This is to certify that the

dissertation entitled

G1/S PHASE REGULATION OF THYMIDINE KINASE
FOLLOWING SERUM INDUCTION OF QUIESCENT CELLS

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

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Date Aug 8, 1990

G1/S PHASE REGULATION OF THYMIDINE KINASE FOLLOWING SERUM INDUCTION OF QUIESCENT CELLS

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1990

ABSTRACT

G1/S PHASE REGULATION OF THYMIDINE KINASE FOLLOWING SERUM INDUCTION OF QUIESCENT CELLS

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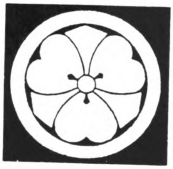
The regulation of expression of the thymidine kinase gene was investigated in serum stimulated cells previously growth arrested and synchronized by contact inhibition and serum deprivation. TK enzyme activities are low in G0, remain low throughout G1 and increase as cells enter S phase. In CV1 cells, the increase of endogenous TK mRNA levels parallels the onset of DNA synthesis and TK enzyme activities after SV40 infection and serum stimulation. In TK^- cells stably transfected with the human TK gene, TK enzyme activities showed the same pattern as endogenous TK after serum stimulation (increasing as the cells enter S phase) indicating that the gene is functional and that sequences required for serum induced expression are linked to the gene.

Various human TK cDNA constructs with different promoters were transfected into Rat3 TK^- cells and assayed for mRNA and enzyme/protein levels in serum stimulated and resting cells. Results from these experiments suggested: 1) The body of the TK cDNA is sufficient for serum induced expression of TK enzyme. 2) The TK gene is regulated posttranscriptionally and transcriptionally. 3) The promoter used to transcribe the TK mRNA can determine the levels of TK mRNA present during G1 and S. However, TK enzyme/protein levels are not strictly

dependent on the promoter or TK mRNA levels present. TK enzyme/protein levels rise concurrently with the onset of DNA synthesis regardless of abundant TK mRNA present.

Deletion mutants of the human TK cDNA were also transfected into Rat3 cells. Results from these experiments suggested: 1) The entire 5' untranslated region, the authentic AUG, nucleotides coding for the first 16 amino acids, and up to 430 nucleotides of the 3' untranslated region are not required for serum induced expression of the TK enzyme.

Levels at which TK may be post-transcriptionally regulated were investigated in the system where TK mRNA levels are uncoupled from TK enzyme/protein levels. Polyadenylation, nuclear/cytoplasmic mRNA transport, sequestering of TK mRNA as a nonpolysomal fraction, and polysomal distribution of TK mRNA were examined in G1 and S phase. No dramatic differences were found at any of these levels between G1 and S phase.




ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Susan E. Conrad. Thanks goes also to the members of my committee and to Drs. Ron Patterson and Richard Schwartz.

I would like to extend personal thanks to, Beryl, JD, Uncle Don, Idah, Holger, and CPM.

A note of thanks should also go to the departments of Biochemistry and Microbiology and Public Health.

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INTRODUCTION

LITERATURE REVIEW

GENE REGULATION

The regulation of gene expression has proven to be complex. Control of gene expression occurs at multiple levels including gene transcription, mRNA metabolism and translation, and protein modification and degradation.

Transcriptional regulation

Transcription is perhaps the best characterized and most commonly studied level of gene regulation. Accurate and efficient transcription is controlled by promoters which are DNA regions located immediately upstream of the transcription start site. Promoters contain conserved DNA elements, such as the TATA box (AT rich sequence) required for accurate initiation of transcription, and the CAAT box (CCAAT) and GC boxes (GGGCGG) required for efficient transcription. Enhancers are another class of DNA sequences responsible for regulating the level of transcription. Unlike promoters, which function in one direction, enhancers function in both orientations, independent of distance upstream or downstream from the gene. For a review on eukaryotic transcription see Gluzman (1).

Transcription can be regulated by various stimuli via elements located within enhancers and promoters. Induction of transcription occurs during viral infection (2), by steroid

hormones (3), heavy metals (4), heat shock (5), differentiation (6), and serum stimulation (7). Serum stimulated regulation of thymidine kinase (TK) gene expression is the subject of this thesis. When appropriate, examples will be cited that relate to serum stimulation or cell cycle dependent regulation of gene expression. Serum stimulation of quiescent cells transiently activates transcription of the c-fos gene. An element located within the promoter is necessary for this transcriptional activation (8). These elements, termed serum response elements (SREs), are found in c-fos and other genes. SREs are the sites of serum response factor (SRF) binding (9) and consequent constitutive or serum induced activation of transcription. Recent evidence suggests that posttranslational modification of SRF, or association of SRF with other factors is involved in serum induced activation of transcription (10). Also, transcriptional regulation of histones during the cell cycle appears to involve changes in the levels of factors present in trans, which interact with transcriptional promoter elements (11,12).

Postranscriptional regulation

For the purposes of this thesis, posttranscriptional control of gene expression will be emphasized. Posttranscriptional regulation involves mRNA processing (ie. capping of the 5' end, polyadenylation and splicing), nuclear-cytoplasmic mRNA transport, mRNA stability, translation initiation, elongation and termination, and posttranslational

regulation.

Capping and the 5' leader

Most eukaryotic mRNAs are thought to be cap-methylated (ie. addition of 5'-methylguanosine through a 5'-5' triphosphate linkage) upon transcription. Although evidence exists indicating that capping is required for efficient translation, capping may not necessarily be required for efficient translation of all mRNAs (13). Shatkin et al.(14) have isolated a cap binding protein (CBP) which regulates the translation of capped and uncapped mRNAs (15). CBP complex (also known as eIF-4F) is now known to play an integral role in mRNA-ribosome binding in translation initiation (16-19). There is evidence to suggest that CBP complex mediates mRNA binding to the ribosome by unwinding the 5' secondary structure of the mRNA (20,21). Cordell et al. (22) have found that translational control of nonallelic rat 1 and rat 2 insulin found in pancreatic tumors is mediated by structural modifications of the 5' end of rat 2 insulin mRNA. Their results suggest that rat 2 insulin mRNA is not capped and therefore not efficiently translated.

As part of the scanning model of translation initiation, cap methylation of mRNA, AUG sequence context, leader length, and secondary structure can dramatically effect translatability (23-26). For example, the translational efficiencies of SV40 early transcripts are regulated through their 5' untranslated regions (utrs) during the SV40 lytic

cycle (27). The early-early (E_E) and late-early (L_E) transcripts use different initiation sites, creating leaders with one or two putative AUG initiation codons in the L_E transcripts. In vitro translation of these SV40 early transcripts have shown that the presence of one or two AUG initiation sites in the leaders of L_E RNAs inhibits efficient translation (from a downstream initiation codon for T antigen).

Polyadenylation

Most mRNAs are polyadenylated at the 3' end as transcription terminates. The 3' ends of RNA polymerase II transcripts are processed in the nucleus by a multiprotein complex [for review see Humphrey and Proudfoot (28)]. The complex cleaves the 3' end of mRNA and polyadenylates to a maximum length of about 260 nucleotides in humans (29). A complex of poly A binding protein (PAB) bound to mRNA is required during mRNA processing (30). Precisely how gene expression is regulated by poly A-PAB functions is largely unknown and controversial. Poly A-PAB functions have been implicated in mRNA nucleocytoplasmic transport (31), cytoplasmic poly A shortening (29), mRNA stability (32), and translation (33).

Examples of modulation of poly A tail length during stimulation of gene expression include vasopressin and insulin regulation. During hyperosmotic stress, vasopressin mRNA levels, protein synthesis, and secretion increase. The

increase in vasopressin mRNA levels is correlated with poly A tract lengthening from ≈250 to ≈400 nucleotides (34). In insulinoma cells, responsiveness to glucose is associated with a change in insulin mRNA size. The poly A tail is 120-140 nucleotides longer in glucose treated cells. No change in steady state insulin mRNA levels was found while secreted insulin levels increased (35).

Studies have been described that suggest poly A tracts are necessary for nuclear-cytoplasmic transport (31). However others have shown that poly A^- mRNA can be transported in vivo (36). Transported poly A^- mRNA may be rapidly degraded in the cytoplasm after transport and the poly A^- mRNA found in the cytoplasm may have been poly A^+ during transport (37). It is also possible that the poly A^- mRNA found in the cytoplasm contains small regions of adenine which bind PAB giving it poly A-PAB functions (38,39).

Initially the poly A tail was thought to be a stabilizer for mRNA (40). In many cases, the poly A tail is removed before mRNA degradation takes place. PAB stabilizes mRNA by preventing the poly A tract from being removed (32). However, for many mRNAs no correlation exists between an mRNA's stability and the length or presence of the poly A tail (29,37,41,42). Therefore, it uncertain how poly A-PAB is involved in mRNA stability.

Similarly, there are conflicting results on how the poly A tract of mRNA effects translation. Translational

efficiencies of mRNAs can be increased by the presence of a poly A tail (29). Translation of poly A^+ mRNA appears to require PAB (33,43). However, naturally occuring poly A^- mRNAs can be efficiently translated in vivo (29). More recently, Sachs and Davis (39) have convincingly shown in S. cerevisiae, that PAB is required for poly A tract shortening in the cytoplasm and ribosomal 60S subunit dependent translation initiation. Their results were obtained by blocking transcription of PAB or through the use of a temperature sensitive PAB mutation to deplete PAB, and by isolating suppressor mutations of a PAB mutation that affect the 60S ribosomal subunit.

mRNA splicing

Another posttranscriptional nuclear event, mRNA splicing, can also be regulated. Pre-mRNA processing may simply be blocked as is the case during heat shock (44). Alternative splicing is well documented in eukaryotes, and produces different mature mRNAs and therefore different protein products from a single primary transcript (45). Control can be conferred by another type of alternative splicing where transcripts retaining introns are untranslated. Differential splicing of introns has been found in genes regulated during development in Drosophila (46,47). For example, the transformer gene is regulated during sexual development in D. melanogaster by differential splicing of its RNA transcripts (48). The transformer transcripts are translatable when all

introns are spliced out, and are not translated when at least part of one intron is retained. Other systems similarly regulated include genes in sea urchin (49) and human (50).

Nuclear-cytoplasmic mRNA transport

Transport of mRNA from the nucleus to the cytoplasm is a fundamental and essential process for mRNAs targeted for translation, yet relatively little is known about the mechanism of transport and its control. It has been proposed that the transcription apparatus is bound to the nuclear envelope, localized for efficient mRNA transport (51). Also, some examples exist which suggest that transport and transcription are somehow associated. For example, during adenovirus infection, most host cellular mRNAs are blocked from transport (52), but certain cellular genes (such as the hsp70 gene family and the β-tubulin gene family) that are transcriptionally induced by the viral protein E1A are able to escape or override the viral transport block (53).

Within the cytoplasm mRNA can be sequestered, degraded, stabilized or, modified to enhance or inhibit translation. Examples of sequestration from translation include: The translational inhibition of preexisting mRNAs during heat shock in Drosophila (54) (these mRNAs are not degraded); the sequestering of some skeletal muscle mRNAs as mRNPs (ribonucleoprotein) and their release during differentiation (55-57).

mRNA stability

Messenger RNA stability is regulated in many systems including during differentiation, viral infection, heat shock, and serum stimulation of quiescent cells. Often, mRNA turnover is also coupled to translation. During adenovirus lytic infection, the early viral mRNAs that are rapidly degraded early in infection are stable late in infection (58). It appears that a 72 kd viral DNA binding protein is necessary for the rapid turnover of early viral mRNA. When the 72 kd protein is not present, the early viral mRNA half life increases 3-5 fold (59).

The stability of hsp70 mRNA is also regulated during adenovirus infection. While transcription of hsp70 continues, its mRNA levels decrease, suggesting enhanced degradation. During heat shock, however, hsp70 mRNA stability increases by approximately 10 fold. When cellular protein synthesis is blocked, hsp70 mRNA is also stabilized, implicating a heat labile protein in its turnover (60).

An instability element has been identified in c-<u>fos</u> mRNA, and in a number of unstable mRNAs. The 3'AU rich element promotes removal of poly A and degradation of the mRNA (61). This removal and degradation seems to require translation. The presence of protein synthesis inhibitors stabilizes the mRNAs.

In other systems, the correlation between translation and mRNA stability have been well defined. A classic example is

that of β-tubulin autoregulation. The concentration of unpolymerized β-tubulin subunits determines the level of β-tubulin mRNA. This regulation was found to occur in the cytoplasm (62). In the presence of free β-tubulin subunits, the β-tubulin mRNA is destabilized, but only if it is being translated. For β-tubulin RNAs to be degraded, they must be polysome bound and translation through 41 codons must occur. In terms of the specificity of mRNA sequences required, 13 nucleotides, corresponding to the 4 amino-terminal amino acids, are necessary and sufficient for this cotranslational regulation (63). Furthermore, Cleveland and coworkers have shown that the 13 nucleotides of the β-tubulin mRNA are not recognized, rather that the corresponding amino-terminal nascent tetrapeptide is recognized by a proposed autoregulatory factor(s) (64). By this interaction, either a ribosome associated RNase is activated, or the RNA is exposed in a favorable conformation for nonspecific RNases.

Cell cycle regulated expression of histone mRNA is also at least in part conferred through regulation of mRNA stability. Histone mRNAs are stabilized during S phase. This stabilization appears to be mediated through the 3' terminal stem-loop structure (65,66). There is also evidence to suggest that translation is coupled to histone mRNA degradation. Proper degradation requires translation through 300-500 nucleotides upstream from the termination site, either to position a ribosome-bound 3' exonuclease or to melt

secondary structure to create a favorable substrate for nucleases (67). It has been proposed that free histone proteins activate histone mRNA decay (68).

Translation initiation

Translational regulation can be defined as a change in the efficiency of translation of a particular mRNA. Translational efficiency is dependent on a number of factors including the rates of initiation and elongation, the number of active ribosomes per mRNA, the proportion of polysomal mRNA, termination, and modulation of factors involved at each step [for a review see (69)].

Translational control mediated through changes in the initiation rate is the most commonly studied mode of regulation. Previously described levels of post-transcriptional processing or modification of mRNA can ultimately manifest themselves at the level of translation. For example, regulated capping, polyadenylation, processing, transport, sequestration, mRNA stability, etc., might alter the translation efficiency of a given mRNA. This change in translational efficiency is most often due to a change in the rate of initiation. A schematic of translation initiation and factors involved are depicted in Figure 1. Regulation of eIF2 and assembly of Met-tRNA$_i$·40S complex, eIF4F, eIF4A, and eIF4B interaction with mRNA (69), scanning, mRNA structure, and modification of factors involved in initiation, can all be involved.

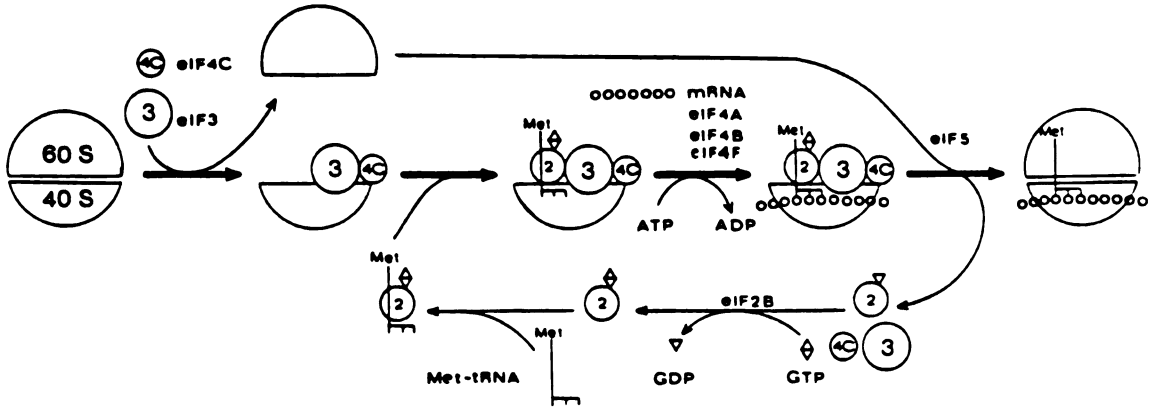

Figure 1. Pathway of initiation of protein synthesis. Figure taken from (69).

Regulation at the level of translation initiation include the involvement of ribonucleoproteins (RNPs) and translational control RNAs (eg. U-rich RNAs, 4S RNA). U-rich RNAs have been identified in many eukaryotic cells and tissues (70-73) as inhibitors of translation. Similarly, the mRNP associated translational control RNA found in embryonic skeletal muscle is able to inhibit translation of skeletal muscle mRNA by binding to the mRNA at the 5' and 3'poly A ends (74,75). However, this translational control RNA from embryonic skeletal muscle is a factor required for message-specific initiation of myoglobin synthesis (76). Furthermore, a 4S RNA found as an RNP in embryonic chick muscle (77,78) inhibits translation. The proteins are not inhibitory (79), but

studies suggest that the 4S RNA inhibits initiation by interfering with binding of mRNA to 43S initiation complexes (80).

The best characterized example of translational regulation by covalent modification of an initiation factor is that of eIF-2 α phosphorylation. Phosphorylation of eIF-2 α is correlated with overall inhibition of translation initiation. However, certain mRNAs such as viral and heat shock protein mRNAs are preferentially translated when eIF-2 α is phosphorylated (81). Phosphorylation of eIF-2α increases under many conditions by activation of various kinases: 1) In heme or iron deprived reticulocytes (82); 2) During serum deprivation (83); 3) Interferon treated cells increase levels of an eIF-2 α kinase, which is activated when followed by infection by many viruses (82); 4) During various stressed states, including heat shock (84).

Other covalently modified translation factors include phosphoproteins, such as, eIF-2 β, eIF-4B, eIF-4E, eIF-5, subunits of GEF and eIF-3, and ribosomal S6 protein (85-89). Modifications other than phosphorylation also occur, such as the lysine of eIF-4D which is changed to hypusine (90). A subunit of eIF-4F is cleaved upon poliovirus infection of human cells (91).

Translation elongation and termination

Examples also exist of regulation during elongation and termination. For example, translation of a gag-pol fusion

protein requires termination suppression or frameshifting for the synthesis of the Rous sarcoma virus reverse transcriptase (92). Translation elongation can also be regulated. Translation of secreted proteins are blocked early in chain elongation by the signal recognition particle (SRP). The SRP recognizes the signal peptide on nascent secretory proteins and blocks their translation. Depending on the presence of certain endoplasmic reticulum factors involved in secretion, such as the SRP receptor docking protein and its interaction with the arrested SRP bound-signal peptide/ribosome, the translational block can be released (93,94). Also, during heat shock, nonheat-shock mRNAs remain polysome bound but are translationally blocked (95).

Posttranslational regulation

Finally, posttranslational control, including protein processing and degradation has been demonstrated. Many hormones are processed posttranslationally to produce the mature, active form. Insulin, parathyroid hormone, glucagon and somatostatin are among these hormones (96-99). Processing can also mean activation or inactivation of mature proteins. For example, many proteins are phosphorylated posttranslationally. During LPS induction of NF-kB, a nuclear enhancer binding protein, a posttranslational modification mediated by protein kinase C appears to be involved (100). Posttranslational degradation is also common for proteins that need to be rapidly cleared. During the cell cycle, genes such

as thymidylate synthase (TS) (101) and TK (102) that are expressed during S phase, are regulated posttranslationaly after S phase. TS and TK proteins are either inactivated or rapidly degraded after S phase.

DNA SYNTHESIS PROTEINS

Proliferating cells go through cell cycles consisting of various phases. The phases are defined as G1 (gap prior to DNA replication), S (DNA synthesis), G2 (gap prior to mitosis), and M (mitosis). Quiescent cells are in a state linked to G1, named G0 (quiescence). Cells in G0 are biochemically distinct from those in G1. Serum deprivation and contact inhibition arrests cells in G0. The molecular basis of the transition from quiescence to proliferation in the eukaryotic cell has been studied by inducing quiescent cells to proliferate by the addition of crude serum or purified mitogenic factors. Infection by Papova viruses, such as SV40 and Polyoma can also induce G0 cells to enter S phase (103).

Initiation of DNA synthesis or transition from G1 to S phase, is among the important steps in cell cycle regulation. During G1, cells grow, prepare for DNA synthesis and determine whether to progress to S or return to G0. G1 is of varying lengths in different cell types. G1 has been divided into various phases (competence, entry, progression, and assembly) by positions or points (C, V, and R) from G0 to S [for review

see (104)].

A complete chromosomal copy within the nucleus is synthesized during S phase. S phase also varies in length between species and between different cell types within a species (105). Synthesis of nucleotide precursors, enzymes involved in DNA replication, and histones are involved in this transition from G1 to S phase. Peak levels of most of these proteins appear during S phase.

Many of the genes which encode the S phase specific proteins are expressed under cell cycle controls. Activities of these enzymes and protein levels increase as cells enter S phase (106). The activities of some enzymes, such as DNA polymerase α (107), topoisomerase I (108), DNA ligase (109) and DNA methylase (110) increase 2-4 fold during S phase. In contrast, enzyme activities of thymidine kinase (111), thymidylate synthase (112), ribonucleotide diphosphate reductase (113), dihydrofolate reductase (114), deoxycytidine deaminase (115), and levels of histone (116) increase greater than 10 fold during S phase. Thymidine kinase (117), thymidylate synthase (112), ribonucleotide diphosphate reductase (118), and dihydrofolate reductase (119) activities remain high throughout S and G2 phases until sometime after mitosis, when activities rapidly decline. The pattern of expression of these enzymes suggests multiple levels of regulation during the cell cycle. The genes encoding these enzymes offer models for molecular analysis of cell cycle

regulated genes.

Among the S phase specific genes investigated (DHFR, TS, histones and TK), no clear picture has yet formed of how the expression of these genes is regulated. However, a few general conclusions can be made. The rates of transcription of these genes have now been shown to increase from G1 to S phase during serum stimulation (120-123). At the posttranscriptional level, an increase in mRNA stability has been shown for all these genes (124-126, Stewart, C. unpublished results). However, variabilities exist in how increases in the rate of transcription and mRNA stability can quantitatively account for increases seen at the protein level. Other posttranscriptional events are regulated for these genes. These include mRNA processing, translational efficiency and posttranslational degradation. What is known of the regulation of several of these S phase specific genes will be discussed in more detail below.

Dihydrofolate reductase

One well studied, cell cycle dependent gene is the dihydrofolate reductase (DHFR) gene. Posttranscriptional regulation of DHFR has been well defined (127), but transcriptional regulation during the cell cycle has been unclear (120) until recently, when a transient peak in the rate of transcription was shown at the G1\S boundary (128). The body of the DHFR cDNA has been shown to be sufficient for cell cycle regulated expression, although deletion of the

first intron decreased the transformation efficiency of DHFR$^-$ CHO cells to DHFR$^+$ (129). Specifically, the 3' end confers this regulation and is dependent on the poly A addition sites used (130). An increase in DHFR mRNA stability has been shown in S phase mouse cells (124).

Thymidylate synthetase

Another enzyme involved in nucleotide precursor synthesis, thymidylate synthetase (TS) has also been investigated. In human and mouse TS, both transcriptional and posttranscriptional cell cycle dependent regulation has been established. However, an increase in TS mRNA stability can account for most of the increase seen in TS enzyme activities (125,131,132). In Saccharomyces cerevisiae, cell cycle dependent regulation appears to be mainly at the level of transcription (121). Posttranslational regulation which destabilizes the activity of the TS enzyme has also been demonstrated following S phase (101).

Histones

Histones are a family of S phase specific proteins. All five classes of histone genes increase their transcription concurrently with the onset of DNA synthesis. In mammalian histone genes, DNA elements and DNA binding factors involved in S phase specific transcription can be specific to the class of histone, but the overall structure of promoters are similar (122,133-137). The work of Heintz and others (11,12,138,139) have led to the postulation that the factors binding to the

class specific elements may be regulated by an overall cell cycle dependent mechanism.

In eukaryotic histone systems studied to date, posttranscriptional controls also exist. Human histones (126) as well as mouse histones (140) are regulated by changes in mRNA stability during the cell cycle. The 5' leader of human H3 histone mRNA is involved in coupling of its stability to DNA replication (141). Luscher et al (142) have found that a RNA processing factor becomes limiting in G1 arrested cells. In their mammalian cell cycle mutant, histone mRNA levels are regulated by 3' RNA processing. Yeast histones have also been determined to be regulated by transcriptional and postranscriptional mechanisms during the cell cycle (143,144).

THE THYMIDINE KINASE ENZYME

Thymidine kinase (TK; ATP;thymidine 5'-phosphotransferase, EC2.7.1.21) is another well studied cell cycle regulated enzyme whose expression is elevated concommitantly with DNA synthesis. TK enzyme levels are low throughout G1 and increase dramatically during S phase. TK is an enzyme in the pyrimidine salvage pathway. It converts dT to dTMP, catalyzing the transfer of gamma phosphate of ATP to dT and analogs of dU. Some of its inhibitors are, dTTP, dT, dCMP, BUdR, FUdR, and IUdR.

TK genes and cDNAs have been cloned from many eukaryotes and viruses. The TK^+ phenotype can be selected for (in

hypoxanthine-aminopterine-thymidine) or against (eg. with FUdR). The availability of *in vitro* mutagenesis, DNA mediated gene transfer, TK enzyme assays, and antibodies against the TK protein aid in the study of the regulation of the TK gene.

An isozyme of the cytoplasmic TK enzyme was discovered when a mitochondrial TK was found in 5-bromodeoxyuridine resistant mutant cells still expressing around 2% of the total cellular TK activity in the mitochondrial fraction, when no activity was detected in the cytoplasm (145). Cytoplasmic TK differs from the mitochondrial form in isoelectric point, sedimentation coefficient, ribonucleoside 5'-triphosphate donor specificity (GTP, CTP, UTP are utilized efficiently by the mitochondrial form, but not by the cytoplasmic TK) polyacrylamide electrophoresis mobility, pH optimum, Km value (146) and dCTP inhibition (the mitochondrial form is effectively inhibited) (147). Mitochondrial TK remains relatively constant throughout the cell cycle while the activity of the cytoplasmic form parallels changes in DNA synthesis (148).

High levels of TK are found in human and animal tumors, cell cultures transformed by oncogenic viruses, and fetal tissues. In normal adult tissues, high TK activity is found in tissues with rapidly dividing cell populations (eg. small intestine, thymus, spleen and bone marrow), and low TK activity is found in non-dividing cells in such tissues as pancreas, testes, lactating mammary gland, lung, brain, and

kidney. Serum stimulation of quiescent cells, DNA tumor virus infection, hormone-stimulation, phytohemagglutinin-stimulation of lymphocytes, rat liver regeneration, and compensatory hypertrophy and hyperplasia in kidney increase TK enzyme activity (149).

The human cytoplasmic TK gene has been mapped to chromosome 17 (150), and the mitochondrial TK gene to chromosome 16 (151). TK^- mouse fibroblast cell mutants (LM TK^-) were isolated in 1963 (152). Other mouse, human, rat, Chinese and Syrian hamster, and bacterial TK^- mutant cell lines have since been isolated (153-155).

The chicken TK gene was cloned by plasmid rescue in 1980 by Perucho and coworkers (156). The Chinese hamster TK gene (157) and mouse TK gene (158) have also been isolated. A functional human TK gene was cloned by gene rescue from a genomic library of TK^- mouse L cells transformed to the TK^+ phenotype with HeLa cell DNA. A 1.6 kb XhoI\EcoRI fragment was found to hybridize to a 1.5 kb human mRNA (159). Lin et al. (160), Lau and Kan (161), and Stuart et al. (162) have also isolated the human TK gene.

The TK protein has been purified to various degrees from HeLa cells, rat Yoshida sarcoma, rat colon adenocarcinoma, regenerating rat liver, human adult liver, calf thymus, and mouse sarcoma (163-169). More recently the human TK enzyme was purified to near homogeneity from Hela cells by ion-exchange and affinity chromatography (170).

The molecular weight of native human TK is 96,000 daltons, consisting of 4 subunits. The subunit molecular weight is 24,000 daltons on SDS-polyacrylamide gels, comigrating with TK activity. This subunit molecular weight of 24,000 daltons correlates well with molecular weights of approximately 25,000 daltons predicted from sequencing of human (171), hamster (172), mouse (157), and chicken (173) cDNAs.

REGULATION OF THYMIDINE KINASE GENE EXPRESSION

At the time the work in this thesis was initiated, very little was known about the regulation of TK gene expression. At that time TK activity was found to increase and decrease periodically, coinciding with the start and end of DNA synthesis (174). The increase in TK enzyme activity observed in synchronized mammalian cells was dependent on protein synthesis (155,175). The enzyme stability during these periods did not vary significantly, indicating that changes in TK protein degradation were not the source of changes in enzyme activity. Instead, it appeared that changes in enzyme activity reflected alterations in TK protein synthesis. Further experiments using hydroxyurea to inhibit DNA synthesis suggested that the beginning of TK synthesis was independent of DNA synthesis. However, termination of TK synthesis, which occurs approximately at the begining of mitosis, does require DNA synthesis. Termination of TK synthesis is also regulated

by a post-transcriptional mechanism that in turn requires RNA synthesis in late S or G2 (174).

It was also known that cell cycle specific regulation of TK is retained in LTK^- cells transfected with human metaphase chromosomes or the cloned human TK gene, indicating that determinants regulating S phase specific induction are associated with the gene (117,159).

Within the last decade, the TK gene has been isolated from mouse, chicken, hamster and human cells. The corresponding cDNAs and promoters have also been cloned, sequenced, and characterized (176,172,173,171,162,177). We and others have used these as molecular probes to study the regulation of TK during various growth states of the cell.

Regulation of TK gene expression has been studied in a variety of systems utilizing several different experimental approaches. These have included examination of the control of the endogenous mouse, chicken, hamster, monkey and human TK genes, as well as the control of transfected cloned TK genes in heterologous cell systems. Growth state-dependent control of TK gene expression has been examined during the cell cycle and in differentiating cells. Also, within the category of cell cycle control of TK gene expression, there are differences in the strategies employed for manipulation of the growth state of the cells. For example, some studies have examined TK expression in cycling cells, while others have examined expression in growth-arrested cells stimulated to re-

enter the cell cycle. The diversity of these systems and strategies makes it difficult to make generalized statements regarding the importance of various levels of regulation of TK gene expression. From these studies, it appears that TK gene expression is regulated at multiple levels ranging from transcriptional to post-transcriptional controls.

We have shown that TK mRNA levels increase 15-20 fold from G0 to S phase in serum stimulated CV1 (simian kidney) cells. This increase corresponds to an increase in TK enzyme activity (162). A transient 6-7 fold increase in the rate of transcription of the TK gene was also found in CV1 cells at the G1/S phase boundary (123).

Rates of TK gene transcription have been measured in populations of quiescent and dividing cells in which TK mRNA and enzyme levels increase at the onset of S phase. Transcription of the TK gene may increase by as much as 11 fold as cells enter S phase.

In serum stimulated murine 3T3 cells, TK mRNA levels have been shown to increase 20-fold from G0 to S. This increase was reported to be due in part to a 2 to 4-fold stimulation in the rate of transcription (178). Nuclear run-on assays performed by Lieberman et al. (176) show similar results, with less than a 2-fold increase in transcription of the transfected mouse TK gene. However, an 11-fold increase in the rate of transcription of the TK gene in mouse L929 cells (wildtype TK^+) was shown (176).

The regulation of expression of the TK gene during estrogen stimulated growth in the human adenocarcinoma cell line MCF-7 appears to be an exception. In the MCF-7 cells transcriptional regulation is the principle level of control of TK during stimulated growth. Changes in human TK enzyme activity are accounted for by changes in steady state levels of message and transcription (179). On the other extreme, although the levels of chicken TK mRNA in avian cells and tissues correlate with cell division, no differences were found in chromatin structure, methylation pattern or rate of transcription of the TK gene in dividing or non-dividing cells. Thus the growth state dependent control of chicken TK shows no signs of transcriptional regulation (180).

Most of the work on the TK promoter has been on the human TK gene. Bradshaw (159), Lin et al (160), Lau and Kan (161), and Stuart et al. (162) have independently isolated the human TK gene. These human TK genes and cloned TK cDNAs (171,162) have been used to identify the promoter.

Deletion mutants of the human TK promoter assayed for transfection efficiency in TK^- cells defined a functional promoter to within 83 bp upstream of the mRNA cap site. The promoter contains GC rich sequences, a CAAT box, and an AT rich region (177). Lipson et al. (181) have also shown that the minimal 83bp retains partial S phase specific regulation by determining TK mRNA levels in ts13 TK^- cells (a temperature sensitive cell line which arrests in late G1 at the

restrictive temperature) stably transformed with various TK promoter deletion constructs expressing TK mRNA. Deletion to a 63bp promoter removes one CCAAT element and diminishes TK mRNA expression and S phase specific regulation. The human TK promoter has also been linked to the bacterial CAT gene and assayed for enzyme activity as a measure of TK promoter strength in L cells. Deletions and mutations have identified a promoter with two-third strength to 139 bp upstream of the mRNA cap site containing a GC, a CCAAT, and TATA element (182) Nuclear DNA binding proteins have been found to bind to two inverted CCAAT boxes within the human TK promoter in a cell cycle dependent manner (183). Binding studies have shown a factor (homologous to nuclear factor Y, which binds to MHC II $E\alpha$ gene promoter) to bind to both CCAAT elements (182).

The importance of transcriptional regulation of the TK gene and the TK promoter in cell cycle dependent expression is supported by the results discussed above. However, in most systems studied the increase in the rate of transcription of the TK gene can not account for all of the increase in TK enzyme levels seen during S phase and in some systems TK mRNA levels are expressed independent of regulated TK enzyme/protein levels. These results suggest the presence of other posttranscriptional levels of regulation.

Stabilization of TK mRNA appears to occur in several systems. TK mRNA half life was approximately 2-3 fold greater during S phase than during quiescence (178, Stewart, C.

unpublished results). Regulated nuclear TK RNA processing has also been proposed. Mature TK message begins to accumulate in the nucleus at the G1/S phase boundary while during G0 very little mature TK mRNA was detected in the nucleus and cytoplasm. This suggests a block in processing during G1 which is released during S (184). In Chinese hamster K12 cells temperature sensitive for DNA synthesis, the mutation blocks progression through G1 to S phase and affects transcription and/or posttranscriptional processing required for TK (115).

Results from a number of laboratories have shown that the elements sufficient to confer growth state dependent regulation of the TK enzyme are intragenic or are contained within the TK cDNA (123,176,185-188). We and others have placed the TK cDNA under the control of its own promoter and various heterologous promoters, such as the HSV-TK and SV40 early promoters. TK enzyme levels were regulated when the TK cDNA was expressed from these promoters. Furthermore, results from experiments utilizing a variety of TK deletion constructs have shown that the polyadenylation signal and most of the 3' utr are unnecessary for growth state dependent regulation of TK expression (176,185,187). Although TK intervening sequences and 3'utr sequences may not be necessary for regulated TK expression, they may be involved in determining the efficiency of TK expression. There is evidence to indicate that TK intervening sequences and sequences 3' of the

poly A addition site affect efficiency of transformation of LTK$^-$ cells by hamster TK minigenes (172).

Baserga and coworkers (189) have examined the induction of TK mRNA levels in cell lines carrying various chimeric constructs of the TK gene. When under the control of various heterologous cellular promoters, TK mRNA levels were highest in G1 arrested cells, after heat shock, independent of the phase of the cell cycle. However, they observed cell cycle regulated expression of TK mRNA levels when the TK cDNA was under the control of the SV40 viral promoter. When under the control of the TK promoter, the bacterial CAT gene is maximally expressed during S phase. However, TK enzyme activity is consistantly high during S phase independent of steady state TK mRNA levels. These investigators have therefore suggested that the TK promoter is important for regulation of TK mRNA levels, because cell cycle regulated expression of TK mRNA levels were lost when the promoter was replaced by other cellular promoters. They also suggest that translational controls determine TK enzyme activity levels.

We have similarly shown that TK mRNA levels are dependent on the promoter used to transcribe the message, as the SV40 early promoter directs a different pattern of TK mRNA induction than do the HSV-TK and wild type TK promoters. However, for all of these promoters, TK enzyme levels are low during G1 and high during S phase, independent of TK mRNA levels (185). When TK mRNA is expressed under the control of

the SV40 early promoter TK mRNA levels are induced within two hours after serum stimulation of resting cells, but it is not until S phase that TK enzyme/protein levels increase. These results also support the presence of posttranscriptional controls in the regulation of TK.

In addition, our experiments utilizing various heterologous promoters to control the expression of TK cDNA and various TK cDNA deletions have shown that TK cDNA sequences are directly or indirectly involved in regulating the expression of the TK enzyme. However, the role of the TK cDNA in the transcriptional regulation of TK remains unclear. Perhaps when TK mRNA is transcribed from a weak promoter such as the HSV-TK promoter, sequences within the TK cDNA can affect the expression of TK mRNA levels. Alternatively, these TK cDNA sequences may be acting posttranscriptionally, for example, in altering TK mRNA stability. When a strong promoter such as the SV40 early promoter is controlling the expression of TK mRNA, the transcriptional or posttranscriptional effects conferred by the TK cDNA may simply be overpowered. At what level the TK mRNA regulates the growth state dependent expression of the TK enzyme also remains unanswered. The expression of TK mRNA and TK enzyme/protein levels was unaffected in our TK cDNA deletions of the 5'utr, authentic AUG, nucleotides coding for the first 16 amino acids, and most of the 3'utr. These results (Chapter 2) and those discussed in Chapter 3 suggest that this

posttranscriptional regulation acts at a level after translation initiation.

There are other systems where TK mRNA levels do not drastically increase from non-growing to growing states. For example, the decrease in chicken TK mRNA levels during myoblast differentiation was low and variable, but enzyme activity and protein levels were regulated efficiently, indicating that TK is regulated by decreased translation or increased degradation of TK protein (190). The polysomal distribution of chicken TK mRNA does not change during muscle cell differentiation. Therefore, TK synthesis is regulated at the translational level that does not effect the polysomal distribution of TK mRNA (191).

Sherley and Kelly have purified TK enzyme from Hela cells and produced polyclonal antibody (170). They have utilized this antibody to show that in cycling Hela cells, changes in enzyme activity correlates with TK protein levels. However, TK mRNA levels show only small changes during the cell cycle and the rate of TK protein synthesis increases 10 fold from G1 to S phase. Also, upon cell division TK protein is rapidly degraded, decreasing TK to basal G1 levels (102).

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STATEMENT OF THE PROBLEM

In tissue culture cells made quiescent by serum deprivation and contact inhibition, serum stimulation causes the cells to leave G0 and enter G1 and S phases, with TK mRNA and protein/enzyme levels being induced at the onset of S phase. The overall goal of this research project was to determine the level(s) of regulation in the expression of the human TK gene during G1 and S phase. Specifically, potential posttranscriptional regulation occuring before and during translation were investigated.

The isolated human TK gene transfected into TK^- cells still maintains regulation during G1/S phase. Therefore the human TK gene contains element(s) which respond to cell cycle controls. In the present studies, the regions of the TK gene containing the putative element(s) were investigated with respect to posttranscriptional controls. These regions of the gene included the promoter and the cDNA (exons, and 5'and 3' untranslated regions). Results from these investigations will contribute to a better understanding of DNA elements involved in the cell-cycle dependent regulation of gene expression.

CHAPTER 1

INDUCTION OF THYMIDINE KINASE

Results presented in this chapter represent the author's contributions to the publications referenced as references 1 and 9

The specific experiments that the author was involved in are listed below:

- -RNA-Northern analysis
- -^{3}H-dT pulse labeling
- --DNA content determination
- -^{35}S-methionine protein labeling
- --Immune precipitation
- --SDS-PAGE
- -TK enzyme assays
- -Expression of TK enzyme and mRNA in Rat 3 cells transfected with TK cDNA constructs, herpes simplex virus TK gene, and the human TK gene.

CHAPTER 1

INTRODUCTION

To initiate a molecular investigation of thymidine kinase (TK) as a model enzyme that is regulated during the cell cycle, the human genomic TK locus and a functional TK cDNA clone were isolated by Stuart et al.(1). Utilizing established procedures (2,3), DNA from a human transformed cell line GM638 was transfected into mouse LTK^- cells and selected for TK expression in HAT medium (containing Hypoxanthine, aminopterin and thymidine). Secondary transformants were established by isolation and transfection of genomic DNA isolated from the initial transformants into LTK^- cells. Secondary transformants containing human DNA were identified by southern analysis of genomic DNA, using the human Alu family of repeats (4) as a probe. One strongly Alu-hybridizing cell line was used to make a library in lambda charon 30 from which 7 overlapping clones that hybridized to the Alu probe were isolated. When lambda clones were transfected individually into Rat3 TK^-cells, no TK^+ colonies were detected. Several pairs of phage, however, gave rise to TK^+ colonies when co-transfected into cells. Restriction maps of these clones were determined (Figure 1), and are in agreement with other published maps of the human TK locus (2,3,5). For details on the cloning of the human TK locus see Stuart et al. (1).

A functional TK cDNA clone was isolated from a cDNA library (6) made from poly A^+ RNA of GM639 cells (1). The cDNAs were cloned into an expression vector containing the pBR322 origin of replication and β-lactamase gene, and the SV40 early promoter, splice donor and acceptor, and polyadenylation signal (Figure 2A). One of the longest cDNAs homologous to the TK gene, pHuTkcDNA7 contained a 1.5 kb insert, in agreement with the known size of TK mRNA. pHuTKcDNA7 also was able to transfect Rat2 TK^- cells to TK^+ and to express TK activity to high levels.

Mapping experiments indicated that there were a minimum of 3 introns in the TK gene based on the regions of the lambda clones that hybridized to the cDNA. An approximate length of 14.5 kb was also determined for the gene. The direction of transcription of the TK gene was shown based on the known orientation of the cDNA insert in the expression vector (Figure 1).

Using single copy subclones either from within the genomic locus or TK cDNA, a series of experiments were initiated looking at the induction of TK enzyme activities, mRNA, and DNA synthesis in serum stimulated and SV40 infected CV1 cells. Results from these experiments showed that TK mRNA levels increase 10 to 20-fold in serum induced and SV40 infected CV1 cells. Regulation at various stages of RNA metabolism could account for these changes in TK mRNA levels. In turn, these increases in steady state mRNA levels could

account for the increases seen in TK enzyme activity. In both serum induced and SV40 infected cells, the induction of TK mRNA and enzyme occur as the cells enter S phase.

To determine whether the rate of TK gene transcription is regulated during serum induction and SV40 infection, nuclear run-on transcription assays were performed (9). The results of an extensive time course experiment spanning the G1/S phase boundary showed a 6 to 7-fold increase in the rate of TK gene transcription in serum stimulated cells. In SV40 infected cells virtually no transcriptional induction was detected.

In an attempt to identify DNA sequences required *in cis* for the regulation of TK gene expression, regulation of the human TK gene (genomic) and various TK cDNA constructs was studied after transfection into Rat 3 TK^- cells. TK mRNA and TK enzyme activity were assayed every 12 hrs. after serum stimulation. TK cDNAs expressed from either the human TK promoter or the SV40 early promoter repeatedly showed similar mRNA and TK enzyme induction patterns. In these experiments, TK mRNA and enzyme levels increased in parallel by 12 hr post-serum stimulation. These results, and those of others (7,8) utilizing another heterologous promoter, the herpes virus TK promoter, led us to believe that sequences within the body of the cDNA were sufficient to confer growth dependent regulation of TK. A more extensive time course early in G1 would reveal that this conclusion is true for regulation of the TK protein and not for TK mRNA. These experiments which address the

roles of the promoter used to transcribe TK mRNA and TK cDNA sequences during serum induction are presented in Chapter 2.

Since the increase in the rate of transcription (6-7 fold) found (9) in serum stimulated CV1s does not account for all of the changes seen in mRNA levels (10-20 fold), neither temporally nor quantitatively, transcriptional regulation was unlikely to be the only mechanism involved. Posttranscriptional regulation was strongly suggested by the apparent normal cell cycle dependent behavior of the TK cDNA hybrids in culture.

MATERIALS AND METHODS

DNA Transfections. DNA transfections were done according to the method of Wigler et al.(10). DNA was added as a $CaPO_4$ precipitate to 5 x 10^5 cells, and TK^+ colonies were selected in hypoxanthine-aminopterin-thymidine (HAT) media. After 2-3 weeks colonies were picked or pooled into cell lines.

Nucleic acid hybridizations. Hybridizations to RNA Filters (Northerns) were in 50% formamide-3x SSC-5x Denhardt solution-50mM Na phosphate (pH 6.8)-5% dextran sulfate-0.1% SDS-50 μg/ml denatured salmon sperm DNA-approximately 10^6 cpm of ^{32}P-labeled probe per ml. Hybridizations were at 42°C for 15-20 h. Filters were washed at room temperature in 2x SSC-0.1% SDS once then 2-3 times in the same solution at 65-68°C. For high background additional washes in 0.1x SCC-0.1% SDS followed.

Viral infection of tissue culture cells. CV1 (African green monkey kidney) cells were grown to confluence in Dulbecco's Modified Eagle's Medium supplemented with 5% calf serum and 5% fetal calf serum. Cells that had been confluent for 48 h. were infected with SV40 at a multiplicity of infection (MOI) of 5. Culture medium was removed, then virus was added in 1 ml of serum-free medium per culture dish. Cells were incubated at 37°C, 5% CO_2 for 1 h., with shaking every 15 min. At the end of the 1 h. incubation, the medium containing virus was removed, and was replaced with the original culture medium. Mock infected cells were treated with serum free medium for 1h. At various times after infection, cells were harvested for RNA, protein, or DNA synthesis analysis.

Poly A^+ RNA isolation and Northern blot analysis. Polyadenylated (poly A^+) RNA was isolated from tissue culture cells as follows: Cells were washed with phosphate buffered saline (PBS) without calcium and magnesium, then lysed with 1ml of lysis buffer (0.5M NaCl, 10mM Tris-pH 7.5, 1mM EDTA, 1% SDS, 200 μg proteinase K per ml) per 100mm plate. The cell lysate was scraped from the plate and passed 3 times through a 21-gauge needle to shear chromosomal DNA. Additional proteinase K was added to 100 μg/ml and the lysate was incubated at 37°C for 30-60 min. Approximately 0.1ml (packed volume) of oligodeoxythymidylate-cellulose was added per plate, and the RNA was batch bound by shaking at room

temperature for 1h. The mixture was then loaded into a small column and washed with 20 column volumes of loading buffer(0.5M NaCl, 10mm Tris, 1mM EDTA, 0.2% SDS) followed by 20 column volumes of the same buffer containing 0.1M NaCl. RNA was eluted with 2 column volumes of 10mM Tris-1mM EDTA and ethanol precipitated with tRNA carrier.

RNA was resuspended in sample buffer (50% formamide, 1x running buffer, 2.2M formaldehyde), heated at 60°C for 5 min, and electrophoresed on 1.2% agarose gels containing 2.2 M formaldehyde. RNA extracted from equal numbers of cells was run in each lane. Running buffer was 20mM MOPS (morpholinepropanesulfonic acid) pH 7-1mM EDTA-5mM sodium acetate. Gels were washed in water after electrophoresis and then in 20x SSC for 30 min. RNA transfer to nitrocellulose filters was in 20x SSC.

TK enzyme extraction. Cells to be assayed for TK activity were harvested by a modification of the method of Johnson et al. (11). Two 100-mm plates of confluent cells were washed with cold PBS, and cells were removed from the plate with a rubber policeman in 1 ml PBS. The cells were pooled, pelleted, and suspended in 200 μl of NonidetP-40 (NP40) reagent (50mM Tris-HCl pH8.0, 3.6mM ß-mercaptoethanol, 0.5% NP40). The lysed suspension was vortexed, the nuclei were pelleted, and the supernatants were frozen at -70°C in two 100 μl aliquots.

TK assay. TK activity was determined by a modification of the methods of Ives et al. (12) and Johnson et al. (11).

Either 5, 10, or 20 μl of the thawed cell extract (brought to a 20 μl volume, if necessary, with NP40 reagent) was added to 60 μl of reaction buffer to yield a final concentration of 50mM Tris-HCl(pH8.0), 15mM NaF, 3.6mM ß-mercaptoethanol, 5mM ATP, 2.5mM $MgCl_2$, 0.08mM cold thymidine, and 50 μCi of ^{3}H-thymidine (specific activity: 20Ci/mmol) per ml. The reaction was incubated at 37°C for 10, 20, or 30 min and stopped by immersing for 2-3 min in a boiling water bath. Control reactions without ATP were included at both zero and 30 min. Ten or twenty μl samples of the reactions were spotted in duplicate on Whatman DE81 anion-exchange filter paper as follows: +ATP, t=30 min; -ATP, t=30 min; -ATP, t=0; and for a total count of ^{3}H-thymidine available, +ATP, t=30 min, without washing. The filters were dried, washed 2 times in 1mM ammonium formate and once in methanol, and dried. The disks were then placed in scintillation vials, and the dTMP product was eluted by adding 1ml of 0.1M HCl-0.2M KCl and shaking for 20-30 min. Ten ml of liquid scintillation fluid was added per vial, gently shaken for 2-4 h, and counted for ^{3}H.

TK activities are expressed in nmol of deoxythymidine converted to dTMP per minute per μg of extract protein:

$$\text{TK unit} = \frac{[(\%\ \text{conversion})(6.6\text{nmol of dT in 80 } \mu\text{l of rxn})]}{[(\text{reaction time}=30')(\ \mu\text{g of protein in rxn})]}$$

$$\%\ \text{conversion} = \frac{[(\text{cpm of +ATP, t=30'}) - (\text{cpm of -ATP, t=0})]}{[\text{cpm of unwashed control}]}$$

The protein concentrations in the extracts were determined by either the Bradford (13) or the Lowry (14) protein assays, using standard curves of purified gamma-globulin or bovine serum albumin, respectively. NP40 did not interfere with either assay in the 8-40 μg/ml range of protein. The amount of enzyme activity observed was directly proportional to both the concentration of the enzyme and the elapsed time of reaction up to conditions converting 50% of the substrate to product.

DNA pulse-labeling. To measure DNA synthesis, serum-starved or stimulated cells on 100mm plates were labeled in 1ml of media with 1 μCi of ^{3}H-deoxythymidine and $4x10^{-7}$M uridine for 1h. Labeled cells were then washed with PBS, trypsinized in 2ml, spun down, and resuspended in 0.5 ml of PBS. Aliquots were counted on a hemacytometer. Two 100 μl aliquots were precipitated onto fiber glass filters (Whatman GFC) with 5% trichloroacetic acid (TCA). The precipitated material was washed with 5% TCA, then 95% ethanol, dried, and counted for ^{3}H in 10 ml of aqueous liquid scintillation fluor. The remaining 300 μl of cells in PBS were assayed for DNA content by the diphenylamine colorimetric assay (15). The cells were incubated for at least 30 min in 0.1% SDS-0.1mg of proteinase K per ml at 37°C, ethanol precipitated, and resuspended in TE before the assay was performed. Results were expressed as cpm ^{3}H incorporated per μg DNA or per cell.

Radiolabeling of proteins. Cells were labeled in 1 ml

methionine free media supplemented with 50 μCi ^{35}S-methionine at various times post-infection. After a 45 min incubation, cells were washed 2x in ice-cold PBS and once in T-Ag wash buffer (0.137M NaCl, 20mM Tris-HCl pH 9.0, 1X Ca^{2+}-Mg^{2+} salts). Cells were then lysed on the plate by addition of 1ml of extraction buffer (T-Ag wash, 10% glycerol, 1% NP40, 1mM phenylmethyl sulfonyl fluoride) and incubated at 4°C for 20 min. The lysates were then scraped from the plate, centrifuged, and the supernatants were stored at -70°C.

Immunoprecipitation. The lysate (1ml) was added to 80 μl of a 50% suspension of protein A-Sepharose. Anti-T-Ag antiserum was added, and this mixture was kept on ice and vortexed every 5 min for 30 min. The Sepharose-antibody-protein complex was centrifuged in a microcentrifuge, and the pellet was washed once with PBS, twice with wash buffer (0.5M $LiCl_2$, 100mM Tris-HCl pH6.8), twice with 1% deoxycholate-1% NP40 in Tris-buffered saline, and finally twice in PBS. The final pellet was dried in a vacuum dessicator, and 50 μl of protein sample buffer (50mM Tris HCl, 2% SDS, 5% β-SH-ethanol, 10% glycerol, 0.001% Bromophenol Blue) was added.

SDS-polyacrylamide gel electrophoresis. Samples were heated to 100°C for 3min and spun in a microcentrifuge. The supernatants were resolved by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel by the procedure of Laemmli (16). The gel was stained, destained, fluorographed, dried, and exposed to X-ray film at -70°C.

TKcDNA constructs. pHuTKcDNA7 and p5'TKcDNA, two TKcDNA clones capable of expressing TK after transfection into eukaryotic cells, are diagrammed in Figure 2. pHuTKcDNA7 is the original TKcDNA clone that was isolated from the Okayama and Berg library (6) and contains the SV40 early promoter, an SV40 splice donor and acceptor, the SV40 late polyadenylation signal, and virtually the entire HuTKcDNA sequence. p5'TKcDNA was derived from pHuTKcDNA7 by replacing the SV40 promoter and splice signals with the 5' region from the genomic human TK locus. The 5' genomic region was linked to the cDNA at an Sma I site within the first exon of the gene. These two plasmids were transfected into Rat 3 TK^- cells, and TK^+ transformants were selected and propagated both as pools of approximately 50 colonies each and as clonal cell lines derived from single colonies.

RESULTS

As discussed in the introduction section, the human TK gene locus and a functional human TK cDNA clone were isolated by Stuart et al. (1). These clones were necessary to begin a molecular analysis of TK gene regulation during the cell cycle. TK enzyme activity was assayed in quiescent and serum stimulated Rat 3 TK^- cells transfected with overlapping genomic lambda clones at various times post serum stimulation. TK enzyme activity was cell cycle regulated, increasing with the onset of S phase. TK enzyme activity routinely increased

by 12 h after serum addition, as did TK activity in Rat 1 cells, the parental TK^+ cell line (data not shown). As negative controls, Rat 3 cells and Rat 3 cells transfected with a cloned herpes virus TK gene were used. (The herpes simplex virus TK gene is not cell cycle regulated.) TK activity remained at basal levels after serum stimulation of Rat 3 cells. Herpes TK activity remained constitutive at a level slightly higher than basal (data not shown). These results indicated that DNA elements necessary for normal regulation were associated with the gene. Cellular components required for serum induction of the transfected TK gene were also present.

SV40 induction of TK. Papovaviruses such as SV40 and polyoma induce both DNA synthesis and many enzymes involved in DNA synthesis. With the availability of DNA probes for TK, a molecular analysis was initiated on the induction of TK in virally infected cells.

Poly A^+ mRNA was isolated at various times from 0 to 48 hr following SV40 infection of confluent CV1 cells. RNA from equal numbers of cells from each time point was electrophoresed on 1.2% formaldehyde-agarose gels, blotted onto nitrocellulose, and probed with a ^{32}P-labeled subclone containing a 1.4kb fragment from the coding region of the TK gene (fragment is underlined in Figure 1) as described in Materials and Methods. The results are shown in Figure 3A. An increase in TK mRNA levels was seen at 24hr. A duplicate

Northern blot hybridyzed with an SV40 probe is shown in Figure 3B. SV40 early mRNAs are detected at 12hr and SV40 late mRNAs are detected at 24 hr post infection. By densitometer scanning the autoradiogram in Figure 3A, TK mRNA induction was quantitated to be approximately 15-fold by 48 hr (Figure 4). TK enzyme activities were also assayed in extracts of cells infected concurrently with those used for mRNA isolation. The results of these experiments showed that TK enzyme induction followed the same pattern as TK mRNA induction (Figure 4). Measurements of ^{3}H-thymidine incorporation determined that the onset of S phase was by 24 hr postinfection (Figure 4).

T antigen expression and production during TK induction. It has been shown that SV40 T antigen is necessary for TK induction during infection (17). To determine the temporal relationship between T antigen expression and TK mRNA induction, duplicate Northern blots containing poly A^{+} RNA isolated every 4 hr after SV40 infection were hybridized to TK and SV40 probes. TK mRNA induction begins by 16-20 hr (Figure 5A). The same blot was rehybridized with a c-myc probe to determine TK mRNA increases relative to c-myc mRNA (Figure 5B) and quantitated by scanning densitometry (Figure 6). c-myc mRNA levels increase within a few hours after serum stimulation (18,19). In this experiment, SV40 infection of CV1 cells also induced a 3-fold increase in c-myc mRNA levels by 4hr, and remained relatively constant thereafter. Thus the

TK mRNA induction is not likely to be due to an increase in the total level of cellular RNA. A duplicate Northern blot hybridized to an SV40 probe is shown in Figure 5C. In this experiment early and late SV40 mRNAs appear by 12 and 16 hr respectively. In other experiments early mRNA has been detected by 8 hr.

To assay for the presence of SV40 T antigen protein, cells infected concurrently with those harvested for mRNA were labeled with ^{35}S-methionine. Proteins immunoprecipitated with anti-T-antigen antibody were electrophoresed on a 10% SDS polyacrylamide gel. T antigen appears approximately 8-12 hr before the increase in TK mRNA (Figure 5D).

Serum induction of TK. The kinetics of serum and SV40 induction of TK mRNA levels were compared in parallel time course experiments. Confluent CV1 cells were serum stimulated with fresh media containing 10% serum or infected with SV40 at an MOI of 5. PolyA$^+$ mRNA was isolated at various times following induction, and assayed for TK mRNA relative to c-myc and ß-2 microglobulin mRNAs. In serum stimulated cells, TK mRNA began to increase by 12 hr (Figure 7) coinciding with the onset of S phase. This increase in mRNA levels was followed by an increase in TK enzyme activities (data not shown). A significant increase in TK mRNA levels was not seen until 16 hr in SV40 infected cells, as seen in previous experiments (Figure 3, Figure 5).

TK induction in cell lines transfected with human TK cDNA constructs. The cDNA constructs shown in Figure 2 and described in Materials and Methods were transfected into Rat 3 TK^- cells and selected in HAT media for TK^+ transformants. Cell lines were derived from both pools of approximately 50 colonies and from single colonies. The resulting cell lines were tested for their ability to regulate TK expression in an S phase specific manner by measuring TK mRNA and enzyme levels in resting and serum stimulated cells. The regulation of TK mRNA levels was investigated by isolating $polyA^+$ RNA at 0, 12, and 24 hr after serum stimulation and quantitating this RNA by Northern blot analysis as described in Materials and Methods. In Figure 8A the three cell lines containing pHuTKcDNA7 were as follows: cell lines 1C and 2A were derived from single colonies, and cell line m was derived from a pool of colonies. In Figure 8B the results are shown from cell lines containing p5'TKcDNA; D3 and A1 were derived from single colonies, and m was derived from pooled colonies. All of these cell lines showed increased levels of TK mRNA by 12 hr after serum addition, and similar results were obtained with several other transfected cell lines tested (data not shown). Rat 1 TK^+ and Rat 3 TK^- cell lines were also included in this experiment as positive and negative controls. The results of TK enzyme assays on the cell lines described above are given in Table 1. These results are in agreement with the mRNA data, with peak levels of TK enzyme activity at either 12 or 24 hr after serum

stimulation. But again, TK mRNA and TK enzyme activities were not assayed between 0 and 12 hrs. post serum stimulation. Thus, both pHuTKcDNA and p5'TKcDNA appeared to be regulated with the cell cycle after serum induction of transfected cell lines, suggesting that sequences within the body of the cDNA were sufficient to confer S phase specific TK mRNA and TK enzyme expression upon these hybrid genes. An alternative explanation was that the SV40 early promoter was itself regulated in an S phase specific manner. Although we had not tested this hypothesis directly, experiments in the laboratory of N. Heintz had indicated that this promoter was not activated during the transition of cells from G1 into the S phase (N. Heintz, personal communication). We later showed that the SV40 early promoter was in fact induced as cells progressed from G0 to G1. This effect became the foundation for experiments presented in Chapter 2.

DISCUSSION

Molecular cloning of the human TK gene and isolation of the TK cDNA (1) and their use in measuring TK mRNA levels in serum stimulated and SV40 infected CV1 cells began and led to the focus of this study. Our laboratory's mapping results are generally in agreement with others who have isolated this gene (2,3,5). Differences in restriction sites are seen at the 3' end and in the large intron. Three out of the four maps contain the BamHI site at the 3' end of the gene. These

differences may be due to artifacts of the cloning process or from natural polymorphisms of the gene. The isolated cDNA was used to determine the direction of transcription, minimum number of introns, and the general positions of the 5' and 3' ends. More importantly, for this study, the cDNA was crucial for initiating deletion mutant and hybrid gene experiments to address the regulation of TK gene expression.

As mentioned previously, DNA tumor virus infection or serum stimulation of quiescent cells increases TK enzyme activity levels by about 20 fold (17,20). These stimuli are among many factors which affect the growth state of cells. In the case of cells growth arrested by contact inhibition and/or serum deprivation, cells go from a quiescent G0 state to G1 then to S phase. Although there is evidence to indicate that the induction of TK is not completely dependent on DNA synthesis (21), its increase in activity is strongly correlated with the onset of DNA synthesis. In our initial experiments, SV40 infection of contact inhibited CV1 cells produced an increase in TK mRNA levels between 16-20 hr postinfection which parallels the onset of DNA synthesis. T antigen, the viral protein shown to be necessary for induction, appears 8-12 hr before TK mRNA starts to accumulate. Serum stimulation of quiescent CV1 cells shows an increase in TK mRNA by 12 hr postinduction, which also parallels the onset of DNA synthesis. For CV1 cells, it takes 8-12 hr after serum stimulation or T antigen appearance for

DNA synthesis to begin. We hypothesize that the time required for the virus to infect and express its early proteins causes the delay of TK induction in this system. These results show that TK mRNA levels increase dramatically in serum stimulated and SV40 infected cells. These changes in the steady state levels of TK mRNA can account for most if not all of the induction in TK enzyme activity.

The results discussed so far indicated that TK mRNA levels were regulated transcriptionally and/or postranscriptionally. A 6-7 fold increase was measured in the rate of transcription of the TK gene at the onset of S phase (9). A similar increase was reported for the DHFR gene in mouse cells (22). However, other experimenters studying cell cycle regulation of DHFR have shown that the increase seen in DHFR mRNA levels during S phase is due to differential mRNA stability (23) and that the 3' end of the gene is required for regulation (24).

Several facts indicated that TK mRNA might be regulated postranscriptionally as well as transcriptionally: 1) It was unlikely that the 6-7 fold increase in the rate of transcription (lasting at most 3 hr) could account for the 10-20 fold increase in TK mRNA levels (lasting for at least 12 hr). 2) Results suggest that the half life of TK mRNA is approximately 2-3 fold longer during S phase than in G0 or G1 phase cells (Stewart,C., unpublished results). 3) Whether the human TK promoter or the SV40 early promoter was used to

express the human TK cDNA, TK mRNAs showed induction at 12 hours after serum stimulation. This suggested that sequences present in the cDNA were sufficient to confer cell cycle regulation upon these constructs. This was supported by results obtained in other systems where hybrid TK genes expressed from heterologous promoters were regulated (7,8).

Posttranscriptional regulation might involve differential TK mRNA metabolism. During S phase TK mRNA may accumulate to high levels due to changes in mRNA stability, transport, and processing. Differences in nuclear processing are unlikely to play major roles in our system because p5'TKcDNA contains no intervening sequences. Furthermore, the TK protein might be independently regulated of TK mRNA. For example, the translational efficiency of TK mRNA may change from G1 to S phase regardless of TK mRNA levels present. In the next chapters, evidence for posttranscriptional regulation of TK and how regulation of TK mRNA may be independent of TK enzyme as well as some possible levels of posttranscriptional regulation are addressed.

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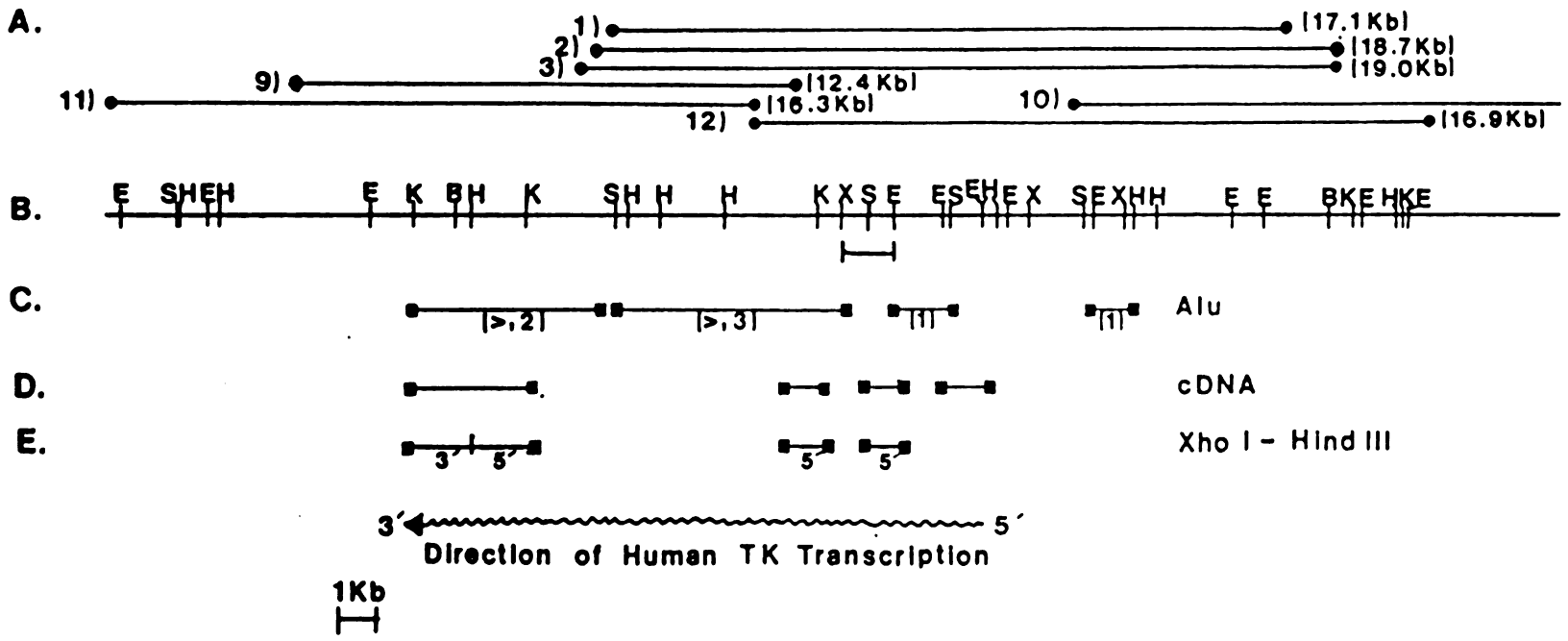
Figure 1. Restriction map of the human TK locus. A). Seven overlapping clones from the human TK locus were mapped by restriction enzyme/blot hybridization analysis. The relative position of these clones are shown. Cotransfection of clones 1+11, 1+9, and 11+12 gives rise to TK^+ colonies. B). Restriction enzyme map of the human TK locus. Enzymes used were: E, EcoRI; S, SstI; H, HindIII; K, KpnI; B, BamHI; X, XhoI. The 1.4-kb XhoI-EcoRI fragment used as probe in Northern analyses is underlined. C). Fragments within the TK locus that hybridize to a human Alu repeat probe (Blur-8). Numbers below the line indicate the minimum number of Alu repeats within that fragment. D). Fragments within the human TK locus that hybridize to the human cDNA clone pHuTK-cDNA7. E). Two fragments from within the cDNA clone were used to map the direction of transcription of the TK gene. The 5' probe was a 1.03-kb XhoI-HindIII fragment, and the 3' probe was a 0.52-kb XhoI-HindIII fragment. The XhoI sites in both cases were located in the vector DNA. (1)


A.
1)
2)
3)
9)
11)
12)
10)
17.1Kb
18.7Kb
19.0Kb
12.4Kb
16.3Kb
16.9Kb
B.
C.
>,2
>,3
1
1
Alu
D.
cDNA
E.
3'
5'
5'
5'
Xho I - Hind III
3'
5'
Direction of Human TK Transcription
1Kb

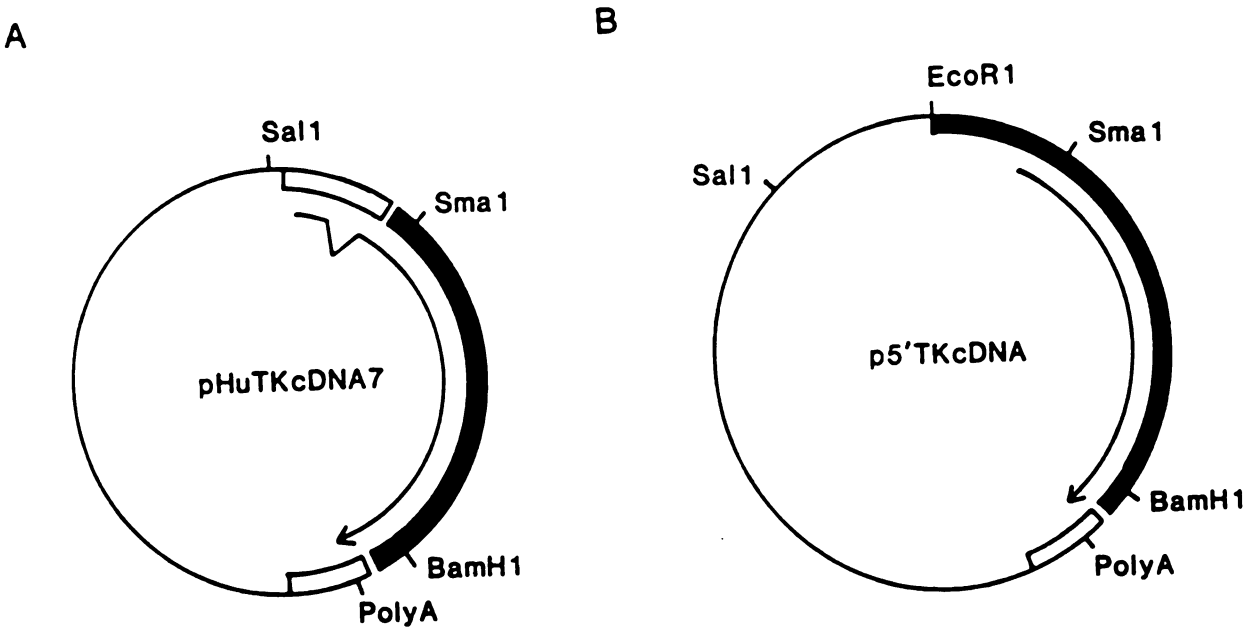


Figure 2. Structures of cDNA clones capable of expressing TK after transfection into TK$^-$ cells. **A**). pHuTKcDNA7 expresses the human message from the SV40 early promoter and contains the SV40 splice doner and acceptor and polyadenylation signal. **B**). p5'TKcDNA expresses the TK mRNA from the human genomic TK promoter, but still retains the SV40 polyadenylation signal from pHuTKcDNA7. For details of these constructions see Materials and Methods. Symbols: (▬) HuTK sequences, (▭) SV40 sequences, (—) pBR322 sequences.

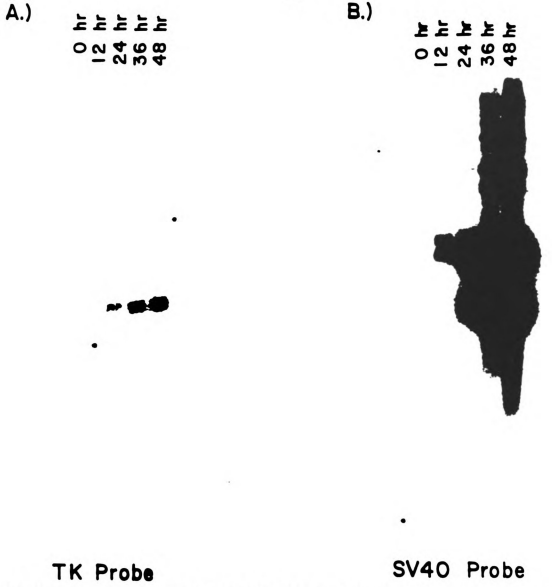


Figure 3. Northern blot analysis of SV40-infected CV1 cells. Confluent plates of CV1 cells were infected with wild-type SV40 at an MOI of 5 at zero time. Poly A^+ mRNA was isolated from cells at 12-h intervals after infection. RNA from equal numbers of cells was electrophoresed on 1.2% formaldehyde gels and transferred to nitrocellulose filters. Duplicate Northern blots were hybridized to **A**). pHuTK 1.4 probe and **B**). SV40 probe.

Figure 4. SV40 induction of TK in simian CV1 cells. Confluent plates of CV1 cells were infected with SV40 at an MOI of 5 at zero time. At 12-h intervals after infection, samples were taken for TK enzyme assays, TK mRNA analysis, and pulse-labeling of total DNA. TK enzyme activity is expressed as follows: 1 unit = 1 nmol of deoxythymidine converted to dTMP/min per μg of protein. (●) TK units x 10^4. TK mRNA levels were estimated from densitrometer tracings of the Northern gel shown in Fig. 3A. (△) Relative TK mRNA levels. DNA was labeled *in vivo* for 60 min with 1 μCi of ^{3}H-thymidine per ml. Counts incorporated were determined by using acid precipitations, and DNA concentrations were determined by a DNA colorimetric assay (diphenylamine reaction). (○) Specific activity of DNA (counts per minute per μg). The first increase in specific activity seen at 24 h post infection indicates the onset of S phase. Although DNA synthesis seems to precede TK induction, this is misleading since the DNA synthesis measurements are pulse-labelings where as TK mRNA and enzyme levels are steady state measurements.

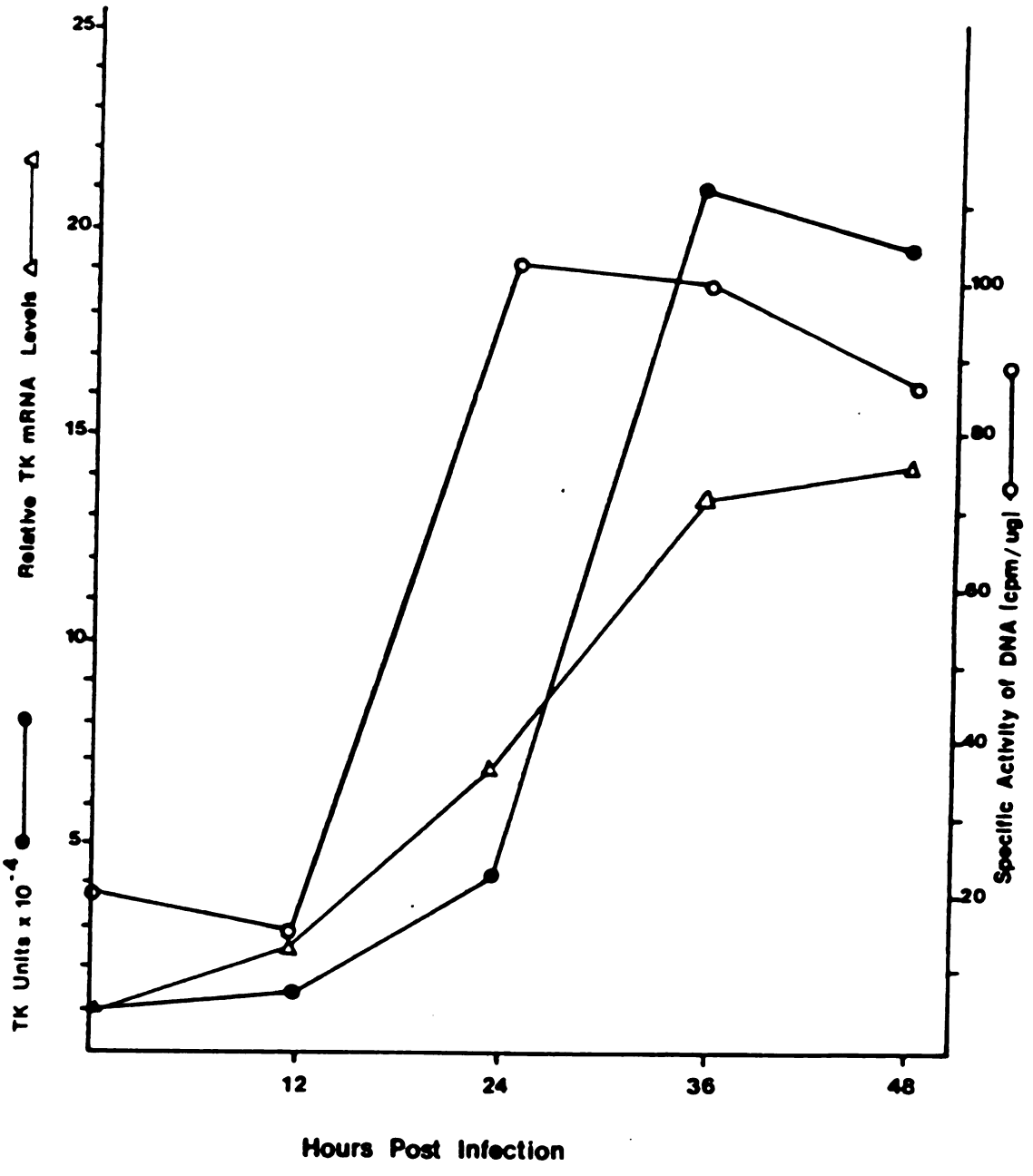
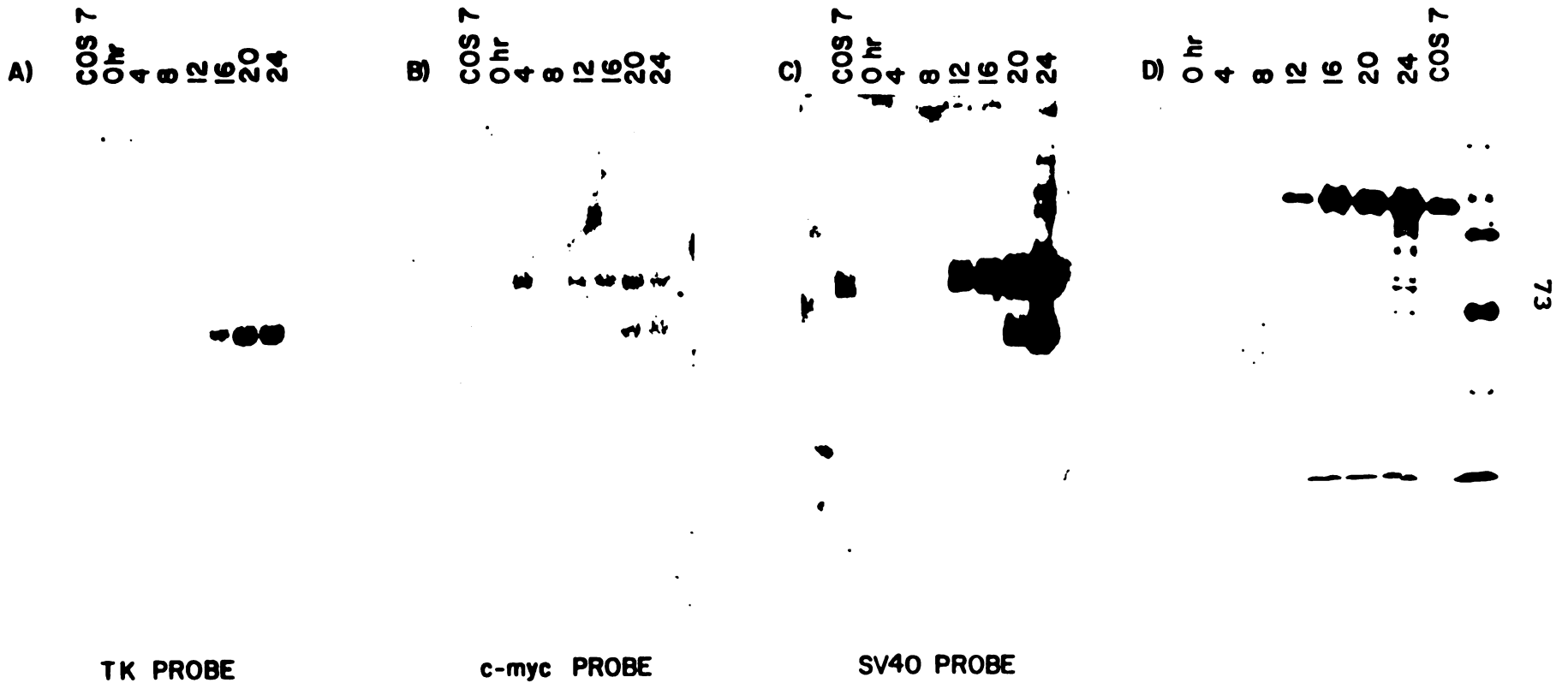

TK Units x 10^{-4}
Relative TK mRNA Levels
Specific Activity of DNA (cpm/ug)
Hours Post Infection
25
20
15
10
5
100
80
60
40
20
12
24
36
48

Figure 5. Twenty-four-hour time course of SV40-infected CV1 cells. Confluent CV1 cells were infected with SV40 as described (Fig. 4). Plates were harvested at 4-h intervals for mRNA and T-antigen analyses. Also included as a control are RNA and protein from COS7 cells, which constitutively express the SV40 early genes. A). Hybridization with pHuTK 1.4 probe. B). Rehybridization of A). with a c-myc probe. C).Duplicate blot of A). hybridized with SV40 probe. D). Acrylamide gel of immunoprecipitated proteins pulse-labeled for 60 min with ^{35}S-methionine.


A)
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0 hr
4
8
12
16
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24
B)
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0 hr
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C)
COS 7
0 hr
4
8
12
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D)
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4
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12
16
20
24
COS 7
TK PROBE
c-myc PROBE
SV40 PROBE

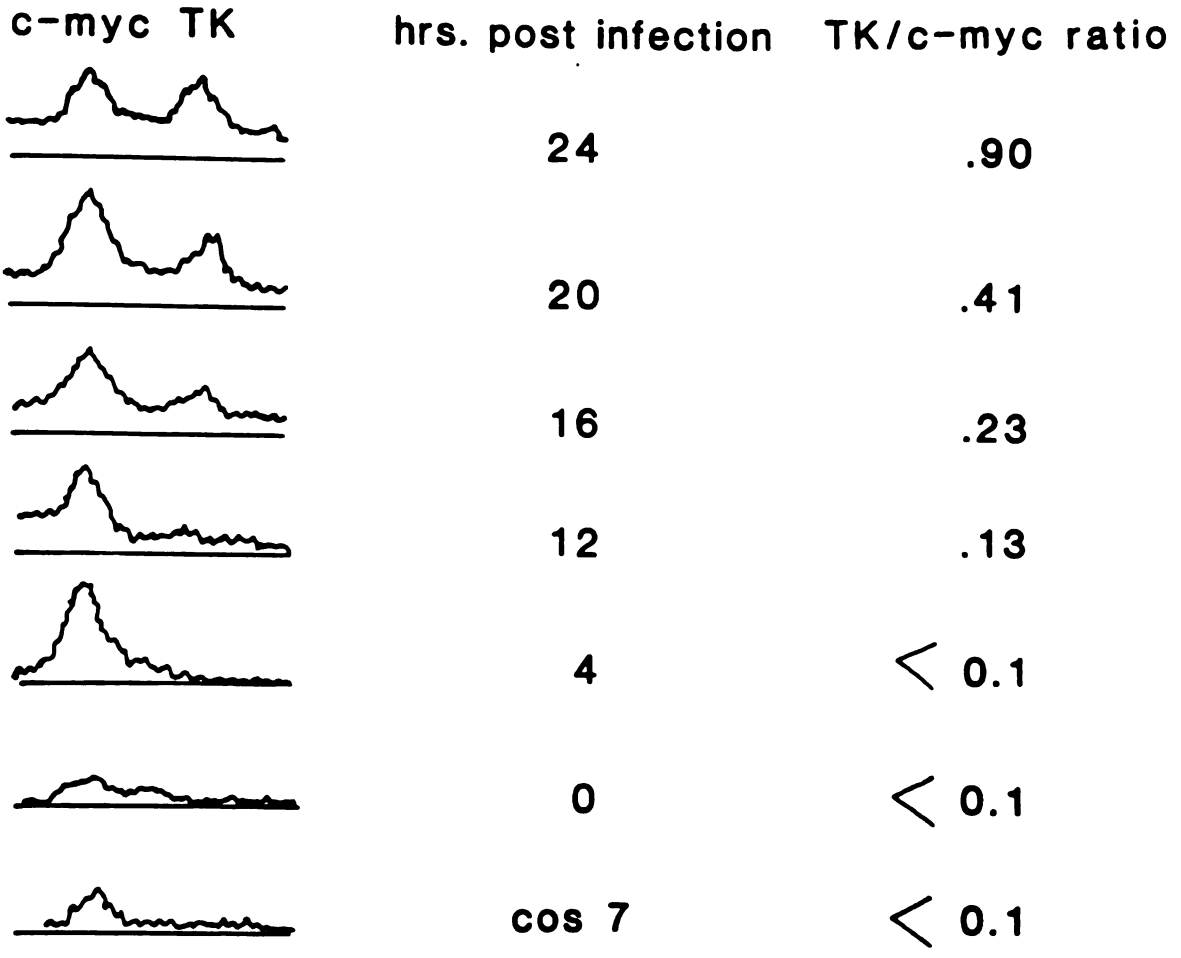

Figure 6. Densitometer tracing of autoradiogram in Fig. 5B. The ratio of TK/c-myc RNA is shown.

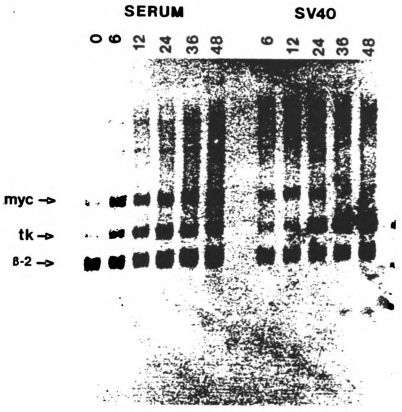

Figure 7. Comparison of serum and SV40 induction of CV1 cells. Confluent CV1 cells were induced to reenter the cell cycle either by addition of fresh media containing 10% serum or by infection with SV40 (MOI=5). At various times (6, 12, 24, 36, and 48 h) after treatment, poly A^+RNA was prepared, and RNA from equal numbers of cells was electrophoresed on a denaturing agarose gel as previously described. The RNA was transferred to nitrocellulose fiter paper and hybridized with a mixture of TK and c-myc probes. The same filter was later hybridized with a human β-2 microglobulin probe. The picture above shows the two films superimposed on one another.

Figure 8. Northern blot analysis of TK poly A^+ RNA in cell lines transfected with TK cDNA constructs. Rat-3 TK^- cells were transfected with either pHuTKcDNA7 or p5'TKcDNA, and HAT^r colonies were selected. Transfected cell lines were growth arrested and serum stimulated as described in Materials and Methods, and poly A^+ RNA was prepared at 0, 12, and 24 h after serum addition. RNA was analyzed by Northern blot analysis and probed with an HuTKcDNA probe. A). Rat-3 (TK^-) control and cell lines containing pHuTKcDNA7. Cell lines 1C and 2A were derived from isolated colonies, and m was derived from a pool of approximately 50 colonies. B). Rat-1 (TK^+) control and cell lines containing p5'TKcDNA. Cell lines D3 and A1 were derived from single colonies, and m was derived from a pool of approximately 50 colonies.

A

pHuTKcDNA7

Rat3	m	1C	2A
0 12 24	0 12 24	0 12 24	0 12 24

B

p5'TKcDNA

Rat1	m	D3	A1
0 12 24	0 12 24	0 12 24	0 12 24

Table 1. TK enzyme assays in cell lines transfected with HuTK cDNA constructs[a]

Time (h) after serum stimulation	TK units (x 10^4)						
			pHuTKcDNA7			p5'TKcDNA	
	Rat-3	Rat-1	Mass	1C	2A	Mass	D3	A1
0		3.4	3.7	1.2	0.9	1.1	2.0	0.3
12		8.5	7.4	13.3	6.4	7.1	11.1	1.6
24	0.003	9.2	3.9	7.2	4.6	12.6	4.1	3.7
36		11.8	2.7	1.2	0.9	0.7	0.8	0.9

[a] The cell lines described in the legend to Fig. 8 were growth arrested and serum stimulated as described. At 0, 12, 24, and 36 h after serum addition TK extracts were prepared, and enzyme assays were performed as described. One TK unit is defined as the amount of enzyme required to convert one nanomole of deoxythymidine to dTMP per microgram of protein per minute of reaction at 37°C.

CHAPTER 2

INDEPENDENT REGULATION OF THYMIDINE KINASE mRNA AND ENZYME LEVELS IN SERUM STIMULATED CELLS

PUBLISHED MANUSCRIPT

Ito, M. and Conrad, S.E. (1990) J. BIOL. CHEM. 265:6954-6960.

CHAPTER 2

INTRODUCTION

The activity of the cytoplasmic form of the thymidine kinase enzyme (EC2.7.1.21) has been shown to be dependent on the growth state of the cell in a variety of eukaryotic systems. The level of thymidine kinase enzyme activity is low in quiescent (G0) phase tissue culture cells synchronized by serum deprivation. Following the addition of fresh serum, the activity remains low throughout G1, but increases dramatically as the cells enter S phase (11). We and others have investigated the molecular basis of this regulation, and have found that expression of the gene is controlled at multiple levels. Several lines of evidence indicate that the thymidine kinase gene is transcriptionally regulated in serum stimulated cells. First, the increase in enzyme activity seen in serum stimulated cells is paralleled by an increase in the level of thymidine kinase mRNA (21). In addition, nuclear run-on transcription assays have shown that the rate of transcription of the gene increases approximately 5-7 fold at the G1/S boundary (15,20). Finally, several groups have shown that the thymidine kinase promoter confers an S phase specific pattern of expression to heterologous linked genes (1,12).

Although the results described above demonstrate that the thymidine kinase gene is transcriptionally regulated in serum stimulated cells, they do not show conclusively that

transcriptional regulation can account totally for the observed induction of either mRNA or enzyme activity. In fact, several lines of evidence suggest that post-transcriptional regulation may also play an important role in the regulation of both mRNA and protein levels. The results from several labs suggest that the half life of thymidine kinase mRNA is approximately 2-3 times longer during S phase than in G0 or G1 phase cells (4, C. Stewart, unpublished results). In addition,, Gudas et al. have reported that changes in nuclear processing of TK pre-mRNA occur between G1 and S phase cells (8). Experiments using recombinant TK cDNA clones expressed from various promoters have also suggested that post-transcriptional mechanisms may be important for the regulation of thymidine kinase mRNA levels. In previous studies, we and others have compared the expression and regulation of human, mouse, hamster and chicken TK cDNA clones transcribed from both the endogenous thymidine kinase promoters and heterologous viral (SV40 early or HSV-1 TK gene) promoters. In our studies, cell lines containing human thymidine kinase cDNAs expressed from the endogenous promoter or the SV40 early promoter were isolated by transfection and selection for thymidine kinase activity, and the patterns of mRNA and enzyme expression were examined in quiescent and serum stimulated cells. When mRNA levels were examined at 0, 12 and 24 hours following serum addition, a similar pattern of expression was seen with both constructs, leading to the

conclusion that sequences within the cDNA were conferring this pattern of regulation (20). Similar experiments using both the SV40 early and HSV-1 TK gene promoters were also done with the hamster (14), chicken (16) and mouse (9,15) thymidine kinase genes, and led to similar conclusions. Additional experiments utilizing cellular promoters, however, suggest that this conclusion may be incorrect, and that the promoter may in fact determine the pattern of TK mRNA, but not enzyme, expression (25). Taken together, the results discussed above suggest that the increase in thymidine kinse enzyme activity in cells released from G0 arrest by the addition of fresh serum can be largely accounted for by changes in the rate of TK mRNA synthesis and the metabolism of TK mRNA.

In contrast to the results in cells released from G0 arrest, evidence suggests that changes in mRNA levels are not the major factor that account for fluctuations in TK protein and enzyme levels in continuously cycling cells. Sherley and Kelly (19) have shown that TK mRNA levels are only moderately (2-3 fold) regulated in cycling cells, while protein and enzyme levels are highly regulated. Two mechanisms appear to account for this observation: the protein is rapidly turned over during a brief interval early in G1, and the mRNA is not efficiently translated during G1. In a related set of experiments, Gross and Merrill have reported that TK protein levels are not proportional to mRNA levels in differentiating myoblasts (6), and that this difference is due to

translational regulation (7).

In the present report, we have investigated the relative role of transcriptional and post-transcriptional regulation in TK mRNA and protein/enzyme levels in serum stimulated cells. We have conducted an extensive series of experiments comparing the patterns of mRNA, protein and enzyme expression among constructs where a human TK cDNA is transcribed from the human TK promoter, the HSV-1 TK gene promoter and the SV40 early promoter. Our results indicate that while the promoter does affect the pattern of mRNA expression, it has a minimal effect upon the pattern of protein or enzyme expression. Thus, even when high levels of TK mRNA are synthesized during G1, protein and enzyme levels do not increase until approximately 8 hours after serum stimulation. In order to investigate the molecular basis of this regulation, we have constructed mutant (deletion) TK cDNA clones expressed from the SV40 early promoter. Experiments with these mutants indicate that this mode of regulation is not dependent upon TK 5' untranslated sequences, the authentic TK translation start site or the first 16 amino acids of coding sequences, suggesting that regulation does not occur at the level of translation initiation. Regulation is also independent of the HuTK polyadenylation site and approximately 430 bp of the 3' untranslated region of the cDNA.

MATERIALS AND METHODS

Cell culture and serum stimulation. Rat3 TK- cells (23) were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum (Hyclone). To obtain synchronous populations of cells, cells were plated in medium plus serum so that they would reach confluence in 4 to 5 days. Upon reaching confluence, the medium was removed and replaced with fresh medium plus 0.1% serum for 24-36 hours. For serum stimulation the medium on arrested cells was changed at time 0 to fresh medium containing 10% serum.

Transfections into Rat3 cells. Transfections into Rat3 cells were performed as described by Wigler (26). TK+ colonies were selected in HAT (hypoxanthine-aminopterin-thymidine) medium, and after 2-3 weeks plates were either stained or pools of 50-100 colonies were collected and propagated for further analysis.

Thymidine kinase enzyme extraction and assays. Thymidine kinase enzyme extractions and assays were done as described previously (20,21).

RNA extractions and Northern blotting. Total RNA was prepared from tissue culture cells by a modification of the method of Favaloro et. al. (5). Cells were washed once with phosphate buffered saline (PBS), and were then lysed on the plate with 1ml of lysis buffer (10mM Tris, pH 7.5, 12mM EDTA, 150 mM NaCl, 1% SDS, 200 μg/ml proteinase K). Cellular DNA was sheared by passage through a 21 gauge needle 2-3 times,

and the lysate was incubated at 37°C for 1 hour. The mixture was then extracted with a 1:1 mixture of phenol:chloroform, and nucleic acids were precipitated from the aqueous phase with 2.5 volumes of ethanol. DNA was removed by digestion with RNase free DNase (Boeringer Mannheim) in 400 μl of a reaction buffer containing 10mM Tris, pH 7.5, 1mM EDTA, 10mM $MgCl_2$, and either RNAsin or Vanadyl Ribonucleoside Complex (VRC) to inhibit RNase. RNA was then precipitated with ethanol, and in cases where VRC was used it was removed by washing the RNA pellet with 20% sodium acetate. RNA was then pelleted once again, and resuspended in 10mM Tris, pH 7.5, 1mM EDTA. The concentration of RNA was determined by absorbance at 260 nanometers, and appropriate amounts of RNA (usually 10 or 20 μg) were ethanol precipitated and analyzed by Northern blot analysis as described previously (20,21).

Quantitation of RNA levels from autoradiographs. The levels of mRNA expression were determined by densitometer scanning of autoradiographs using a laser densitometer (LKB, UltroScan XL).

SDS-polyacrylamide gel electrophoresis and Western blotting. Proteins in enzyme extracts were electrophoresed on 12% SDS-polyacrylamide gels according to the procedure of Laemmli (13). The amount of protein in the extracts were determined using a Bradford assay (3). Proteins in the gel were transferred to nitrocellulose by the Western blotting procedure (24), with transfer being done at 4°C overnight at

15V. The nitrocellulose filters were blocked in 1% nonfat dried milk in PBS for 2 hours at room temperature and then at 4°C overnight. Incubation with a 1/500 dilution of the B3b primary antibody (provided by Dr. T. Kelly) was for 1 hour at room temperature. The filter was then subjected to three 10 minute washes in PBS. Antibody detection was performed using a biotinylated secondary antibody/streptavidin-alkaline phosphatase kit purchased from Bethesda Research Labs. Incubation with the secondary antibody was in TBST plus 1% gelatin, and the rest of the procedure was performed as recommended by BRL.

RESULTS

Role of promoter sequences in the regulation of TK mRNA levels. A variety of human TK cDNA constructs (diagrammed in Figure 1A-C) that express the human TK cDNA from either the HuTK, the SV40 early or the HSV-1 TK gene promoter were used to transfect Rat3 (TK-) cells to a TK+ phenotype. In each case pools of 50-100 stable transfectants were propagated and used to analyze the patterns of TK gene expression in serum starved and stimulated cells. Figure 2 shows the pattern of mRNA expression at 0, 2, 6, 12 and 24 hours following serum stimulation in cells transfected with Hu-TK, SV-huTK, and HSV-huΔScaTK. As previously reported, and in agreement with the pattern of expression of endogenous thymidine kinase genes, expression of the Hu-TK construct is low in G0 cells,

remains low throughout G1, and increases approximately 13 fold by 12 hrs following serum addition. Cells transfected with HSV-hu ScaTK show a similar pattern of expression, with induction occurring at 12 hrs. Since the basal levels of mRNA are higher in GO and G1 in these cells than in cells transfected with Hu-TK, the magnitude of the induction is reduced to approximately 4 fold. The pattern of thymidine kinase mRNA expression in cells transfected with SV-huTK, however, is dramatically different. Although the level is lowest in G0 phase cells, it increases approximately 3 fold by 2 hours following serum stimulation, and remains high throughout the time course of the experiment.

These results are consistent with the promoter contributing significantly to the pattern of thymidine kinase mRNA expression in this system. In the case of the human TK promoter, expression is low throughout G1, and increases as the cells enter S phase. The activity of the HSV-1 TK gene promoter is believed to be independent of the growth state of the cell, since cells transfected with the intact HSV-1 TK gene show no cell cycle regulation of the TK mRNA (9). This constitutive promoter activity therefore gives rise to higher levels of TK mRNA in G0 and G1 phase cells transfected with HSV-hu ScaTK. The activity of the SV40 promoter is low in G0 phase cells, but increases rapidly upon serum addition (H. Roehl and S. Conrad, manuscript in preparation).

Role of promoter sequences in the regulation of thymidine kinase enzyme levels. In order to compare the regulation of thymidine kinase enzyme expression to that of mRNA, we have measured thymidine kinase enzyme activities in quiescent and serum stimulated cells using the pools of Hu-TK and SV-huTK transfected cells described above. In our experience, activity in terms of actual TK units varies somewhat from experiment to experiment, and from cell line to cell line. In order to compare the magnitude and kinetics of induction between cell lines, we have therefore plotted relative thymidine kinase activities in Figure 3A. For each cell line and experiment, the level of activity at each time point is normalized to the activity at t=0, which is defined as 1.0. The results of these experiments indicate that enzyme levels can be uncoupled from mRNA levels during G1. The most dramatic demonstration of this is seen in the case of cells transfected with SV-huTK, where enzyme levels remain low until 10-12 hours following serum stimulation, while the mRNA is fully induced by 2-4 hours (Figure 2B).

One possible problem in the interpretation of these results is the size heterogeneity of the mRNA produced in SV-huTK transfected cells (Figure 2B). Since multiple RNA species are produced, it is possible that only some give rise to functional thymidine kinase enzyme, and that these functional mRNAs are in fact growth regulated (i.e. remain low throughout G1 and increase as the cells enter S phase).

Although we have not examined the source of the heterogeneity in detail, we hypothesized that it was due to sequences in the vector, such as the SV40 splice donor and acceptor, or the G/C tails used in the original cDNA cloning. In order to remove these sequences, we constructed a new cDNA clone, designated SV-huΔScaTK (Figure 1D), which joins the SV40 early promoter directly to a Sca1 site 30 bp into the 5' untranslated region of the human thymidine kinase cDNA. Rat3 cells were transfected with this construct, and pools of transfectants were examined for thymidine kinase mRNA and enzyme expression at 0, 2, 4, 12 and 24 hrs after serum stimulation. The results of this experiment, shown in Figures 3B (enzyme) and 4A (mRNA), confirm the original results with SV-huTK; mRNA levels increase 5 fold by 2 hours and then remain high until 12 hrs, while enzyme levels remain low until at least 4 hrs and increase by 12 hrs. As predicted, the mRNA produced by SV-huΔScaTK is of a uniform size, and shows no evidence of the heterogeneity seen with the original SV-huTK construct.

The results presented above, that thymidine kinase enzyme activity is low during G1, even in the presence of high levels of mRNA, are consistent with the proposal of Sherley and Kelly (19) that thymidine kinase mRNA is not efficiently translated during G1. It was also possible, however, that the protein was present but inactive in our experiments. We therefore performed Western blotting analyses to examine the level of thymidine kinase protein directly. Aliquots of the SV-hu

ΔScaTK extracts assayed in Figure 3B were subjected to SDS-polyacrylamide gel analysis on 12% gels. The proteins in the gel were transferred to nitrocellulose, and thymidine kinase protein was detected using the anti-human thymidine kinase polyclonal antibody of Sherley and Kelly (18). The results of this experiment, shown in Figure 5, indicate that thymidine kinase protein levels parallel the enzyme activity, and are uncoupled from mRNA levels.

Role of sequences within the HuTK cDNA in G1/S phase regulation of the gene. The results presented above, and those of others (7,19), suggest that the thymidine kinase gene is subject to translational regulation during the cell cycle; specifically thymidine kinase mRNA is not efficiently translated during G1. In order to investigate the mechanism of this regulation, we have begun to examine the sequences within the cDNA that are required for regulation. As previously noted, SV-huΔScaTK has deleted the first 30 bp of the cDNA. We have also constructed a new cDNA clone, SV-huΔSmaTK (Figure 1E), which contains the SV40 early promoter joined via a ClaI linker to an SmaI site within the cDNA. The resultant thymidine kinase gene has deleted the entire 5' non-coding sequences, the authentic thymidine kinase AUG, and the first 16 amino acids of the protein. We believed that deletion of these amino acids would not abolish enzyme activity, since several viral enzymes that have been analyzed are missing this portion of the protein (2). Translation of the remaining

TK coding sequences in SV-hu ΔSmaTK begins at an in frame AUG provided by the Cla1 linker.

SV-huΔSmaTK and SV-huΔScaTK DNAs were transfected in parallel into Rat3 cells and TK^+ colonies were selected. In several independent experiments, these two DNAs transformed Rat3 cells with approximately the same efficiency, indicating that the enzyme activity is not seriously impaired by the deletion of the first 16 amino acids (data not shown). Pools of transfectants were grown to confluence, serum starved and induced by the addition of medium with fresh serum, and the levels of thymidine kinase mRNA (Figure 4), enzyme (Figure 3) and protein (Figure 5) were assayed in parallel at 0, 2, 4, 12 and 24 hrs after serum addition. The results of these experiments indicate that the two mutant genes show the same pattern of regulation, which is also indistinguishable from the pattern seen with SV-huTK, although the absolute levels of RNA, protein and enzyme are often higher with the Sma deletion. It is also noted that, as expected, the protein produced by this gene is of lower molecular weight than the 24 kD wild-type protein (18).

The data presented above indicates that the overall patterns of mRNA and enzyme expression are similar with SV-huTK, SV-huΔScaTK and SV-huΔSmaTK. In order to confirm and extend these results, we repeated these experiments by conducting an extensive time course on newly transfected cells (transfected in parallel) containing either SV-huTK, SV-hu

ΔScaTK or SV-huΔSmaTK. Two independent pools of cells from each new transfection were propagated, and analyzed in parallel for TK mRNA and enzyme activity at two hour intervals after serum stimulation. By performing each step of the experiment in parallel, we are able to eliminate possible differences due to variability from experiment to experiment. In addition, we have noted some change in the regulation of transfected TK genes dependent upon how long the transfected cells have been passed. The results of these experiments, shown in Figure 6 (mRNA) and Table 1 (enzyme and mRNA) confirm that TK mRNA and enzyme levels are uncoupled during G1 with all of these constructs. In this experiment the mRNA was induced a maximum of 3-5 fold within 2-4 hours of serum addition, while enzyme levels were induced from 18-80 fold, with the highest levels seen at either 12 or 14 hours. These results indicate that sequences at the 5' end of the thymidine kinase cDNA, including the 5' untranslated region, the translation initiation site, and the first sixteen amino acids are not required for S phase specific induction of the gene.

We have also investigated the role of sequences in the 3' untranslated region of the cDNA in regulation of enzyme expression. Since all of our constructs terminate in an SV40 polyadenylation site, it was known that polyadenylation at the authentic HuTK site was not required for regulation. We have constructed two additional cDNA clones, SV-huΔScaTKΔBam and SV-huΔScaTKΔHind, that have deletions of approximately 60 and

430 bp of the 3'untranslated region, respectively. These clones were transfected into Rat3 cells, and tested for serum inducibility at 0, 2, 4, 12 and 24 hours as described above. The pattern of mRNA and enzyme induction of these genes was similar to that seen previously, with mRNA levels being induced by 2-4 hours while enzyme levels are not induced until 12-24 hours (Table 2).

DISCUSSION

Although cell cycle and growth regulated expression of the thymidine kinase enzyme have been well documented, determining the molecular mechanisms responsible for this regulation has proven difficult. In fact, multiple mechanisms, including regulation at the level of transcription, RNA processing, RNA metabolism, translation and post-translation, have been proposed to occur under a variety of conditions. For the purpose of this discussion, it is useful to distinguish between the regulation of thymidine kinase mRNA and thymidine kinase protein/enzyme levels.

At the level of thymidine kinase mRNA, it has been clearly demonstrated that levels are low in cells arrested in either the G0 or G1 phase of the cell cycle. When these cells are released from the block, TK mRNA levels remain low throughout G1, rise dramatically as the cells enter S phase, remain high for some time, and then gradually decline. Evidence from several labs has suggested that both

transcriptional and post-transcriptional mechanisms can account for these changes in mRNA levels. Transcriptional induction of the gene has been demonstrated both by nuclear run-on transcription assays (4,15,20) and by linking the thymidine kinase promoter to the body of non-cell cycle regulated genes (1,12). It has also been proposed that sequences within the thymidine kinase cDNA contribute to the regulation of mRNA levels in cells released from growth arrest. This conclusion was based upon the fact that mRNA levels were regulated when the thymidine kinase cDNA was expressed from a number of heterologous promoters, including the SV40 early promoter and the herpes simplex virus thymidine kinase gene promoter (9,14,15,20). More recent experiments using cellular promoters (25) have challenged the regulatory role of sequences within the cDNA, since the results indicated that the promoter does in fact play an important role in the regulation of thymidine kinase mRNA, but not enzyme, levels. The role of transcriptional regulation in the modulation of thymidine kinase mRNA and enzyme levels in continuously cycling cells is unclear, and several groups have reported minimal or no changes in thymidine kinase transcription rates or mRNA levels in cycling cells (19). In addition, it has been reported that thymidine kinase protein and enzyme levels do not parallel mRNA levels in cycling HeLa cells (19), or in cells undergoing differentiation (6). Thus, in these cases regulation appears to occur largely at a translational or

post-translational level.

The results presented in this report address the issues of the role of the promoter in serum induction of thymidine kinase mRNA and enzyme, and the uncoupling of mRNA and enzyme/protein levels. Our results are consistent with the promoter playing a major role in the regulation of mRNA levels for this gene. We have shown that the patterns of mRNA expressed from either the human thymidine kinase, the HSV thymidine kinase or the SV40 early promoters are quite different in serum stimulated cells. The major difference between this and previous studies, where the opposite conclusion was reached, is that an extensive time course was done in these experiments, while earlier experiments only compared growing to resting, or G0 to S phase cells. The question of whether sequences within the cDNA have any effect on mRNA levels, perhaps by altering RNA stability, remains an open one. Our results with cells containing HSV-huΔScaTK are consistent with the proposal that sequences within the human cDNA play a role in mRNA regulation, since the level of mRNA increased from G1 to S phase. No such increase is seen, however, in cells transfected with SV40 promoter constructs. The high levels of mRNA produced during G1 in these cells may override the mechanisms that normally function at the level of mRNA.

In contrast to the regulation of mRNA levels, the levels of thymidine kinase protein and enzyme activity during growth

arrest/stimulation are essentially independent of the promoter from which the cDNA is expressed. This fact is best demonstrated in the case of cells containing the cDNA expressed from the SV40 early promoter, where protein levels remain low until 8-10 hrs following serum stimulation, although mRNA levels are high throughout G1. These results indicate that expression of thymidine kinase gene is regulated at a translational and/or post-translational level during G1 in cells released from serum starvation. If this regulation is translational, as would be suggested by the work of others (6,19), the fact that sequences at or near the translation start site are not required suggests that it does not occur at the level of translation initiation. This is consistent with other observations showing no detectable change in the pattern of polysomes containing TK mRNA in G1 vs S phase (M. Ito, unpublished results) or growing vs. differentiated (7) cells.

Taken together these results suggest a pattern of regulation of the thymidine kinase gene in cycling and growth arrested cells. In continuously cycling cells, mRNA levels are not stringently controlled, while protein levels are. The results of Sherley and Kelly (19) indicate that the major modes of regulation in this case involve both translational and post-translational mechanisms. There are also moderate changes in mRNA levels in cycling cells, and these may be due to either transcriptional or post-transcriptional mechanisms. As cells are removed from the cycle, either by removal of

serum or by differentiation (6), the level of protein declines relatively rapidly, and mRNA more slowly, and cells in G0 therefore contain little thymidine kinase mRNA or protein. In the "normal" case, with the endogenous TK promoter, both mRNA and protein levels remain low throughout G1 in serum stimulated cells, and increase only as the cells enter S phase. Again, this induction is the result of both transcriptional and post-transcriptional controls. Thus it appears that a major mode of regulation in this system is at the level of RNA. However, even when this "normal" regulation is perturbed by expressing the thymidine kinase gene from a strong promoter that is expressed during G1 phase, the second level of regulation still functions and keeps protein levels low until the beginning of S phase. Thus the cell has evolved a complicated machinery for regulating the levels of this enzyme.

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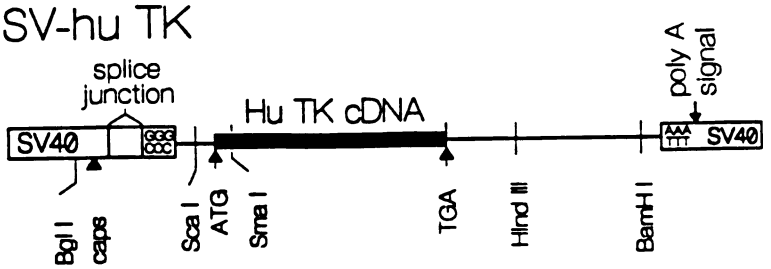
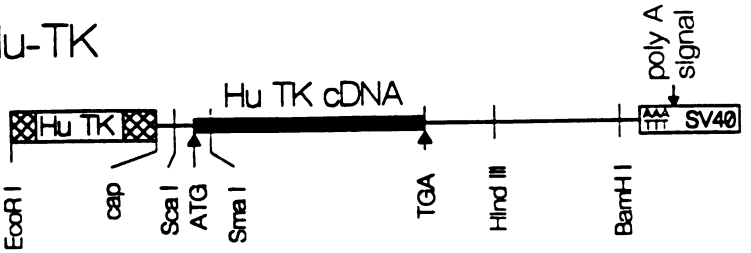
Figure 1. Structures of human thymidine kinase cDNA clones. **A: SV-huTK** (formerly designated pHuTKcDNA7); This cDNA clone was isolated from the Okayama and Berg expression library (17), and contains virtually the entire human cDNA expressed from the SV40 early promoter. It also contains an SV40 splice donor/acceptor, an SV40 polyadenylation signal, and the G/C and A/T tails used in cloning. **B: Hu-TK** (formerly designated p5'TKcDNA); This cDNA clone was derived from SV-huTK by replacing the SV40 early promoter with the human thymidine kinase promoter joined to the cDNA at an Sma1 site within the first exon of the gene. **C: HSV-huΔScaTK;** The HuTK cDNA expressed from the herpes simplex virus thymidine kinase gene promoter. A BamH1-BglII fragment containing the HSV promoter was joined to the HuTK cDNA at a Sca1 site within the 5' untranslated region of the cDNA, thereby deleting 30 nucleotides from the cDNA. **D: SV-huΔScaTK;** The SV40 early promoter (PvuII-HindIII fragment) was joined via BglII linkers to the Sca1 site in the thymidine kinase cDNA described above. **E: SV-huΔSmaTK;** The SV40 early promoter joined via a Cla1 linker to an Sma1 site within the thymidine kinase cDNA. This constructs deletes 17 amino acids of coding sequence, and adds an in-frame AUG from the Cla1 linker.

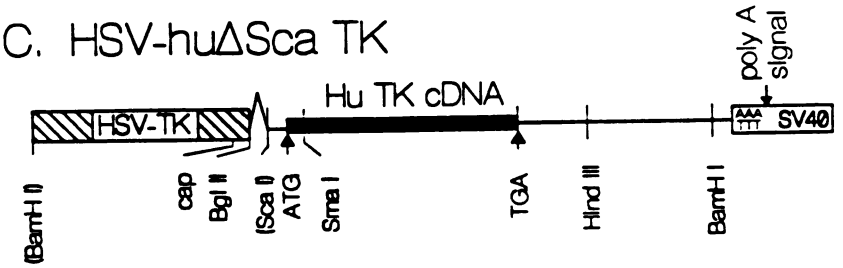
A. SV-hu TK



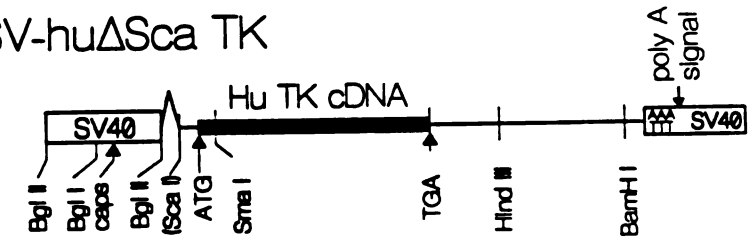
B. Hu-TK



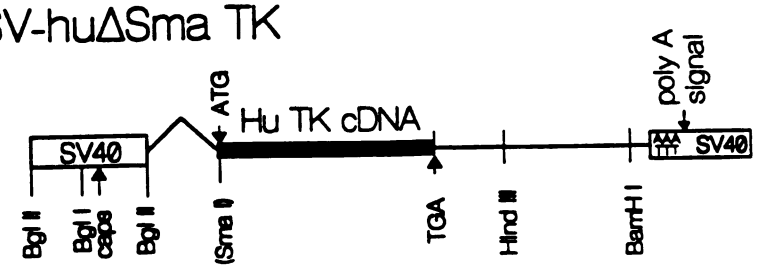
C. HSV-huΔSca TK



D. SV-huΔSca TK



E. SV-huΔSma TK



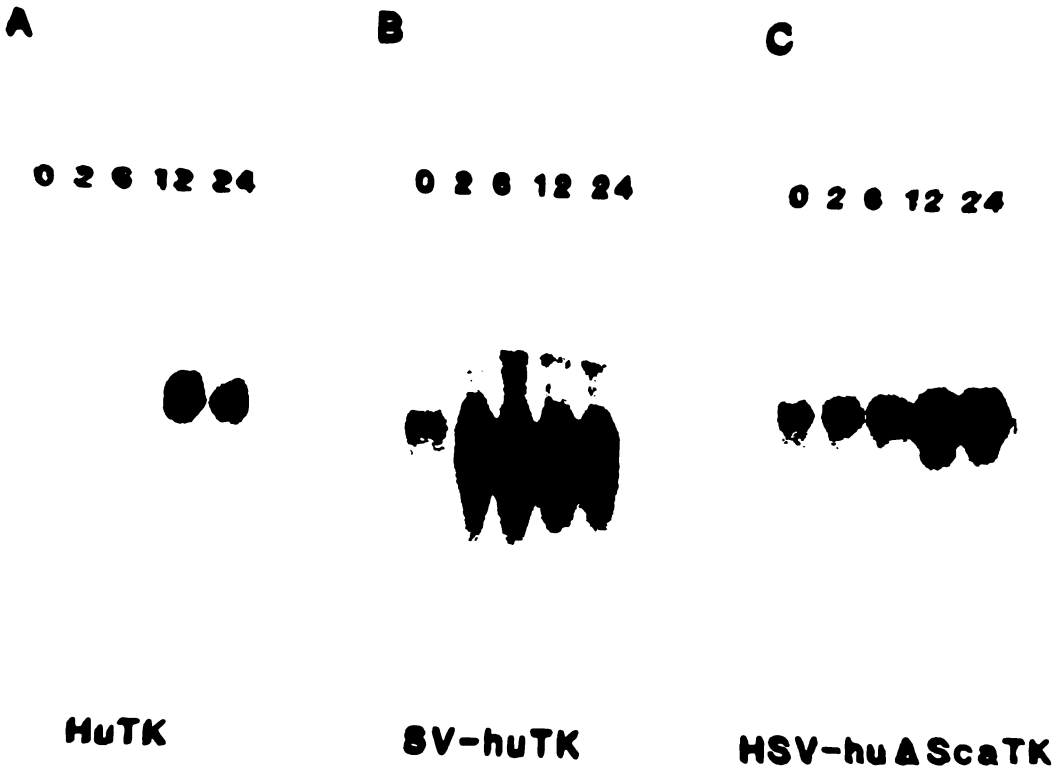
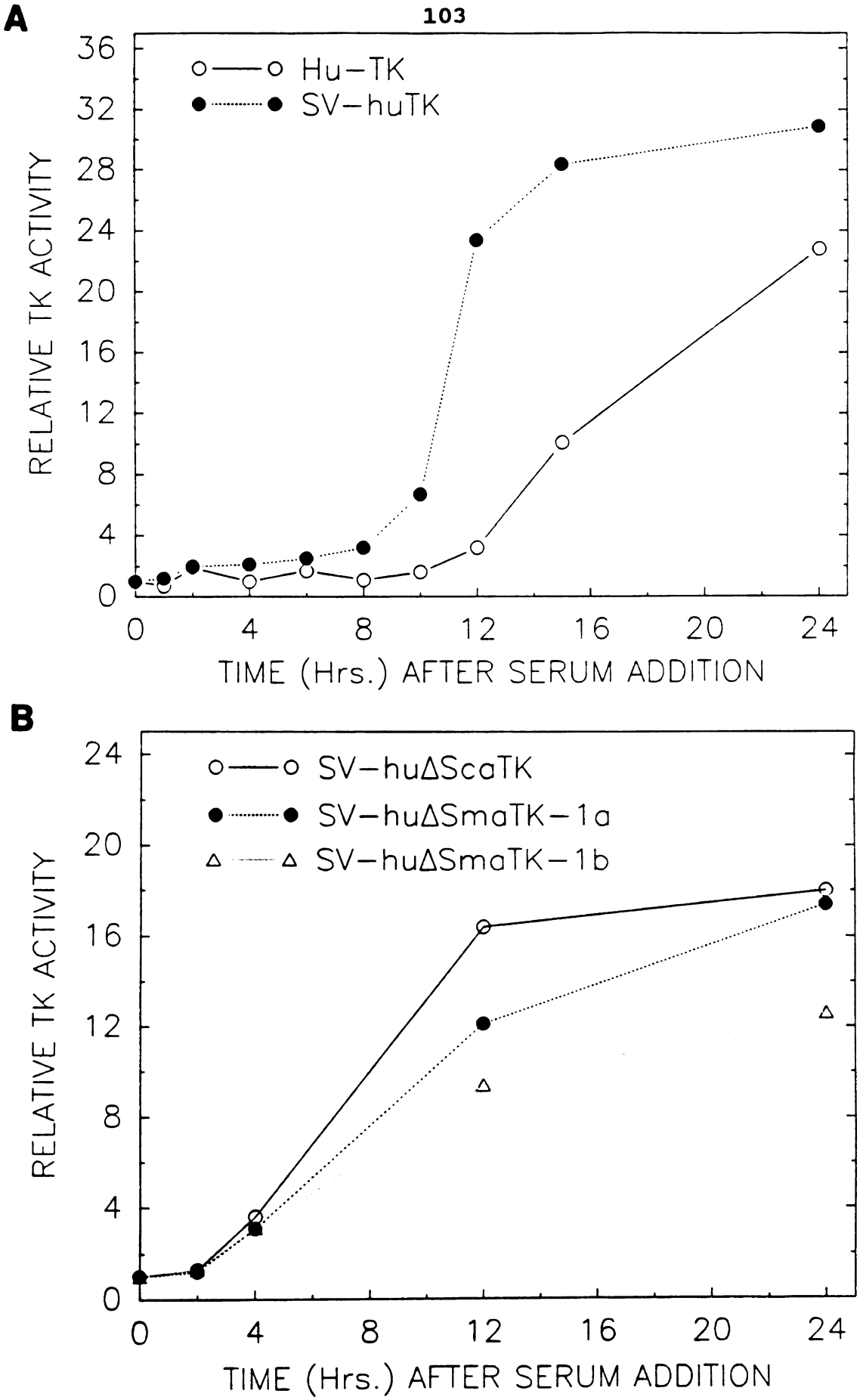


Figure 2. Northern blot analysis of thymidine kinase mRNA levels in serum stimulated Rat3 cells transfected with various HuTK cDNA constructs. Total RNA was prepared at the times indicated after serum stimulation, and 20 ug of RNA was analyzed per time point by electrophoresis on a 1.2% formaldehyde agarose gel. Hybridization was performed with a ^{32}P-labelled Sma1-BamH1 fragment from within the HuTK cDNA.

Figure 3. Thymidine kinase enzyme regulation in Rat3 cells transfected with various cDNA constructs. Thymidine kinase enzyme extracts and assays were performed as described in Materials and Methods. In each case relative thymidine kinase activities are plotted, with the level of activity in each cell line at t=0 being defined as 1.

A: Cell lines transfected with Hu-TK or SV-huTK. Each point is the average of 2 experiments.

B: Cell lines transfected with the indicated DNAs. SV-huΔSmaTK-1a and 1b were separate pools of cells transfected with independent DNA preparations.


A
Hu–TK
SV–huTK
RELATIVE TK ACTIVITY
0
4
8
12
16
20
24
28
32
36
TIME (Hrs.) AFTER SERUM ADDITION
B
SV–huΔScaTK
SV–huΔSmaTK–1a
SV–huΔSmaTK–1b
RELATIVE TK ACTIVITY
0
4
8
12
16
20
24
TIME (Hrs.) AFTER SERUM ADDITION

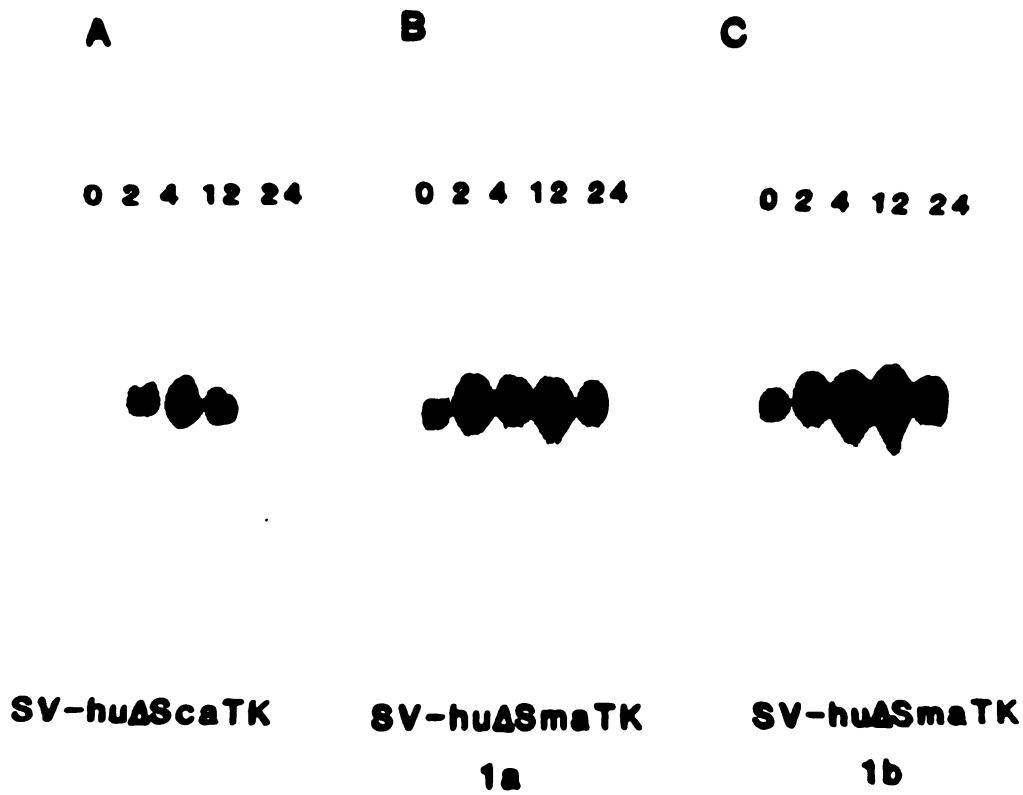


Figure 4. Northern blot analysis of thymidine kinase mRNA levels in serum stimulated Rat3 cells transfected with cDNAs expressed from the SV40 early promoter. Total RNA was extracted from the indicated cell lines in parallel with the TK enzyme extracts described in Figure 3B. 20 μg of RNA was analyzed at each time point.

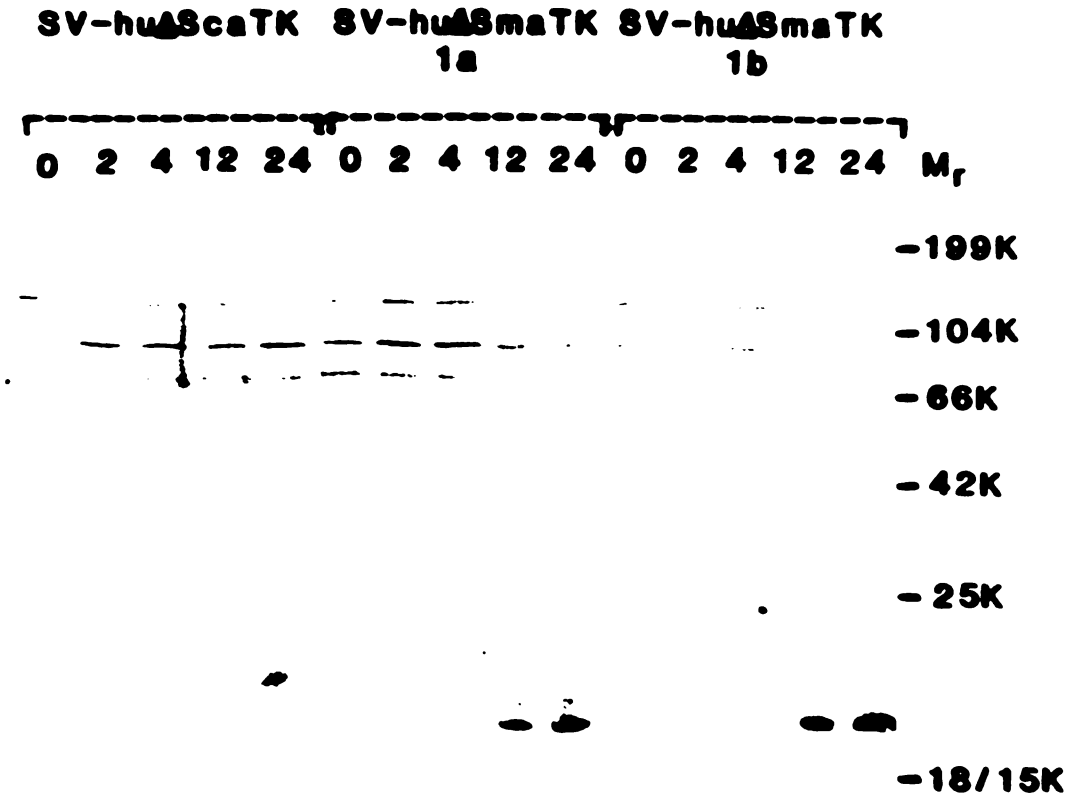


Figure 5. Western blot analysis of thymidine kinase protein levels in serum stimulated Rat3 cells transfected with cDNAs expressed from the SV40 early promoter. Extracts were the same TK enzyme extracts described in Figure 3B. Either 200 μg (SV-huΔScaTK) or 150 μg (SV-huΔSmaTK) of protein were analyzed per lane. Electrophoresis, blotting and probing was done as described in Materials and Methods.

Figure 6. Northern blot analysis of thymidine kinase mRNA levels in an extensive time course in serum stimulated cells. RNA was extracted as described, and 20 μg of RNA was analyzed per lane.

0 2 4 6 8 10 12 14 24 0 2 4 6 8 10 12 14 24
SV-huTK
0 2 4 6 8 10 12 14 24 0 2 4 6 8 10 12 14 24
SV-huΔScaTK
0 2 4 6 8 10 12 14 24
SV-huΔSmaTK

Table 1. Relative[a] mRNA and enzyme levels in Rat 3 cells transfected with TK cDNAs

	SV-huTK				SV-huΔScaTK				SV-huΔSmaTK			
	(1)[b]		(2)		(1)		(2)		(1)		(2)	
Time	RNA[c]	enz[d]	RNA	enz	RNA	enz	RNA	enz	RNA	enz	RNA	enz
0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
2	3.0	1.4	2.2	1.0	2.2	0.9	3.7	0.7	1.2	2.0	1.2	1.8
4	2.3	2.1	2.1	2.3	3.1	1.8	2.3	3.0	1.9	6.2	2.5	3.4
6	3.4	1.3	2.8	2.7	2.7	3.9	2.7	4.7	2.8	12.8	2.0	3.8
8	2.4	14.8	2.8	10.3	3.9	29.7	2.7	4.6	2.5	15.9	3.0	6.5
10	2.2	21.1	1.5	6.6	4.4	36.5	2.2	16.5	2.9	14.9	2.9	17.0
12	1.2	9.5	1.9	8.9	5.0	39.9	2.5	63.2	1.8	82.2	3.2	14.3
14	1.8	28.8	2.4	52.7	5.0	54.4	1.3	16.2	1.9	17.8	2.9	18.5
24	0.9	14.3	1.1	5.1	2.7	38.5	0.8	11.0	0.9	13.0	1.0	6.5

[a] Thymidine kinase units were calculated as described (20,21). For each experiment the number of TK units or level of TK mRNA in quiescent (t=0) cells was defined as 1. The values for all other time points are expressed relative to that value.

[b] For each DNA construct, cell lines (1) and (2) were derived from independent pools of cells.

[c] Relative mRNA levels were determined by scanning autoradiographs (Figure 6) as described in Materials and Methods. For each value, several different exposures of the gel were scanned and the values were averaged.

[d] The values for each time point are the average of enzyme assays done on two independent extracts.

Table 2. Relative mRNA and enzyme levels in rat cells transfected with 3'-deleted TK cDNAs

	SV-ΔScaTKΔBam		SV-ΔScaTKΔHind	
Time	RNA[a]	enz[b]	RNA	enz
0	1.0	1.0	1.0	1.0
2	2.5	0.9	2.5	1.1
4	2.1	1.7	3.6	2.3
12	1.4	10.8	4.9	16.9
24	1.3	24.9	4.1	15.3

[a] Relative TK mRNA levels were determined as described in the legend to Table 1. Each value is the average of two independent experiments using the same pools of cells.

[b] Relative TK enzyme levels were determined as described in the legend to Table 1. Each value is the average of 10 independent experiments.

CHAPTER 3

POSTTRANSCRIPTIONAL LEVELS OF REGULATION OF TK DURING G1 AND S PHASE

CHAPTER 3

INTRODUCTION

The molecular basis of the regulation described in Chapter 2, that is, the regulation of TK enzyme/protein levels independent of TK mRNA levels, is the focus of this chapter. Our results from the TK cDNA deletion mutants indicated that the 5' untranslated region, the authentic TK AUG, coding sequences for the first 16 amino acids, and approximately 430 bp of 3' untranslated sequences were not required for this regulation. These deletions also suggest that certain levels of regulation are not likely to be involved. At the 5' end of the TK mRNA, the untranslated sequences, the authentic AUG, and the first 16 codons have been deleted, strongly suggesting that regulation is not likely to be at the level of initiation of translation. Translation through the 16 codons specific to TK (producing a nascent peptide) also does not seem to be required or coupled to another level of regulation. In addition, up to 430 bp of the 3'end of the gene, including the TK polyadenylation site and signal have been deleted and replaced with an SV40 polyadenylation signal without changing the regulation of TK message and enzyme levels. Specific polyadenylation at the authentic TK site or mRNA stability and secondary structure dependent on 3' untranslated sequence elements are therefore unlikely levels of regulation. This

Chapter addresses several possible mechanisms by which posttranscriptional regulation could be occurring in this system.

First, a relatively simple level of control could be the differential polyadenylation of TK mRNA in G1 and S phase. This was unlikely since the polyadenylation of TK mRNA is independent of the authentic TK polyadenylation site and context. The TK cDNA constructs used in this system are missing sequences 3' of the site of cleavage and polyadenylation but have an SV40 polyadenylation signal at its 3' end. Although the function of the eukaryotic 3'poly(A) tail is yet not well understood, it has been implicated in nuclear-cytoplasmic transport (1), mRNA stability (2), and translational efficiency (3).

Another possible level of regulation might be at the stage of mRNA nuclear-cytoplasmic transport or compartmentalization (4). Transport of TK mRNA out of the nucleus may be blocked during G1. Alternatively if TK mRNA is being transported to the cytoplasm during G1, it may be present in a nonpolysomal fraction. Even if TK mRNAs are in the polysomal fraction during G1 and S phase, they may not be translationally active during G1. One way of demonstrating the translational activity of an mRNA is based on its distribution on polysomes. Noticable shifts in the average number of ribosomes per TK message could explain the low levels of TK protein detected during G1 in the presence of

abundant message. An important assumption in this case would be that the polysome profiles reflect active ribosomes. Any of these levels of control would prevent TK mRNA from being efficiently translated during G1.

In order to address these issues, we have attempted to examine the levels of TK mRNA in nuclear/cytoplasmic and polysomal/nonpolysomal fractions during G1 and S phase. In addition we have examined the polysome profiles of TK mRNA containing polysomes. Our results show no major differences between the 2 phases of the cell cycle.

MATERIAL AND METHODS

Isolation of poly A^+ mRNA. See Materials and Methods in Chapter 1.

Nuclear and cytoplasmic RNA isolation. Cytoplasmic RNA was isolated by modifications of the procedures of Favaloro et al. (5) and Gay et al. (6). Cells were washed with ice-cold PBS and lysed with 300 μl of C/N lysis buffer (0.14M NaCl, 1.5mM $MgCl_2$, 10mM Tris-HCl pH 8.6, 0.5% NP40, 10mM vanadyl-ribonucleoside complexes [VRC]). The lysate was transferred to microfuge tubes, vortexed, and spun at 4°C for 5 min in a microfuge to pellet nuclei. An equal volume of 2x PK buffer (200mM Tris-HCl pH 7.5, 24mM EDTA, 300mM NaCl, 2% SDS, with 200 μg/ml proteinase K) was added to the supernatant, and the extract was incubated at 37°C for 30-45 min. The lysate was then phenol-chloroform (50:50) extracted, and ethanol (2.5

volumes) precipitated. Nuclear RNA was isolated by lysing the nuclei in 1x PK buffer, shearing DNA through a 21 gauge syringe and incubating at 37°C for 45-60 min. The nucleic acid was purified by phenol-chloroform extraction and ethanol precipitation. DNA was then digested with RNase free DNase (Boeringer Mannheim) in 10mM Tris pH 7.5, 1mM EDTA, 10mM $MgCl_2$, and VRC. The RNA was extracted again with phenol-chloroform, ethanol precipitated, rinsed with 20% sodium acetate (to remove VRC) and reprecipitated.

RNAs were resuspended in 10mM Tris pH 7.5, 1mM EDTA and were quantitated by determining absorbance at 260 nm.

Polysomal and nonpolysomal RNA isolation. Cells were washed 2x in ice cold phosphate buffered saline (PBS) with 100 μg/ml cycloheximide. Cell counts were performed in a hemocytometer. Several more washes in RSB (10mM Tris pH 7.4-7.5, 10mM NaCl, 3mM $MgCl_2$, with 100 μg/ml cycloheximide) preceded lysis in 0.5% w/v NP40, RSB or polysome buffer (25mM Tris pH 7.6, 25mM NaCl, 5mM $MgCl_2$ with 100 μg/ml cycloheximide). The nuclei were pelleted at 4°C for 5-10 min in a microcentrifuge or for 10 min at 9-10K rpm in a RC2 Sorvall centrifuge. The supernatant (cytoplasmic fraction) was centrifuged in RSB with 100 μg/ml cycloheximide at 39K rpm for 90 min in a L8-70M Beckman Ultracentrifuge using a Beckman SW50.1 rotor, to pellet the polysomal fraction. The polysomal pellet was resuspended in polysome buffer by careful vortexing and homogenizing with a sterile glass rod, being careful to

keep the polysomes at around 0°C. The supernatant (nonpolysomal fraction) and polysomal fractions were then digested with proteinase K by adding equal volumes of 2xPK buffer with 200 μg/ml proteinase K and incubating at 37°C for 30-45 min. RNA was purified by phenol-chloroform extraction and ethanol precipitation. Optical density at 260 and 280nm was measured to quantitate the RNA and to assess its purity. This procedure was primarily a modification of those of Eschenfeldt (7).

Polysomal profiles. After the polysomal pellet was resuspended in polysome buffer (see above), optical density was read at 254 nm to approximate OD units (of polysomes) loaded per gradient. Linear sucrose gradients (15-40%) in polysome buffer were used to analyze the polysomes. Most of the gradients were centrifuged in an SW 50.1 rotor at 45 K rpm for 35-45 min. Some were centrifuged in an SW 41Ti rotor at 38 K rpm for 75-90 min. Gradient fractions (0.3ml each) were monitored continuously for absorbance at 254 nm as they were collected from the top. The fractions were denatured and slot-blotted onto nitrocellulose by a modification of a protocol by Theodorakis and Morimoto (8). Equal volumes of 2x NaPF (2M NaCl, 80mM sodium phosphate pH 7.0, 12% formaldehyde) were added to gradient fractions, heated at 65-68°C for 10 min and equilibrated to room temperature before applying to nitrocellulose. The slots were then rinsed several times with 10x SSC. Hybridization to ^{32}P labeled DNA probes was as

previously described (Chapter 1).

Quantitation of TK mRNA present in the various RNA fractions. The isolated RNA fractions were either run on formaldehyde agarose gels and blotted or directly bound to nitrocellulose using a slot blot manifold (BRL or Schleicher & Schuell). Equal amounts of RNA or polysomes quantitated by optical density, yields from equal numbers of cells or equal volumes from equal plates of cells were loaded on a gel or slot blotted per sample. The nitrocellulose filters were hybridized to TK and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) probes. The GAPDH gene (10,11) was used as an internal control. The levels of message were determined by scanning densitometry as described (Chapter 2).

Fluorescence activated cell sorter (FACS) analysis. Stained Rat 3 and Rat 3 cells transfected with TK cDNA constructs were sorted for their relative DNA content in a Ortho Cytofluorograph System 50. Cells were stained with acridine orange following a modification of a protocol by Darzynkiewicz (9). Plates (100 mm) of confluent cells at various times after serum stimulation were resuspended in 1 ml of media. To 200 μl of the cell suspension 400 μl of buffer A [0.1% Triton X-100, 0.2M sucrose, 0.0001M EDTA, 0.02M citrate phosphate buffer pH 3.0 (40 mM citric acid monobasic, 20 mM sodium phosphate dibasic)] was added and samples were stored at 4°C ready to be analyzed. Before analysis, 400 μl of buffer B [0.1M NaCL, 0.01M citrate phosphate buffer pH 3.8

(9 mM citric acid monobasic, 10 mM sodium phosphate dibasic), 20 μg/ml acridine orange] was added to the sample.

RESULTS

Induction patterns of TK mRNA in poly A^+ mRNA and total cell RNA. Poly A^+ mRNA was isolated at 0, 2, 4, 12, and 24 hours after serum stimulation from pooled cell lines of Rat 3 cells transfected with the SV-huTK and HuTK cDNA constructs discussed previously. Poly A^+ mRNA from equal numbers of cells at each time point were analyzed by Northern blot analysis as previously described (MATERIALS AND METHODS-Chapter 1). The autoradiogram in Figure 1 shows that poly A^+ TK mRNA is induced with the same pattern as total TK mRNA (RESULTS-Chapter 2). Poly A^+ TK mRNA and total TK mRNA induction profiles are compared in Table 1. For TK message transcribed from the SV40 promoter (SVTK), poly A^+ TK mRNA is abundant and maximal at 2 hour, no different from total TK mRNA induction patterns. Poly A^+ TK mRNA transcribed from its own promoter (HuTK), is not induced until 12 hours, again similar to total TK mRNA induction patterns. No great differences in the amount of polyadylated TK mRNA transcribed from the SV40 promoter were detected during G1 and S phase.

Polysomal and nonpolysomal TK mRNA in the cytoplasm. As briefly discussed in the introduction, although TK mRNA expressed from the SV40 early promoter is abundant in the cytoplasm during G1, it is either not being translated or the

enzyme is rapidly degraded after translation. In order to look for evidence of translational regulation, the fraction of TK mRNA that is polysomal in G1 and S phase cells was analyzed. Polysomal and nonpolysomal RNAs were isolated, and quantitated as described in Materials and Methods. GAPDH was used as an internal reference. GAPDH was used as an internal reference because of its constitutive expression in a variety of cell lines (10), and its constant levels throughout the cell cycle (11). The RNAs were slot-blotted as shown in Figure 2 for experiment 1. A summary of the results are presented in Table 2. Data were calculated as polysomal mRNA divided by the sum of polysomal and nonpolysomal mRNA. Values are expressed as percent polysomal mRNA (%P). The internal reference, GAPDH shows not much of a shift from G1 to S phase. The %P values for TK are somewhat more ambiguous, in experiment 1, the shift from 49 to 69% is no different than for GAPDH (43 to 64%). In experiment 2, the TK %P values change from 88 to 47% during G1 and S phase. In experiment 3, the average values from two separate RNA samples are from 77% during G0, 56 to 84% from G1 to S while GAPDH values are not different throughout. There is no dramatic increase in polysomal TK mRNA present during S relative to G1.

Polysome distribution of TK mRNA during G1 and S. Since there is no apparent dramatic change in the fraction of polysomal TK mRNA present during G1 and S phase, the distribution of TK and GAPDH mRNAs on polysomes were assessed

during G1 and S phase. TK mRNA detected in the polysomal fraction during G1 may be on monosomes halted at initiation or in some other TK mRNA/protein complex. Serum stimulated Rat 3 cells transfected with TK cDNA constructs (SV-huTK and SV-hu ΔScaTK) expressed from the SV40 early promoter were lysed; polysomes were prepared and sedimented on 15 to 45 % sucrose gradients. The absorbance profiles (A_{254}) of polysomes from these cells are shown in Figure 3A-B (R3-SV-huΔScaTK) and Figure 4A-B (R3-SV-huTK). Equal volumes of each fraction of the sucrose gradients were denatured, bound to nitrocellulose in duplicate, and hybridized to ^{32}P-labeled TK and GAPDH probes. The autoradiograms of theses RNA slot blots are shown in Figures 5 (R3-SV-huΔScaTK) and 6 (R3-SV-huTK). mRNA levels in each fraction were quantitated by scanning densitometry. TK and GAPDH mRNA distributions on polysomes during G1 and S are shown in Figure 3C-F (R3-SV-huΔScaTK) and Figure 4C-F (R3-SV-huTK). The two different Rat 3 cell lines containing SV-hu ΔScaTK and SV-huTK were used to see if the 5'utr deleted in SV-huΔScaTK (see Fig.1, Ch.2) and extra SV40 sequences present in SV-huTK (see Fig.1, Ch.2) would show differences in the polysome distribution of TK mRNA. The similarity in distribution of TK mRNA on polysomes during G1 and S suggests that TK mRNA is as efficiently translated during G1 as in S. More specifically, the average number of ribosomes on TK mRNA during G1 and S phase do not significantly change (Fig. 3C-D, 4C-D). However, without studying the rate of initiation,

elongation and termination, conclusions about actual translational efficiences can not be made. GAPDH, the internal reference, also appears not to change in its mRNA distribution on polysomes (Fig.3E-F,4E-F). The slight shift seen in the TK mRNA polysomal profiles from G1 to S is at most 2 fractions, corresponding to a few ribosomes. In other GAPDH mRNA polysomal profiles (not shown) such a shift was also seen. Furthermore, no noticible differences in the distribution of TK mRNA on polysomes were detected between the cell lines containing SV-huΔScaTK and SV-huTK.

FACS analysis of Rat 3 cells serum stimulated for G1 and S phase studies. The experiments comparing TK regulation between G1 and S phase were conducted between 2-5 hr. and 14-16 hr. respectively, after serum stimulation. The method of synchronization was by contact inhibition and/or serum deprivation, which arrested cells in G0. Previous measurements of DNA synthesis indicated that ^{3}H-thymidine incorporation could be detected by 6 hr and peak incorporation occurred after 12 hr. For measuring DNA content and determining the percentage of cells in G1/S/G2-M, FACS analysis was performed on acridine orange stained whole cells. Rat cells were analyzed every 2 hr after serum stimulation for 24 hr. The results are shown in a series of graphs (Fig.7). The x axis reflects relative fluorescence and the y axis is relative cell number. The fluorescence corresponding to peak cell number does not begin to shift until 6 hr. By 20 hr the

peak cell number has broadened, indicating that a considerable number of cells have finished S and, are in G2 or M. Depending on the cell density at arrest and how long the cells have been in quiescence, the time taken to enter S phase can vary. Also, some cells do not complete mitosis as seen at 22 and 24 hr. However, the majority of the Rat 3 cells were in G1 from 2 to 5 hours, and in S phase by 12-15 hours post serum stimulation. These results show that cells were assayed during G1 and S phase.

DISCUSSION

Postranscriptional regulation appears to be involved in serum induced expression of TK enzyme in the system used for these studies. TK mRNA is transcribed from the SV40 early promoter in Rat 3 cells upon serum stimulation of quiescent cells. TK mRNA is abundant during G1 and S phase, however, TK enzyme/protein levels remain low until S phase. The experiments described in this chapter have been directed towards examining potential levels of posttranscriptional regulation in this system. Possible explanations for the low levels of TK protein present during G1 include polyadenylation, inhibition of nuclear/cytoplasmic transport, and decreased association with polysomes during G1. It was found that none of these regulatory mechanisms appear to be involved in the expression of TK in this system, where TK mRNA and enzyme/protein levels are uncoupled (12).

Polyadenylation was examined as a potential regulatory event by assaying steady state levels of polyadenylated and total TK mRNA during G1 and S phase. The TK mRNA present during G1 and S phase is polyadenylated and poly A^- TK mRNA levels are either negligible or follow the same induction pattern as poly A^+ TK mRNA. No obvious differences between poly A^+ and total RNA were seen in the induction pattern of TK mRNA, although small differences in poly A tail length would not be detected. Also, it is unlikely that the TK mRNAs present during G1 are regulated by polyadenylation at the SV40 polyadenylation site present in the cDNA constructs. Very little is known about what role(s) the poly A tract plays in cell cycle regulated genes. However, mRNA metabolism can be regulated through changes in poly A tail length during development and physiologic stress (13-15).

Another nuclear processing event, splicing of intervening sequences, can not be regulated in this system because TK mRNA is transcribed from cDNAs (without introns). However, Gudas et al. (16) have shown that nuclear processing of native TK RNA is regulated between G1 and S phase in murine 3T3 cells. They have observed that incompletely processed nuclear TK RNA accumulates during G1, and as the cells enter S phase mature TK mRNA appears in the cytoplasm. This could be due to the absence of factors necessary for processing in arrested cells.

Since TK mRNA appears in the cytoplasm early in G1 (data not shown) nucleocytoplasmic transport of TK mRNA appears to

occur in G1. Low TK enzyme/protein levels in G1 are unlikely to be due to lack of available cytoplasmic TK mRNA.

Translational regulation was addressed by investigating the association of TK mRNA with polysomes. Polysomal to nonpolysomal ratios and distribution of TK mRNA on polysomes during G1 and S phase were not dramatically different. The distribution of TK mRNA on polysomes during G1 and S phase were also similar for cells transfected with TK cDNA constructs with (SV-huTK) or without (SV-huΔScaTK) the first 30 bp of the 5'utr of the TK cDNA. Several similar systems exist that do not exhibit tight control over TK mRNA levels, while TK enzyme/protein levels are tightly regulated. In myoblasts induced to differentiate, transfected chicken TK activity decreases dramatically but TK mRNA levels do not change (17). This decrease in TK activity was found to be associated with a 10 fold decrease in the rate of TK protein synthesis. They also found no change in the TK mRNA distribution on polysomes (18). In cycling Hela cells, again TK mRNA levels do not significantly change throughout the cell cycle but TK enzyme/protein levels increase during S phase (19). This increase was shown to be caused by a 10 fold increase in the translationl efficiency of TK mRNA. In addition, TK is regulated posttranslationally upon cell division by rapid degradation.

The deletion mutants studied in Chapter 2 predicted and support these results. The 5' untranslated region, associated

with translation initiation in leader length and secondary structure (20,21), as well as the authentic AUG and 16 codons were deleted without affecting TK mRNA and TK enzyme/protein levels. Thus, regulation at the level of translation initiation and dependence on translation through the first 16 codons is unlikely. Similarly, the 3' untranslated region, associated with, mRNA processing, transport, stability and translational efficiency, was deleted without effect. If the rate of TK protein synthesis does increase during S phase, these results and those of others suggest that it is not due to any function associated with polyadenylation, transport, mRNA stability, translation initiation, or TK mRNA distribution on polysomes. Remaining possibilities include translational elongation, termination, ribosome pausing (22), and posttranslational modification and degradation.

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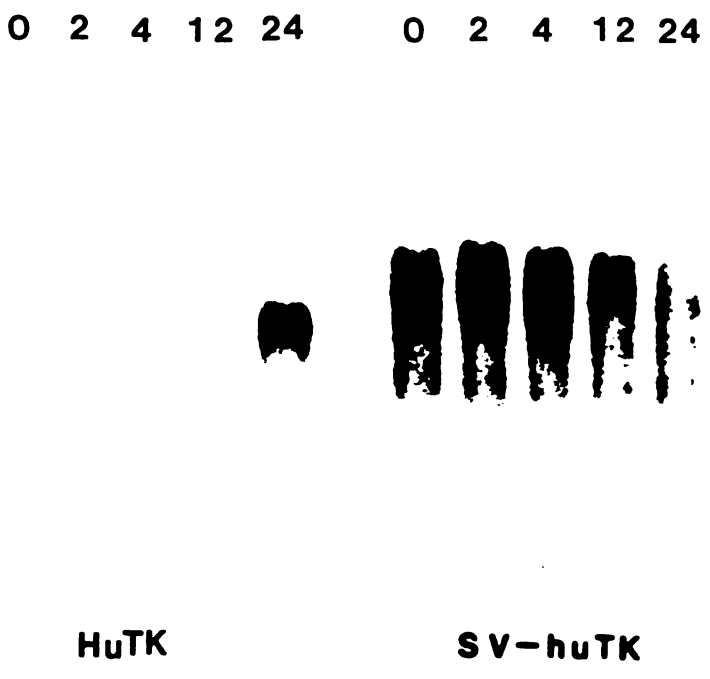


Figure 1. Poly A^+ mRNA Northern blot analysis of SV-huTK and Hu-TK transfected into Rat3 (TK^-) cells. At 0, 2, 4, 12, and 24 hours post-serum stimulation of serum deprived, growth arrested cells, poly A^+ mRNA was prepared by oligo-dT cellulose binding. RNA from equal numbers of cells at each time point were subjected to Northern analysis as described in Materials and Methods.

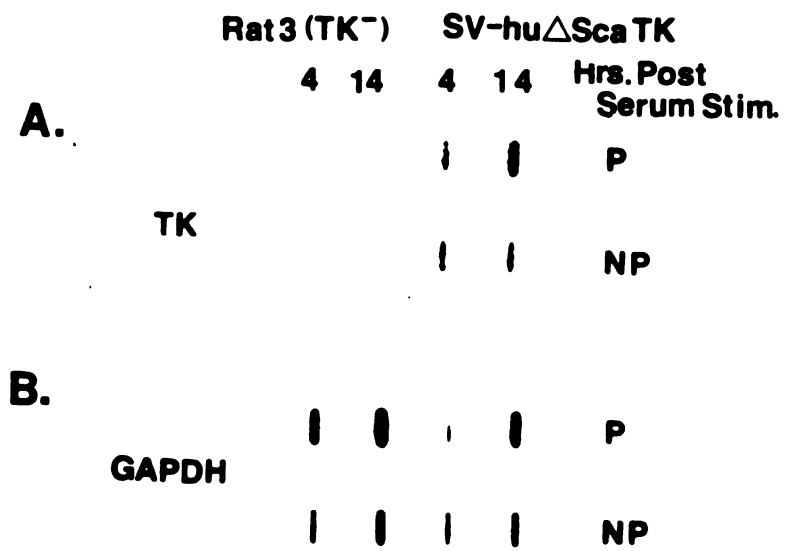


Figure 2. Polysomal and nonpolysomal RNA during G1 and S phase. Polysomal and nonpolysomal RNA was isolated at 4 and 14 hrs after serum stimulation from RAT3 (TK^-) and R3SV-hu △ScaTK cells. RNA was slot blotted as described in Materials and Methods and quantitated for TK (A.) and GAPDH (B.) in polysomal and nonpolysomal fractions.

Figure 3. Distribution of TK mRNA on polysomes during G1 and S phases in R3-SV-huΔScaTK cells. Polysomal profiles of TK and GAPDH mRNAs were determined in Rat 3 cells stably transformed with SV-huΔScaTK. Polysomes were isolated and sedimented on 15-45% sucrose gradients and fractionated as described in Materials and Methods. A,B) Absorbance (A_{254}) profile of polysomes fractionated on sucrose gradients during G1 (A) and S (B) phases. The direction of sedimentation is from left to right. The arrow indicates the approximate position of the 80S (monosome). C,D) TK mRNA polysomal distribution during G1 (C) and S (D) phases. The y-axis represents % of total TK mRNA detected in each fraction (x-axis). E,F) Internal reference, GAPDH mRNA polysomal distributions during G1 (E) and S (F) phases. The time (hrs) after serum stimulation is indicated in ( ).

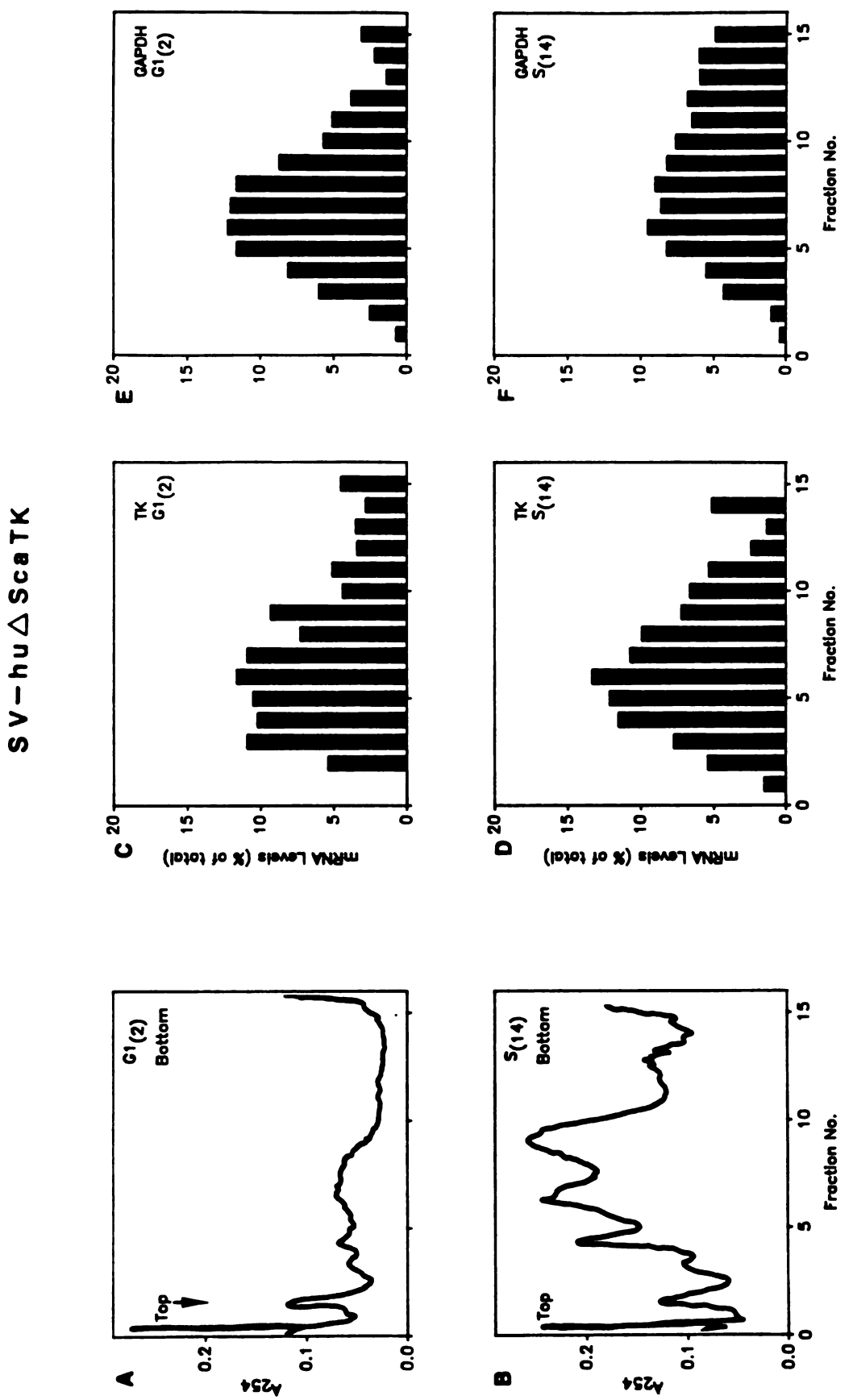
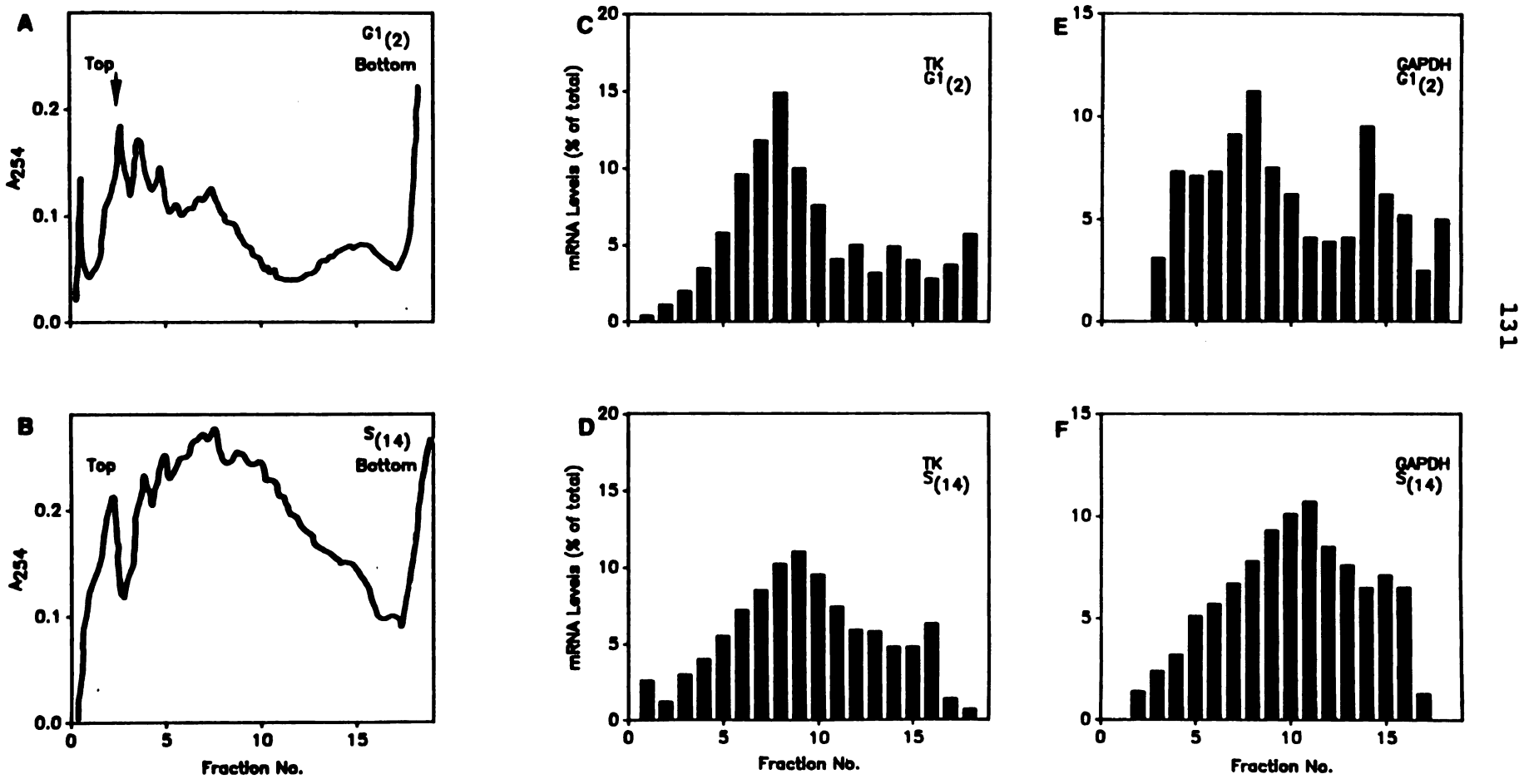

SV-huΔScaTK
A
G1(2)
Bottom
Top
A254
0.2
0.1
0.0
B
S(14)
Bottom
Top
A254
0.2
0.1
0.0
0
5
10
15
Fraction No.
C
TK
G1(2)
mRNA Levels (% of total)
20
15
10
5
0
D
TK
S(14)
mRNA Levels (% of total)
20
15
10
5
0
0
5
10
15
Fraction No.
E
GAPDH
G1(2)
20
15
10
5
0
F
GAPDH
S(14)
20
15
10
5
0
0
5
10
15
Fraction No.

Figure 4. Distribution of TK mRNA on polysomes during G1 and S phases in R3-SV-huTK cells. Polysomal profiles of TK and GAPDH mRNAs were determined in Rat 3 cells stably transformed with SV-huTK. Polysomes were isolated and sedimented on 15-45% sucrose gradients and fractionated as described in Materials and Methods. A,B) Absorbance (A_{254}) profile of polysomes fractionated on sucrose gradients during G1 (A) and S (B) phases. The direction of sedimentation is from left to right. The arrow indicates the approximate position of the 80S (monosome). C,D) TK mRNA polysomal distribution during G1 (C) and S (D) phases. The y-axis represents % of total TK mRNA detected in each fraction (x-axis). E,F) Internal reference, GAPDH mRNA polysomal distributions during G1 (E) and S (F) phases. The time (hrs) after serum stimulation is indicated in ( ).

SV-huTK


A
G1(2)
Top
Bottom
A254
0.0
0.1
0.2
B
S(14)
Top
Bottom
A254
0.0
0.1
0.2
0
5
10
15
Fraction No.
C
TK
G1(2)
mRNA Levels (% of total)
0
5
10
15
20
D
TK
S(14)
mRNA Levels (% of total)
0
5
10
15
20
Fraction No.
E
GAPDH
G1(2)
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5
10
15
F
GAPDH
S(14)
0
5
10
15
Fraction No.

Figure 5. Polysomal gradient fractions during G1 and S phase in R3-SV-huΔScaTK cells. The fractions were slot blotted and hybridized to detect TK and GAPDH mRNA. The direction of sedimentation is shown from top to bottom. The polysomal gradients shown in Figure 3A and 3B are shown slot blotted here.

SV−huΔScaTK

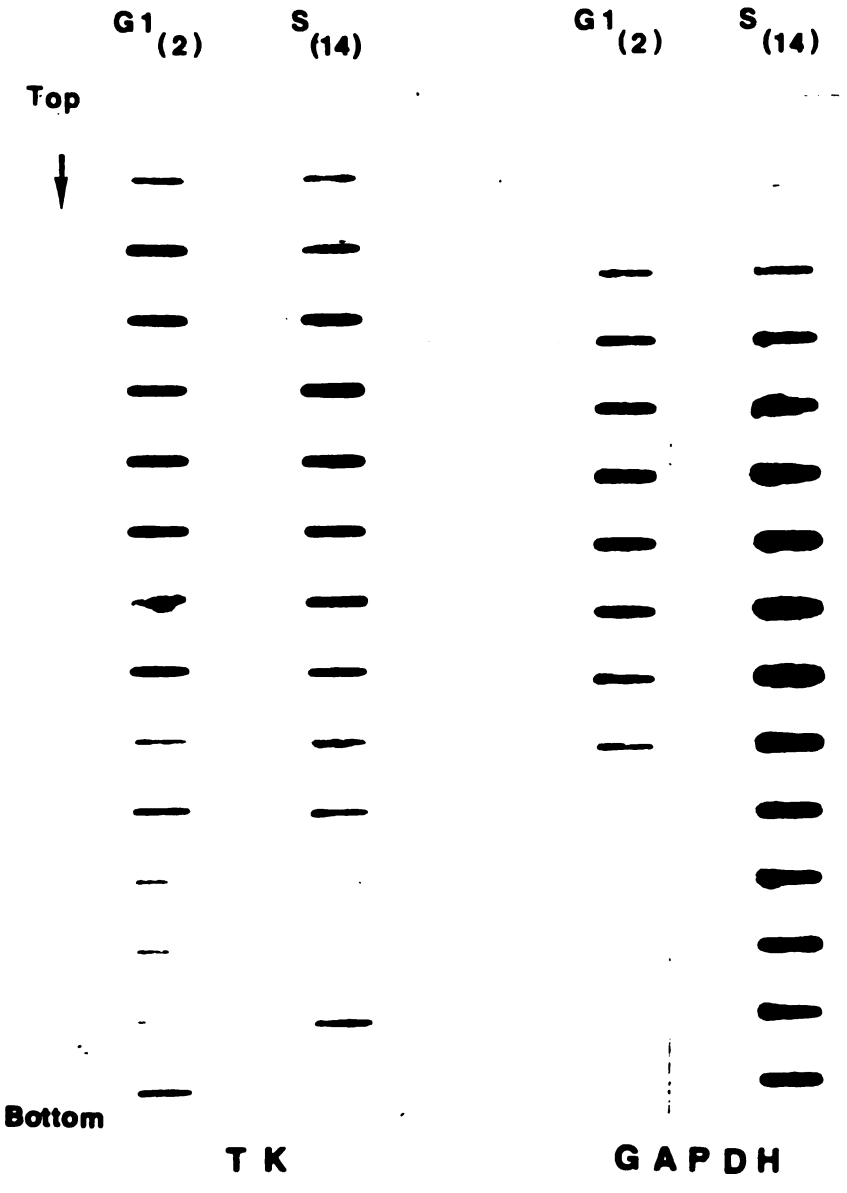

Figure 6. Polysomal gradient fractions during G1 and S phase in R3-SV-huTK cells. The fractions were slot blotted and hybridized to detect TK and GAPDH mRNA. The direction of sedimentation is shown from top to bottom. The polysomal gradients shown in Figure 4A and 4B are shown slot blotted here.

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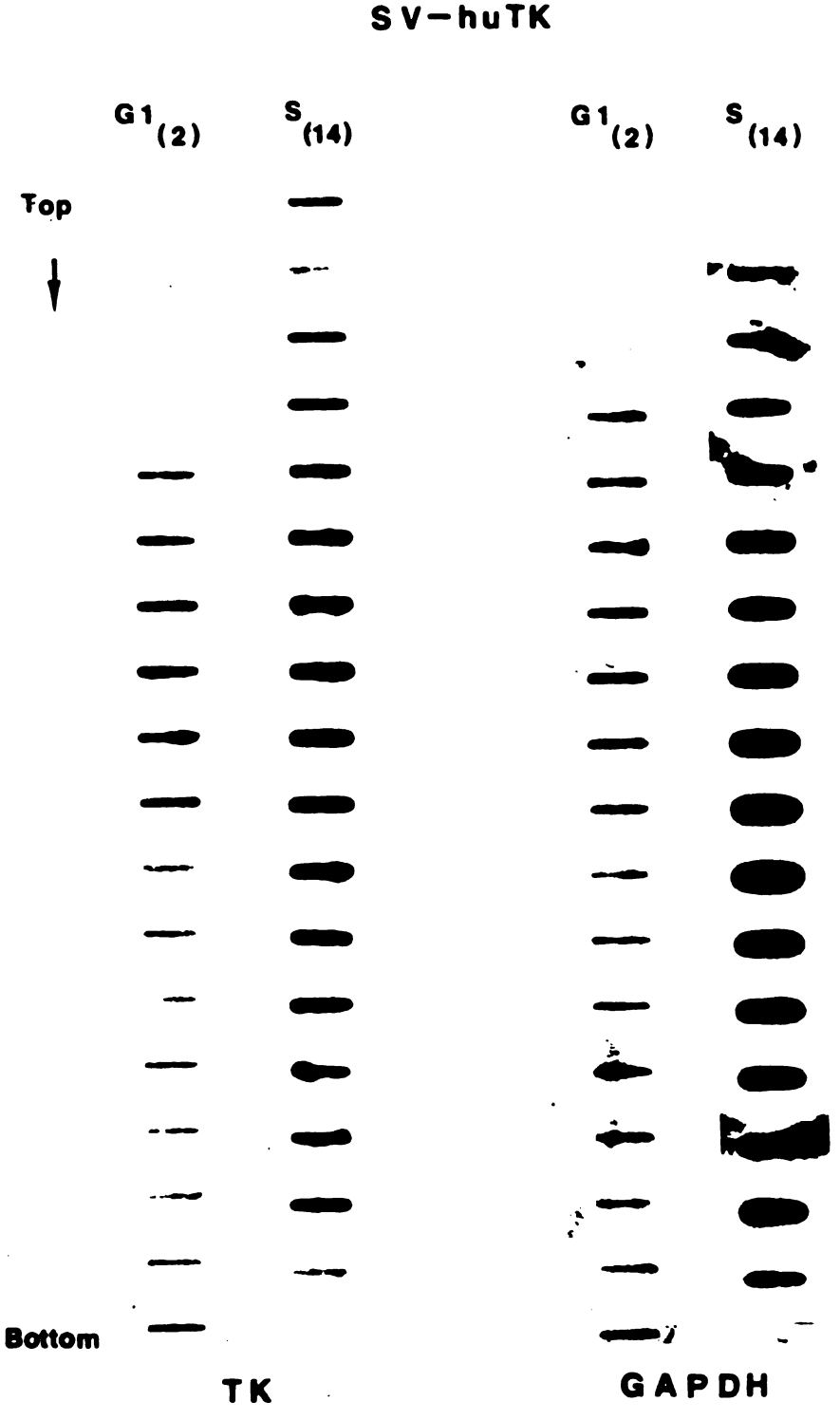

Figure 7. FACS analysis of DNA content in serum induced quiescent rat cells. DNA content was determined in rat cells transfected with various TK cDNA constructs. The results presented here were determined for SV-huΔScaTK. Cells were stained with acridine orange and analyzed as described in Materials and Methods. The y-axis on all panels, represents relative cell number at relative fluorescence (x-axis). Hours post serum induction are indicated at the top-right corner of each panel.

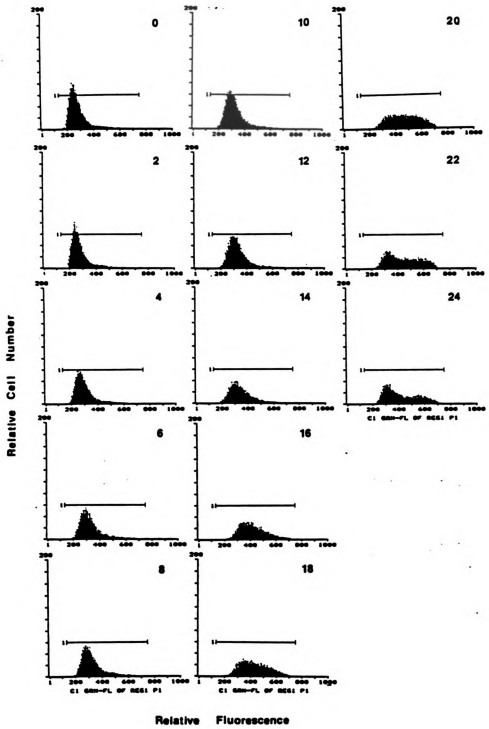
0
10
20
2
12
22
4
14
24
6
16
8
18
Relative Cell Number
Relative Fluorescence

Table 1. Relative* poly A^+ TK mRNA verses total TK mRNA levels during G1 and S phases.

Hrs.post serum stim.	SV-huTK Total[a] (1)	(2)	SV-huTK Poly A^{+b}	Hu-TK Poly A^+
0	1.0	1.0	1.0	1.0
2	3.0	2.2	2.0	1.3
4	2.3	2.1	2.1	0.9
12	1.2	1.9	1.3	4.6
24	0.9	1.1	0.7	18.6

* The level of TK mRNA in quiescent (t=0) cells was defined as 1. The values for all other times are expressed relative to that value.

[a] Relative total mRNA levels were determined by scanning autoradiograghs as in Table 1-Chapter 2 for cell lines (1) and (2), derived from independent pools of cells.

[b] Relative poly A^+ mRNA levels were deterimined by scanning autoradiographs (Fig.1) as described under Materials and Methods. For each value, several different exposures of the gel were scanned and the values were averaged.

Table 2. Polysomal TK mRNA during G1 and S phase.

Expt. #	mRNA	%Polysomal mRNA			
		R3-SV-huΔScaTK		Rat 3(TK-)	
1[a]		$G1_{(4)}$*	$S_{(14)}$	$G1_{(4)}$	$S_{(14)}$
	TK	49	69	-	-
	GAPDH	43	64	66	60
2[b]		$G1_{(5)}$	$S_{(15)}$	$G1_{(5)}$	$S_{(15)}$
	TK	88	47	-	-
	GAPDH	80	82	95	83
		R3-SV-huΔScaTK			
3[c]		$G0_{(0)}$	$G1_{(4)}$	$S_{(14)}$	
	TK	77	56	84	
	GAPDH	82	86	88	

[a] %P: (polysomal mRNA) P + (Total =nonpolysomal mRNA + P) x 100

[b] Values were calculated as for Expt. 1, but represent an average from 3 serial dilutions of each sample.

[c] Values were calculated as for Expt. 1, but represent an average from 3 serial dilutions for each of 2 separate samples.

* Approximate phase in the cell cycle; (Hr after serum stimulation).