1 cells. At present, we do not understand the differences in the results obtained from the CV1 and Rat-1 cell systems.

Figure 7. Luciferase and endogenous TK enzyme inductions in Hybrid-Luc Rat-1 cells infected with mutant Ad T/dl309 viruses. Confluent, serum-starved cells were infected with recombinant adenoviruses expressing either wild-type (WT) or mutant (2803, 2831, 2809. 2811, 2827, K1) forms of SV40 T-Ag. Both luciferase and endogenous TK enzyme activities were determined in whole cell extracts prepared at 0 and 72 h after infection, as described in Materials and Methods, and fold induction at 72 h, relative to activity at 0 h, was calculated. 72 un = uninfected cells maintained in low serum medium throughout the 72 h infection period. **(A)** Average luciferase inductions are shown for four independent colonies. Bars are grouped by colony, and the identities of the viruses used and colonies tested are given below the graph. The graph is a composite, with each bar representing an average level of luciferase induction determined from multiple infections. Averages were calculated from duplicate plates infected in one experiment (n = 2). Error bars reflect the standard error of the mean. **(B)** A comparison of the patterns of luciferase and endogenous TK enzyme inductions in one experiment with one infected clonal cell line (Hybrid-Luc Colony 15). Each bar represents the level of induction attained in that one experiment only. All data was taken from the same experiment.


A
Fold Luciferase Induction
WT
2803
2831
K1
2809
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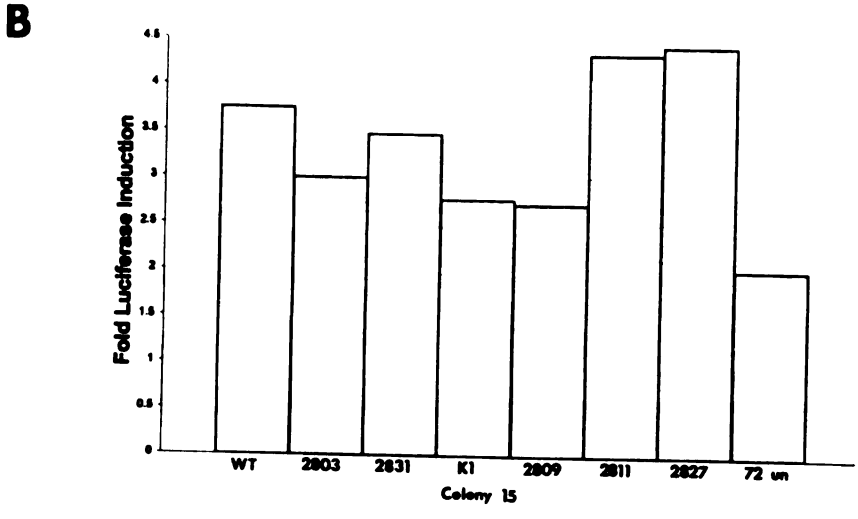
2827
72 un
Colony 5
Colony 12
Colony 14
Colony 15


B
Fold Luciferase Induction
WT
2803
2831
K1
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2811
2827
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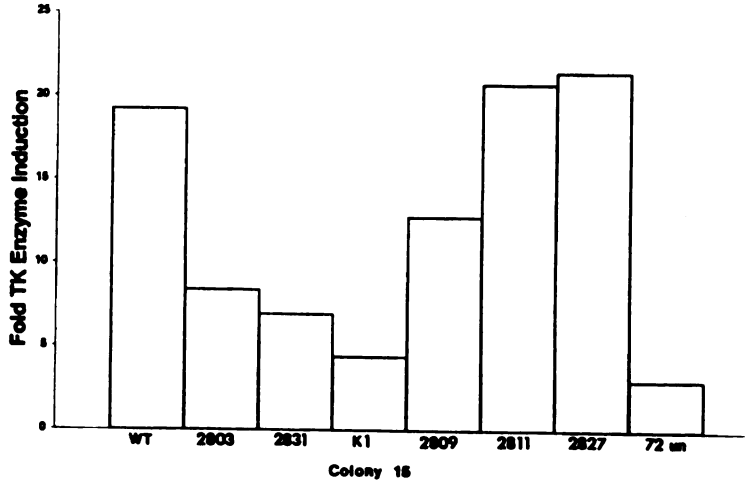
Colony 15


Fold TK Enzyme Induction
WT
2803
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2827
72 un
Colony 15

Figure 7.

Discussion

The experiments described in this chapter were aimed at defining, more precisely, regions of the human TK promoter required for mediating G1/S regulation in SV40-infected cells. Infection of quiescent CV1 cells with SV40 virus results in an S-phase-specific induction in TK enzyme activity (47), mRNA levels (47), and transcription rate (40) attributable to the actions of the viral replicative protein, large T Antigen (37). Previously, we showed that a human TK promoter fragment truncated to 67 bp upstream of the transcriptional start site was sufficient to confer S phase-dependent expression to a linked *Neo* gene in SV40-infected CV1 cells (40). In this study, we have analyzed the expression of a TK-Neo hybrid gene containing a full-length, 444 bp promoter fragment internally deleted of sequences between -135 and -67 bp, in stably transfected serum-starved CV1 cells that were subsequently serum-stimulated or infected with SV40 virus. We detected a six- to eightfold increase in *Neo* mRNA levels at G1/S in SV40-infected, but not serum-stimulated CV1 cells, confirming our previous result that sequences downstream of -67 are sufficient to confer SV40-mediated, but not serum-mediated, regulation. These results were surprising, as several sequences upstream of -67 resemble binding sites for the transcription factor E2F and have been found to be required for serum-mediated regulation (25) and important in T-Ag-mediated TK promoter induction in rat cells (Chapter 2, this dissertation). This prompted us to examine more closely the response of individual TK promoter regions to SV40 T-Ag.

To do this, we separated the TK promoter into individual fragments containing upstream (-135 to -65 bp) and downstream (-67 to +30 bp) sequences. TK-Luc hybrid genes were constructed and tested in stably transfected Rat-1 cells for activation in response to infection with a recombinant adenovirus encoding wild-type or mutant forms of SV40 T-Ag. Contrary to our expectations, neither upstream nor downstream promoter

fragments were activated by T-Ag, indicating that, in this system, these regions alone are insuffucient for conferring regulation.

The results presented here, then, appear to contradict the previous findings in CV1 cells: 67-Neo was induced in SV40-infected CV1 cells, but 67-Luc was not induced in Ad T/dl309-infected Rat-1 cells. With the exception of the reporter gene, the hybrid gene constructs are identical. While it is possible that sequences within the *Luc* gene are interfering with promoter regulation, this is unlikely, particularly since 135-Luc has been shown to be induced to relatively high levels.

A number of other possibilities exist. First, the difference may reside with the two virus preparations used and the corresponding mechanisms for delivery and production of T-Ag. SV40 infection of CV1 cells results in a productive infection with multiple rounds of viral DNA replication and eventual production of high levels of T-Ag. In contrast, the recombinant adenoviruses used here are nonreplicating viruses in the absence of the adenovirus E1A and E1B proteins. In addition, a small deletion at the SV40 Ori prevents SV40-directed replication of the T-Ag coding sequences. Thus, the recombinant adenoviruses serve as vectors for the delivery of T-Ag sequences to the cell interior, where they are transcribed and translated but not replicated. Regardless of the cell type infected, under these conditions, T-Ag protein levels most likely do not reach the levels eventually attained in SV40-infected CV1 cells.

To determine if the discrepancy in our results was due to viral differences in T-Ag delivery and/or expression, T-Ag mRNA levels were directly compared following infection of cells with either SV40 or the recombinant adenovirus. A pool of CV1 cells stably transfected with 67-Neo was infected with both wild-type SV40 and wild-type Ad T/dl309 at MOIs of 15 and 200, respectively. The protocols for infection were as described in Materials and Methods for the two viruses. Total RNA was then prepared at 0, 24, and 48 hours following infection with SV40 and 0, 24, 48, and 72 h following infection with Ad T/dl309 (SV40-infected cells will have lysed by 72 h after infection), and equal

amounts of RNA (20 μg) were electrophoresed through a 1.2% agarose-formaldehyde gel, blotted overnight, then probed for T-Ag mRNA. The Northern blot results are shown in Figure 8.

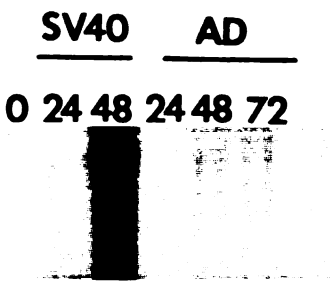

Figure 8. Comparison of T-Ag mRNA levels in SV40-infected and Ad T/dl309-infected CV1 cells. A pool of cells containing the 67-Neo hybrid gene construct was serum-starved then infected with either SV40 (MOI = 15) or wild-type Ad T/dl309 (MOI = 200). Total RNA was prepared at the indicated times (hours) after infection and analyzed by Northern blotting, as described in Materials and Methods. The blot was hybridized with a T-Ag probe.

Clearly, T-Ag message is more abundant at 48 h following SV40 infection than at 72 h following Ad T/dl309 infection. However, in the previous experiments with SV40-infected CV1 cells, *Neo* mRNA levels were measured no later than 24 h following

infection, and, at this point, T-Ag message levels are similar to levels present 72 h following infection with the recombinant adenovirus. A direct comparison of T-Ag mRNA levels in SV40-infected CV1 cells and Ad T/dl309-infected Rat-1 cells was not done, so it is still possible that T-Ag is not expressed at such high levels in Rat-1 cells. Based upon these results, though, it seems unlikely that differences in T-Ag delivery and expression are responsible for the discrepancy regarding the regulation of the 67 bp promoter.

A parallel blot containing the same set of RNAs was also probed with a ^{32}P-labelled DNA fragment corresponding to sequences within the *Neo* gene. This was done in an effort to both reproduce the 67-Neo induction first observed in infected CV1 cells and to verify that *Neo* mRNA levels are increased in parallel upon infection with Ad T/dl309. This would have provided sound evidence that the discrepant results were not due to use of a different virus. The *Neo* probe, however, recognized the cognate RNA species very weakly, too weak, even upon longer exposure, to allow for a qualitative comparison in mRNA levels (data not shown). A photograph of the Northern gel before blotting ascertained that all RNAs were intact and that equal amounts had been loaded. In addition, a human β2 microglobulin probe failed to recognize the corresponding mRNA species and signals with a human TK probe were weak. Taken together, these results suggest that an undetermined technical problem, most likely with probe preparation, was interfering with recognition of the specific mRNAs. Information concerning *Neo* expression upon infection with the two different viruses was, therefore, unattainable.

A second possibility for the discrepancy in our results is the involvement of a cell type-specific factor in regulation. This factor would be present in CV1 cells, the permissive host for SV40 infection, but absent in Rat-1 cells, a nonpermissive cell line uninfectable by SV40. While this particular aspect of the problem was not addressed, previous experiments in this lab have alluded to a cell type-specific factor(s) involved in human TK promoter activity. In particular, it was found that *Neo* mRNA levels were

expressed at constitutively low levels in serum-stimulated Rat-3 and CV1 cells stably transfected with 67-Neo, but expressed at constitutively high levels in analogous serum-stimulated NIH 3T3 transfectants (39). While none of these cell lines were regulated in an S phase-specific manner, in accordance with the established inability of sequences downstream of -67 to mediate regulation in serum-stimulated cells, the differences in the expression of the *Neo* mRNA between the different cell lines suggests the action of cell type-specific factors.

Finally, it is possible that the observed induction of 67-Neo in CV1 cells was not actually T-Ag-dependent. UV-irradiated SV40 virus was not tested in those experiments, so the increase in mRNA detected could have been the result of T-Ag-independent activation mechanisms. Work presented in Chapter 2 of this dissertation supports the hypothesis that most of the T-Ag-independent induction seen is the result of viral-dependent activating events, such as signal transduction triggered by virus binding to cell surface receptors, rather than, for example, the presence of a 293 cell-derived transactivating factor in the virus preparations. This was apparent in that background induction levels were quite significant in cells infected with UV-irradiated virus but low in extracts from cells treated with a 293 cell lysate ("mock-infected" cells). An attempt was made to address this issue in that, in the above Northern blot analyses, RNAs prepared from cells infected with a UV-irradiated SV40 virus were also included. A decrease in *Neo* mRNA levels with UV-irradiated virus, compared to untreated virus, would have verified dependence upon T-Ag for induction of promoter activity. The result of this analysis was also undetermined, however, due to the failure of the *Neo* probe to recognize *Neo* mRNA. Thus, at this point, the differences between these experiments in unclear.

The inability of T-Ag to convincingly activate the upstream (-135 to -67 bp) promoter sequences, as illustrated in the experiments with the Hybrid-Luc construct, was also disappointing, particularly since experiments from Chapter 2 indicate the E2F-like sequences within this region are important for T-Ag-mediated induction of a 135 bp

promoter (-135 to +30 bp). While an E2F protein is apparently involved in regulation of the murine TK promoter (11, 36), its role in regulation of the human TK promoter is unclear. It appears from our results that Hybrid-Luc is not activated by T-Ag in this system. Results, however, were borderline; in some cases, it appeared that a small amount of specific activation was occurring, while in others, induction above background was not evident. It is possible that T-Ag mediates a slight stimulation through these elements, but that the corresponding increases in luciferase induction are masked by the high levels of background induction inherent in these experiments. In any case, it is obvious that the upstream promoter elements are not themselves sufficient for maximal induction of the TK promoter by T-Ag; additional sequences are required.

Since 135-Luc is activated to high levels by T-Ag, it is evident that cooperation between the two promoter domains is required for maximal T-Ag-mediated induction in Rat-1 cells. Alternatively, an important protein binding site may have been destroyed upon splitting of the promoter into the upstream and downstream domain fragments. Results from another lab have verified the presence of a protein binding between the two inverted CCAAT elements (Dr. Ken Lipson, personal communication). As this is exactly the region destroyed upon division of the 135 bp promoter, this protein may represent an important cellular factor required for TK promoter activation in Rat-1 cells. The identity of this protein and its role in TK regulation have not been determined.

In conclusion, sequences between -135 and +30 bp are required for regulation of the human TK promoter in Rat-1 cells expressing SV40 large T-Ag. The identities of required specific sequence elements, in addition to the upstream E2F-like binding sites, remain unclear, although the TATA box may be a potential regulatory elememt. Since the individual upstream and downstream promoter domains were not activated by T-Ag in this system, the effects of mutations within the upstream E2F-like binding sites or downstream TTTAAA element were not able to be tested. Analysis of the role for TTTAAA in human

TK promoter regulation will have to await, for example, the site-directed mutagenesis of 67-Neo in the CV1 system or 135-Luc in the Rat-1 system.

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Summary and Conclusions

An analysis of both the human TK promoter and SV40 T-Ag protein was conducted to identify regions within each involved in T-Ag-mediated trans-activation of TK. To facilitate this analysis, a series of human TK promoter-luciferase hybrid genes (TK-Luc) were constructed and stably transfected into Rat-1 cells. Cells were then infected with recombinant adenoviruses expressing either wild-type or mutant forms of SV40 T-Ag, and luciferase activity was measured as an indicator of promoter activity. Using this method, regions within both the TK promoter and T-Ag protein have been identified as being important for activation, and a model for TK promoter regulation can be postulated.

It was first established that activation of a TK promoter fragment containing sequences from 135 bp upstream to 30 bp downstream of the transcriptional start site (-135 to +30) was largely dependent upon viral early gene expression (presumably T-Ag), since luciferase induction levels following infection with UV-irradiated virus preparations were only 30% of levels attained with untreated, wild-type virus. The residual level of induction, however, indicates the existence of other T-Ag-independent activation mechanisms. A likely source is the activation of gene expression due to signal transduction events triggered by virus binding to cell surface receptors.

Second, it was shown that T-Ag protein domains containing p53-binding, helicase, and ATPase activities are not required for TK promoter stimulation, but that the T-Ag pRb/p107 binding domain is involved. Specifically, a T-Ag mutant unable to bind pRb was severely defective in its ability to induce expression from the 135 bp wild-type TK promoter. In addition, deletion of TK promoter sequences resembling binding sites for the transcription factor E2F abolished promoter activation by wild-type T-Ag, indicating that these promoter sequences are important for trans-activation. More importantly, these

results suggest that cellular factors such as pRb, p107, and members of the E2F family of transcription factors are involved in TK promoter regulation.

Based upon these results, a model for TK promoter activation both during the normal cell cycle and following SV40 infection can be postulated. Specifically, TK may be transcriptionally repressed during G1 due to the binding and inactivation of E2F-like factors by pRb (and/or p107). Phosphorylation of pRb/p107 at G1/S would result in the dissociation of these complexes, releasing transcriptionally-active E2F and relieving TK promoter repression. Alternatively, inhibitory complexes containing pRb or p107 would be dissociated by the binding of SV40 T-Ag to pRb/p107, leading to activation of cellular gene transcription.

Previous work in this laboratory has suggested that alternative TK promoter sequences (besides the E2F-like binding sites) are utilized for promoter activation in SV40-infected cells. Specifically, studies with CV1 cells stably transfected with TK promoter-Neo hybrid genes showed that sequences between -67 and +30 bp were sufficient for promoter induction in SV40-infected but not serum-stimulated cells. This result is significant in that the putative E2F sites are located upstream of -67, between -88 and -113 bp. In this dissertation, this result has been confirmed by studying the expression of a TK promoter-Neo hybrid gene containing promoter sequences between -444 and +30 bp, but deleted of sequences between -135 and -67 bp (encompassing the E2F-like sites), in stably transfected SV40-infected CV1 cells. A six- to eightfold induction in Neo mRNA levels was detected, suggesting that, in CV1 cells, promoter elements downstream of -67 bp (which mainly include the TTTAAA element and one inverted CCAAT box) are sufficient to mediate activation in response to T-Ag.

Therefore, to further investigate involvement of the individual upstream (-135 to -67 bp) and downstream (-67 to +30 bp) TK promoter domains in T-Ag-mediated trans-activation, the promoter was subdivided, and the expression of TK-Luc hybrid genes containing the separate promoter regions was studied in stably transfected Rat-1 cells

infected with the recombinant adenovirus expressing wild-type SV40 T-Ag. Surprisingly, T-Ag was incapable of activating expression from either promoter half, indicating that, in this system, neither sequences downstream of -67 bp, nor the E2F-like binding sites alone, are sufficient for stimulation of TK promoter activity by T-Ag; both promoter domains are required. The discrepancy between these results and those obtained in CV1 cells is, at present, not understood.

While these particular results are disappointing, the data implicating pRb/p107 and E2F-like factors as regulators of TK transcription is significant. Since the human TK gene is highly regulated throughout the cell cycle, being specifically activated at the G1/S interface, and, since TK is a cellular promoter naturally induced following infection with SV40 virus, the link established between the SV40 T-Ag pRb-binding domain and the TK promoter E2F sites strongly implicates an E2F/pRb (or p107) interaction as a fundamental component of cell cycle regulatory mechanisms governing the G1/S transition.

An important challenge for the future, then, would be to identify an interaction between an E2F family member and the human TK promoter. While proteins have been shown to bind to the E2F-like sites within the human TK promoter, their identities remain unknown, and evidence for direct stimulation of human TK promoter activity by E2F is weak. Isolation and characterization of the proteins recognizing the putative TK E2F binding sites would be a priority for future work.

Another focus for future studies would be identification of additional TK promoter elements required for activation by T-Ag. A particularly attractive candidate is the TTTAAA element, since adenovirus E1A protein has been shown to activate gene transcription by interacting with proteins bound to TATA boxes, and a direct interaction between SV40 T-Ag and TBP has been detected. Site-directed mutagenesis studies could be effectively employed to investigate the role of this element

Finally, it would be interesting to further investigate the T-Ag protein domains involved in TK activation, since, as indicated above, important clues to cellular factors

involved can be obtained. Mutations within the first 100 amino acids of the T-Ag protein would be of particular interest, since these regions have been found to interact with a number of cellular proteins, such as TBP, the transcription factor TEF-1, and p300. Analyses of two mutations within this region of T-Ag were attempted in the studies described in this dissertation, and were unsuccessful due to the instability of the mutant T-Ag proteins. If, however, stable mutants could be acquired, exciting information regarding regulation of the human TK gene, in particular, and common cell cycle control mechanisms, in general, could be obtained.