This is to certify that the

dissertation entitled

A Study of the Regulation of

Mammalian Thymidine Kinase Genes

presented by

Christine Joyce Stewart

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

Susan E. Conrad
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Date May 20, 1988

A STUDY OF THE REGULATION OF MAMMALIAN THYMIDINE KINASE GENES

By

Christine Joyce Stewart

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

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ABSTRACT

A STUDY OF THE REGULATION OF MAMMALIAN THYMIDINE KINASE GENES

By

Christine Joyce Stewart

The regulation of the cytoplasmic thymidine kinase (TK) gene occurs in a cell cycle growth-phase dependent manner. It is known that cells are mitogenically induced to enter the cell cycle by either addition of serum growth factors or by infection with the papovavirus, SV40. I have studied the regulated expression of the TK gene using both types of induction of quiescent mammalian tissue culture cells. TK mRNA and enzyme levels are low in resting G_0/G_1 phase simian CV-1 cells, but increase dramatically by late S/G_2 phase. To determine whether an increase in the rate of transcription was mediating this increase, nuclear run-on transcription assays were performed at various times after serum stimulation or SV40 infection of quiescent CV-1 cells. Assays performed at time points spanning the G_1/S phase interface showed a 6-7 - fold increase in serum induced cells, but only a 2.5-3.5 - fold increase in SV40 infected cells. Studies with Actinomycin D treated synchronized cell cultures showed that the TK messenger RNA is extremely stable when

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the drug is added in G_0/G_1, S and G_2 phase, as well as SV40 infected cells in S phase. Results from experiments with 3′ end deletion mutants of a TK mini-gene contruct suggest that a poly-adenylation recognition site is necessary for the mediation of TK mRNA stability, while the relatively long 662 base pair 3′ untranslated region of the TK gene is not. TK mRNA and enzyme levels were studied into and beyond the G_2 phase of the cell cycle following serum stimulation. Withdrawal of serum during G_2 phase caused a decrease in TK enzyme activity, but the mRNA level remained unaffected. Cessation of DNA synthesis early in S phase by the addition of hydroxyurea caused TK enzyme and mRNA levels to increase more slowly than control levels, and both kept increasing up to 50 hours post serum stimulation. The promoter region of the human TK gene was sequenced and two transcription start sites were mapped by S1 nuclease analysis. A 40 base pair homology common to the hamster and human TK promoter regions was found. Taken together, these results suggest multiple levels of regulation for the thymidine kinase gene.

To Mom and Dad for the support, understanding and encouragement that made this possible.

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Acknowledgements

I would like to thank the members of my committee; Dr. Ron Patterson, Dr. Barb Sears, Dr. Larry Snyder, and Dr. Jerry Dodgson for all of their help throughout the past four and a half years. Thanks also to my advisor, Dr. Sue Conrad for her support.

Thanks to Moriko Ito for all the hard work, great discussions, and especially for being such a good friend and helping me through all the rough times.

Thanks to Paul Boyer for help with sequencing and S1 mapping experiments, for being such an incredibly loyal friend, and especially for feeding me during the late night shifts.

Thanks to the cousins--Don P. and Tom for teaching me how to handicap and helping to improve my golf game.

Thanks to the members of the Brubaker lab for adopting me and showing me that science can actually be a heck of a lot of fun.

Last, but not least, thanks to Rick Mehigh for believing in me and for all the love and encouragement throughout the final phases of my stay here.

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INTRODUCTION

The S phase dependency of the cytosolic form of the thymidine kinase (TK) enzyme has been known for many years. Only recently, with the use of the cloned TK gene as a probe, have we been able to begin to elucidate how this enzyme is regulated at the molecular level. At the time the studies described here were begun, very little was known about the synthesis or metabolism of TK mRNA in the cell, or how these processes affected the regulation of the cognate enzyme. Previous studies using synchronized cell cultures had shown that increases in TK enzyme activity in the cell cycle were dependent upon both protein and RNA synthesis, but not DNA synthesis. Therefore, our first step in the approach to this problem was to investigate the steady state levels of TK mRNA produced throughout the cell cycle, and to attempt to correlate this to the enzyme activity. Since it was also well known that infection with papovaviruses such as SV40 produced an induction of DNA synthesis enzymes similar to serum growth factor addition, Appendix 1 describes the investigation of the nature of TK mRNA induction using both these mitogenic factors to stimulate growth of quiescent cells.

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The results of our preliminary experiments showed that both TK mRNA and enzyme were regulated in a similar manner under both of these conditions, and this suggested that TK regulation might be occurring at the level of transcription. Chapter 2 and appendix 2 describe an analysis of the rate of transcription in both serum induced and SV40 infected cells. Since the results from these experiments indicated that there might be multiple levels of regulation, chapter three describes an attempt to determine the half-life of the messenger RNA throughout the various phases of the cell cycle. In an attempt to help further understand TK regulation in the cell cycle, a more detailed characterization of the fate of the message and enzyme at late times in the cell cycle is also included in this chapter.

The last chapter includes a description of the subcloning and DNA sequence analysis of the genomic promoter region of the human TK gene. An analysis of this region was necessary to begin to understand the molecular mechanisms that might mediate transcriptional cell cycle regulation of this gene.

Finally, a summary and conclusions section has been presented at the end. This section is a summation of the results, and attempts to describe areas of research that could be undertaken in the future.

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Chapter 1.

LITERATURE REVIEW

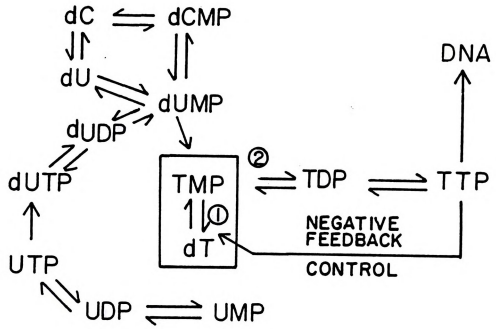
Biochemical Aspects of Thymidine Kinase

Deoxythymidine kinase (ATP:deoxythymidine 5' phosphotransferase, EC 2.7.1.75) is an enzyme in the pyrimidine salvage pathway for DNA synthesis which catalyzes the reaction:

Deoxythymidine + NTP --> dTMP + NDP (24).

Typically, the nucleoside phosphorylated is deoxythymidine, but thymidine kinase (TK) will also catalyze the phosphorylation of deoxyuridine and of several halogenated pyrimidine deoxyribonucleoside analogs of dT: flouro-deoxyuridine (dFu), triflourothymidine (F_3dT), and iododeoxyuridine (dIU). Therefore, the specificity for the nucleoside acceptor is that it be a uracil in which the substituent on carbon 5 of the pyrimidine is hydrogen, a methyl group, i.e. deoxythymidine or any of several halogens (72). All of these analogs are known to be competitive inhibitors of the phosphorylation reaction when either dT or dU are employed as nucleoside receptors (24,68). The main reactions in the formation of deoxythymidine triphosphate, and ultimately DNA synthesis, are shown in figure 1 (79). Considering enzymes 1 and 2, thymidine kinase and TMP kinase, respectively, evidence shows that TMP kinase is the

Figure 1 Some of the reactions involved in the synthesis of precursors of deoxythymidine triphosphate required for DNA synthesis. The boxed area indicates the reaction catalyzed by thymidine kinase. Numbers in circles: 1 is thymidine kinase, 2 is TMP kinase.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critical rate controlling enzyme for TTP synthesis, while TK is more of a "salvage" enzyme (24,50,68,79). The presence of the TK gene is actually of benefit to the cell, which functions by using less energy (ATP) for synthesis of dTMP than biosynthesis of TTP by the de novo pathway.

Thymidine kinase has been reported to be present in a wide range of animal, plant and microbiological forms (yeast and other fungi being notable exceptions) and is also coded for by many viral genomes (64,68,79,101). Even the bacteriophage T4 carries a functional thymidine kinase gene, and has been shown to induce activity of TK upon infection of a mutant of E. coli lacking this activity (15,30). The enzyme has been partially purified from a variety of sources (24,27,68,72,79). The enzyme in E. coli was purified approximately 1200 fold and its properties investigated by Okazaki and Kornberg in 1964 (72). The TK enzyme from E. coli is an allosteric protein which consists of two monomeric subunits of 42,000 daltons each. These subunits are extremely temperature sensitive, but become quite insensitive in the dimeric form (68,72). The molecular weights of animal cell enzymes are about 80-100,000 daltons as estimated by gel filtration with Sepharose G100 or G200. The cytosolic TK from human placenta has been purified to homogeneity. The molecular weight obtained by gel filtration and sucrose density ultracentrifugation is 92,000. The

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subunit molecular weight is 44,000, suggesting that the enzyme is a homodimer in its native state (27).

The enzyme has also been purified and studied in detail in a variety of other microorganisms such as Tetrahymena pyriformis and Chlorella pyrenoidosa, as well as in a host of eukaryotic viruses; notably the herpesviruses such as HSV types I and II, EBV and CMV (38). In plants, it has been foundin Zea mays, in roots of sprouting seedlings and in Trillium (68,79). However, it is TK associated with mammalian tissue that has been studied the most extensively.

TK from both mammalian tumors and E. coli has been shown to be quite stable, particularly in the presence of thymidine (79). There is also fairly general agreement that the metal ion Mg^{++} is absolutely necessary for TK activity (24,33,79). The requirement for Mg^{++} can be partially fulfilled by Mn^{++}, Co^{++}, Ca^{++} and Fe^{++}, but none are as effective as Mg^{++}, and higher concentrations are inhibitory in the presence of Mg^{++} (24). With the highly purified E. Coli enzyme, maximum activity was obtained in the presence of both Mg^{++} and Mn^{++}(72). The pH optimum is reported to range from 7.5 to 8.5 in a variety of sources but as pH drops to 7.0, approx. 25% of enzyme activity is lost (24,33).

One important mechanism of control of the TK enzyme in vivo, as well as in vitro, involves a regulatory feedback

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mechanism in which TK activity drops upon accumulation of its end product, TTP. Okazaki and Kornberg (72) demonstrated this in E. coli, and also discovered an activation of the enzyme by a number of nucleoside di- and triphosphates, such as dCDP, that would accumulate in the absence of TK activity. This same effect has been noted in a variety of mammalian systems as well, including rat embryo, normal adult and regenerating rat liver, matched human normal and cancer tissues, and uninfected or virus infected LM or CV-1 cells (68,79). TDP has also been shown to be inhibitory to TK activity in regenerating rat liver, although TTP was described as the more "potent" of the two inhibitors (11). The inhibition appears to be competitive with thymidine, and is also strongly modulated by the concentrations of both substrates, ATP and thymidine (11,33). A variety of other nucleoside triphosphates, including UTP, dCTP, dGTP and dATP, were found to have no inhibitory effect on thymidine kinase (11). Other nucleoside kinases, such as dCMP kinase, are also inhibited by high concentrations of their end products (68). Therefore, enzymes such as thymidine kinase can be seen as a component of a larger homeostatic cellular mechanism for growth control if deoxyribonucleic acid synthesis takes place at critical concentrations of cellular deoxyribonucleoside triphosphate levels.

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TK Isozymes

It is now well known that two distinct forms of eukaryotic TK exist--the cytoplasmic form of the enzyme (ctTK) and the mitochondrial form (mtTK). The mitochondrial form of the enzyme was first discovered by Berk and Clayton in 1973 when they found that a mutant mouse cell line resistant to 5-bromodeoxyuridine, designated $LMTK^-$, still expressed about 2% thymidine kinase activity in the mitochondrial fraction while lacking all activity in the cytosol. Further evidence showed that when TK^- cells are grown in media containing [^{3}H]-thymidine, mitochondrial DNA incorporates tritium, while nuclear DNA does not (8). Also, ctTK and mtTK were shown to differ with respect to electrophoretic mobility, Km, sedimentation coefficient, pH optimum, phosphate donor specificity and inhibition by dCTP (44,91). The two enzymes have characteristic expression during cell growth. In rapidly growing cultures of established cell lines, ctTK accounts for approximately 96-99% of total activity, with mtTK accounting for the rest. In more slowly growing xeroderma pigmentosa cells, however, one finds the mtTK accounting for almost 45% of activity (44,45). CtTK activity is also regulated with the growth state of the cell--its activity declines rapidly as cells enter stationary phase, while mtTK expression is constituitively expressed (38). Some studies have shown a correlation of expression between the

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TKs in fetal and adult tissue. In fetal and tumor tissue, the ctTK is the major molecular form, yet in some adult human and rat tissues, the mtTK accounts for most of the cellular TK activity (12,22,91). One of the most useful ways to distinguish the two forms in tissue extracts is to calculate the ratio of enzyme activity obtained with either CTP or ATP as phosphate donor, as mtTK can efficiently utilize CTP, while ctTK cannot (22). Although the TK isozymes are found in different subcellular compartments, they are both encoded by nuclear DNA. Willecke et. al. in 1977 were able to assign human mtTK to chromosome 16 by the use of human-mouse somatic cell hybrids which allowed all human chromosomes to be analyzed. In contrast, the human ctTK, has been shown to reside on chromosome 17 (23,100).

Expression of TK in the Cell Cycle

As mentioned in the last section, the activity of ctTK is a growth stage, or cell cycle, dependent phenomenon. Since TK is a DNA synthesis enzyme, it follows that to understand it's regulation, one must first understand a typical mammalian cell cycle.

By cytological observation of the cell, the cycle can be broken down into two basic parts: interphase and mitosis. Interphase itself can be divided into three parts each defined by the status of the chromosomes. The G_1

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(first gap) period is defined as the time during which the daughter cells from mitosis retain a diploid set of chromosomes (52). As chromosomes are released from their condensed mitotic state, specific regions of the genome become accessible to RNA polymerases, and RNA and protein synthesis resume at a rapid rate. In particular, the enzymes for DNA metabolism (such as TK) and many proteins associated with cell differentiation functions begin to be synthesized in G_1 (70). The crucial control events for the regulation of growth therefore seem to reside in G_1. Evidence suggests a restriction point or commitment point in mid to late G_1 phase, where a decision is made whether to initiate DNA synthesis and undergo cell division or to cease proliferation (75,102). This process appears to be serum growth factor dependent (51). In 3T3 cells, processes immediately responsive to serum are completed at approximately 3.2 hours into G_1, after which progression to mitosis can be completed in the absence of serum growth factors (101,102).

Growth factors present in serum interact through specific surface receptors. The biochemical steps that transfer the mitogenic signal from the receptor to the responding genes have not been resolved, although numerous reactions which are possibly involved have been characterized (76). Therefore, it remains to be seen what exact effect serum induction provides for the

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initiation of DNA synthesis in the mammalian cell cycle. Cells leave G_1 when they begin to replicate their chromosomes, and the period in which DNA synthesis takes place is termed S phase. At the end of S, a cell has replicated its entire genetic complement and therefore possesses two diploid sets of chromosomes (52). Cells remain in this state for the G_2 period until mitosis begins. At the end of G_2, when the cells enter mitosis, chromosome condensation occurs (52,70).

Somatic cells which have ceased to divide are usually found in G_1. This is true of most differentiated cells and of cells in culture that display contact inhibition (94). Cells that have stopped dividing are described as being in the G_0 state. It is not yet understood if G_1 and G_0 represent genuinely different states of the cell or whether G_0 is a special and reversible example of the G_1 state (52).

The time spent in each phase of the cell cycle is characteristic of the particular cell type. A diagram of a typical mammalian cell cycle with a 24 hour doubling time is shown in figure 2 (88).

In order to study the cell cycle and characterize its biochemical events, one has to be able to obtain a synchronous population of cells in culture. There are several ways to achieve synchrony in a population : blocking by drugs, serum or amino acid

Figure 2 Cell cycle of a mammalian tissue culture cell with a 24 hour doubling time.

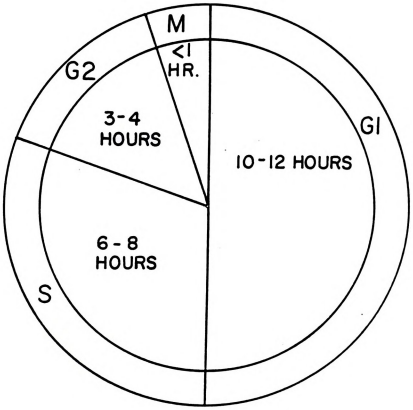

FIGURE 2

depletion, density dependent arrest by growth to confluence, and the more recently popular methods of mitotic shake off or cell elutriation by size. Using these techniques a variety of laboratories have shown that the expression of TK enzyme activity appears to be an S-phase dependent phenomenon.

S phase dependency was seen in many early studies on TK expression, in which assay for enzyme activity was compared to the various cell cycle stages. Littlefield in 1965 demonstrated a periodic appearance of TK activity in cultured mouse fibroblasts (L cells) synchronized with 5-FUdR. After release from the block, a step-wise increase of TK activity was seen to preceed and then accompany the first wave of DNA synthesis, and a second increase was seen as coincident with the second period of DNA synthesis (59). In amethopterin synchronized HeLa cells, Stubblefield (89) found a very similar rise and fall of enzyme activity during a single growth cycle. He was, in fact, the first to suggest that this enzyme might be produced periodically as the nucleus of the cell acquires competence for DNA synthesis. Eker (21) also found a positive correlation between the growth rate of human liver cells and the activity of thymidine kinase. In rapidly growing, continuously cycling cells, TK activity is high compared to the levels of TK found in stationary phase cells.

Gradually, a view began to emerge which supported

the theory that thymidine kinase might play an important role in the control of, or in being controlled by, deoxyribonucleic acid synthesis and tissue growth. In 1967 an attempt was made by Stubblefield and Murphree (90) to relate TK activity to the growth phase of the cell cycle after blocking with colcemid and subsequent release. They characterized an S phase dependent induction of TK enzyme activity which peaked in late S and G_2 phases and dropped dramatically at the beginning of the G_1 phase of the next cell cycle. This group and another (58) also ruled out the possibility of a soluble inhibitor for TK activity in G_1 or S by mixing cell extracts from G_1 and late S phase cells which gave an activity value averaging that of either phase. The inference was therefore made that some other type of activation mechanism was necessary for TK induction.

Mittermayer et. al. (69) performed an elegant study showing that the increase in enzyme activity was probably due to <u>de novo</u> synthesis of TK enzyme. By treating cells with Actinomycin D or Puromycin, they were able to show delayed or altered increases of TK enzyme activity levels in the mouse L-cell cycle. The suggestion was made that during the G_1 or presynthesis phase of the cell cycle, messenger RNAs are synthesized for these enzymes, which are in turn required for the following DNA synthesis phase. A similar approach was taken by Johnson et. al.

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(35) who demonstrated through the use of Actinomycin D at various times post serum stimulation of 3T6-fibroblasts that the induction of TK enzyme activity in the cell cycle was dependent on transcription. TK was also shown to be dependent on protein synthesis and the presence of serum growth factors.

Interestingly, even though TK is important for DNA synthesis, the induction of TK activity was shown by Bello in 1974 to be insensitive to the presence of hydroxyurea, even though DNA synthesis was inhibited by 98%. Termination of enzyme activity was, however, dependent upon DNA replication, as 59% or more of the cellular DNA was required to be replicated for efficient shut-off of TK activity. Using a more finely detailed study, Bello then demonstrated that termination of TK enzyme activity is caused by a post transcriptional block in enzyme synthesis which, in turn, is dependent upon RNA synthesis either during G_2 or late S phase (7). In addition, direct estimates of the rate of TK enzyme synthesis suggested that the enzyme is synthesized about 10 times faster during periods of increasing enzyme activity.

The expression of TK is not only cell cycle dependent, but also appears to be dependent on the type of tissue in which it is being expressed. Several studies which made use of freshly excised tissues from inbred rats showed a rather extreme variability in expression.

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All fetal and neoplastic tissues have much higher TK activities than the cognate adult tissue, and adult tissues which proliferate (erythropoetic in this case) have the highest activities in adult tissue (38,58,61). These studies suggest the possibility of a tissue specific regulatory element associated with the thymidine kinase gene.

There is only limited knowledge of what type of signal is inducing thymidine kinase activity. Yang and Pardee (101) have demonstrated that both Epidermal Growth Factor (EGF) and insulin like growth factor I (IGF-1; somatomedin C) are capable of inducing thymidine kinase activity in cells rendered competent for progression. IGF-1 was necessary only when high levels of transcription were required by the cells, so there is no time where IGF-1 is required and transcription is no longer needed for progression. It is unclear as to what exact effect IGF-1 is producing in these cells, or on the thymidine kinase gene.

TK Molecular Structure

Most studies of thymidine kinase in more recent years involve the use of the cloned cytoplasmic TK gene as a probe for TK gene expression. A variety of TK genes have been cloned, including the human (10), hamster (53), chicken (66), mouse (31), Herpes (64) and Vaccinia virus (99). Interestingly, the mitochondrial form of the gene has not yet been cloned or characterized.

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We can now compare the Chinese hamster, human, chicken and vaccinia virus TK proteins, the genes for each of which has been isolated and sequenced very recently. Each gene contains seven exons, and predicts a protein with molecular weight of approximately 25,500 (10,53). The catalytically active TK has an estimated mw of 90,000 and migrates as a single band of 44,000 when seen by SDS-PAGE (27). Covalent modification of the protein after translation may be a possible explanation for this difference. The least conserved portion of the TK protein is at the carboxy terminus. When compared to the TK gene of hamster, chicken, and vaccinia virus, the human TK amino acid sequence has 90%, 70%, and 60% homology, respectively. The human sequence shows no extended homology at either the nucleotide or amino acid sequence level to the TK gene of herpes simplex virus type 1 (64). Two regions named "A" and "B", have been found by computer analysis to represent regions of high homology and are of high hydrophobicity. It remains to be seen whether A or B may be important in TK catalysis as domains which perhaps constitute substrate binding sites or participate directly in the thymidine phosphorylation reaction (53). However, neither A nor B contain the conserved sequence:

$$\text{GLY-X-GLY-X-X-GLY-(X)}_n\text{-LYS}$$

which has been described as a necessary core element

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for various ATP binding proteins (18). The human, hamster and chicken TK genes have long 3' untranslated regions, as does the mouse TK gene, and each contains a similar polyadenylation signal with reference to sequence and placement (10,53,56,66). Both the human TK gene and the chicken TK gene have 3' untranslated regions of 662 bp long, while in the hamster, the intranslated region is 491 bp and in the mouse it is 422 bp. The 5' promoter region of the human TK gene has been sequenced and characterized, as has the hamster promoter (53), by our lab and others (48,49,chapter 4). Further comparison and discussion of the 5' region can be found in chapter 4.

Studies on the regulation of the cloned gene have shown that the sequences which are responsible for its expression and regulation seem to be closely associated with the gene. Bradshaw (9) demonstrated cell cycle regulation of TK enzyme activity by transfection of a 16 kb human genomic lambda clone into mouse LTK^- cells. Similar data had already been obtained by Schlosser et. al. in 1981 (85) with cells transformed with human metaphase chromosomes. The cloned Chinese Hamster TK gene was also seen to be cell cycle regulated (54), as was the mouse TK gene (31). These transfection/selection studies are made possible as the exhibition of a strong cell-cycle dependent regulation of TK actually seems not to be essential for the

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growth of cells in culture, since TK^- cells may be selected for. These cells grow well in normal media but can be easily characterized by their inability to grow in a selective medium--HAT. This medium contains hypoxanthine, thymidine and aminopterin - a drug which inhibits dihydrofolate reductase which causes a block in the main pathway of thymidine phosphate and purine nucleotide synthesis (31). Therefore, the existence of a system which allows for selection of cells which produce active TK upon transfection makes it possible to study the molecular basis of TK regulation via various mutations added to the gene.

The extent and duration of TK induction has been seen to differ among the various transfected TK genes. In an interesting study by Schlosser et. al. (85), TK^- cells were transfected with metaphase chromosomes or genomic DNA fragments from human, hamster, mouse and rat cell lines and selected for TK^+ phenotype. In cells synchronized by serum manipulations, the human transfectants varied from a 3.7- to a 45-fold induction level in three separate experiments, however, the timing of the induction was always similar. In each case, there was a lengthy G1 period of about 8 hours following serum addition to growth-arrested cells, followed by an increase in TK activity which peaked at 22-24 hours. Hamster TK was induced 6.5-fold, mouse by 90-fold, rat 15-fold, and a

herpes TK gene was not induced. In contrast, cells synchronized by mitotic selection showed a 45-fold increase in mouse TK levels and a 30-fold increase of human TK--niether selection showing a very long G1 period as the cells were not previously arrested. All of these experiments showed a close correlation between TK enzyme activity and cellular DNA synthesis. In contrast, not much variability was seen with the hamster TK gene transfected into Rat 4 TK^- cells. A 2.5 fold increase in activity was seen in serum refreshed cells, while a 7-fold increase was noted in cells which had been confluent and arrested and then stimulated to grow by replating and refeeding (54). The chicken TK gene has shown extreme variability in the level of activity from one transfected clone to another as a 9.4-fold to 86-fold difference in induction was observed between six assayed clones (67). An isolated human TK lambda clone has been seen to be induced 12-fold by Bradshaw in 1983, peaking by 40 hours after serum addition in LTK^- cells transfected to TK^+ (9). Similarly, studies with a TK "mini gene" carrying the genomic 5′ promoter and 3′ genomic untranslated region have shown TK to be induced by 5-7 - fold by 12 - 24 hours in Rat3 TK^- cells transfected to TK^+ (Moriko Ito--unpublished data).

Many reasons exist for the variability in TK activity observed between clonally isolated transfectants of the

same TK gene. Differences probably exist with relation to the number of copies of the gene integrated into each genome, the position effects on transcription probably differ, and variations in extent of methylation between each transfectant are just a few possibilities.

In contrast, the endogenous TK genes show much less variability in their extent of enzyme induction from one assay to another. A very typical 6-fold induction by mid to late S phase has been reported for TK genes from Chinese hamster (54,90), Rat embryo (1), and mouse L cells (69). A lower 3-fold level has been reported for mouse fibroblasts (16), and higher levels of 13-15-fold in HeLa cells (89), 10-15-fold in Balb/c3T3 cells (101), 13-17-fold in mouse 3T3 (16), 50-fold in mouse 3T6 cells (31), and a 10-15-fold increase in simian TK activity (Appendix 1) have all been reported. Obviously, variations in the levels of induction of the various genes may be explained by the fact that each is being assayed in a variety of cell lines which have differing cell cycle lengths. Also, a variety of methods were used to synchronize these cells: butyrate (31), colcemid (90) and amethopterin (89) were used in some assays, while serum deprivation and refreshment were used in others (Appendix 1,1,16,101), while a simple measurement of mass cultures in either the resting or

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logarithmically growing state accounted for the low 3-fold induction reported in mouse fibroblasts (58).

The mechanism of induction of the TK gene had not been elucidated at the time the experiments reported herein were begun. A variety of studies that made use of various drugs to block protein, RNA and DNA synthesis were undertaken and showed a general dependence on protein and RNA synthesis, but not on DNA synthesis (7,16,35,38,69). For the most part, other studies which made use of probes to look at TK RNA levels were done concurrently with our own and are mentioned in later sections.

Other Cell Cycle Regulated Genes

The expression of many other S phase-specific genes has been examined. Some of the best characterized are the replication dependent histone genes. During S phase in HeLa cells, these histone RNAs have been shown to be induced to approximately 15-fold above the G1 phase level. Both an average 2-5 fold increase in transcription rate and an average five-fold increase in mRNA stability between G1 and S seem to mediate this induction (87). The timing and extent of induction of each of the S phase dependent histones seem to be very similar one to the other, both transcriptionally and post-transcriptionally. For example, the human H4 histone gene is transcribed 3-10 fold more efficiently in nuclear extracts from S phase HeLa cells (17) than in extracts from non-S phase cells, while the

mouse H3 histone gene increases by 1.5-2.5-fold in S phase MEL cells (2), and 5-fold in mouse 3T6 fibroblasts (19). Mutational analysis of a variety of histones has shown that sequences 5′ to these genes are important for their cell cycle dependent expression, (4,17), while 3′ sequences have been shown to be important for post-transcriptional regulation (14,60).

In contrast to the effect on TK message, when S phase cells are treated with DNA synthesis inhibitors the level of histone mRNAs drop rapidly. This is due to a destabilization of the message, which can be prevented or counteracted by treating cells with protein synthesis inhibitors. This suggests that histone mRNA stability is sensitive to changes in DNA synthesis and that a labile protein may be involved in its degradation (86). However, it has been suggested that changes in steady state mRNA levels may be due to stabilization of transcripts in the nucleus since transcription doesn't increase greatly and the incorporation of [^{3}H]-uridine pulses into cytoplasmic H3 mRNA does rise sharply during the transition from G1 to S and then begins to fall sharply as cells approach G2 (2). Obviously, there appear to be multiple levels of control for the induction of the S-phase dependent histones.

The same situation appears to be true for the dihydrofolate reductase (DHFR) gene. It has been shown that

the level of DHFR mRNA is 10-fold higher in growing cells than in resting cells (36). Specifically, within 2 hours into S phase and continuing throughout the duration of S, there is a 90% increase in DHFR specific activity. This increase is the result of new DHFR molecules initiated after the cell is physiologically committed to DNA replication, and the maximum peak of activity coincides with the maximum rate of DNA synthesis (62). A rather complex set of regulatory controls seem to mediate DHFR induction. Leys et. al. (55) suggest that DHFR levels are mediated solely by changes in the stability of transcripts located in the nucleus, and by the use of continuous labelling experiments they were able to show that in growing cells most DHFR transcripts were converted to mRNA, whereas in resting cells the majority were rapidly degraded in the nucleus. On the other hand, Farnham and Schimke (25) were able to detect a transcriptional burst of DHFR at the beginning of S phase which was seven times higher than G1 phase levels. The peak was quite transient, and remained low through the remainder of S and into G2. DHFR appears to be another "housekeeping" gene which is cell cycle regulated by multiple levels of controls.

Another example of this type of "multiple level" regulation would be the thymidylate synthase (TS) gene product, whose activity increases 20-fold by 30 hours

following serum addition to mouse 3T6 fibroblasts (71). This increase was shown to be due to an increase in the de novo synthesis of the enzyme, and the induction is controlled both by an increase in transcription and in RNA half-life (34,71). Interestingly, when cells are stimulated in the presence of DNA synthesis inhibitors, TS activity increases in the same manner as in control-stimulated cells (71). Like TK, TS gene expression therefore appears not to be coupled to DNA replication.

Ribonucleotide Reductase is a DNA synthesis enzyme which exhibits a rather different type of cell cycle regulation. This enzyme has a very short half-life (about 2 hours) in the cell and its production ceases as soon as DNA synthesis is completed (96). In contrast, Gelbard et. al. (28) showed that deoxycytidine monophosphate deaminase activity appears to be more specific to the M phase of the cell. Enzyme activity decreases after cell division and starts to rise during S phase, reaching a peak at 16-18 hours after the first mitosis, when the number of cells in DNA synthesis decreases and some cells start to enter into mitosis. In both cases, studies have shown that the rise and fall of these enzymes is dependent on changes in the rate of synthesis and degradation of the enzyme during the cell cycle rather than on a process of activation and inactivation (28,96). Like both TK and TS, dCMP deaminase activity in cells treated with

hydroxyurea exceeds that in cells not treated with the drug (28). Again, this is another example of a DNA synthesis enzyme whose regulation appears not to be dependent upon DNA synthesis.

Of course, not all cell cycle regulated genes are specific to the S-phase of the cell cycle. Examples of some G1 phase dependent genes include the c-myc and c-fos genes, both of which are induced transcriptionally very shortly (0-2 hours) after serum addition to confluent growth arrested cells (29,83,97). Both of these proto-oncogenes have been implicated in cell growth control, so the placement of their transcriptional induction in early G1 is significant. Both proteins are nuclear and are post-translationally modified (5). The kinetics of the steady-state levels of these mRNAs are very different. c-fos mRNA appears 15 minutes after serum stimulation and decreases below detectable levels within 60 minutes, while c-myc appears at 2 hours post-stimulation and this level remains invariant throughout the rest of the cell cycle in the constant presence of serum growth factors (93). Interestingly, following removal of serum growth factors, the c-myc mRNA level rapidly declines, yet the cells can proceed through the cell cycle which has already been initiated (37). These data place both c-fos and c-myc into the catagory of genes whose products seem to be necessary in G1 phase.

One recent discovery involving DNA synthesis enzymes in the cell cycle may be significant in elucidating the mechanism of their regulation. An unlocalized aggregate of enzymes including DNA polymerase-a , ribonucleotide reductase, TS, TK and DHFR was initially reported by Baril et. al. (6) in Novikoff tumor cells. The possible physiological significance of this complex was then elucidated by Reddy and Pardee (80) by sedimentation profile analysis of these enzymes on sucrose density gradients. Lysates of cells in S phase, but not in G1 phase, contained a major fraction of these enzymes that sedimented rapidly on sucrose gradients, and these aggregated complexes were localized to and active in the nucleus of the cells. The possibility that the regulation of these enzymes may somehow be related to the formation of this complex has not yet been determined, however, it does point out that at one point in the cell cycle the activities of these enzymes are physically tied together.

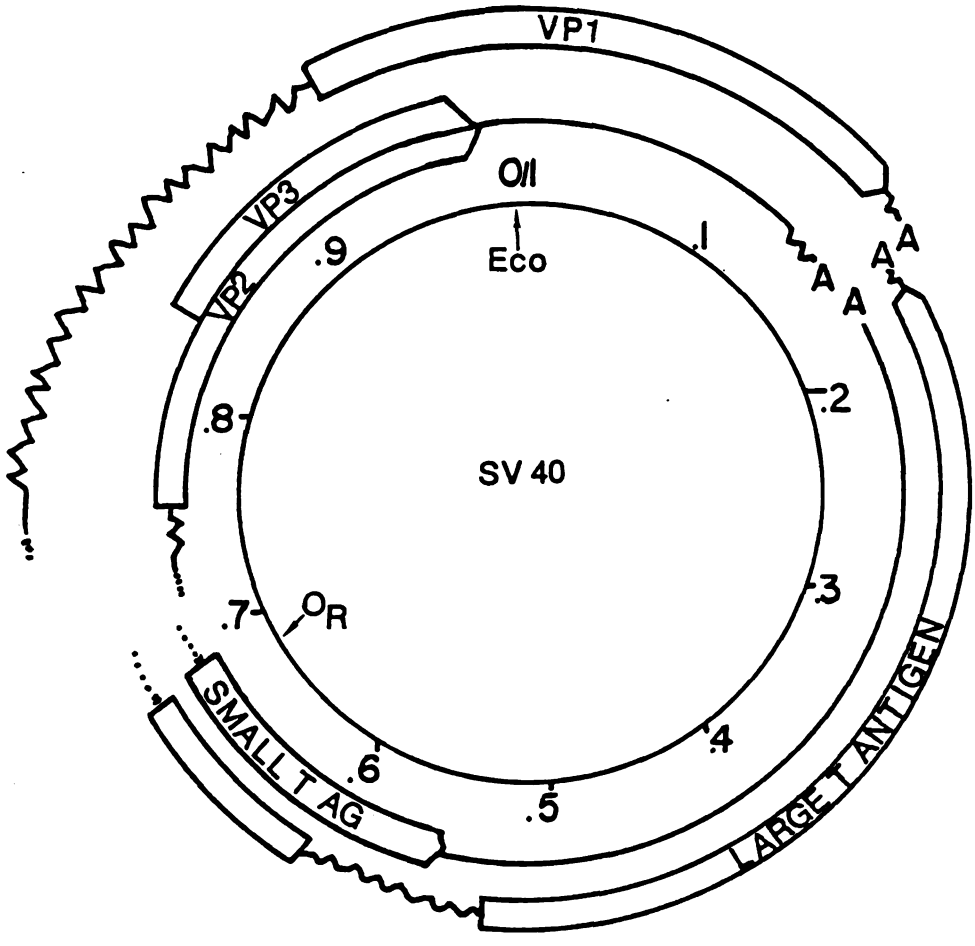
Viral Induction of TK Activity

One as yet unmentioned inducer of thymidine kinase, as well as other DNA synthesis enzyme activities in the cell, is infection with a variety of eukaryotic viruses such as SV40, Polyoma and Adenovirus (20,26,38,42,54,77). Specifically, SV40 and Polyoma viruses have been of interest, as they are DNA tumor viruses which contain small, well characterized genomes of

approximately 5 Kb in length. The growth cycle of these viruses is lengthy compared to other DNA containing animal viruses. Specifically, the SV40-eclipse period during productive infection lasts for 20 to 24 hours. Total infectious virus then increases for the next 40 to 48 hours. Vacuolation can be seen by about 60 hours, and by 72 hours virtually all of the cells in the culture display typical cytoplasmic vacuolation (42). Therefore, the properties of SV40, its small genome and lengthy infection cycle make this virus a good model for the study of virus-host cell interaction and subsequent regulation of host cell genes.

Figure 3 shows a molecular map of the SV40 genome, as well as each of its known RNA transcripts. SV40 DNA is a double stranded circular molecule. It's gene expression is temporally regulated at what appears to be the level of transcription of the viral genes (65,95). The first transcripts to be expressed are the early mRNA's--encoding the large and small T-antigens. They are transcribed from only one of the two strands of viral DNA, designated the "E" strand, the complementary DNA strand being the late or "L" strand (57). Figure 3 shows that the 5' ends of these early mRNAs are near the origin of DNA replication and the polyadenylated 3' ends lie about halfway around the circular genome. The two proteins encoded by this region are a large T polypeptide of 90-100,000 daltons

Figure 3 Schematic representation of the SV40 genome. "Ori" is the origin of replication. The open box-arrows indicate the coding positions of mRNAs, with the arrowheads pointing in the 5'-3' direction.


VP1
VP3
VP2
O/I
Eco
.1
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.3
.4
.5
.6
.7
.8
.9
SV 40
OR
SMALL T AG
LARGE T ANTIGEN
A
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FIGURE 3

and a small T polypeptide of 17-22,000 daltons which have been found by tryptic peptide and immunological analysis to be related to each other (95). The molecular basis for the difference can be seen in figure 3--large T antigen mRNA splices out a sequence which contains the small-T translation termination signal. The three proteins encoded by the L strand are expressed late in infection and are designated VP1, VP2 and VP3; they have been shown to be polypeptides which are utilized as viral capsid antigens. Their transcripts also represent about one-half of the viral genome and are different due to differential splicing of internal sequences. However, since the late viral genes are not expressed until after host cell deoxypyrimidine kinases are induced, the viral T-antigens will be concentrated on as they are potentially involved in some form of mitogenic stimulation.

Immunoflourescence studies have localized the large T antigen to the nucleus (92), while the small-T antigen has been shown to be found largely in the soluble fraction of cytoplasmic extracts (32). One of the known functions of large T antigen is that it is a DNA binding protein which specifically binds to the SV40 origin of replication. Recent evidence shows a specific protein-DNA interaction which utilizes the consensus pentanucleotides GAGGC and GGGGC (82,84). This is

significant as the SV40 regulatory region contains tandem T-antigen binding sites adjacent to and including the origin of DNA replication. Binding of large T antigen to these areas of the SV40 genome allows large T to regulate its own synthesis by reducing the rate of synthesis of E strand RNA. Binding at the origin is also crucial for the initiation of viral DNA replication (95). Temperature sensitive mutants of the A gene--or the gene which encodes large T antigen--are denoted tsA. These mutants have been extremely useful in determining many of the large T antigen's possible functions. Temperature shift up experiments with tsA mutants show that SV40 DNA molecules which have already initiated synthesis will complete their replication, but no new initiation will take place. Also, if tsA mutants are maintained at the nonpermissive temperature from the moment of infection onward, the high levels of L-strand transcription found during the late phase are not attained (3). Therefore, a large-T antigen-viral DNA interaction is required for the regulation of transcription of viral DNA.

It has been demonstrated for the appearance of T-antigen and for total DNA synthesis that individual cells in an infected culture probably begin viral DNA synthesis in a highly asynchronous manner (95,98). Studies of infection of synchronized cell cultures have shown that cells must pass through the end of the G1

phase and the beginning of S phase before viral DNA synthesis can begin and the viral late genes can be transcribed (74). Whether this requirement is related to the induction of host cell enzymes mentioned later or is of another nature is not known. Since resting cells are stimulated upon infection to pass through G1 into S, the asynchrony in the events of viral infection may be a direct result of asynchrony in the response of cells to this type of stimulation (95).

Cellular DNA synthesis has also been shown to be stimulated in cultures of GMK (African Green Monkey Kidney) cells productively infected with SV40. A stimulated incorporation of [^{3}H]-Thymidine is first detected at about 16 hours after SV40 infection. These cultures continue to incorporate [^{3}H]-Thymidine at a high rate for at least 50 hours post-infection. The rate of incorporation into DNA by 32-34 hours is 3-4 times greater than in non-infected, serum stimulated cultures (42). Slightly different results were obtained in another lab with SV40 infected CV-1 cells and AGMK cells, as the rate of cellular DNA synthesis after infection with SV40 was seen to increase by 8-10 - fold. The induction of cellular DNA synthesis began at about the same time as viral DNA synthesis (15-20 hours post-infection) (81). The incorporation of [^{3}H]-Thymidine into DNA is stimulated not only in cell cultures productively infected with SV40 but also in

cultures abortively infected with the virus. In noninfected mouse kidney cultures (a non-permissive cell for SV40) only 2-5% of the cells incorporate [^{3}H]-Thymidine, while 20-25% of SV40 infected cells do so (40).

The finding that DNA synthesis is initiated in cell cultures infected with SV40 suggests that the enzymes of cellular DNA metabolism might be induced by this virus. This has proven to be the case, as increases in thymidine kinase, DNA polymerase-a, DHFR and thymidylate synthetase activities also occur at the time that DNA synthesis is stimulated in monkey kidney cell cultures productively infected with SV40 (40,42,43, Appendix 1). However, the activities of deoxycytidylate deaminase and thymidylate kinase do not increase in these cells. In other cultures infected with papovaviruses, additional enzyme activities have been studied. The activities of uridine kinase, thymidylate phosphatase, deoxyadenylate kinase, and deoxycytidylate kinase have been found not to change appreciably post-infection (20,41,42). Interestingly, the mitochondrial form of thymidine kinase is not induced following SV40 infection of CV-1 cells. However, a different form of the cytoplasmic TK enzyme has been located in the mitochondria, and this form is also enhanced in activity in the infected cells (46).

Enzyme changes also occur in cells abortively infected

with SV40 virus. Four of the enzymes of DNA metabolism (deoxycytidylate deaminase, TK, thymidylate kinase, and DNA polymerase-a) have been studied in mouse kidney cell cultures infected with SV40 (40), and all showed dramatic increases. The enhanced activities were first detected about 16 to 24 hours after infection. The DNA polymerase activity of infected cells was 8-fold higher than non-infected cultures at 48 hours and remained elevated for at least 72 hours. TK activity was 8-fold greater than the non-infected counterparts at 30 and 40 hours post-infection and declined to about 4-fold by 72 hours. By 44-45 hours post-infection, the activities of thymidylate kinase and dCMP deaminase were 2-3 - fold and 2-5 - fold greater, respectively, than their non-infected controls (40).

Experiments with puromycin and cycloheximide have shown that de novo protein synthesis is required if SV40 induced enzyme increases are to occur. Both drugs inhibit TK, DNA polymerase and DHFR activities in SV40 infected monkey kidney cell cultures (26,42). These same studies also showed that if protein synthesis inhibitors are added after these enzymes have been induced, further increases are blocked, and the removal of the drugs permits a renewal of enzyme synthesis.

Actinomycin D studies in SV40 infected CV-1 cells have also been very interesting. Addition of this drug at 2

hours post-infection completely prevents TK enzyme induction. If actinomycin D is added at 10-14 hours post-infection, a partial induction of TK activity takes place. If the drug is added at 17-21 hours post-infection, almost normal levels are induced, suggesting that most of the TK mRNA required for the enzyme induction is made by 17 hours post - SV40 infection (39,43).

In cultures of CV-1 cells, the activity of TK enzyme was seen to increase by 15-20 - fold post infection, and this increase was shown to be paralleled by a 15-fold increase in TK steady state mRNA levels. Similar results (10-15 - fold enzyme induction and mRNA induction) were seen with serum induced cells by 12 and 24 hours (Appendix 1). Interestingly, the Michaelis constant (Km) of the TK enzyme appears to be approximately 2-3 - fold higher in SV40 infected cells than in non-infected stimulated controls (42). This may account for the perceived differences encountered in the CV-1 cell cultures between serum and virally induced TK enzyme activity.

Finally, the large T-antigen of SV40 has been implicated as the protein responsible for TK induction in virally infected cells. One study which made use of ts mutants of the various SV40 gene products has shown that only tsA mutants (of large T antigen) grown at the non-permissive temperature were incapable of inducing TK activity, as well as the other aforementioned genes

(78). Another supporting study also implicates the SV40 early region genes as being responsible for TK induction. 1-b-D-arabinofuranosylcytosine (ara-C) is a potent inhibitor of DNA synthesis and cell growth. It has been shown to inhibit the synthesis of SV40 late region antigens, but not the large T-antigen (13). Ara-C also slightly induces TK activity when added to cells in culture, but when these cells are infected with SV40, a much greater additive effect is seen to occur (42). Other evidence shows that the appearance of large T-antigen protein in the CV-1 cell following infection occurs prior to the induction of TK mRNA and protein levels (Appendix 1).

Similar studies which make use of tsA mutants have also shown that large T-antigen is the oncogene product required for the initiation and maintainance of the transformed state (63,73). Yet transformation does not appear to be a primary function of SV40 large T-antigen in its normal host. We can see that because of its small genome size and genetic content, SV40 must rely on cellular encoded proteins for the replication of its DNA genome. Large T appears to us to be the virally encoded protein which not only is required for viral replication but induces the cell to ignore existing regulatory signals and enter into its cycle so that viral DNA can be replicated to a high copy number, but in concert with cellular DNA

as the S phase is reached. If a complete viral life cycle is completed, infectious virus particles are produced and the cell dies (42). Transformation events most likely arise in a cell non-permissive for viral DNA replication, when viral DNA becomes integrated into the host genome and continues to produce its oncogenic signal such that a transformed or "cancer" cell would be produced.

The study of a DNA synthesis enzyme such as TK can be useful as a model system for other S phase dependent genes. The studies which follow are an attempt to elucidate the cellular mechanism(s) which regulates the TK gene in cells which are mitogenically induced to enter into the cell cycle via addition of serum growth factors, as well as viral infection with SV40. The use of such model enzyme systems may help to elucidate the mechanism by which viruses such as SV40 can override normal cellular controls and stimulate a quiescent cell to replicate its genetic content.

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CHAPTER II

(ARTICLE)

Evidence for Transcriptional and Post-Transcriptional Control of the Cellular Thymidine Kinase Gene

by

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Published in Molecular and Cellular Biology
Volume 7, pages 1156-1163

ABSTRACT

We have studied the cell-cycle regulated expression of the thymidine kinase (TK) gene in mammalian tissue culture cells. TK mRNA and enzyme levels are low in resting, G_0 phase cells, but increase dramatically (10-20-fold) during S phase in both serum stimulated and SV40 infected cells. In order to determine if an increase in the rate of TK gene transcription is responsible for this induction, nuclear run-on transcription assays were performed at various times after serum stimulation or SV40 infection of growth arrested simian CV-1 cells. When assays were performed at twelve hour intervals, a small (2-3-fold) but reproducible increase in TK transcription was detected during S phase. When time points were chosen to span the G_1/S interface a larger (6-7-fold) increase in transcriptional activity was observed in serum stimulated but not in SV40 infected cells. The large increase in TK mRNA levels and the relatively small increase in transcription rates in growth stimulated cells suggest that TK gene expression is controlled at both a transcriptional and post-transcriptional level during the

mammalian cell cycle. In order to identify the DNA sequences required for cell cycle regulated expression, several TK cDNA clones were transfected into Rat-3 TK^- cells and their expression was examined in resting and serum stimulated cultures. These experiments indicated that the body of the TK cDNA is sufficient to insure cell cycle regulated expression regardless of the promoter or polyadenylation signal used.

INTRODUCTION

An understanding of the regulation of genes encoding products involved in DNA replication is an important step towards identifying the elements that are involved in, and possibly necessary for, the control of the eukaryotic cell cycle. We have used the thymidine kinase (TK) gene as a model system to study cell cycle controls, since TK activity is closely linked to the growth state of the cell. There is a low level of TK enzyme activity in resting cells, and upon serum stimulation this activity increases dramatically in parallel with the onset of DNA synthesis (9). TK enzyme activity is also induced to the same or a greater extent by infection with papovaviruses such as SV40 (12,19,24), and again the induction coincides with the onset of DNA synthesis. The viral protein reported to be responsible for this induction is large T antigen, which is also required for the initiation and maintenance of transformation by this virus.

We are interested in the mechanism(s) by which these two mitogenic agents, serum and SV40, induce S phase regulated genes such as TK, and in the relationship between SV40's ability to affect expression of these genes and its ability to immortalize and/or transform cells.

Our approach to this problem has been to examine the molecular basis for the regulation of TK gene expression in serum stimulated and SV40 infected tissue culture cells. We have previously demonstrated (24) that the increase in TK enzyme activity seen in serum stimulated and SV40 infected CV-1 cells is paralleled by an increase in the steady state levels of TK mRNA, and that in both cases the increase occurs during S phase. In our current analysis we have extended our study of TK gene regulation by examining the rates of TK transcription in resting, serum stimulated and SV40 infected CV-1 cells. In addition, we have used several hybrid TKcDNA constructs to begin to identify the DNA sequences required for cell cycle regulated expression of the gene.

The expression of several other S phase specific genes, including several histone genes, the dihydrofolate reductase (DHFR) gene, and the thymidylate synthase (TS) gene have been examined, and in each case a complex pattern of regulation has been observed. In the case of the histone genes, there is good evidence that both transcriptional and post-transcriptional controls occur (1,7,21,22). Post-transcriptional control of DHFR expression is well documented (14,15), but the existence of transcriptional control has been controversial (20,26).

A recent report suggests that there is a transient burst of DHFR transcription at the G_1/S boundary, but that the increased transcriptional level is not maintained throughout S phase (2). A study of TS gene expression has reported the existence of both transcriptional and post-transcriptional controls (8).

Experiments with both the chicken (16) and hamster (13) TK genes have suggested that regulation occurs at a post-transcriptional level, and that the regulation is independent of the promoter used to transcribe the gene. In the experiments reported here we show that sequences within the human TKcDNA are sufficient to confer cell cycle regulated expression on hybrid minigenes transfected into Rat-3 TK^- cells, suggesting that post-transcriptional regulation is occurring. Using nuclear run-on transcription assays to measure rates of transcription, however, we have been able to demonstrate a 6-7-fold increase in TK gene transcription at the G_1/S interface in serum stimulated CV-1 cells. Thus, regulation of TK gene expression, like that of histones and DHFR, appears to occur at multiple levels.

MATERIALS AND METHODS

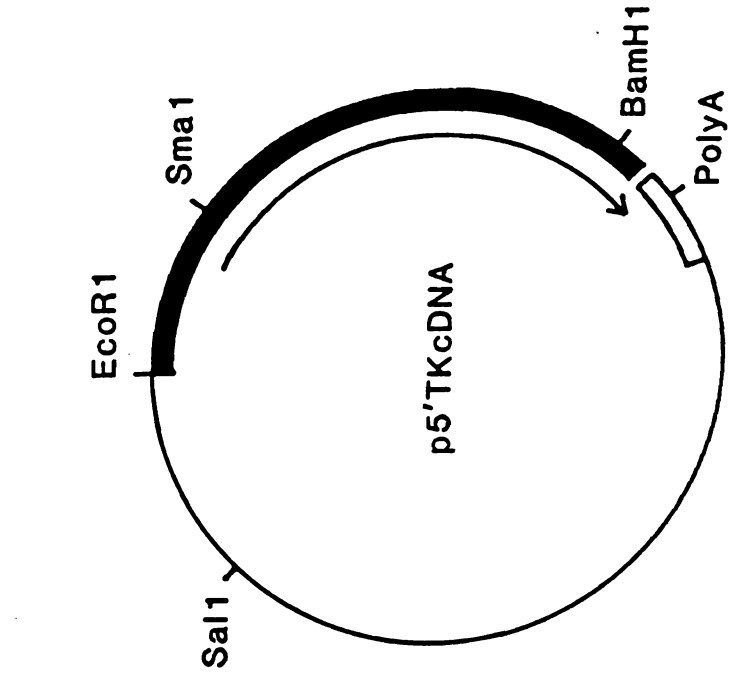
Cell culture, serum stimulation and viral infections. The CV-1 African Green Monkey kidney cell line was grown in Dulbecco's modified Eagle's medium (DME) supplemented with 5% calf serum and 5% fetal bovine serum (both from Hyclone). Rat-3 TK^- cells (25) were grown in DME supplemented with 10% calf serum. In order to obtain synchronous populations of cells, cells were plated in medium plus 10% serum so that they would reach confluence in 4-5 days, and they were held at confluence for an additional 48 hours to insure that the population was truly growth arrested. For serum stimulation, the medium on arrested cells was changed at time 0 to fresh medium containing 10% serum. For viral infections, medium was removed and saved from plates of arrested cells, and SV40 was added at a multiplicity of infection (MOI) of 15 in a total volume of 0.6 ml per 100 mm plate and incubated with occasional shaking for one hour. At the end of this infection, the original (depleted) medium was added back to the cells, and incubation was continued until the time indicated.

Northern blots, DNA pulse labelings and TK enzyme extractions and assays were performed as described previously (24).

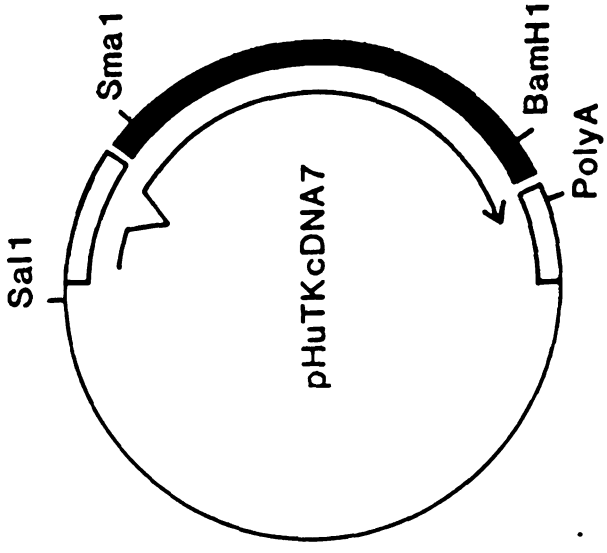
Plasmid constructions. pHuTKcDNA7 and p5'TKcDNA, the two TK cDNA clones capable of expressing TK after transfection into eukaryotic cells, are diagrammed in Figure 1. pSP64 Bam-Sma TK contains the 1.2 kb BamH1-SmaI fragment from within the TK cDNA in pHuTKcDNA7 subcloned into the polylinker in pSp64. The human β2 microglobulin cDNA clone was a gift of Dr. Hsiu-Ching Chang, and contained the β2 cDNA cloned in the Pst1 site of pBR322. pSpβ2 contains this Pst1 fragment subcloned into pSp64. A plasmid containing a genomic BamH1 fragment from within the human c-myc locus was a gift of Dr. Hsing-Jien Kung. A Sal1-EcoR1 fragment from this clone was subcloned into pSp65 to give rise to pSpmyc. By genomic blotting, this clone was shown to hybridize to repetitive human and monkey DNA. pSplink2A contains the chicken histone H3.2 cDNA subcloned into an Sp6 vector, and was a gift from Dr. Jerry Dodgson. All fragments subcloned into Sp6 vectors were inserted in an orientation relative to the Sp6 promoter that allows for transcritpion of the complementary (anti-sense) message.

Sp6 vector transcription. Complementary RNA transcripts were made using fragments subcloned into either pSp64 or pSp65 (ProMega Biotech). Reactions were

Figure 1. Structures of cDNA clones capable of expressing TK after transfection into Tk^- cells. pHuTKcDNA7 expresses the human TK message from the SV40 early promoter, and also contains an SV40 splice donor and acceptor and polyadenylation signal. p5'TKcDNA expresses the TK mRNA from the human genomic TK promoter, but still utilizes the SV40 polyadenylation signal from pHuTKcDNA7. For details of these constructions see Materials and Methods. ▬ = HuTK sequences, ▭ = SV40 sequences, ——— = pBR322 sequences.


BamH1
PolyA
Sma1
EcoR1
p5'TKcDNA
Sal1

B


Sma1
BamH1
PolyA
Sal1
pHuTKcDNA7

A

FIGURE 1

performed as described by Green et al. (3) with the following modifications. NaCl was added to the transcription buffer to a concentration of 5 mM, 10 units of Sp6 polymerase (ProMega) were added per 100 ul reaction, unlabeled ribonucleotides were added at 0.5 mM each and the reactions were incubated at 40°C. The amount of RNA transcribed was quantitated by running one standard volume against various known amounts of control RNA on agarose formaldehyde gels and staining with ethidium bromide.

RNA and DNA dot blots. Complementary RNA transcripts and DNA plasmids were bound to nitrocellulose filters using a Dot Blot Manifold (BRL). For each dot, 5 ug of RNA was resuspended in 15 ul of diethyl pyrocarbonate treated dH_2O. Five ul of formaldehyde was added, the RNA was heated to 65°C for 10 minutes, and then 130 ul of 20X SSC was added and the RNA was immediately added to the dot blot apparatus. For DNA, plasmid DNAs were linearized by restriction enzyme digestion and ethanol precipitated. After resuspension in 25 ul dH_2O, 5 ul of 2M NH_4OH was added and the sample was boiled for 3 minutes at 90°C and quickly quenched on ice. Next, 20 ul of ice cold 5 M NaCl was added, and the solution was vortexed and added to the dot blot apparatus where it was immediately washed with 2X

SSC. Filters were air dried and baked under vacuum at 80°C.

Transcription in isolated nuclei. Nuclear run-on transcription assays were performed essentially as described by Paul Robbins (personal communication) and Greenberg and Ziff (4) with the following modifications. Confluent, growth arrested CV-1 cells were either serum stimulated or infected with SV40 virus at an MOI of 15 at various times pre-harvesting. Upon harvesting, cells were washed once in PBS, once in hypotonic buffer (20 mM TRIS pH 8.0, 5 mM $MgCl_2$, 6 mM $CaCl_2$, 0.5 mM DTT), placed on ice for 5 minutes, and lysed on plates with NP40 lysing buffer (0.6 M sucrose, 0.2% NP40, 0.5 mM DTT). The lysate was scraped off the plates with a rubber policeman, and nuclei were pelleted in a clinical centrifuge at 2000 rpm. The nuclei were washed once in hypotonic buffer and were then incubated at 26°C for 45 minutes in a reaction mixture containing 50 mM Hepes (pH 8.0), 90 mM NH_4CL, 6 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.1 mM EDTA, 12% Glycerol, 0.4 mM rNTPs (-UTP) and and 100 uCi $^{\alpha 32}$P-UTP (3000 Ci/mmole) (NEN). The newly labeled transcripts were treated with an SDS-Proteinase K solution {(0.3% SDS, 100 mg/ml yeast tRNA, 100 ug/ml Proteinase K in TK (10 mM TRIS, 1 mM EDTA)} containing 100 units/ml of RNasin (ProMega) for 20 minutes

at 37°C, extracted with H_2O saturated phenol, and ethanol precipitated. Prehybridization, hybridization and post-hybridization washes were performed according to Groudine et al. (6). Equal numbers of incorporated counts from each time point were hybridized to filter-immobilized probes for 36 hours. Blots were exposed to X-ray film with screens at -70°C.

Transfections into Rat-3 cells were performed as previously described (24).

RESULTS

TK mRNA induction in CV-1 cells. We have previously reported (24) that TK mRNA levels increase 10-20-fold after either serum stimulation or SV40 infection (MOI=5) of CV-1 cells, with the levels of RNA peaking at 12 hours after serum stimulation or 24 hours after viral infection. In our current experiments we have increased the MOI to 15, since we have determined by immunofluorescence that a higher percentage of cells (100%) stain positively for T antigen at 24 hours after infection with virus at the higher MOI (data not shown). Poly A^+ RNA was prepared from serum stimulated or SV40 infected cells at 0, 12, 24 and 36 hours following treatment and analyzed by Northern blot analysis as described in Materials and Methods. The

results of this experiment, shown in Figure 2, are similar to those described previously (24). TK mRNA levels are low in resting cells, and peak at 12 hours following serum stimulation or 24 hours following SV40 infection (Figure 2A). In addition, the TK mRNA accumulates to a higher level in the virally infected than in the serum stimulated cells. As an internal control for the amount of RNA in each lane, the filter in figure 2A was re-hybridized with a human $\beta 2$ microglobulin probe, since it has been shown that the level of $\beta 2$ mRNA is relatively constant throughout the cell cycle (11,17). The results of this experiment, shown in figure 2B, confirm that approximately equal amounts of mRNA were present in each lane.

Measurements of transcription rates in nuclei isolated from CV-1 cells. In order to determine if the increased levels of TK mRNA seen in serum stimulated and SV40 infected CV-1 cells are due to increased rates of transcription of the gene, we have performed nuclear run on transcription assays as described in Materials and Methods. Figure 3 shows the results from two sets of assays, one done with serum stimulated and one with SV40 infected cells. Nuclei were prepared at 0, 12, 24 and 36 hours following treatment, RNA was transcribed, purified and hybridized to filters containing complementary RNA to

Figure 2. Northern blot analysis of TK mRNA levels following serum stimulation or SV40 infection of CV-1 cells. Confluent, growth arrested CV-1 cells were induced to re-enter the cell cycle by the addition of fresh serum or by infection with SV40 as described in Materials and Methods. Poly A^+ RNA was prepared from two plates of cells at 0, 12, 24 and 36 hours after induction, and RNA from equal numbers of cells at each time point was subjected to Northern blot analysis. (A) Hybridization with pSp64 Bam-Sma TK, which contains a 1.2 kb fragment internal to the TK cDNA. (B) Rehybridization of the filter in (A) with a human $\beta 2$ microglobulin probe.

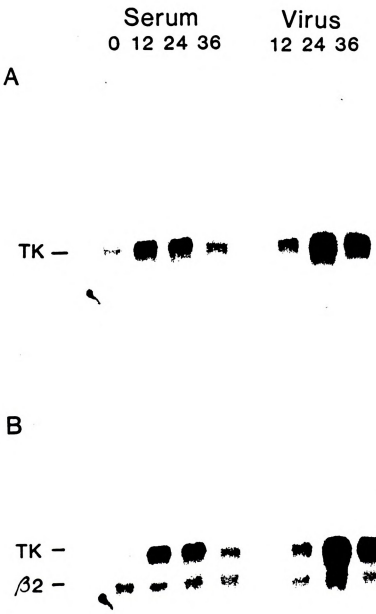


FIGURE 2

the human TK gene, the chicken H3.2 histone gene, the human c-myc gene, and RNA transcribed from the Sp6 vector as an internal negative control. Details of the plasmids and methods used to synthesize the cRNAs are given in Materials and Methods. We have found that the use of complementary RNA on the filters greatly increases the sensitivity of these assays, and allows us to measure TK transcription in CV-1 cells. In the case of SV40 infected cells, plasmid pJY1 DNA, which contains a linear copy of the SV40 genome inserted at the BamH1 site of pBR322, was bound to the filters and used as a positive control. After hybridization and washing the filters were exposed to x-ray film, and the resulting autoradiographs are shown in figures 3A and 3B. In order to quantitate the amount of RNA bound, each dot was cut out after autoradiography and counted for ^{32}P. These values, given as cpm/dot, are also shown in figures 3A and 3B.

In serum stimulated cells there was approximately a 3-fold increase in TK transcription at 12 hours, the level dropped by 24 hours and continued to decrease at 36 hours (Figure 3B). Transcription from the histone gene also increased approximately 3-fold at 12 and 24 hours, and decreased at 36 hours. The low number of counts hybridized to the H3.2 cRNA is presumably due to the fact

Figure 3. Nuclear run-on transcription assays at twelve hour intervals following serum stimulation or viral induction of CV-1 cells. Growth arrested CV-1 cells were induced to re-enter the cell cycle by addition of fresh serum or infection with SV40 as described in Materials and Methods. Nuclei were prepared at twelve hour intervals after induction and used to conduct transcription assays as described in Materials and Methods. In each case the results are shown both as autoradiograms of the hybridizations and as graphs of cpm hybridized per dot. TK (●), H3.2 (□), c-myc (0) and SV40 (△). (A) Results of hybridizations with RNA prepared from SV40 infected cells. 3.0×10^6 cpm of labelled RNA from each time point were added to each hybridization. (B) Results of hybridizations with RNA prepared from serum stimulated cells. 2.2×10^6 cpm of labeled RNA from each time point were added to each hybridization.

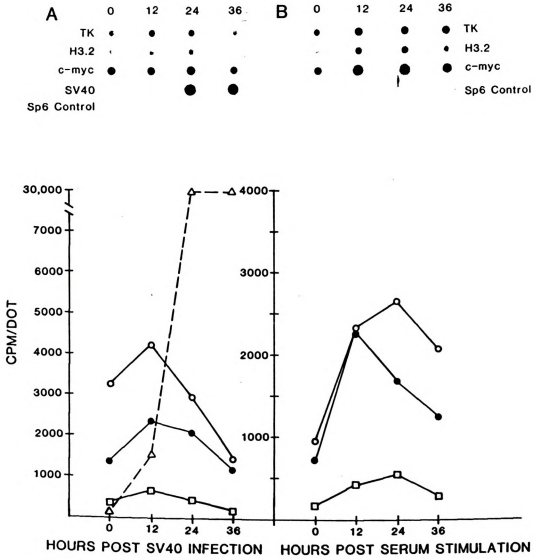

A
0
12
24
36
TK
H3.2
c-myc
SV40
Sp6 Control
B
0
12
24
36
TK
H3.2
c-myc
Sp6 Control
30,000
7000
6000
5000
4000
3000
2000
1000
4000
3000
2000
1000
CPM/DOT
0
12
24
36
HOURS POST SV40 INFECTION
0
12
24
36
HOURS POST SERUM STIMULATION

FIGURE 3

that the chicken and simian genes are not very homologous. The pattern of transcription from the c-myc gene is similar to that seen for both TK and H3.2, with a 3-fold increase at 12 and 24 hours. No hybridization to the Sp6 control was seen in this or any other experiment. The patterns of transcription in the SV40 infected cells was similar to that seen in serum stimulated cells, with small increases at 12 and 24 hours (Figure 3A). The obvious exception is the PJY1 control, which shows that SV40 transcription begins by 12 hours, and reaches very high levels at 24 and 36 hours after infection. It appears from these results that changes in the rate of TK gene transcription are not responsible for the large increase in TK mRNA seen during S phase in serum stimulated and SV40 infected CV-1 cells. In fact, the rate of transcription appears relatively constant throughout the cell cycle in these experiments.

Transcription at the G1/S phase interface. Although the experiments reported in the previous section did not detect any large changes in TK gene transcription during the cell cycle, it seemed possible that the time points chosen were not those where TK transcription was at a maximum. It was recently shown that transcription of the mouse DHFR gene peaks at the G_1/S phase interface, where a

7-fold increase over the level found in resting cells was seen. This increase occurs just prior to or concomitantly with the initiation of DNA synthesis in serum stimulated mouse 3T6 cells containing multiple amplified copies of the DHFR gene (2). We therefore decided to measure TK transcription rates at and around the G_1/S interface in serum stimulated and SV40 infected CV-1 cells. We have previously shown that DNA synthesis is induced by 12 hours following serum stimulation and 24 hours following SV40 infection of CV-1 cells (24). In order to determine the exact onset of DNA synthesis, cells were pulse labeled for one hour at 1 hour intervals surrounding the approximate initiation of S phase, and incorporation was monitored as described in Materials and Methods. The results of these experiments, shown in figure 4, indicated that DNA synthesis begins between 8-9 hours after serum stimulation and 17-18 hours after SV40 infection.

Nuclear run-on transcription assays were performed at 0,6,7,8,9,10,11 and 12 hours post serum stimulation in order to span the G_1/S phase boundary. The assays were performed as described above, except that the c-myc cRNA was omitted and cRNA to a human β2-microglobulin cDNA was included on the filters as a control for a gene that is expressed at relatively constant levels throughout the

Figure 4. Measurements of DNA synthesis in serum stimulated and SV40 infected cells. CV-1 cells were arrested and then serum stimulated or infected with SV40 (MOI=15) as described in Materials and Methods. At the indicated times after treatment, they were labeled with ^{3}H-thymidine for 1 hour, and the cpm incorporated/cell was determined by TCA precipitation of a given number of cells.

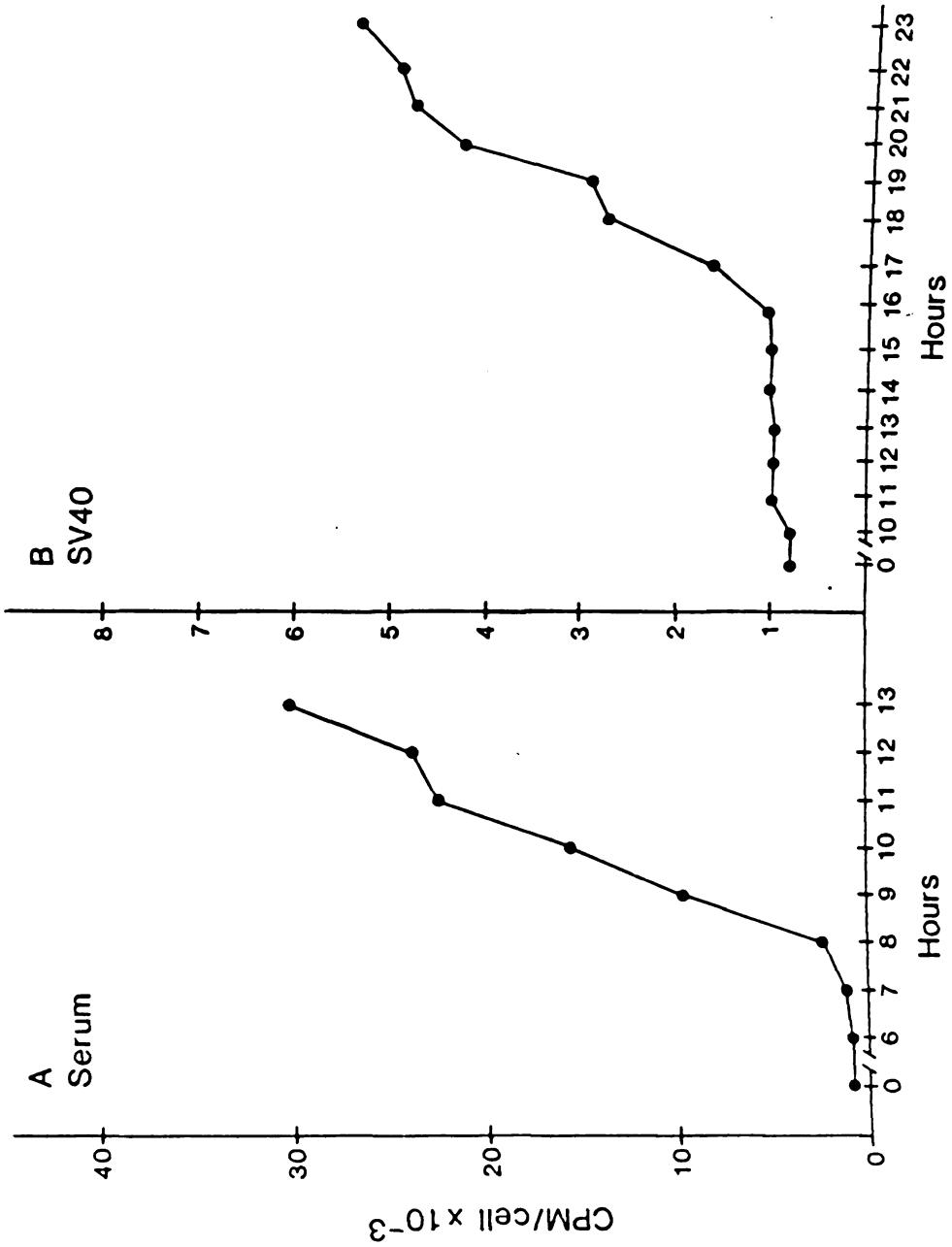

A
Serum
B
SV40
CPM/cell x 10^-3
Hours
Hours
0
10
20
30
40
1
2
3
4
5
6
7
8
0 6 7 8 9 10 11 12 13
0 10 11 12 13 14 15 16 17 18 19 20 21 22 23

FIGURE 4

cell cycle. Figure 5 shows that there is a sharp increase in TK transcription at 9 hours following serum stimulation, and that the level falls by 11 and 12 hours. We have quantitated these results by comparing the levels of TK transcription to the $\beta 2$ microglobulin levels at each time point and determined that there is a 6-7-fold increase in TK transcription at the 9 hour time point (Figure 5B and Table I). The H3.2 histone gene again shows a small increase at the 9-12 hour time ponts, and the $\beta 2$ microglobulin control is relatively constant throughout the time course. This experiment has been repeated, and each time the TK gene showed approximately a 6-fold increase in transcription at the G_1/S boundary (Table I). These results indicate that the TK gene is transcriptionally regulated in serum stimulated cells, with the rate of transcription showing a sharp but transient increase as the cells enter S phase.

We next examined TK transcription at the G_1/S boundary in SV40 infected cells. CV-1 cells were infected with SV40 at an MOI of 15, and transcription assays were performed at 0,17,18,19,20,21,22 and 23 hours after infection. The results of this experiment, shown in Figure 6 and Table I, were somewhat surprising. While the transcription of the $\beta 2$ microglobulin control was

Figure 5. Nuclear transcription assays performed at one hour intervals spanning the G_1/S boundary in serum stimulated CV-1 cells. Nuclei were prepared at the times indicated following serum stimulation, and used for transcription assays. 2.0×10^6 cpm of labeled RNA were added to each hybridization.
(A) Autoradiogram showing results of hybridization.
(B) Graphical analysis of the results in (A). TK (●), H3.2 (□), and β-2 microglobulin (△).

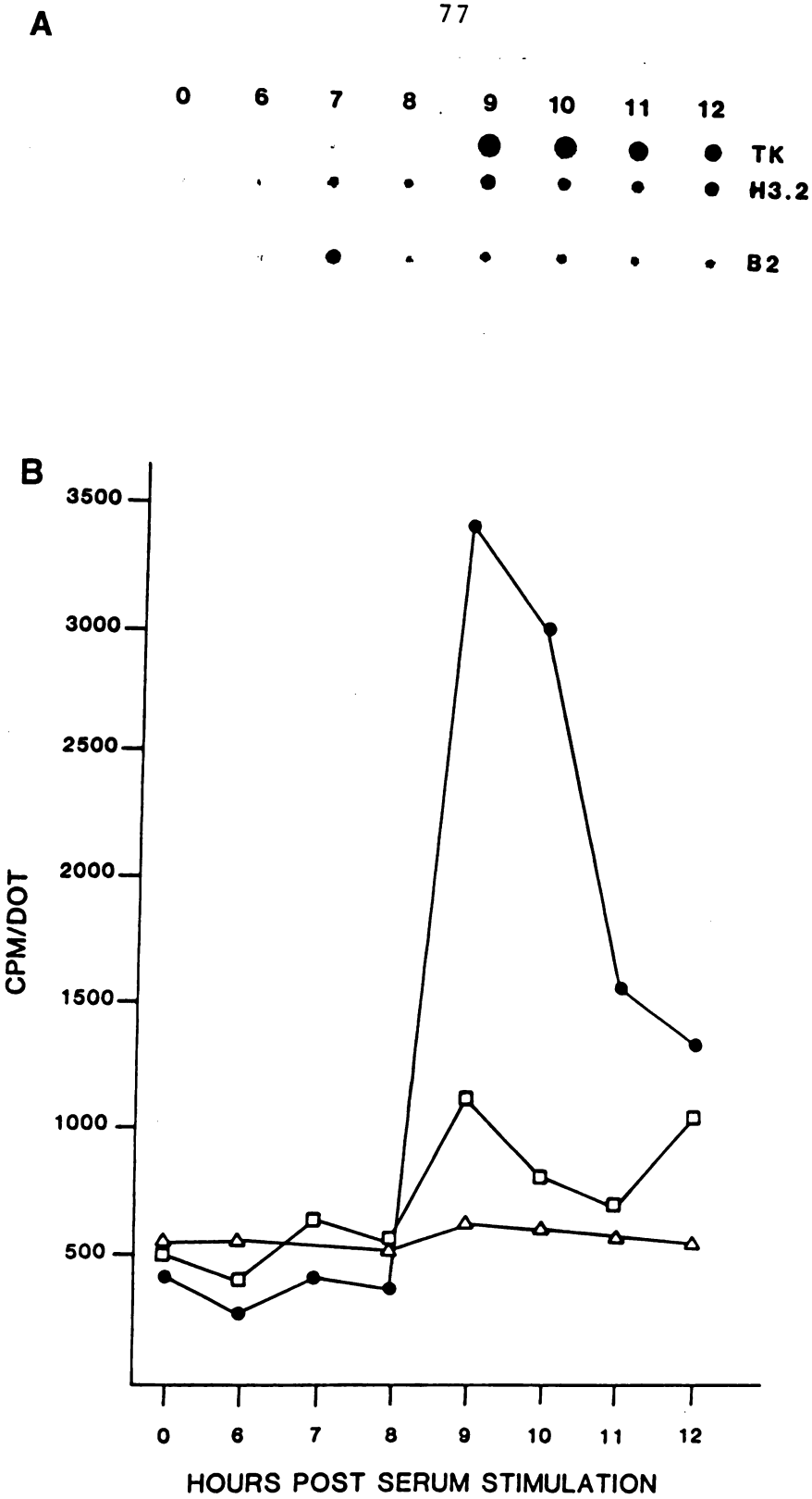

A
0
6
7
8
9
10
11
12
TK
H3.2
B2
B
3500
3000
2500
2000
1500
1000
500
CPM/DOT
0
6
7
8
9
10
11
12
HOURS POST SERUM STIMULATION

FIGURE 5

Figure 6. Nuclear transcription assays performed at one hour intervals spanning the G_1/S boundary in SV40 infected CV-1 cells. Nuclei were prepared at the times indicated following viral infection, and used for transcription assays. 2.5×10^6 cpm of labeled RNA were added to each hybridization.
(A) Autoradiograms showing the results of hybridization. (B) Graphical analysis of the results in (A). TK (●), H3.2 (□), β-2 microglobulin (△) and SV40 (△---△).

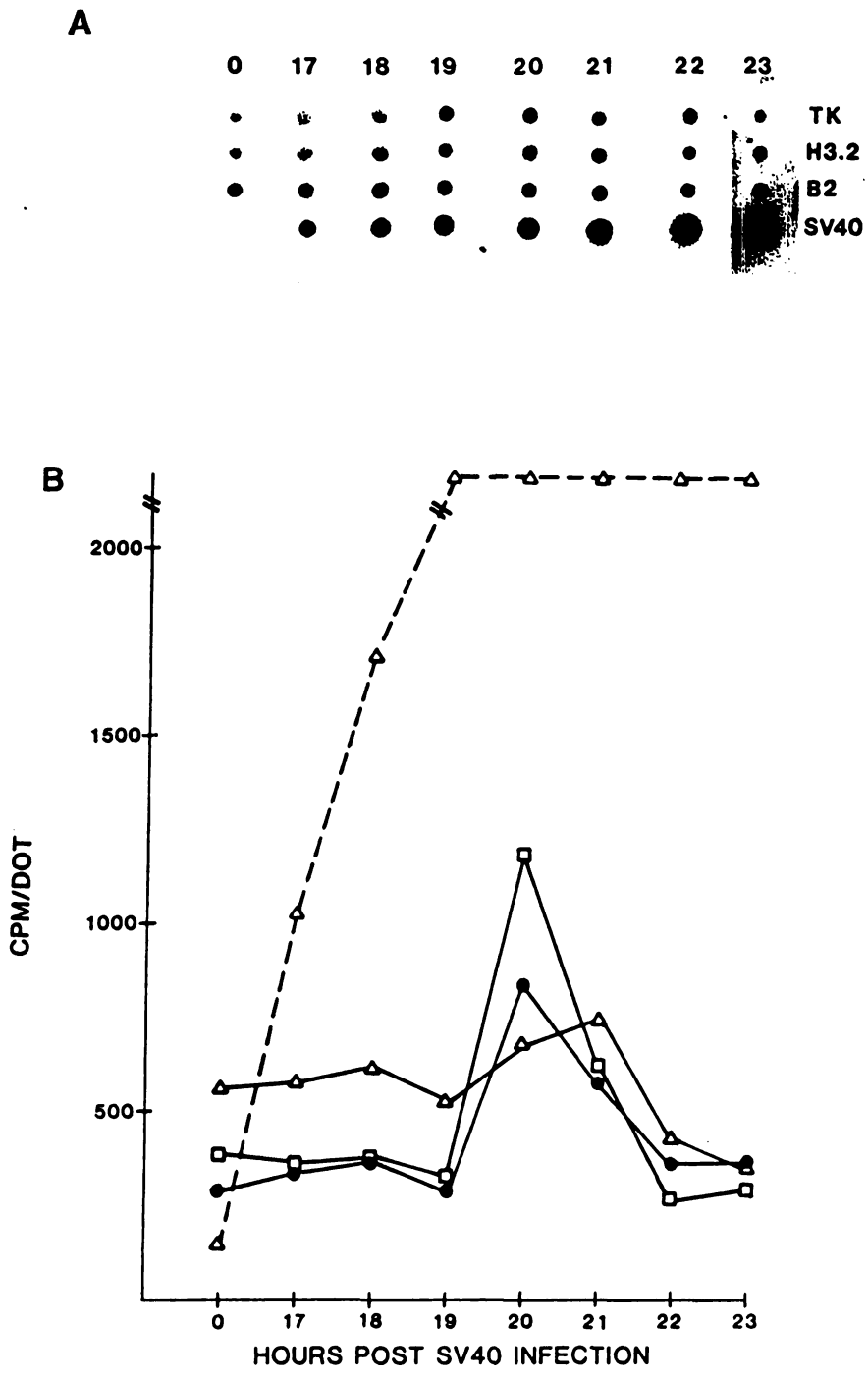

A
0
17
18
19
20
21
22
23
TK
H3.2
B2
SV40
B
2000
1500
1000
500
CPM/DOT
0
17
18
19
20
21
22
23
HOURS POST SV40 INFECTION

FIGURE 6

relatively constant throughout the time course, transcription of both TK and histone H3.2 showed a small increase at 20 hours. The magnitude of the increase in TK transcription was significantly less than in serum stimulated cells, however, peaking at less than 2-fold the resting cell level. This experiment was repeated, and no significant increase in TK transcription was seen (Table I).

One explanation for our inability to detect transcriptional induction of TK following SV40 infection is that we missed the correct time point, and that induction was occurring prior to the 17 hour time point. In order to examine this possibility, we measured the levels of TK mRNA and transcription at one hour intervals from 12-18 hours post-infection. The results of these experiments, shown in Figure 7 and Table I, indicate that although TK mRNA starts to accumulate by 14 hours, no transcriptional induction is detected at any of the time points tested. These results are in contrast to those measuring mRNA levels (24 and Figure 2), where TK mRNA accumulates to a higher level in virally infected than in serum stimulated cells, and suggest that SV40 is utilizing an alternate mechanism for inducing high levels of TK mRNA.

Figure 7. Measurements of TK polyA$^+$ RNA levels and transcription rates in SV40 infected cells. Resting CV-1 cells were infected with SV40 (MOI=15) at t = 0, and at the times indicated plates were harvested for either the preparation of polyA$^+$ RNA or nuclear transcription assays.

(a) Northern gel analysis of polyA$^+$ RNA hybridized to TK and β-2 microglobulin probes.

(b) Hybridization of ^{32}P-RNA synthesized in isolated nuclei to dot blots containing 5 ug of TK, β2 and Sp6 cRNA and 5 ug of PJY1 (cloned SV40) DNA.

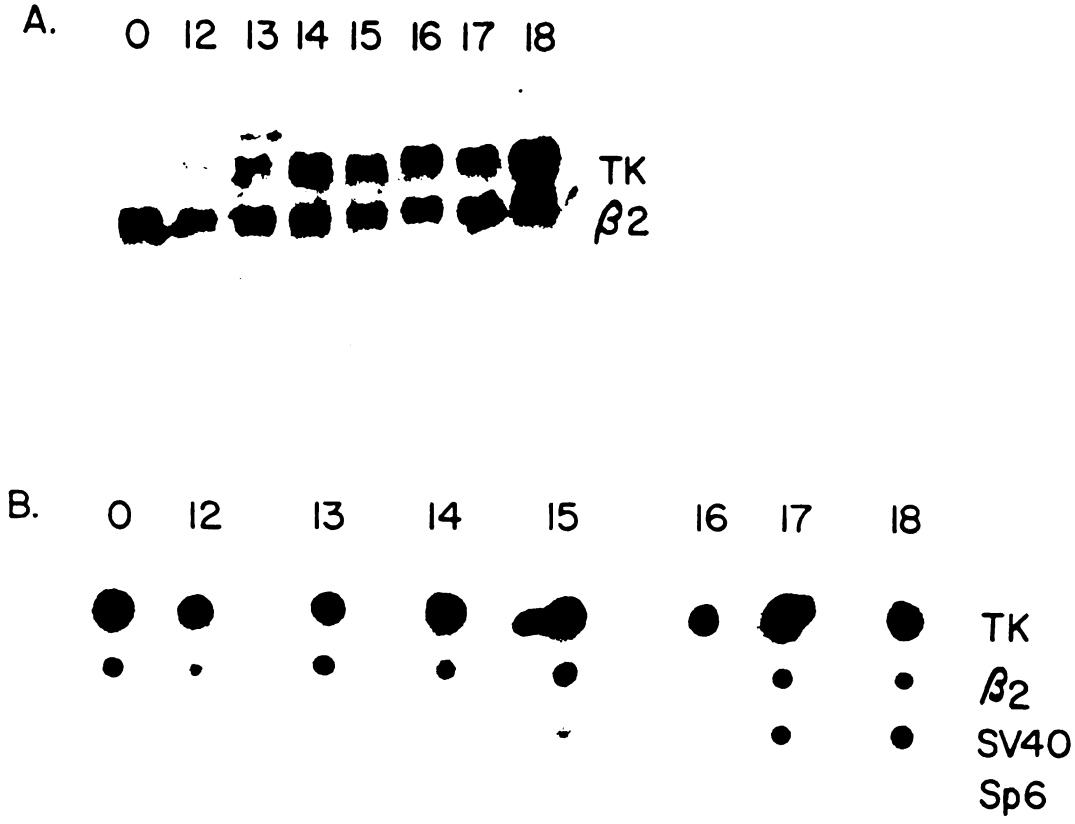


FIGURE 7

TABLE 1

[a]Transcription assays were performed as described in Materials and Methods. In order to quantitate the level of TK transcription, dots containing ^{32}P-labeled RNA hybridized to either TK or β-2 microglobulin cRNA were cut out and counted in a scintillation counter. The ratio of (cpm hybridized to TK)/(cpm hybridized to β-2) was then calculated for each time point, and the ratio at time 0 was defined as 1.0. Levels of TK transcription at the other time points within one experiment are expressed relative to this ratio.

TABLE 1. Levels of TK gene transcription in serum stimulated and SV40 infected CV1 cells[a]

Serum Experiment				SV40 Experiment				
Hours post-serum stimulation	1	2	3	Hours post-SV40 infection	1	2	3	4
0	1.0	1.0	1.0	0	1.0	1.0	1.0	1.0
6	0.7	1.2	2.7	12			0.9	0.8
7	0.5	3.9	3.7	13			0.8	0.4
8	0.9	6.7	5.2	14			0.3	0.8
9	6.7	4.6	2.7	15			0.5	0.6
10	5.9	3.9	4.7	16			0.5	0.7
11	3.4	4.3	4.2	17	1.0	2.0	0.5	0.9
12	3.1	1.4	2.7	18	1.0	1.4	0.7	0.8
				19	1.1	0.3		
				20	1.4	1.6		
				21	1.1	1.4		
				22	1.0	1.5		
				23	1.3	0.4		

TK induction in cell lines transfected with human TK cDNA contructs. In order to identify the DNA sequences required in cis for the regulation of TK gene expression, we have isolated several different TK cDNA contructs and studied their expression after transfection into Rat-3 TK^- cells. These plasmids, which are diagrammed in Figure 1, are as follows: pHuTKcDNA7 is the original TK cDNA clone that we isolated from the Okayama and Berg library (18), and contains the SV40 early promoter, an SV40 splice donor and acceptor, the SV40 late polyadenylation signal and virtually the entire HuTK cDNA 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 SmaI 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.

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 poly A^+ RNA at 0, 12

and 24 hours after serum stimulation and quantitating this RNA by Northern blot analysis as described previously (24). The results of these experiments are shown in Figure 8. In Figure 8A three cell lines containing pHuTKcDNA7 were examined, with cell lines 1C and 2A being derived from single colonies and cell line m being derived from a pool of colonies. In Figure 8B the results are shown from cell lines containing p5'TKcDNA, where D3 and A1 are derived from single colonies and m from pooled colonies. All of these cell lines show increased levels of TK mRNA by 12 hours 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 II. These results are in agreement with the mRNA data, with peak levels of TK enzyme activity being seen at either 12 or 24 hours following serum stimulation. Thus, both pHuTKcDNA and p5'TKcDNA are cell cycle regulated after serum treatment of transfected cell lines, indicating that sequences within the body of the cDNA are sufficient to confer S phase specific expression upon these hybrid genes. An alternative explanation would be

Figure 8. Northern blot analysis of TK polyA$^+$ 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 hours following serum addition. RNA was analyzed by Northern blot analysis, and probed with a HuTK cDNA probe. (A) Rat-3 TK$^-$ negative control and cell lines containing pHuTKcDNA7. Cell lines 1C and 2A were derived from isolated colonies, and m 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 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

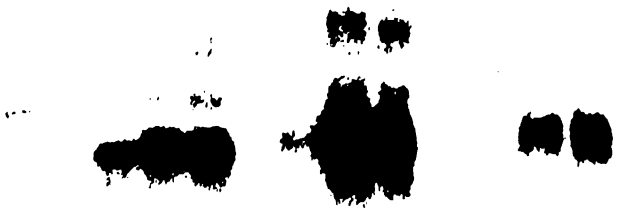


FIGURE 8

TABLE II: TK enzyme assays in cell lines transfected with HuTK cDNA constructs[a]

Hours post serum stimulation	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	.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]Table II. TK enzyme assays from cell lines transfected with TK cDNA constructs. The cell lines described in the legend to figure 6 were growth arrested and serum stimulated as described in Materials and Methods. At 0,12 and 24 hours after serum addition, TK extracts were prepared and enzyme assays performed as described previously (24). Data is presented as TK units x 10^{-4}, where one unit is defined as the amount of enzyme required to convert one nmole of dT to dTMP per microgram of protein per minute of reaction at 37°C.

that the SV40 early promoter is itself regulated in an S-phase specific manner. Although we have not tested this hypothesis directly, experiments in the laboratory of Dr. N. Heintz have indicated that this promoter is not activated during the transition of cells from G_1 into S phase (N. Heintz, personal communication).

DISCUSSION

The results presented here suggest that the thymidine kinase gene is regulated at both transcriptional and post-transcriptional levels in serum stimulated cells. Transcription of the gene is low in resting cells, and remains low during G_1 after serum stimulation. As the cells enter S phase, which is at approximately 8-9 hours following serum stimulation, there is a 6-7-fold increase in the rate of TK gene transcription. Transcription drops to 3-fold the level in resting cells by 12 hours following serum addition, and continues to decrease at 24 hours. These results are similar to those reported for the DHFR gene in mouse cells, where there is a 7 fold increase in transcription at the G_1/S boundary (2). In contrast, no sharp increase in TK transcription was detected in SV40 infected CV-1 cells in these experiments, although the TK mRNA accumulates to higher levels in virally infected than

in serum stimulated cells. Thus the mechanisms of induction by these two mitogenic agents appears to differ in some or all aspects. This was also suggested by earlier experiments, where it was shown that induction of DHFR by polyoma virus was not sensitive to the same inhibitors as was induction by serum (10). The molecular basis of these differences remain to be determined.

There are several reasons why we believe that the increased transcription seen in serum stimulated CV-1 cells cannot completely account for the increase in TK mRNA levels. First, the transcriptional induction is both shorter and of lesser magnitude than the change in mRNA levels. The transcriptional induction peaks at approximately 6-fold the level found in resting cells, and persists for only about 3 hours, while the mRNA levels increase 10-20-fold, and remain high for at least 12 hours. Second, the relative levels of mRNA induction in serum stimulated and SV40 infected cells do not coincide with the levels of transcriptional induction. Finally, we have studied TK regulation in cell lines transfected with hybrid cDNA clones that utilize either the human TK promoter or the SV40 early promoter, and have shown that sequences within the body of the cDNA are sufficient to confer cell cycle regulation upon these constructs. These

results are in agreement with those obtained in other labs with both the chicken (16) and hamster (13) chromosomal TK genes, where it was shown that hybrid genes expressed from the Herpes TK promoter showed cell cycle or growth dependent expression. We have considered two possible explanations for the ability of the various cDNA clones to be regulated in an S phase specific manner. The first, and in our opinion most likely, is that TK RNA metabolism differs somehow during S phase so that it accumulates to high levels. Since p5'TKcDNA contains no introns, it seems unlikely that the difference is in nuclear RNA processing, but may be in transport from the nucleus, or in nuclear or cytoplasmic stability. A second possible explanation is that sequences within the TK cDNA confer cell cycle regulation upon an adjacent promoter, whether it be the genomic TK promoter, the herpes TK promoter or the SV40 early promoter. This seems unlikely, however, since even in the case of CV-1 cells with the homologous promoter the level of increased transcription cannot account for the increase in the level of mRNA in all cases. Given these three lines of evidence, it seems likely that both transcriptional and post-transcriptional regulatory mechanisms are operating to increase expression of the TK gene during S phase.

We cannot unambiguously determine from our results whether or not the cDNA construct containing the genomic HuTK promoter is more highly induced during S phase than the one containing the SV40 promoter, which we might expect if transcriptional control plays an important role in the regulation of this gene. Although the pooled colonies containing p5'TKcDNA always show higher levels of induction than the pools containing pHuTKcDNA7, this is not the case for individual cloned cell lines. Since we have not characterized the integrated plasmids in these cells lines, the variations we see may be due to both the number and location of integration sites. In order to quantitatively compare the levels of induction in transfected cell lines, we will either have to use transient expression assays or carefully compare the patterns of integration in different cell lines.

Our results, taken together with the results from other labs, suggest that in general S phase specific genes may exhibit multiple levels of regulation. As mentioned previously, both transcriptional and post transcriptional regulation of histone gene expression has been demonstrated for some time (7,21,22,23). In the case of other S phase regulated genes such as DHFR, TS and TK the existence of transcriptional regulation has been in

question, although post transcriptional control has been demonstrated. In part the difficulty in demonstrating transcriptional control has been that the genes are transcribed at low levels, so the levels of transcription are difficult to measure. Because of this, several investigators used cell lines containing amplified DHFR or TS genes to measure transcription rates. In this report we have been able to measure TK transcription rates in cell lines containing single copies of the gene by binding single stranded cRNA to filters to hybridize to RNA transcribed in isolated nuclei. A similar approach was used by Groudine and Casimir (5) to study regulation of the chicken TK gene. Even with sufficiently sensitive assays, several investigators have failed to detect transcriptional regulation of TK and DHFR gene expression. Our results, and those of Farnham and Schimke (2), suggest that the precise timing of the assay is critical, since the burst of increased transcription is quite transient.

The situation has been further complicated by the fact that hybrid TK genes utilizing heterologous promoters are cell cycle regulated in several systems (13,16), and this has led investigators to conclude that transcriptional control is not occurring. In fact, the presence of dual levels of control is now established for

many of the S phase regulated genes that have been examined. A complete understanding of the complex pattern of regulation of these genes, and of the relative importance of the different regulatory mechanisms, awaits a more detailed analysis. By utilizing the approaches outlined here, that is the construction of hybrid or mutant genes and the study of their transcription and/or RNA metabolism in cells, we should be able to progress quickly towards that goal.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the work of Moriko Ito for the TKcDNA transfection experiments. We would also like to thank Ron Patterson and Karen Friderici for helpful comments on this manuscript.

This work was supported by Public Health Service Grant CA37144. SEC is a recipient of a Junior Faculty Research Award from the American Cancer Society.

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CHAPTER III

(ARTICLE)

Rhythmic Expression of the Cytoplasmic Thymidine Kinase Gene is Regulated by Multiple Levels of Cell Cycle Dependent Controls.

by

Christine J. Stewart and Susan E. Conrad

To be submitted to Molecular and Cellular Biology

ABSTRACT

The regulation of the cytoplasmic thymidine kinase (TK) gene occurs in a cell cycle-dependent manner. TK has been shown to be mitogenically induced by serum growth factors and by infection with papovaviruses such as SV40. We have studied the stability of TK messenger RNA by the addition of a transcriptional inhibitor in the G_0/G_1, S, SV40-S, and G_2 phases of the cell cycle. By addition of Actinomycin D to synchronously growing cell populations, we were able to follow the decay of TK mRNA in these various stages. TK mRNA was relatively stable in all phases: 8-9 hours in G_0/G_1, 20 hours in S, 32 hours in SV40-S and 9-10 hours in G_2. Removal of poly-adenylation recognition sequences destabilized the message ($t < 1$ hour). However, removal of other 3' untranslated sequences had no effect on the rate of decay. TK mRNA and enzyme levels were studied into and beyond the G_2 phase of the first cell cycle following serum stimulation. Withdrawal of serum in G_2 decreased TK enzyme activity, but TK mRNA remained relatively unaffected. Addition of

hydroxyurea in early S phase did not affect the initial induction of TK enzyme or mRNA. However, termination of both TK mRNA steady state levels and enzyme activity kept rising until extremely high levels were seen at late times post serum stimulation, as compared to controls. These results suggest multiple levels of regulation for the TK gene in various phases of the cell cycle.

INTRODUCTION

The activity of the cytoplasmic thymidine kinase enzyme (EC 2.7.1.75) has been shown in a variety of systems to be dependent on the specific growth state of the eukaryotic cell. In rapidly growing, continuously cycling cells, thymidine kinase (TK) activity is high compared to the levels found in stationary phase or quiescent cells (9). Characteristically, fetal and neoplastic tissues show much higher TK activities than the cognate adult tissue, and adult tissues which proliferate have the highest activities of adult tissue (18, 29). In tissue culture cells synchronized via serum deprivation or drug blocking with butyrate, colcemid, amethopterin or aphidicolin, TK activity is low in G_0/G_1 phase cells. Following serum refreshment or drug removal, TK activity increases dramatically from levels seen in G_1 phase by mid-to late-S phase (1, 6, 13, 44, 45, 46, 49), and this induction commences with the onset of DNA synthesis (16, 44). In contrast, the genetically distinct mitochondrial form of the enzyme shows no variation in activity throughout the cell cycle (1, 23). By treating tissue culture cells with RNA or protein synthesis inhibitors, the increases in cytoplasmic TK enzyme activities were shown to be dependent upon both RNA and protein synthesis

(16, 34). Addition of DNA synthesis inhibitors does not affect the induction of TK enzyme activity, but a certain proportion of the genome must be replicated for efficient termination of TK activity (3, 16, 19).

Another method of induction of TK activity is by infection with papovaviruses such as simian virus 40 (SV40) (20, 21, 22). As in normal cell cycle progression, TK induction in infected cells coincides with the onset of DNA synthesis. Use of various temperature sensitive mutants of SV40 has shown that the A gene product, or large T-antigen, is responsible for the induction of TK (38). Large T-antigen is also necessary for efficient lytic viral replication and is required for the initiation and maintenance of transformation (31, 37, 47).

The induction of TK by serum growth factors and by SV40 infection appears to occur at multiple levels. We have previously demonstrated (44) that increases in TK enzyme activity appear to be paralleled, at least temporally, by an increase in steady state levels of TK mRNA in both systems. By using nuclear run-on transcription assays, we have shown that there is a 6- to 7-fold increase in TK transcription in serum stimulated simian CV-1 cells at the G_1/S phase border (43). A smaller 3-3.5-fold increase in transcription has also been found at the beginning of S phase in SV40 infected CV-1

cells (42). Other studies have shown that the chicken, hamster and mouse TK genes are regulated at a post-transcriptional level (12,13,26,27). TK regulation was found to be independent of the promoter used to transcribe the gene. Finally, induction of TK enzyme activity depends upon protein synthesis and may also be dependent upon the constant presence of serum growth factors (16, 34).

Multiple levels of regulation also have been found for other S phase specific genes. The replication-dependent histone genes are regulated by both transcriptional and post-transcriptional mechanisms (2, 5, 7, 8, 29, 40). The dihydrofolate reductase (DHFR) gene, another DNA synthesis enzyme, shows a pattern of transcriptional induction similar to the TK gene (10. 17). Post-transcriptional control of DHFR has also been reported (28). Thymidylate synthetase is another DNA synthesis enzyme where both transcriptional and post-transcriptional control has been reported (15, 35).

The purpose of our present study is to more thoroughly investigate the nature of post-transcriptional regulation of TK expression. We report a very stable TK message with approximate half-lives of 8-9 hours in the G_0/G_1 phase, 20 hours in S phase, and 9-10 hours in the G_2 phases of the cell cycle, as well as an extremely long half-life of 32 hours in SV40 infected cells. The

sequences responsible for this stability have been localized to the 3' end of the mRNA, and the regulation of TK termination as cells progress through the cell cycle also has been examined.

MATERIALS AND METHODS

Cell Culture

Simian CV-1 (African Green Monkey Kidney) cells were grown to confluence in 1X Dulbecco's Modified Eagle's Medium containing 5% fetal calf serum (FCS) and 5% calf serum (CS). For synchronization, the medium was removed after the cells reached confluence and was replaced by medium containing 0.1% FCS + CS. To obtain synchrony, cells were allowed to incubate for 48 hours to cause a uniform arrest in G_0/G_1. For viral infections, SV40 was added at this time at a multiplicity of infection (MOI) of 15. Infections were done by removing medium, infecting for 1 hour with a concentrated viral stock, and then replacing the original 0.1% serum-containing medium. For serum stimulations, new medium containing 5% FCS + 5% CS was added. At various times after induction with either SV40 or serum, plates were harvested for RNA, protein or DNA synthesis analysis. Rat3 TK^- cells (43) were grown in 1X DME with 10% CS. Serum stimulations were performed as described for CV-1 cells.

DNA pulse labelling

To obtain a profile of the cell cycle, the specific activity of DNA was determined in a separate experiment by pulse labelling one 100 mm plate of cells per time point with 1 uCi of [^{3}H] deoxythymidine (61 Ci/ mmole) and 4 x 10^{-7} M uridine for 1 hour. TCA precipitates were prepared as described (44) and the number of incorporated counts was determined by counting for ^{3}[H] in a scintillation counter. One 100 ul aliquot of cells in 1x PBS was counted on a hemacytometer. Specific activities were calculated as counts incorporated/number of cells.

Preparation of total RNA

Total RNA was prepared from tissue culture cells as follows. Cells were washed twice with phosphate buffered saline (PBS) without calcium and magnesium. Lysis buffer (100 mM Tris 7.5, 12 mM EDTA (Ethylenedinitrilo)-Tetraacetic Acid, 150 mM NaCl, 1% Sodium Dodecyl Sulfate) containing 200 ug/ml of proteinase K was added to each sample in a volume of 2 mls/100mm plate. DNA in the cell lysate was sheared by passage through a 22-gauge needle, transferred to a tube, and fresh proteinase K was added to 100 ug/ml. This solution was incubated at 37^{o}C for 45 minutes, and then extracted twice with 50:50 v/v Phenol:Chloroform. Sodium Acetate was then added to 0.3M and the solution was ethanol precipitated. Samples were spun in a Sorvall RC-2 centrifuge at 10,000 RPM for 20

minutes, the ethanol poured off and pellets allowed to air dry. Pellets were resuspended in 400 ul of RNase-free 10 mM TRIS (7.5), 1 mM EDTA ({Ethylenedinitrilo}-Tetraacetic Acid) (1X TE) and transferred to eppendorf tubes. A solution containing 4 ul of 1M $MgCl_2$, 5 ul of vanadyl ribonucleoside complex (VRC) and 1 ul of a 1 mg/ml solution of Pancreatic DNase was added, and tubes were incubated at 37° C for 30 minutes. Next, 16 ul of 0.5 M EDTA and 20 ul of 20% Sodium Acetate was added, this mixture was extracted twice with 50:50 v/v Phenol:Chloroform and then ethanol precipitated at -70°C. RNA was next pelleted in a microcentrifuge at 4°C for 25 minutes and pellets were dried in a vacuum pump dessicator. Pellets were resuspended in 150 ul of 20% Sodium Acetate and spun for 10 minutes in a microcentrifuge at 4°C. Supernatants were discarded and the remaining pellets were resuspended in 100 ul of 1X TE and then ethanol precipitated after addition of 10 ul 20% Sodium Acetate. For determination of optical density, samples were spun down at 4°C in a microcentrifuge for 25 minutes, drained, dryed and resuspended in 200 ul of 1X TE. One-fifth of each sample was diluted into 1 ml of 1X TE and the optical density was monitored at 260 nm.

Northern Blotting and TK Enzyme Assays

Northern blotting and TK enzyme extraction and TK assays were performed as described previously (44). All

blots were probed with a ^{32}P-labelled internal SmaI-Bam HI TK fragment from p5'TKcDNA (Fig. 1).

Construction of Tk 3' end deletion mutants

Plasmid p5'TkcDNA is a human cDNA clone capable of expressing TK after transfection into eukaryotic cells and was constructed as described previously (43). The plasmid contains the 5' genomic promoter region attached to the TK cDNA and 3' untranslated region in a vector containing the SV40 polyadenylation signal. Figure 1 shows this plasmid, as well as two gross deletions extending from the SV40 polyAdenylation recognition site (leaving this site intact) to a Hind III site (p5'TKcDNA^H) and to a Bam H1 site (p5'TKcDNA^B), both of which are contained in the rather long 662bp 3' untranslated region of the TK mRNA. Also shown is a construction which deletes the SV40 polyAdenylation recognition signal and 60 base pairs of the 3' untranslated region up to the Bam H1 site (p5'TKcDNA^A+). Transfections of these constructs into Rat3 cells were performed as described (43).

Nucleic Acid Hybridizations

Hybridizations to RNA filters and post-hybridization washes were performed as previously described (44). The final wash step with 0.1XSSC-0.1%SDS at 68^{o}C was deleted.

Figure 1 Functional TK cDNAs. Each TK cDNA construct contains the genomic TK promoter region cloned into an EcoR1 (E) to Sma1 (S) site. The body of our TK cDNA is represented by the large open box. The 662 base pair 3' untranslated region is represented by a line and contains two restriction sites used for a gross mutational analysis, Hind III (H) and Bam H1 (B). The smaller open box contains the SV40 polyadenylation recognition sequence. p5'TKcDNA is the full length mini-gene. p5'TKcDNA^B deletes from the poly-A site up to the Bam H1 site, leaving the poly-A site intact. p5'TKcDNA^H deletes up to the Hind III site and also leaves an intact poly-A sequence. p5'TKcDNA^A+ deletes both poly-A and 60 base pairs up to the Bam H1 site.

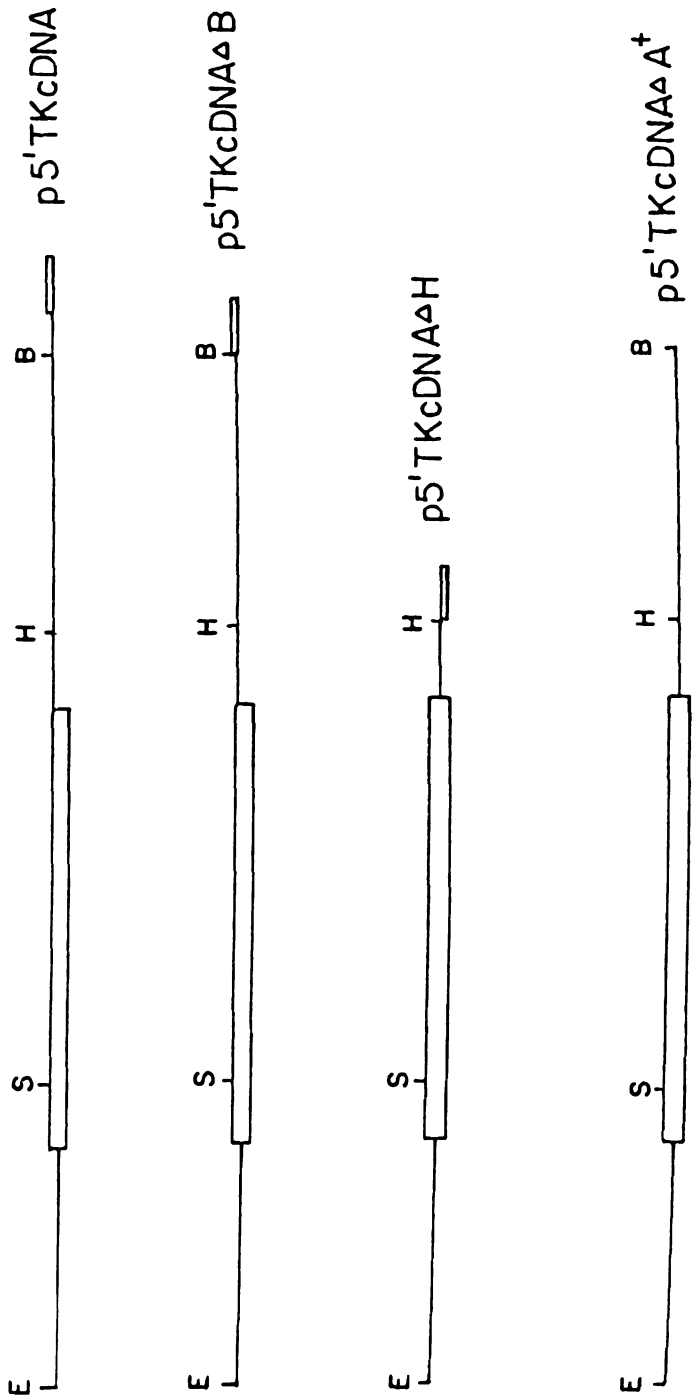

p5'TKcDNA
B
H
S
E
p5'TKcDNAΔB
B
H
S
E
p5'TKcDNAΔH
H
S
E
p5'TKcDNAΔA+
B
H
S
E

FIGURE 1

RESULTS

Determination of TK mRNA half-life in CV-1 cells.

Previously, we have reported on the induction of TK mRNA and enzyme activities in CV-1 cells following either serum-induction or SV40-infection. A 10-20 fold induction was seen in both systems (for both RNA steady state levels and enzyme activity) which peaked by 12-24 hours in serum induced cells and by 24-36 hours in SV40 infected cells. I have also characterized the mechanism of this induction as being partially at the transcriptional level in both systems. At the G_1/S phase border a 6-7-fold transcriptional induction occurs at 8-9 hours post-serum stimulation, and a 3-3.5-fold induction occurs at 14-16 hours post SV40 infection (42). Since the levels of transcriptional induction did not seem to account for the high level of message produced, I now have characterized the extent of the stability of the messenger RNA during various phases of the cell cycle as this was a likely candidate for post transcriptional control. The results of this experiment are shown in figure 2. Stability of the TK message was determined by the addition of 5 ug/ml of Actinomycin D to CV-1 cells in either the arrested (G_0/G_1) state, at 10-12 hours post-serum-induction or at 15-22 hours post-SV40-infection when cells are in S phase. The decay of the TK message was then followed by preparing total RNA at various time points

following addition of drug and analyzing constant amounts of total RNA from each time point on denaturing agarose-formaldehyde gels. Each time course was run twice, and the relative values of all these experiments can be seen in table 1. Samples of our autoradiograms are shown in figure 2. All of our autoradiograms were traced by densitometer and approximate half-lives determined by linear regression analysis. Plots of the decay curves are shown in figure 3. For all of these phases, the half-lives are rather long. In arrested G_0/G_1 phase cells (fig. 2a), TK message half-life is 8-9 hours, while in serum induced S phase cells (fig. 2b), the half-life extends to 20 hours. TK in the SV40-infected cells (fig. 2c) shows an even longer half life of 32 hours. A human c-myc probe was used as a positive control for the inhibition of transcription, since it has a half-life of 30 minutes. c-myc was not detected by 1 hour following addition of drug in any of these studies (data not shown).

Characterization of G_2 Phase in Serum Induced CV-1 cells

In order to characterize the stability of TK mRNA during the G_2 phase of our CV-1 cells, the timing of the S and G_2 phases of the cell cycle in our CV-1 cells needed to be more finely characterized. Figure 4 contains the results of pulse-labelling experiments using [^{3}H]thymidine uptake as an indication of the timing of DNA synthesis.

Figure 2 TK mRNA Stability in CV-1 Cells. The rate of decay of TK message was determined by the addition of 5 ug/ml Actinomycin D to CV-1 cells which were either: (a) non-induced or in G_1 phase, (b) Serum-stimulated and treated with the drug at 12 hours or S phase, (c) infected with SV40 (MOI = 15) with drug added at 15 hours, and (d) serum-stimulated with drug added at 20 hours or G_2 phase. Total RNA was prepared from cells harvested after the number of hours indicated on the abcissa, and constant amounts (30 ug for part a, 10 ug for parts b, c and d) were run in each lane on denaturing agarose-formaldehyde gels. The figure shows autoradiograms of gels blotted to nitrocellulose and probed with an internal SmaI - Bam HI TK fragment.

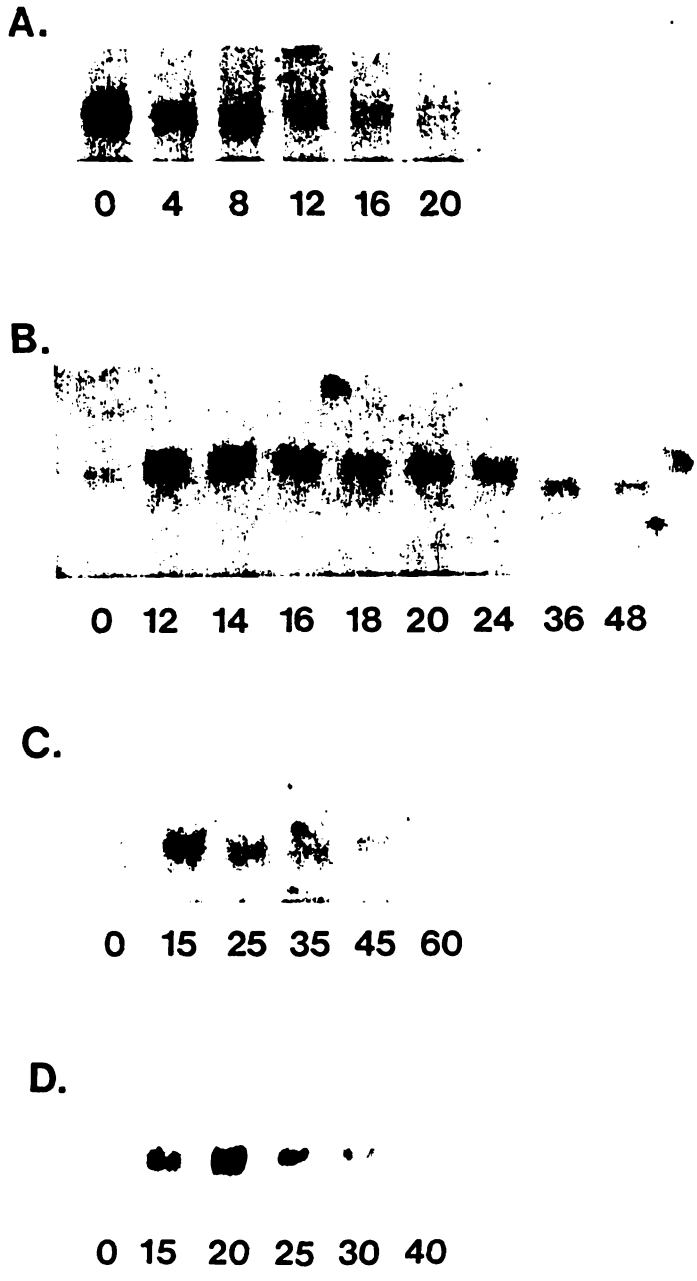

A.
0 4 8 12 16 20
B.
0 12 14 16 18 20 24 36 48
C.
0 15 25 35 45 60
D.
0 15 20 25 30 40

FIGURE 2

TABLE 1

G0/G1			S-Phase			SV40-S			G2		
HRS	Exp 1	Exp2	HRS	Exp 1	Exp2	HRS	Exp1	Exp2	HRS	Exp1	Exp2
0	1.0*	1.0*	0	1.0	1.0	0	1.0	1.0	0	1.0	1.0
2	0.74		12	4.8*	6.3*	15	7.9*		15		14.8
4		0.77	14	4.5		22		25.8*	20	8.8*	28.0*
6	0.62		16	3.8		25	6.5		22	6.4	
8		0.71	18	3.9	3.9	28		17.2	24	6.2	
9	0.57		20	4.1		35	6.3	13.3	25		14.0
12	0.51	0.64	24	3.6	2.1	45	3.3	11.4	26	5.3	
15	0.34		35		1.6	60	2.3		28	4.9	
16		0.49	36	2.1					30	4.7	10.0
20		0.31	48	2.0					32	1.7	
									34	1.9	
									40		1.6
									44	1.2	
									50	0.9	

Table 1. Relative values representing the amount of TK RNA seen at various times post addition of Actinomycin D. Asterisks indicate the time point in each experiment where the drug was added.

Figure 3 Quantitation of the results of the densitometer scan of the autoradiograms in figure 2. The area under the curves was calculated, and plotted with respect to time. The line of best fit and the half-lives were calculated by linear regression analysis (IBM SigmaPlot).

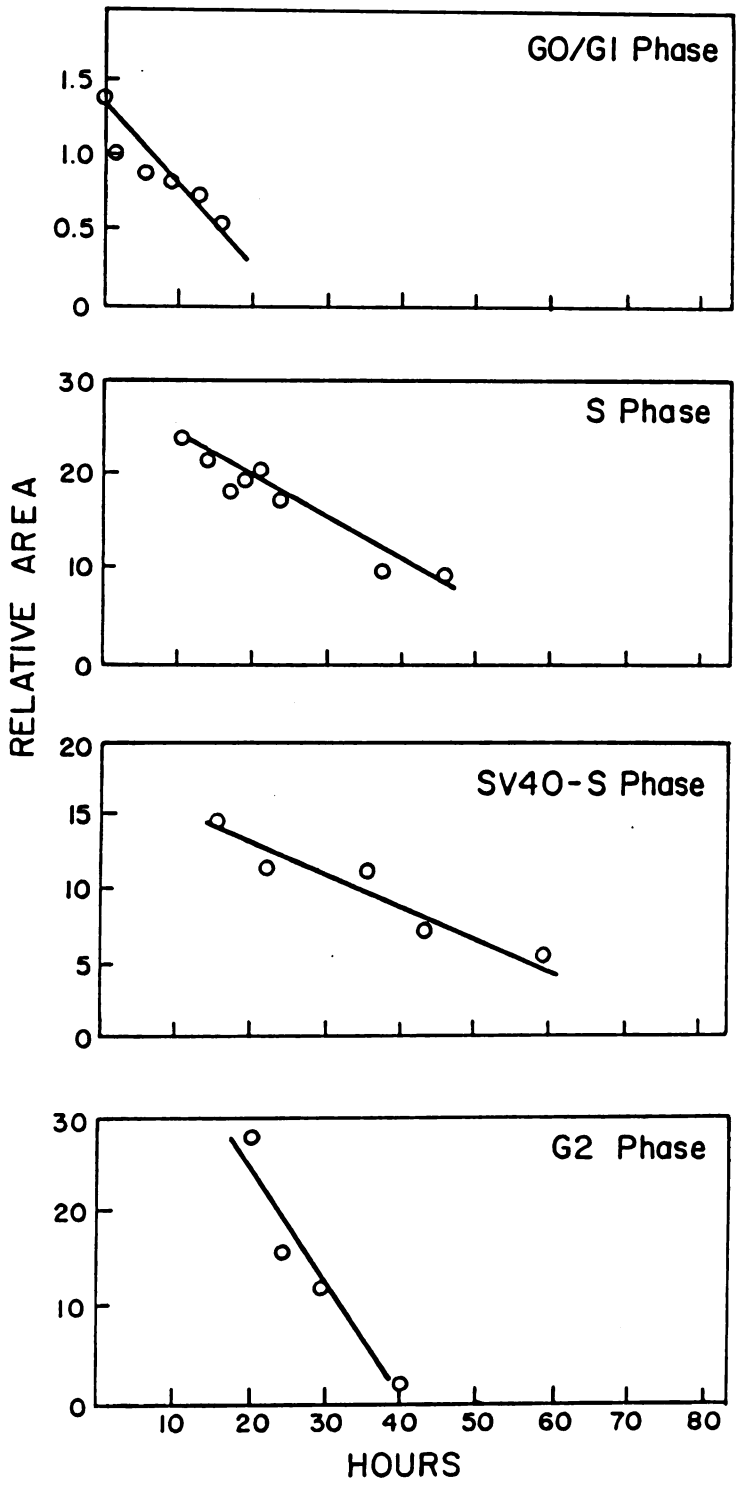

G0/G1 Phase
1.5
1.0
0.5
0
S Phase
30
20
10
0
RELATIVE AREA
SV40-S Phase
20
15
10
5
0
G2 Phase
30
20
10
0
10
20
30
40
50
60
70
80
HOURS

Figure 3

Figure 4 Measurement of DNA synthesis in serum-stimulated CV-1 cells. CV-1 cells were arrested and then serum-stimulated by addition of fresh 1X DME + 5% calf serum and 5% fetal calf serum. At the indicated times (hours indicated on the abcissa), cells were labelled with [^{3}H] Thymidine for one hour, and the counts per minute incorporated per cell were determined by trichloroacetic acid precipitation of a given number of cells.

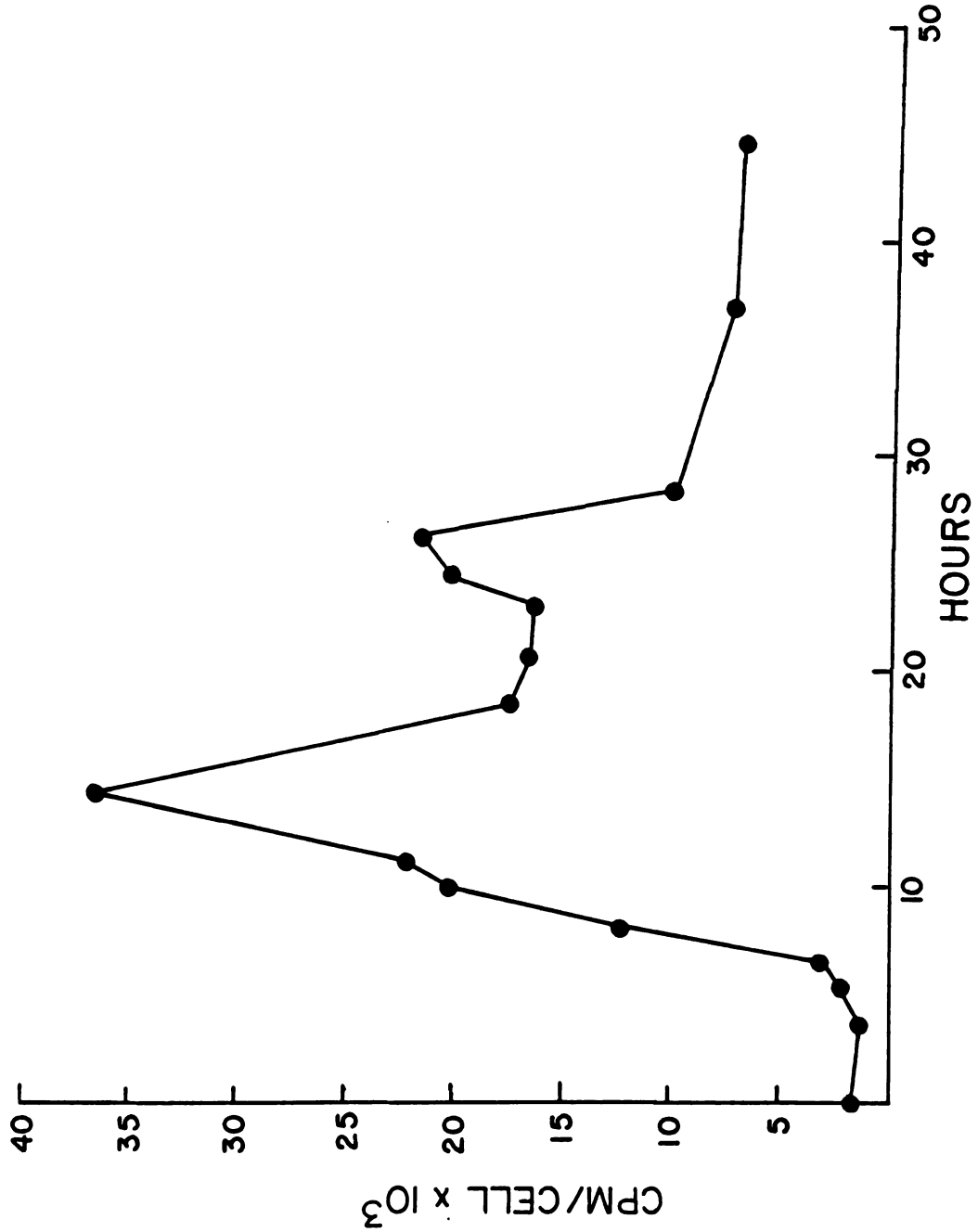


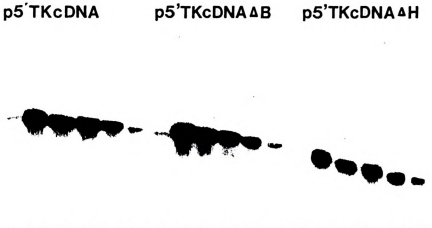
FIGURE 4

Previously, the timing of the G_1/S phase interface had already been characterized (43), and was seen to occur at 8-9 hours post serum stimulation. It can be seen that DNA synthesis (S phase) peaks by 16 hours and then drops off until about 20 hours. The drop off in DNA synthesis signals the beginning of G_2 phase, or the second gap period known to occur before the onset of M phase, or mitosis (24). G_2 in these cells appears to be approximately 4 hours in duration. A second smaller peak of DNA synthesis can also be seen, and this may indicate that even though our starting cultures were confluent, at least some of these cells are capable of mitosis, a very short G_1 phase, and another round of DNA replication. It cannot be ruled out, however, that this secondary peak may also indicate that not all of the cells are induced into S phase at a similar time. Since in the majority of the population the G_2 phase begins at about 19-20 hours post serum stimulation, Actinomycin D was added to cells at 20 hours to determine if the stability of TK mRNA was altered during this time. Figure 2d shows that TK message has a shorter half-life of 9-10 hours in G_2 phase than that of TK in S phase cells.

The human (4), hamster (26), chicken (32), and mouse (13) TK mRNAs all contain relatively long 3′ untranslated regions. This region is 662 base pairs in length in the human system. Due to the convenient placement of several

Figure 5 TK 3'Deletion Mutation Analysis. Time courses involving the mutants shown in figure 1 were performed after transfection and selection for stable transformants as described (43). Cells were serum stimulated at t = 0. Actinomycin D was added to cell lines at t = 11 hours, and total RNA was made at the timepoints indicated (hours). RNA was run on denaturing agarose-formaldehyde gels and blotted to nitrocellulose as described (44). (A) Rate of decay of p5'TKcDNA (containing the full length 3' untranslated region with the SV40 poly-A site), p5'TKcDNA^B (containing a 3' deletion from poly-A to the Bam H1 site) and p5'TKcDNA^H (containing a 3' deletion from poly-A to the Hind III site). (B) Rate of Decay of p5'TKcDNA^A+. This construct is missing a poly-A site and 3' untranslated sequences up to the Bam H1 site. A dot blot manifold was used to add 10 ug of total RNA from each time point to a nitrocellulose filter. Blots from both parts A and B were probed with the internal Sma 1-Bam H1 TK fragment. The apparent decrease in size of the message in part A is actually due to one side of the gel running slightly faster than the other.

A.


p5'TKcDNA
p5'TKcDNAΔB
p5'TKcDNAΔH
0 10 15 20 25 35 0 10 15 20 25 35 0 10 15 20 25 35

B.

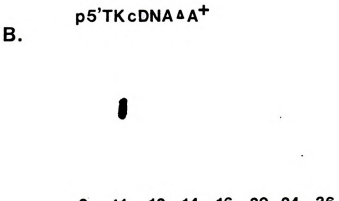

p5'TKcDNAΔA+
0 11 12 14 16 20 24 36

FIGURE 5

restriction sites, we were able to make a set of gross deletions (fig. 1) which allowed us to determine if this region was important for the extended stability of this message. Figure 5a shows a comparison study, in which Actinomycin D was added at 12 hours post serum stimulation to Rat 3 cells which had been transfected with our wild type mini gene (p5'TKcDNA), a deletion mutant extending to the Bam H1 site in the 3' untranslated region (p5'TKcDNA^B) and a deletion mutant extending to the Hind III site in the 3' untranslated region (p5'TKcDNA^H). Densitometer tracings show that the stability of the mutants and of the wild type are virtually the same (data not shown). The apparent decrease in size over time seen on this autoradiogram is due to one side of the gel running slightly faster than the other. All of these mutants still contain the SV40 polyadenylation site, so our final assay was performed on a mutant which deleted this area by gross mutation up to the Bam H1 site (p5'TKcDNA^A+). Figure 5b shows that this message was extremely unstable, as it virtually disappears after addition of the drug. Similar data was obtained by S1 analysis and Northern blotting (data not shown).

Late S/G_2 Phase Regulation of TK

Finally, our attempts to further characterize the regulation of TK expression in the cell cycle have led us to examine the mechanisms by which TK enzyme and mRNA

levels are regulated throughout the cycle. Our previous work focused on the mechanism of induction of TK at the G_1/S phase interface. We would now like to examine how the gene is regulated and/or terminated at the end of the cycle. We have based our studies on two previous reports on TK enzyme regulation. The first, from Johnson et. al. (16), showed that TK enzyme activity declines with a four hour half-life in mouse 3T6 fibroblasts when serum stimulus is removed in S phase. The second study, by Bello (3), showed that induction of TK enzyme activity was insensitive to the presence of hydroxyurea, even though DNA synthesis was inhibited by 98%. Termination of TK enzyme activity was found to be dependent upon DNA replication, since 59% or more of the cellular DNA had to be replicated for efficient termination of TK activity. Figure 6 shows TK enzyme data from serum stimulated CV-1 cells under similar conditions, and figure 7 shows northern blots of the corresponding TK mRNAs. Plotted in figure 6 are TK enzyme activities from cells treated with: a)serum (t=0) and hydroxyurea (t=12), b)serum (t=0) and serum withdrawal (t=20) and c)serum (t=0) alone. Figure 7 shows autoradiograms of the corresponding RNAs run on agarose formaldehyde gels and probed with an internal fragment from our TK cDNA. Finally, figure 8 shows a composite plot of the RNA levels from the autoradiograms in figure 7 as determined by

Figure 6 Late S/G_2 Phase TK Enzyme Activity. The activity of the TK enzyme was determined from CV-1 cell extracts made at the times indicated. All cells were serum stimulated at t = 0 and followed for two consecutive cell cycles. Control (□-·-□), Serum Depleted at t = 20 hours (○---○), and Hydroxyurea treated at t = 12 hours (△—-△). TK activiy was determined as described previously (44).

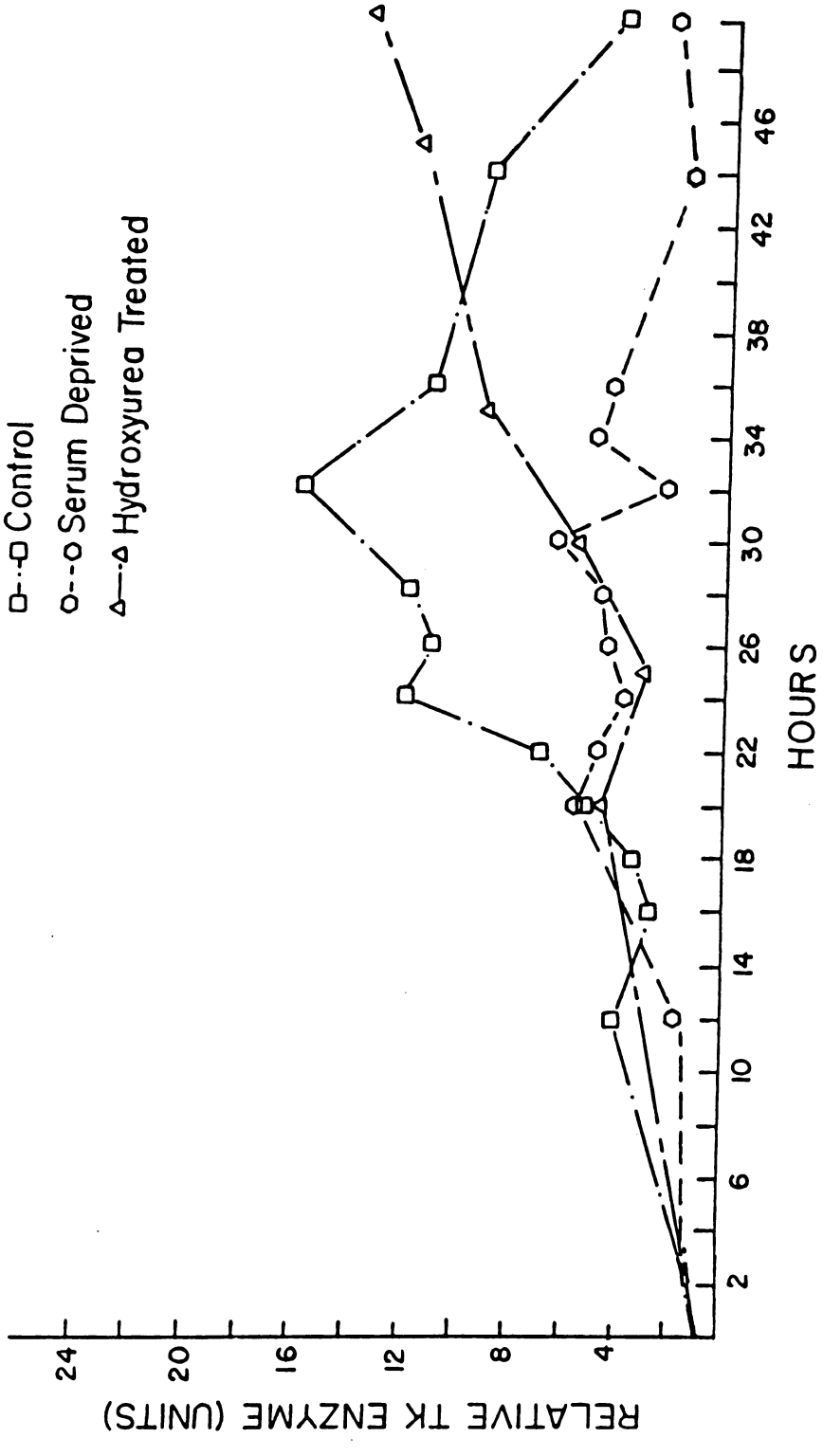

□--□ Control
o--o Serum Deprived
Δ—Δ Hydroxyurea Treated
RELATIVE TK ENZYME (UNITS)
4
8
12
16
20
24
HOURS
2
6
10
14
18
22
26
30
34
38
42
46

FIGURE 6

Figure 7 Late S/G_2 Phase TK mRNA Levels. Total RNA was made as described in Materials and Methods with the same cell populations from figure 5. An equal amount (15 ug) of RNA from each time point was run on denaturing agarose-formaldehyde gels and blotted to nitrocellulose as described (44). All blots were probed with a ^{32}P-labelled SmaI-Bam HI internal TK fragment. (A) Serum stimulated control, (B) Serum depleted at t = 20 hours, and (C) Hydroxyurea treated (0.1 ug/ml) at t = 12 hours.

A.

0 12 16 18 20 22 24 26 28 32 36 40 44 50

B.

0 12 20 22 24 26 28 30 32 34 36 44 50

C.

0 12 20 25 30 35 45 55

FIGURE 7

Figure 8 Densitometer Analysis of Late S/G_2 Phase TK mRNA Levels. The autoradiograms in figure 6 were traced with a densitometer, the area under each curve was calculated, and the value at t = 0 was set to 1.0 and plotted vs. time (hours). Control (□—·—□), Serum Depleted at t = 20 (⬡——⬡), and Hydroxyurea treated (△—-△) values are shown.

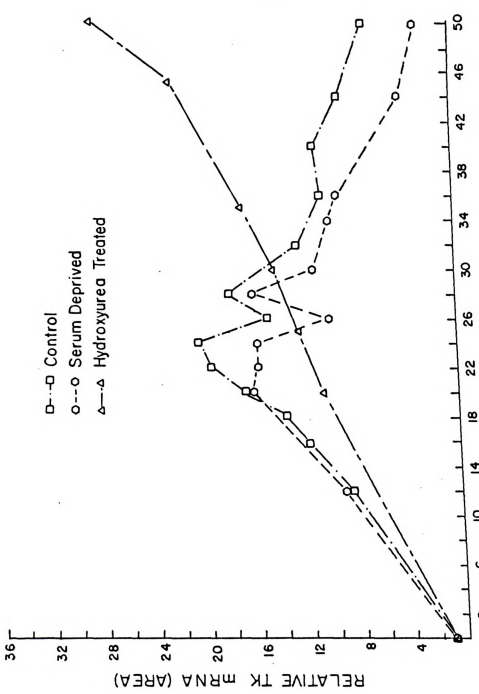

RELATIVE TK mRNA (AREA)
36
32
28
24
20
16
12
8
4
0
2
6
10
14
18
22
26
30
34
38
42
46
50
HOURS
Control
Serum Deprived
Hydroxyurea Treated

FIGURE 8

densitometer scanning. The control TK enzyme activity and RNA levels appear to be somewhat similar. By 24 hours the enzyme activity has increased 12 fold and the mRNA level 20 fold over the level seen at t=0. These levels then fall, and a second peak occurs at 28 hours (for mRNA) and at 32 hours (for enzyme) post-serum-stimulation. Each induction begins during the highest measurable rate of DNA synthesis, and each peak occurs just after. In CV-1 cells which were transferred to serum depleted media at t=20 hours post stimulation, TK enzyme levels never attain the high levels seen in our controls. A 7-7.5 fold induction of activity occurs by 20 hours, and this level falls to 5.5-fold by t=50 hours. In contrast, TK mRNA induction is similar to the control TK mRNA levels. Again, two peaks of induction are seen at approximately the same times, and attain only slightly lower levels of mRNA with a very similar kinetic pattern. The addition of hydroxyurea (0.1 ug/ml) to these cells inhibits DNA synthesis by 97% (data not shown) and has a rather extreme effect on both TK enzyme activity and mRNA levels. Figure 6 shows that TK enzyme levels are slightly reduced at 24 hours from control levels, however the activity level keeps rising even up to 50 hours post-serum-induction, where it (fig. 7 and 8) attains extremely high levels. The initial induction is similar to the control,

however no decrease is seen. TK steady state mRNA levels also keep rising until a 30-fold level is seen by 50 hours.

DISCUSSION

Our present study demonstrates that the mechanisms involved in the regulation of the steady state levels of TK mRNA are dependent upon each phase of the cell cycle in serum-induced CV-1 cells. Throughout the entire cell cycle, TK mRNA appears to have an extended stability. No previous determination of G_1 phase TK message half-life exists. However, the S phase levels that we see are similar to those seen with the human TK gene (48). TK mRNA is less stable in our simian cells when Actinomycin D is added at the start of G_2 phase, while the human TK levels remain high in aphidicolin-treated HeLa cells (48). In contrast, confluent growth-arrested cells which are mitogenically stimulated to enter S phase by SV40-infection show an even greater stability. Our previous results show a temporal dependence on the G_1/S phase interface for transcriptional activation of TK in serum-induced CV-1 cells and later results suggest a smaller activation at this same phase in SV40 infected cells. The molecular basis for these cell cycle dependent regulatory controls has yet to be determined. One piece of evidence now exists for the mediation of the extended

stability of TK mRNA. My deletion analysis at the 3' end of the gene shows that the only absolutely necessary sequence in this area is a functional polyadenylation recognition site. Removal of a large portion of the 3' untranslated region had no effect on stability of the TK message. However, when the polyadenylation site was removed, the TK message was destabilized and had a half-life of less than one hour during S phase in actinomycin D treated Rat3 cells transfected and stably selected for this construct. This is in general agreement with Greenberg (11), who found that poly-adenylated mRNAs turn over approximately once per cell generation time. Also histone genes, which are one of the only sets of mammalian mRNAs which lack polyA sequences, turn over much more rapidly than other types of RNA. The replicative histone variants all have half-lives of only 10-30 minutes (11, 40). The poly-A site has also been shown to be important for the mediation of mRNA stability of the globin gene of Xenopus Oocytes, and many viral genes from Adenovirus (14, 25). Since the poly-A site used in our constructs was the SV40 polyadenylation site, it remains to be seen whether the same situation is true <u>in vivo</u> with the endogenous TK poly-A site intact. Whether the poly-A site in our constructs requires the presence of other TK sequences for the mediation of its stability has yet to be determined, however, it is entirely possible that this

site is not the only site necessary for a stable message.

Further characterization of the cell cycle in CV-1 cells has allowed us to more finely characterize TK mRNA and enzyme levels. By taking time points at two to four hour intervals at late times after serum induction, we were able to observe two peaks in TK mRNA levels, both of which occur just after DNA synthesis levels peak in the first and possibly second cell cycles following serum induction. The second peak in TK mRNA induction may be due to a second transcriptional induction of TK as the cells traverse G_1 into S phase for a second round of DNA replication. Since there is a relatively short time span between the peaks of DNA synthesis, not enough time is allotted for much measurable degradation of TK mRNA. This fact probably accounts for the high levels of TK steady state mRNA levels previously observed at late times post serum stimulation (44), and allows us to now state that the regulation of the thymidine kinase gene does appear to be dependent upon multiple cellular controls.

The two peaks of TK mRNA levels are also observed with cells that are placed in serum depleted medium during the G_2 phase of the first cell cycle. Since the level of induction is only slightly decreased, it is questionable as to whether serum removal has any real effect on TK mRNA levels or its regulation in cells that have been mitogenically stimulated for long periods of

time by the addition of serum growth factors. In contrast, TK enzyme levels appear to be greatly affected by removal of serum, as control levels (Fig 6) are never reached. However, a steady low level of activity is maintained in our cells, in contrast to Johnson (16) who found a rapid decrease in activity by six hours following serum removal from 3T6 cells. It does appear, however, that a constant presence of serum growth factors is required for cells to attain high levels of TK activity, and this effect appears to be a post-transcriptional one. What significance this result has for the in vivo regulation of TK remains to be seen.

The most striking result presented here involves the use of the DNA synthesis inhibitor, hydroxyurea. Inhibition of DNA synthesis by 97% does not prevent induction of either TK enzyme or TK mRNA levels in serum stimulated CV-1 cells, but the termination of this induction is greatly affected. The initial induction itself appears not to be directly influenced by DNA synthesis. Rather, it is probably transcriptionally induced in late G_1 by some G_1-specific mechanism which is somehow inducible by serum growth factors.

The decrease of TK mRNA and enzyme activity appears to be mediated by a different mechanism. It is known that hydroxyurea directly inhibits Ribonucleoside Diphosphate Reductase (RDP Reductase) activity in the cell, and that

cellular dNTP pools are then rapidly depleted. It is also known that dTTP is specifically required in the cell as an allosteric effector for the reduction of GDP to dGDP, and the dGTP generated from dGDP is an allosteric effector for the reduction of ADP to dADP (18). It is therefore possible that TK activity is induced to a high level in hydroxyurea treated cells to produce more dTTP in an attempt to compensate for the loss of RDP reductase function. This cellular induction of TK activity could possibly be used to maintain cellular dNTP pools at a specific level. It is already well known that in a variety of systems a regulatory feedback mechanism exists in which TK activity drops upon the accumulation of its end product, TTP (33, 36, 39). It would therefore seem that since intracellular pools are depleted in hydroxyurea treated cells, the normal regulatory "feedback" mechanism may never become operational. This theory also has significance for our SV40-infected cells. It is known that levels of TK mRNA and enzyme activity are typically much higher in SV40-infected cells than in serum-stimulated cells. SV40-infected cells present a condition which demands rapid DNA replication. If intracellular dNTP pools become extremely depleted during this process, TK activity may be induced to an extremely high extent to help compensate for this situation. The induction may involve an even longer increase in the half-life of the

message, and this may account for the greater half-life seen in our SV40-infected cells. It is also possible that if replication of SV40 DNA continues until cellular lysis occurs, a prolonged half-life of messenger RNAs involved in DNA synthesis may also be a necessary result.

Obviously, these ideas require a more in depth analysis. However, we can state that the thymidine kinase gene is regulated at multiple levels throughout the cell cycle. Both a transcriptional control is operational in G_1/S, while a lengthy half life of the message is seen in all phases. However, the exact mechanism regulating serum dependence of the TK enzyme is not known and must be more fully investigated. The dependence of TK messenger RNA and enzyme activity shut-off on cellular DNA synthesis should also prove to be an interesting area of study.

ACKNOWLEDGMENTS

I would like to thank Richard Mehigh for help in the preparation of figures and Moriko Ito for helpful discussions.

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CHAPTER IV

(ARTICLE)

Sequence Analysis of the Human Thymidine Kinase Gene Promoter Region

by

Christine J. Stewart and Susan E. Conrad

ABSTRACT

The gene for the cytoplasmic thymidine kinase (TK) enzyme has been shown to be regulated in a growth phase dependent manner. Since part of this regulation is known to be at the level of transcription, the promoter of the human TK gene has been analyzed. We have located the human TK promoter in a 900 bp EcoR1 fragment of human DNA. The 900 base pair fragment was sequenced and the promoter sequence localized to a 550 bp EcoR1-Sma1 fragment which was subcloned onto the body of a TKcDNA for further *in vitro* and *in vivo* analysis of its regulation. Nuclease S1 mapping experiments were performed and two transcription start sites were mapped in human 293 cells. Computer sequence analysis has localized a 40 bp homology in the hamster and human TK promoter regions. It is possible that this area may be a thymidine kinase gene specific regulatory element.

INTRODUCTION

Knowledge of how a gene such as TK is regulated during the cell cycle is essential for understanding the process of cell growth on a molecular level. Localizing and sequencing the promoter region of the thymidine kinase gene became important in order to obtain a better understanding of transcriptional regulation of this gene. By comparing the thymidine kinase gene promoter sequence to various other cell cycle regulated genes we hoped to find novel gene specific sequences which may be important for directing the timing and extent of transcription in the cell cycle.

Many consensus sequences have been identified as important for transcription of RNA polymerase II transcribed genes. One of the most well known elements is an A-T rich region, or "TATA" box, typically found at -25 to -30 nucleotides from the transcriptional start site. Mutation analysis of this sequence in many genes has shown that it is important for the correct initiation of transcription (16, 33). An example of some of the Pol II-transcribed genes which do not have a TATA homology are the polyoma virus late genes (31). These late genes

exhibit multiple start sites for transcription (31).

Another DNA sequence that is necessary for maintaining a correct or efficient rate of transcription is the CCAAT box. This element typically resides at -70 to -90 nucleotides from the transcription start site, and has the consensus sequence $^{5'}$-GGPyCAA$^{A}_{T}$CT-$^{3'}$ (2). Mutations in this element in a variety of genes, such as Rabbit β-globin (9) and Xenopus hsp70 gene (3) are known to reduce transcription of these genes. CAAT boxes function in either orientation, as evidenced by studies on the thymidine kinase gene of herpes simplex virus (15, 27). Inverted and/or tandem CAAT boxes have been found in a variety of cell cycle regulated histone genes (5, 18, 29), and more recently in the human thymidine kinase gene promoter (23). The CAAT box may be functioning either independently or in conjunction with other elements (3, 17).

Another important promoter element is the G/C rich sequence. The consensus sequence for this element is $^{5'}$-$^{T}_{G}$GGGCGG$^{GGG}_{AAT}$-$^{3'}$ (22) and like the CCAAT box, it can also function in either orientation (10). This element is common to a host of viral and cellular genes and is typically found in multiple copies in the promoter region.

This element has also been shown to be necessary for an efficient rate of transcription, and is known to bind the transcription factor Sp1 (10, 12).

One of the most potentially interesting elements to be found in the promoter region of any gene, and specifically of the TK gene, would be a gene-specific or gene family-specific sequence such as an enhancer. Finding a sequence with these properties would help us to identify specific transcriptional controls within a particular gene or gene family and may explain how genes such as TK are regulated in the cell cycle. An enhancer can be distinguished from other promoter elements by its ability to function upstream, within or downstream of a gene, as well as to function in an orientation independent manner. Enhancers are cis-acting and usually function to stimulate transcription from either heterologous or homologous promoters (1, 13). Gene-specific regulatory elements which exhibit some or all of the properties of enhancers are often found in groups of genes which are regulated in a similar fashion. For example, cAMP regulated genes contain a well conserved sequence (7, 8, 14, 34) and this element is quite similar to a consensus element of the cAMP responsive genes in procaryotes (11).

In order to more fully explore these possibilities

with the human TK gene, the promoter region of the human TK gene was isolated from a lambda library, characterized by restriction mapping and sequenced. S1 nuclease mapping experiments were performed and the transcriptional start site was mapped. A computer dot-matrix analysis was performed in order to compare the human TK promoter sequence with a variety of other sequences. A 5' non coding element was identified which is highly conserved between the hamster and human thymidine kinase genes.

MATERIALS AND METHODS

Plasmids: Figure 1 from Appendix 1 shows a restriction map of a variety of lambda clones which produced a TK^+ phenotype upon transfection of two or more clones into Rat3 TK^- cells. Part D of this figure shows fragments which hybridized to the human TK cDNA clone pHuTK-cDNA7 (30). The 5' most fragment to which this cDNA hybridized was a 900 base pair EcoR1-EcoR1 fragment. This fragment was isolated and subcloned into the EcoR1 site of pBR322 to give pBRTKpro.

Preparation of DNA Fragments and Sequencing Two fragments were obtained by double digestion of pBRTKpro with EcoR1 and Sma1 (Bethesda Research Laboratories), and

the Sma1 site was mapped to approximately 550 base pairs from the 5′ most (relative to the gene) EcoR1 site. For labellings, the DNA was first cut with either EcoR1 or Sma1, and then labelled at either the 5′ end with T4 DNA kinase and gamma-^{32}P-ATP or at the 3′ end with the Klenow fragment of DNA polymerase I and α-^{32}P-ATP (New England Nuclear). A second cut was then made, and fragments isolated by agarose gel electrophoresis. Sequencing of the 900 base pair fragment was performed by the Maxam-Gilbert procedure (25).

Preparation of Total RNA Total RNA was prepared from 293 cells via the procedure described in chapter 3.

S1 Nuclease Analysis For S1 analysis a 258 bp Hinf1 fragment was isolated from p5′TKcDNA, which is a plasmid containing the 550 base pair EcoR1-Sma1 fragment (found by sequence analysis to encode the TK genomic promoter region) subcloned onto the body of the TK cDNA. Figure 1 shows the map of this plasmid as well as the relevant restriction sites. The Hinf1 fragment was isolated by gel electrophoresis and 5′ end labelled with T4 DNA Kinase and gamma-^{32}P-ATP. 10 ng of labelled DNA was ethanol precipitated with 15 ug of RNA, resuspended in 10 ul of hybridization buffer (80% formamide, 0.4M NaCl, 0.04 M Pipes pH 6.4, and 1mM EDTA). This mixture was heated to

Figure Legends

Figure 1 Structure of p5'TKcDNA. p5'TKcDNA expresses the TK mRNA from the human genomic TK promoter, but utilizes an SV40 polyadenylation signal. Symbols: (■) HuTK sequences, (▭) SV40 sequences, (——) pBR322 sequences. Arrows denote the Hinf1 sites used in the S1 mapping analysis.

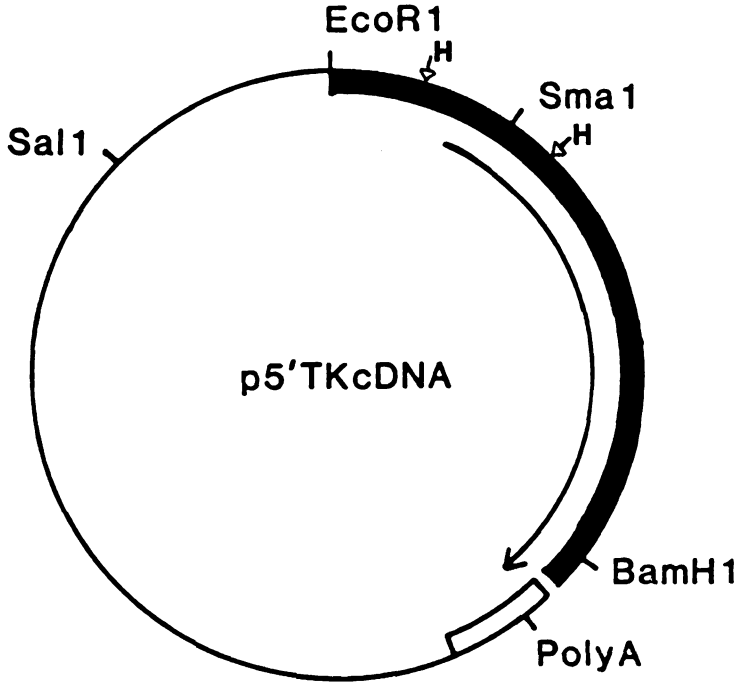

FIGURE 1

90°C for 3 minutes and immediately transferred to 60°C and incubated for 12 hours. The sample was then diluted to 200 ul with 1x S1 salts (0.28 M NaCl, 0.05 M Sodium Acetate pH 4.6, 4.5mM $ZnSo_4$) and aliquoted in equal volumes into 3 separate tubes. S1 nuclease (Pharmacia) was added at 50, 100, and 175 units in each of the three tubes, and incubated at 37°C for 15 minutes. The samples were then extracted with an equal volume of phenol/chloroform (50:50 v/v) and precipitated with ethanol. Samples were resuspended in loading buffer and run on an 8% denaturing polyacrylamide sequencing gel.

RESULTS

To study the human thymidine kinase promoter it was first necessary to isolate the 5′ end of the gene. This was done by localizing the 5′-most area of lambda clones which were known to hybridize to a nearly full length TK cDNA (see appendix 1). A 900 base pair EcoR1 fragment was isolated and subcloned into the EcoR1 site of pBR322 and pSp64. By digesting with SmaI, this fragment could be broken down into two fragments of unequal length for isolation and labelling. The nucleotide sequences of two fragments $^{5'}$EcoR1-SmaI$^{3'}$ (550 base pairs) and $^{5'}$SmaI-

EcoR1$^{3'}$ (350 base pairs) were determined. Figure 2 shows the sequence of the 550 base pair fragment as well as part of the sequence of the 350 base pair fragment. This 350 bp fragment contains the junction between the first exon and intron as determined by comparison with the published cDNA sequence (4).

There are several interesting features of this sequence. There are two 6 base pair A-T rich sequences (TTTAAA), one at -25 and the other at -248. These have been denoted in figure 2 by a box placed around each element. The hexanucleotide GGGCGG is found seven times--twice in the $^{5'}$-GGGCGG-$^{3'}$ position, twice inverted $^{3'}$-GGCGGG-$^{5'}$, twice on the opposite strand in the inverted position (CCGCCC), and once in the $^{5'}$-CCCGCC-$^{3'}$ position on the opposite strand (GC rich elements have been signified in figure 2 by dots placed overhead). Four of these elements reside within a 27 bp inverted repeat element; one copy of each repeat is found on each side of the first A-T rich homology (TTTAAA) (shown in fig. 2 with a line below and arrows above each 27 bp repeat). In studies on the SV40 promoter and the HSV TK promoter only the GGGCGG hexanucleotide sequence (or its inverse) has been shown to bind the Sp1 transcription factor, however the other GC rich elements seen in this promoter have not been shown in

Figure 2 Sequence of the human TK promoter region. A 550 bp $^{5'}$EcoR1-Sma1$^{3'}$ fragment was sequenced via the Maxam-Gilbert technique using base specific chemical cleavages (25). Symbols: (•) indicates G/C rich hexanucleotides, (*) the 40 bp hamster-human homology, (□) A-T rich TATA sequences, (⟶ over sequence) the 9 bp inverted CCAAT sequence, and (⟶ over and ___ under) the 27 bp inverted repeats. Base pair changes or differences noted from other published sequences (4, 24) are indicated by placing the appropriate nucleotide below the nucleotide found in this analysis. X denotes nucleotides not found in the other two sequences.


5'
C TAAATCTA ATAAATG AGC TAACTCCGCC CCAGCCCCTT AGTCCCTCCC TGCAATCCAC
CX X CCCCC
CTACCTCTGC AGACATCTTC TTCCAAGGAA CCTTGCTTGG GAAACCCACA CCAGACACAT
C C
CCATCATGGC GTCTACAGCC GCATGGGCGT GCGTCCCTCT GTTTATATGG CCAGAG CCCG
C
CCTCGCTCCG CCCCTTTAAA CTTGGTGGGC GGACCGAGGC GGGGCTCAGA CCAGGCCCCA
X T
CCCCGATCAG CCACGTACCA TCGCCCTGAT TTCCAGGCCC TCCCAGTCCC TGGGCGCACG
X
TCCCGGATTC CTCCCACGAG GGGGCGGGCT GCGGCCAAAT CTCCCCGCCA GTCAGCGGCC
X
GGCGCGTGAT TGGCCCCATG GCGGCGGGGC CGGCTCGTGA TTGCCCAGCA CGCCGTGCTT
TAAAGCGGTC GGCCCGG GAA CCAGGGGCTT ACTCCCGGAC GGCCTTCGAG AGTACTCCGG
T
TTCGTGAACT TCCCGGAGGC GCA ATG AGC TGC ATT AAC TGC CCA CTG TGC TCC CTT
GCT CCC CCA GCA AGA CCC GGG CAG ATC CAG GTGCGGCGGC CAGCCCTCCG
CGTGGCTGGG GATCAGGTGG TCAGTGGT....3'

FIGURE 2

other systems to bind to Sp1 (12). . The other arrows in figure 2 have been placed above a 9 base pair inverted repeat found by Kreidberg and Kelley (24).
This sequence contains the canonical CCAAT box in an inverted orientation and has been recently shown to be important in the binding of cell cycle regulated transcription factors (23). There are several base pair differences in this sequence compared to the two published sequences. These have been denoted by placing the published alternate base pair below the particular nucleotide in question. An "X" has
been placed where a nucleotide which I found was not reported in either published sequence, and nucleotides contained in the published sequence(s) but not in this sequence are shown placed below the position in which they were found by the other researcher(s).

To correctly identify the 5′ end of the TK mRNA, total RNA from human 293 cells was isolated and analyzed using an S1 nuclease procedure. The probe was a Hinf1 fragment which begins 73 base pairs 3′ of the first ATG and extends to a site approximately 185 bp 5′ (upstream) of the first ATG of the cDNA sequence. Figure 3 shows that the RNA-protected two fragments that differ in size by 7-8 base pairs with similar intensity; this

Figure 3 Autoradiogram of an S1 nuclease experiment run on an 8% polyacrylimide gel. For size comparison the cognate sequenced TK promoter fragment is shown. The sequence position of the first cap site is denoted by an arrow.

FIGURE 3

indicated two equally efficient start sites for the mRNA.

In order to search for sequences that might mediate cell cycle regulation, the human TK promoter sequence was compared to other known promoter sequences of cell cycle-regulated genes and other thymidine kinase genes using the GENEPRO computer program (IBM). The most significant homology was found using a Dot Matrix program in a comparison of the human TK gene promoter to the hamster TK promoter. Figure 4 shows that an extremely homologous 40 base pair region was found at a location 400 base pairs upstream from the first ATG in the human sequence, and 232 base pairs upstream from the first ATG in the hamster sequence. This sequence is shown in figure 2, with an asterisk placed above each homologous nucleotide. The computer search turned up other close approximations (80% homology) of this sequence in 22 cases, but none were contained within the promoter regions of any of these genes. Most were contained in internal sequences. The only gene that had any type of similar function was the Rat PEP Carboxykinase (GTP) gene. None of the genes that are regulated in a manner similar to TK, such as DHFR or thymidilate synthetase contained this sequence. Nine out of the 22 sequences found came from eucaryotic viruses, but the significance of any of this is unclear. Unfortunately,

Figure 4 Dot matrix comparison of the human TK promoter and the hamster TK promoter regions. The 5′ most sequences for each gene begin in the lower left corner. This program compares every 10 base pair stretch in one sequence to every 10 base pair stretch in the other and places a dot on the page in an area signifying a match based on 8 out of 10 possible base pairs matching correctly.

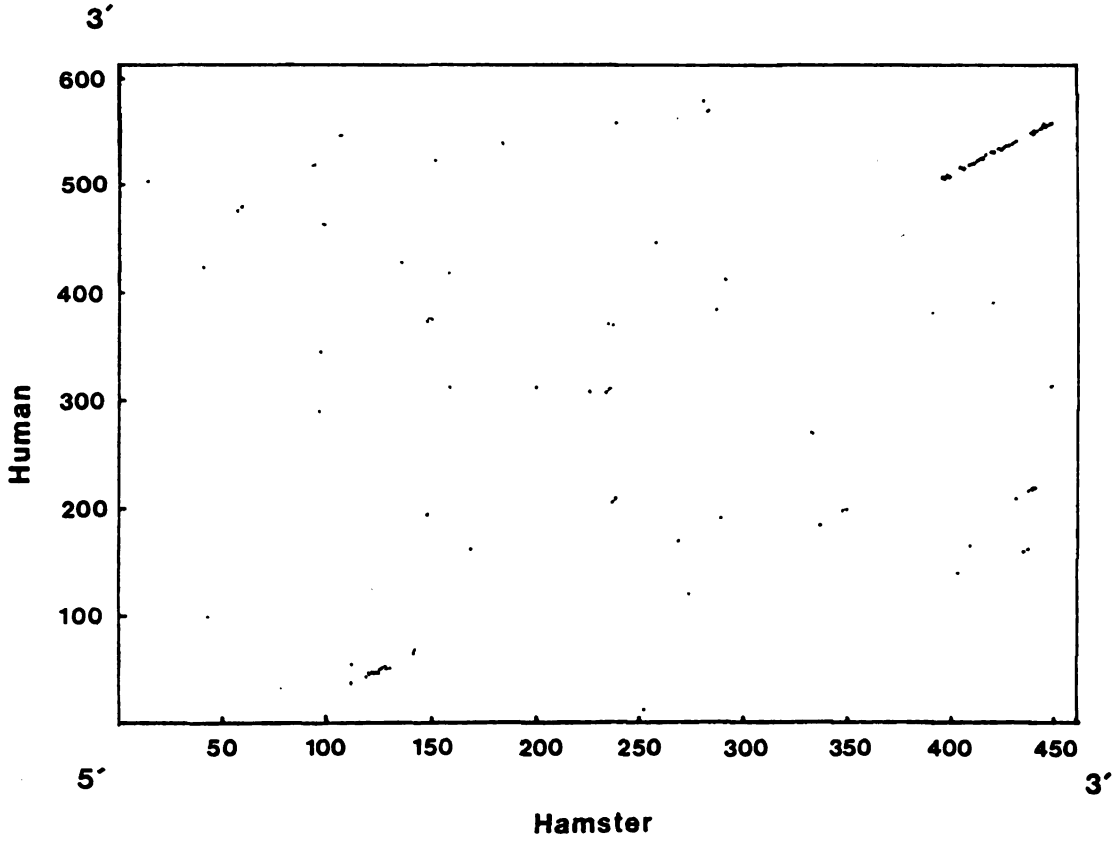
3′
600
500
400
300
200
100
Human
50
100
150
200
250
300
350
400
450
5′
3′
Hamster

FIGURE 4

comparisons to the mouse and chicken TK gene promoters were not possible as these sequences have not yet been published.

DISCUSSION

We have performed a DNA sequence analysis of a 550 base pair genomic region found closely associated with the first exon of the human TK gene. By comparison with the published cDNA sequence (4) it was possible to map the 3' end of this sequence to the first exon of the TK cDNA. This area was subcloned onto the body of our TKcDNA via a common SmaI site, and an internal Hinf1 fragment was used to perform an S1 analysis. The start site of transcription was mapped, and two separate sites were identified. The intensity of these bands on the autoradiogram indicated that each site is used to an equal extent in human 293 cells. This same 550bp region has since been defined as containing the functional promoter region by a deletion mutational analysis. Only 83 bp 5' to the cap site are required for the efficient expression of the gene, as assayed by transfection of a mini-gene

construct into Ltk^- cells and assaying for growth in selective HAT (hypoxanthine-amethopterin-thymidine) media (24). A published analysis of TK promoter mutations and their transformation efficiencies indicates that the A-T rich region nearest the CAP site is not absolutely required for expression, but deletions which remove it seem to reduce promoter function (24). This region is not capable of directing expression in the abscence of other upstream promoter sequences. Similarly, mutation or removal of one or both 9 bp sequences containing the inverted CAAT sequence dramatically decreases the efficiency of TK^+ transformation. This result has also been seen with various globin genes (6, 9) as well as the HSV TK gene (26). Recently, it was shown that both inverted CAAT sites in the human TK promoter form complexes with nuclear DNA-binding proteins (23). The nature of the complexes changes dramatically, as seen by gel mobility shift analysis, as the cells approach S phase. Between 9 and 18 hours the mobility of bands shifted to other areas of the gel. This result correlates well with our previous data (see chapter two) on the timing of the transcriptional increase of the TK gene in the cell cycle. However, it is not yet known whether the CAAT box binding observed by Knight et. al. (23) is truly

resulting in a transcriptional increase. The factor which binds to the TK CCAAT sequence may be similar to the CAAT binding transcription factor (CTF) which has been purified from HeLa cell nuclear extracts (21). CTF was shown to be indistinguishable from nuclear factor 1 (NF-1), and is known to bind the CAAT sequence of the hsp70 gene (28).

The importance of the promoter region in the regulation of cell cycle stage-dependent expression of the thymidine kinase gene has not yet been fully explored. Experiments performed by Travali et. al. (32) clearly show that the promoter region is necessary for cell cycle dependent regulation of TK mRNA levels. When the TK promoter was linked to the bacterial CAT gene, it was expressed at the highest levels in S phase. An HSP-70 promoter from Drosophila linked to the body of the TK cDNA did not show cell cycle dependent expression. Heat shock of cells transfected with this construct showed high levels of TK mRNA, even in G_0 cells. Likewise the calcyclin promoter linked to the TK cDNA showed its highest expression in serum deprived cells. Also, a construct carrying the SV40 promoter linked to the TK cDNA was shown to be cell cycle regulated. Further experiments in our laboratory have shown that there is an induction of TK mRNA levels with our SV40 promoter-TKcDNA construct

between 0-2 hours post serum stimulation, but TK enzyme activity remains S phase dependent (20). It remains to be seen if our cells are inducing the SV40 promoter in early G_1 via some cellular mechanism or if our TKcDNA plays a role in this induction. The experiments of Travali (32), as well others(19, 20, chapter 3) make it clear that a translational and/or post-translational control is/are present which specifies cell cycle dependent expression of TK enzyme activity. What has also become very clear is that the TK promoter region is definitely involved in the mediation of cell cycle dependent expression of TK mRNA steady state levels. Future work should focus on trying to define area(s) of the promoter, such as the 40 bp hamster-human TK promoter homology, which may mediate cell cycle dependent expression.

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SUMMARY AND CONCLUSIONS

The regulation of the cell cycle dependent expression of the thymidine kinase gene was examined. The experiments in Appendix 1 showed us that thymidine kinase (TK) enzyme and mRNA levels are regulated in a cell cycle-dependent manner in CV-1 cells after mitogenic stimulation by addition of serum growth factors or by infection with SV40 virus. In both cases, TK induction paralleled the entry of CV-1 cells into S phase. However, TK mRNA typically accumulated to a higher level in the virus infected cells. Most of the enzyme induction was accounted for by increases in the level of mRNA, and this indicated that the control of TK in the cell cycle might be at the level of transcription of this gene.

The experiments described in Chapters 2 and 3 show that both a transcriptional and at least one type of post-transcriptional control are involved in the cell cycle regulation of TK. The experiments in Chapter 2 describe nuclear run-on experiments which show a very low level of transcriptional induction in both systems. A transient six fold increase in transcription was seen at the G_1/S

phase border in serum-stimulated cells, and a 2.0-3.5-fold increase has been noted at the beginning of S phase in SV40 infected cells. It is possible that the increase is lower in SV40 infected cells because the timing of infection of each cell within each population may be highly variable. This might show up in our assays as a slow but steady increase in transcription even though the actual rate per cell may be much higher. This seems likely as this slow steady type of induction is observed with the mRNA steady state level as observed on agarose gels.

The experiments presented in Chapter 3 begin to focus on possible mechanisms of post-transcriptional regulation of TK. Specifically, mRNA half-lives were examined and were found to be quite long throughout the cell cycle. The 20 hour half-life seen in S phase helps to explain the high levels of mRNA steady state levels seen at later times in the cell cycle. Transcription takes place throughout approximately a six hour period of time, so the 10-20-fold increase in levels of mRNA seen later in S phase and into G_2 are probably a result of the message accumulating over this period of time and remaining intact due to its lengthy half-life.

Our highest measurable levels of TK mRNA occur very

late in the cell cyle and seem to depend upon DNA synthesis for efficient termination of transcription. It is not known how this dependence occurs, and this suggests a possible area of research to pursue. It is known that TK enzyme operates via a feedback mechanism due to the accumulation of its end product TTP. It is possible that intracellular pools of TTP may function to regulate TK mRNA by stimulating transcription when levels fall below a certain limit, and by shutting off expression when TTP levels reach another specific level.

Another possible area of research to pursue would involve the examination of serum-dependence on TK enzyme expression. Our results, as well as others, have shown that the removal of serum growth factors from tissue culture cells has a rather negative effect on TK enzyme activity. We have shown that this is not related to mRNA levels. Therefore, it is possible that serum growth factors induce some type of post-translational modification of the thymidine kinase enzyme which allows it to become active. Removal of serum may abolish this activity.

Finally, the results presented in Chapter 4 will allow future work to progress on the characterization of gene-specific regulatory elements which are involved in

transcriptional control of the TK gene, as well as cell cycle control. The promoter elements and homologies found may be useful in a molecular characterization of sequence elements necessary for cell cycle dependent expression.

APPENDIX I

MOLECULAR AND CELLULAR BIOLOGY, June 1985, p. 1490–1497
0270-7306/85/061490-08$02.00/0

Vol. 5, No. 6

Induction of Cellular Thymidine Kinase Occurs at the mRNA Level

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Received 16 November 1984/Accepted 26 February 1985

The thymidine kinase (TK) gene has been isolated from human genomic DNA. The gene was passaged twice by transfection of LTK⁻ cells with human chromosomal DNA, and genomic libraries were made in λ Charon 30 from a second-round TK⁺ transformant. When the library was screened with a human Alu probe, seven overlapping λ clones from the human TK locus were obtained. None of the seven contained a functional TK gene as judged by transfection analysis, but several combinations of clones gave rise to TK⁺ colonies when cotransfected into TK⁻ cells. A functional cDNA clone encoding the human TK gene has also been isolated. Using this cDNA clone as a probe in restriction enzyme/blot hybridization analyses, we have mapped the coding sequences and direction of transcription of the gene. We have also used a single-copy subclone from within the coding region to monitor steady-state levels of TK mRNA in serum-stimulated and simian virus 40-infected simian CV1 tissue culture cells. Our results indicate that the previously reported increase in TK enzyme levels seen after either treatment is paralleled by an equivalent increase in the steady-state levels of TK mRNA. In the case of simian virus 40-infected cells, the induction was delayed by 8 to 12 h, which is the length of time after infection required for early viral protein synthesis. In both cases, induction of TK mRNA coincides with the onset of DNA synthesis, but virally infected cells ultimately accumulate more TK mRNA than do serum-stimulated cells.

Thymidine kinase (TK) is an enzyme in the pyrimidine salvage pathway that catalyzes the phosphorylation of thymidine to dTMP. Many mammalian cells, including human HeLa cells, contain both cytoplasmic and mitochondrial forms of the enzyme (3), but the cytoplasmic form alone concerns us here. The regulation of the synthesis of TK is interesting because it is typical of that seen for many enzymes involved in DNA metabolism. TK activity is closely linked to the growth state of the cell, being present in rapidly growing but not in resting cells (13). In synchronized populations of cells, the activity is low in resting or G1 phase cells, but increases dramatically 10 to 20 h after the cells are released from arrest by serum stimulation, in parallel with the onset of DNA synthesis and entry into S phase. This induction is not absolutely dependent upon DNA synthesis (13), but does require both RNA and protein syntheses, suggesting that induction may be at the level of transcription. TK can also be induced by infection of resting cells with papovaviruses such as simian virus 40 (SV40) and polyoma (16, 17), and the viral genes required for this induction are the large T antigens (30). Whether viral induction occurs by the same or a different mechanism(s) as serum induction is a question that remains to be answered.

The TK gene provides a useful model system for carrying out a molecular analysis of genes that are cell cycle regulated and induced by viral infection. First, TK shows a great increase in activity (10- to 20-fold) after both serum and viral induction. Moreover, TK enzyme assays are both sensitive and easily performed. We can genetically select both for (hypoxanthine-aminopterin-thymidine media) and against (bromodeoxyuridine media) the TK⁺ phenotype, and many TK⁻ cell lines exist. It has been shown that LTK⁻ cells transfected to a TK⁺ phenotype with heterologous (human, rat, or hamster) chromosomal DNA containing a functional TK gene exhibit normal cell cycle regulation of the gene (31). Recent experiments with a cloned human gene (5) indicate that the sequences required for cell cycle regulation are closely linked to the gene and function after transfection into TK⁻ cells. Thus, this system should offer the chance to dissect the sequences involved in cell cycle-specific gene regulation.

The mechanisms by which the expression of cell cycle-dependent genes is controlled, and by which the papovaviruses override these controls, remain obscure, although many of the initial observations were made more than 15 years ago. To a large extent this is because molecular probes for these genes and their transcripts have not been available. In this paper we report the isolation of both the human chromosomal TK locus and a functional human TK cDNA clone. Isolation of the chromosomal locus has previously been reported by several investigators (5, 19, 22), and our mapping is essentially in agreement with their data. In addition, we have used the cDNA clone to map mRNA coding sequences and the direction of transcription within the locus. We have also used a subclone from within the coding sequence of the gene to monitor TK mRNA levels in serum-stimulated and SV40-infected simian CV1 cells. Our results show that the induction of TK enzyme activity is paralleled by an increase in mRNA levels, indicating that induction may be at the level of transcription.

MATERIALS AND METHODS

DNA transfections. Human genomic DNA was prepared from an SV40-transformed cell line, GM638. DNA transfections were done according to the method of Wigler et al. (35). Briefly, 10 to 20 μg of human DNA was added as a $CaPO_4$ precipitate to 10^6 LTK⁻ cells, and TK⁺ colonies were selected in hypoxanthine-aminopterine-thymidine media. After 2 to 3 weeks colonies were picked and expanded into cell lines.

Construction of genomic libraries. Recombinant phage libraries were constructed and screened as described previously (25). Genomic DNA from secondary transformant cell

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line B4 was partially digested with *Sau*3A, and 15- to 20-kilobase (kb) fragments were purified by centrifugation through 5 to 20% sucrose gradients. Charon 30 DNA was digested with *Bam*HI, and the arms were purified away from the internal fragments. The human DNA was ligated to the λ arms, packaged in vitro, and used to infect *Escherichia coli* K802. Approximately 10^6 phage from this unamplified library were screened by the plaque hybridization method of Benton and Davis (2), using Blur DNA labeled by nick translation as a radioactive probe.

Nucleic acid hybridizations. Hybridizations to DNA filters were performed at 68°C under aqueous conditions in 6× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10× Denhardt solution–0.1% sodium dodecyl sulfate (SDS) with approximately 10 to 20 ng of ^{32}P-labeled probe per ml. Hybridizations were done for 15 to 24 h. For northern blots, hybridizations were done in 50% formamide–3× SSC–5× Denhardt solution–50 mM sodium phosphate (pH 6.8)–5% dextran sulfate–50 μg of denatured sheared salmon sperm DNA per ml–0.1% SDS–approximately 10^6 cpm of ^{32}P-labeled probe per ml. Hybridizations were carried out for 15 to 20 h at 42°C.

After hybridization, all filters were rinsed once at room temperature in 2× SSC–0.1% SDS and then washed two to three times in the same solution at 68°C. In cases where the background was still high after such treatment, an additional wash in 0.1× SSC–0.1% SDS was added.

Viral infection of tissue culture cells. Simian CV1 (African green monkey kidney) cells were grown to confluence in media containing 5% calf serum–5% fetal calf serum. Cells were allowed to remain at confluence for 48 h and were then infected with SV40 at a multiplicity of infection (MOI) of 5. Infections were done by removing media from cells, infecting for 1 h with a concentrated viral stock, and then replacing the original media. Mock-infected cells were treated for 1 h with serum-free media. At various times after infection, plates were harvested for RNA, protein, or DNA synthesis analyses.

Preparation of poly(A$^+$) RNA and northern analysis. Polyadenylated [poly(A$^+$)] RNA was prepared from tissue culture cells as follows. Cells were washed once with phosphate-buffered saline (PBS) without calcium and magnesium. They were then lysed on the plate with 1 ml of lysis buffer (0.5 M NaCl, 10 mM Tris [pH 7.5], 1 mM EDTA, 1% SDS, 200 μg of proteinase K per ml) per 100-mm plate. The lysed cells were scraped from the plate, and cellular DNA was sheared by passage three times through a 21-gauge needle. Fresh proteinase K was added to 100 μg/ml, and the solution was incubated at 37°C for 30 to 60 min. A small amount (~0.1 ml of packed volume per 100-mm plate) of solid oligodeoxythymidylate-cellulose was added, and the RNA was bound in bulk by shaking at room temperature for 1 h. The mix was then loaded into a small column and washed with 20 column volumes of loading buffer (0.5 M NaCl, 10 mM Tris, 1 mM EDTA, 0.2% SDS) followed by 20 column volumes of the same buffer with 0.1 M NaCl. RNA was then eluted with 2 column volumes of 10 mM Tris–1 mM EDTA and ethanol precipitated after the addition of sodium acetate and tRNA carrier.

Precipitated RNA was suspended in gel sample buffer (50% formamide, 1× running buffer, 2.2 M formaldehyde), heated to 60°C for 5 min, and run on 1.2% agarose gels containing 2.2 M formaldehyde. Running buffer was 20 mM MOPS (morpholinepropanesulfonic acid), pH 7–1 mM EDTA–5 mM sodium acetate. Gels were run in 1× buffer plus 2.2 M formaldehyde. After electrophoresis, gels were soaked once briefly in water and then in 20× SSC for 30 min. RNA was transferred to nitrocellulose filters in 20× SSC.

TK extraction. Cells to be assayed for TK activity were harvested by a modification of the method of Johnson et al. (13). Two 100-mm plates of confluent cells were washed with cold PBS and taken up with rubber policemen in 1 ml of PBS per plate. The cells were pooled, pelleted, and suspended in 200 μl of NonidetP-40 (NP40) reagent (50 mM Tris-hydrochloride [pH 8.0], 3.6 mM β-mercaptoethanol, 0.5% NP40). The lysed suspension was vortexed, the nuclei were pelleted, and the supernatants were frozen and stored at −70°C in two 100-μl aliquots.

TK assay. TK activity was determined by a modification of the method of Ives et al. (11) and Johnson et al. (13). 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 50 mM Tris-hydrochloride (pH 8.0), 15 mM NaF, 3.6 mM β-mercaptoethanol, 5 mM ATP, 2.5 mM $MgCl_2$, 0.08 mM unlabeled thymidine, and 50 μCi of [^{3}H]thymidine (specific activity, 20 Ci/mmol) per ml. The reaction mix was incubated at 37°C for 10, 20, or 30 min and stopped by immersing for 2 to 3 min in a boiling-water bath. Control reactions without ATP were included at zero time and 30 min. Ten- or 20-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 = zero; and for a total count of [^{3}H]thymidine available; +ATP, t = 30 min, without washing. The filters were dried, washed twice in 1 mM ammonium formate and once in methanol, and dried. The disks were then placed in scintillation vials, and dTMP product was eluted by adding 1 ml of 0.1 M HCl–0.2 M KCl and shaking for 20 to 30 min. A 10-ml portion of liquid scintillation fluid was added per vial, gently shaken for 2 to 4 h, and counted for ^{3}H.

TK activities are expressed in nanomoles of deoxythymidine converted to dTMP per minute per microgram of extract protein:

$$\text{TK units} = [(\text{percent conversion})(6.6\ \text{nmol of deoxythymidine in 80 } \mu\text{l of reaction mixture})]/[(\text{reaction time})(\text{micrograms of protein in reaction})]$$

$$\text{Percent conversion} = [(\text{counts per minute of +ATP at t = 30 min} - \text{counts per minute of −ATP at t = zero})/(\text{counts per minute of unwashed control} - \text{background})] \times 100$$

The protein concentrations in the extracts were determined by both the Bradford (4) and the Lowry (23) protein assays, using a standard curve of bovine serum albumin in each case. In the 8- to 40-μg range of protein, NP40 (nonionic detergent) did not interfere with the Lowry reaction. 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 determine the specific activity of DNA, confluent cells on 100-mm plates were labeled in 1 ml of media with 1 μCi of [^{3}H]deoxythymidine and 4×10^{-7} M uridine for 1 h. Labeled cells were then washed with PBS, trypsinized in 2 ml, spun down, and suspended in 0.5 ml of PBS. Cells were counted on a hemacytometer at this point. Two 100-μl aliquots were trichloroacetic acid precipitated onto fiber glass filters with 5% trichloroacetic acid, washed with ethanol, dried, and counted for ^{3}H in 10 ml of aqueous liquid scintillation fluor. The remaining 300 μl of the PBS suspension of cells was assayed for DNA content by the

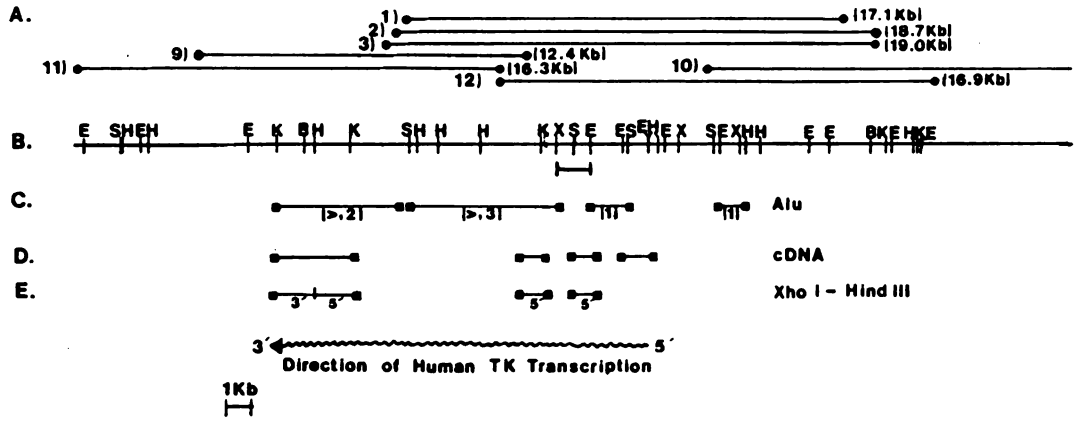

FIG. 1. Restriction map of the human TK locus. (A) Seven overlapping clones from the human TK locus were mapped by restriction enzyme/blot hybridization analysis. The relative positions of these seven clones are shown. Cotransfection of clones 1 + 11, 1 + 9, and 11 + 12 gives rise to TK$^+$ colonies. (B) Restriction enzyme map of the human TK locus. Enzymes used were: E, *Eco*RI; S, *Sst*I; H, *Hin*dIII; K, *Kpn*I; B, *Bam*HI; X, *Xho*I. The 1.4-kb *Xho*I-*Eco*RI fragment used as a 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 *Xho*I-*Hin*dIII fragment, and the 3' probe was a 0.52-kb *Xho*I-*Hin*dIII fragment. The *Xho*I sites in both cases were located in the vector DNA.

diphenylamine colorimetric assay (7). The cells were incubated for at least 30 min in 0.1% SDS–0.1 mg of proteinase K per ml at 37°C, ethanol precipitated, and suspended in TE before the assay was performed.

Radiolabeling of proteins. Cells were labeled in methionine-free media with [^{35}S]methionine at time points every 4 h postinfection. After a 45-min incubation period, cells were washed twice in ice-cold PBS and once in T-antigen wash buffer (0.137 M NaCl, 20 mM Tris-hydrochloride [pH 9.0], 1× Ca^{2+}-Mg^+ salts). Cells were lysed on the plate by the addition of 1 ml of extraction buffer (T-antigen wash, 10% glycerol, 1% NP40, 1 mM phenylmethylsulfonyl fluoride) and incubated for 20 min at 4°C. Cells were scraped from the plate and centrifuged, and the supernatants were stored at −70°C.

Immunoprecipitation. The lysate (1 ml) was added to 80 μl of a 50% suspension of protein A-Sepharose. Anti-T-antigen antisera were added, and this mixture was kept on ice and vortexed every 5 min for a 30-min period. The Sepharose-antibody-protein complex was centrifuged in a microcentrifuge, and the pellet was washed once with PBS, twice with wash buffer (0.5 M $LiCl_2$, 100 mM Tris-hydrochloride [pH 6.8]), twice with 1% deoxycholate–1% NP40 in Tris-buffered saline, and finally twice in PBS. The final pellet was dried, and 50 μl of protein sample buffer was added.

SDS-polyacrylamide gel electrophoresis. Samples were heated to 100°C for 3 min and spun in a microcentrifuge. The supernatants were resolved by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel by the procedure of Laemmli (18). The gel was stained, destained, fluorographed, dried, and exposed to X-ray film at −70°C.

RESULTS

Molecular cloning and sequence organization of the human TK gene. The human TK gene was isolated by using an experimental protocol similar to one used by other investigators (5, 22). Briefly, total human DNA from a transformed cell line, GM638, was used to transfect murine LTK$^-$ cells to a TK$^+$ phenotype. Genomic DNA was prepared from cell lines derived from these primary transformants and used to again transfect LTK$^-$ cells to a TK$^+$ phenotype. Genomic DNA was prepared from cell lines derived from these secondary transformants and examined by blot-hybridization. The radioactive probe used to detect the human DNA in these cell lines was Blur-8, a cloned member of the human Alu family of repeats (12). DNAs from a total of five different secondary transformants derived from the two different primary transformants were digested with either *Bam*HI or *Eco*RI, and the pattern of hybridization to the Blur-8 probe was examined. All five cell lines contained one to four Alu-hybridizing fragments ranging in length from 5,000 to 20,000 base pairs (data not shown). Identical patterns of hybridization were seen to Blur-11, another cloned Alu repeat, but not to a pBR322 control.

Cell line B4, which contains one strong Alu-hybridizing *Bam*HI fragment of approximately 15 kb, was chosen as a source of DNA for molecular cloning of the TK gene. B4 DNA was partially digested with *Sau*3A and cloned into λ Charon 30 as described in Materials and Methods. A library of approximately 10^6 phage was screened with the Blur-8 probe, and seven overlapping phage from the human TK locus were isolated. Endonuclease cleavage maps of the seven λ Charon 30-human clones were determined, and the combination gives a restriction enzyme map of a 40-kb stretch of DNA. This map (except that portion unique to λ clone 10), along with the extent and length of each human TK clone insert, is shown in Fig. 1. That it is an accurate representation of the human TK gene within the secondary transformant B4 genome was confirmed by Southern blot analysis of B4-LTK$^+$ genomic DNA (data not shown). Many similarities exist between this map of a twice-transfected GM638 TK gene and the published restriction endonuclease maps of other human TK gene isolates: the HeLa TK gene

(5, 22) and the placental TK gene (19). A cDNA clone able to express TK activity (see below) was labeled with ^{32}P and hybridized to a blot of a gel containing restriction enzyme digests of λ clones 1, 9, and 11. The resulting pattern of cDNA hybridization, shown schematically in Fig. 1, indicates a minimum of three intervening sequences in the human TK gene and, assuming that pHuTK-c7 contains nearly all of the mature TK mRNA sequence, a maximum gene length of approximately 14.5 kb.

The seven λ clones from the human TK locus were transfected individually and in pairwise combinations 9 + 11, 1 + 9, 1 + 11, and 11 + 12 onto Rat-3 TK^- cells (29), and TK^+ transfectants were selected. Although no one clone contains the intact gene (i.e., gives rise to TK^+ colonies), overlapping phage 1 + 9, 1 + 11, and 11 + 12 do give rise to TK^+ colonies. We interpret these results to show that the overlapping clones can recombine during transfection to generate an intact gene, as has been suggested by others (22). One interesting aspect of our results is that λ phage 11 + 12, which according to our restriction mapping contain little or no overlapping sequence, do give rise to TK^+ colonies upon cotransfection. We suggest that this may be due to recombination between different Alu repeats within the large intron in the TK gene. These results indicate that we have cloned the entire human TK locus on overlapping clones.

Isolation of a functional human TK cDNA clone. A human cDNA library was screened for the presence of cDNA clones homologous to the TK gene. The library used was a gift of H. Okayama and P. Berg; it had been constructed by using poly(A^+) RNA from log-phase GM639 cells, an SV40-transformed human cell line, by a method favoring full-length cDNA copies of mRNAs (28). In addition, the cDNAs were linked to an expression vector containing, along with the pBR322 replication origin and β-lactamase gene, several SV40 transcriptional control sequences, the early gene promoter, a late gene splice donor and acceptor, and a polyadenylation signal, which allow expression of the cDNA insert when the clones are introduced into mammalian cells (Okayama and Berg, personal communication).

In a screening of 2.4×10^6 bacterial colonies, over 200 positive clones were found. Of these, 20 were picked, and of these 16 were successfully isolated. Two clones containing the longest cDNA inserts were tested for function by transfection into Rat-2 TK^- cells, and both were able to stably transform the cells to a TK^+ phenotype at a frequency equal to or greater than that of a chicken TK gene clone (29) used as a control. Since the 1,500-base pair size of the cDNA insert of pHuTK-c7 correlates well with the known TK mRNA size, and since the clone expresses TK activity in mammalian cells at a high level, we believe this cDNA isolate must contain nearly all, if not all, of the sequence of the mature TK mRNA.

The known transcriptional polarity of the cDNA insert with respect to its expression vector allowed the direction of transcription of the human TK gene to be readily determined, and these results are also illustrated in Fig. 1. Since this work was initiated, the DNA sequence of a similar human TK cDNA clone has been reported (6).

Viral induction of simian TK. Infection of confluent mammalian tissue culture cells by the papovaviruses SV40 and polyoma causes several changes in cell metabolism, including the induction of cell DNA synthesis and many of the enzymes involved in DNA metabolism. We have studied the induction of TK activity at a molecular level, using a DNA probe from within the TK coding region. A subclone containing the 1.4-kb *Xho*I-*Eco*RI fragment underlined in Fig. 1 (pHuTK 1.4) was used as a probe for northern blot analysis.

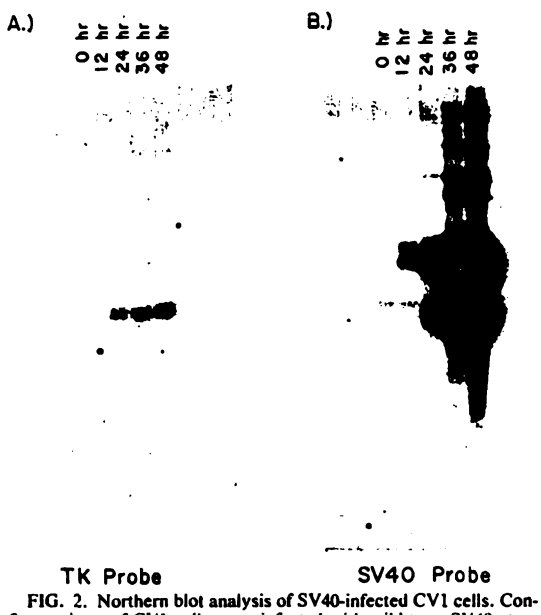

FIG. 2. Northern blot analysis of SV40-infected CV1 cells. Confluent plates of CV1 cells were infected with wild-type SV40 at an MOI of 5 at zero time. Poly(A^+) mRNA was isolated from cells at 12-h intervals after infection. RNA from equal numbers of cells was electrophoresed on 1.2% formaldehyde gels and transferred to nitrocellulose paper. Duplicate northern blots were hybridized to (A) pHuTK 1.4 probe and (B) SV40 probe.

Confluent simian CV1 cells were infected with SV40 virus at an MOI of 5, and poly(A^+) mRNA was prepared at various times from 0 to 48 h postinfection. RNA from equal numbers of cells at each time point was electrophoresed on a 1.2% formaldehyde-agarose gel, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled pHuTK 1.4 probe as described in Materials and Methods. The results of such an analysis are shown in Fig. 2A. The length of the TK mRNA is approximately 1.5 kb, as has been previously reported for human cells (21). The mRNA is barely detectable at zero time, and the first increase is seen at 24 h. Figure 2B shows a northern blot run in parallel hybridized with an SV40 probe. SV40 early and late mRNAs are apparent by 12 and 24 h postinfection, respectively. Densitometer tracings of the autoradiogram in Fig. 2A are plotted in Fig. 3 and show a final level of TK mRNA induction of approximately 15-fold at 48 h. In control experiments, mock-infected cells showed no induction within the 48-h time period (data not shown). Also shown in Fig. 3 is a plot of TK enzyme activities from cells infected in parallel with those used for the northern analysis. As can be seen, both the time course and the extent of enzyme induction are similar to that of the mRNA. Also plotted is [^3H]thymidine incorporation into DNA, indicating the onset of S phase by 24 h postinfection. It has previously been shown (10) that this incorporation represents both viral and cellular DNA synthesis occurring after infection of confluent monkey cells with SV40.

Since it has been reported that the viral protein T antigen is responsible for the observed TK induction (30), we

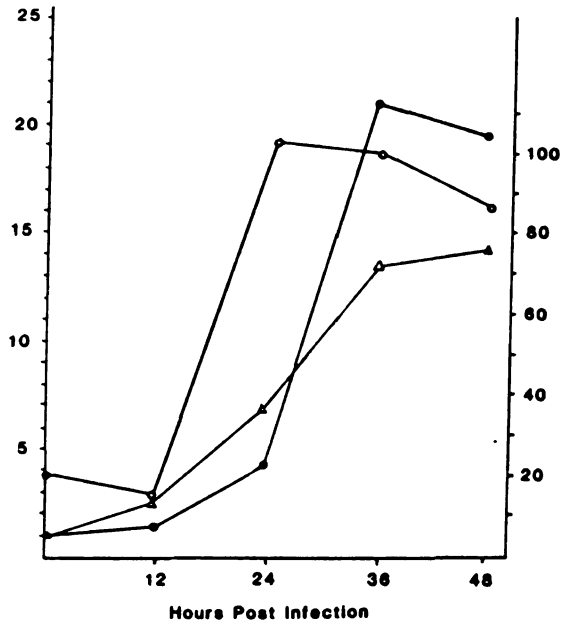

FIG. 3. SV40 induction of TK in simian CV1 cells. Confluent plates of CV1 cells were infected with SV40 at an MOI of 5 at zero time. At 12-h intervals after infection, samples were taken for TK enzyme assays, TK mRNA analysis, and pulse-labeling of total DNA. TK enzyme and mRNA analyses are plotted using the scale on the left; DNA labeling is plotted using the scale on the right. TK enzyme activity is expressed as follows: 1 unit = 1 nmol of deoxythymidine converted to dTMP/min per μg of protein. (●) TK units × 10^{-4}. TK mRNA levels were estimated from densitometer tracings of the northern gel shown in Fig. 2A. (△) 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 microgram). The first increase in specific activity seen at 24 h postinfection indicates the onset of S phase. Although DNA synthesis seems to precede TK induction, this is deceiving since the DNA synthesis measurement is done by pulse-labeling and the TK mRNA and enzyme levels are cumulative.

determined the earliest detectable TK induction relative to the presence of T antigen. A more extensive time course was performed, with time points taken every 4 h postinfection. Duplicate RNA blots were done, and one hybridized to pHuTK 1.4 probe and one hybridized to SV40 probe (Fig. 4). Figure 4A shows that TK mRNA induction may begin by 16 h and has clearly begun by 20 h. Shown in Fig. 4B is the same blot rehybridized with a c-*myc* probe, which shows approximately equal amounts of RNA at each time point except 0 and 8 h. The RNA sample at 8 h has apparently been lost during preparation. This film was scanned by a densitometer, and the amount of TK mRNA relative to c-*myc* mRNA was determined. These results are shown in Fig. 5. It has previously been reported that the levels of c-*myc* mRNA increase very quickly within 2 h of serum stimulation of mouse fibroblasts (8, 15). The levels of c-*myc* mRNA also appear to increase between 0 and 4 h after infection of CV1 cells with SV40 in this experiment. After the 4-h time point, the c-*myc* mRNA level is relatively constant over the course of this experiment, allowing us to say that the increase in TK mRNA levels is not a result of a general increase in the level of cellular mRNA. Figure 4C shows a parallel northern blot hybridized with SV40 probe. In this experiment, early SV40 mRNA appears by 12 h and late mRNA appears by 16 h. In other experiments, SV40 early mRNA has been detected at 8 h postinfection. Also shown in lane 1 is poly(A$^+$) mRNA from COS7 cells, a

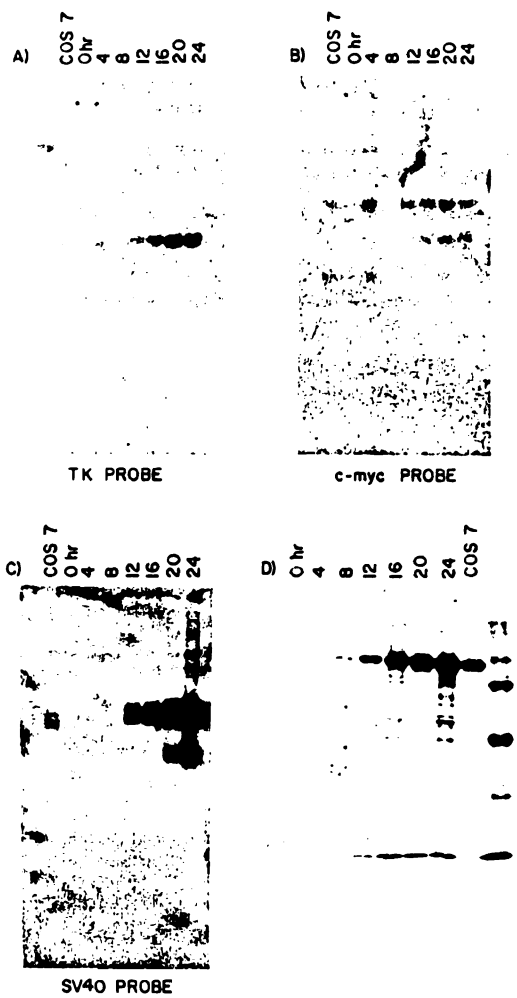

FIG. 4. Twenty-four-hour time course of SV40-infected CV1 cells. Confluent CV1 cells were infected with SV40 as described in the legend to Fig. 3. Plates were harvested at 4-h intervals for mRNA and T-antigen analyses. Also included as a control are RNA and protein from COS7 cells, which constitutively 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.

simian cell line transformed by SV40. This cell line contains only the SV40 early mRNA.

To look directly at SV40 T antigen, SDS-polyacrylamide gels have been run. Cells infected in parallel with those analyzed in Fig. 4 were labeled with [^{35}S]methionine at 4-h intervals after infection. The proteins were immunoprecipitated with anti-T-antigen antibody and electrophoresed on a 10% polyacrylamide gel. The results (Fig. 4D) show that large T antigen first appears at 8 postinfection, approximately 8 to 12 h before the increase in TK mRNA levels.

Since one of our long-term goals is to determine the mechanism by which SV40 induces arrested cells to re-enter the cell cycle, we would like to determine whether the same events occur during G1 in virally infected and serum-stimulated cells. To begin to approach this question, we have compared TK and c-*myc* mRNA levels in cells induced in parallel by either serum stimulation or viral infection. Confluent CV1 cells were treated either by the addition of fresh media containing 10% serum or by infection with SV40 virus (MOI = 5). Poly(A$^+$) RNA was isolated at various times (0, 6, 12, 24, 36, and 48 h) after treatment and analyzed for the levels of TK and c-*myc* transcripts. Since c-*myc* mRNA levels increase soon after serum stimulation of mouse fibroblasts, we have also hybridized with a β-2 microglobulin probe as an additional internal control. (A human β-2 microglobulin clone was a gift of Hsiu-Ching Chang.) The results of this experiment are shown in Fig. 6.

To analyze this data, we have compared the levels of TK and c-*myc* mRNA to the β-2 microglobulin internal control. In serum-stimulated cells, TK mRNA levels are significantly increased by 12 h after treatment and decrease somewhat by 48 h. The earliest time of the induction (~12 h) coincides with the onset of DNA synthesis in these cells, and the time course of mRNA induction parallels that of enzyme induction (data not shown). In virally infected cells, the level of TK mRNA does not significantly increase until 24 h postinfection, but again the increase coincides with the onset of DNA synthesis. We believe that this delay is due to the 8- to 12-h time period required for the virus to infect the cells and express the early viral proteins (Fig. 4). Once this viral induction of TK begins, the magnitude of the response exceeds that seen in serum-stimulated cells.

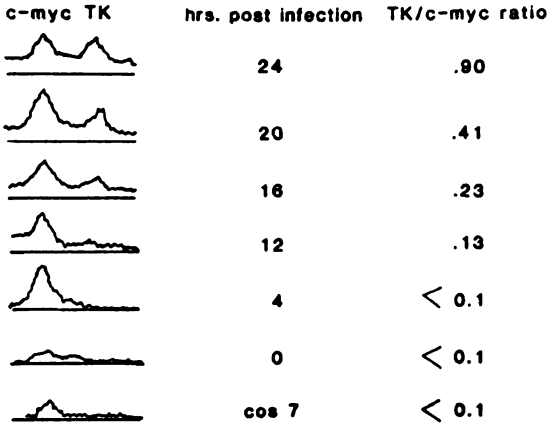

FIG. 5. Densitometer tracing of autoradiogram in Fig. 4B. The ratio of TK/c-*myc* RNA is shown.

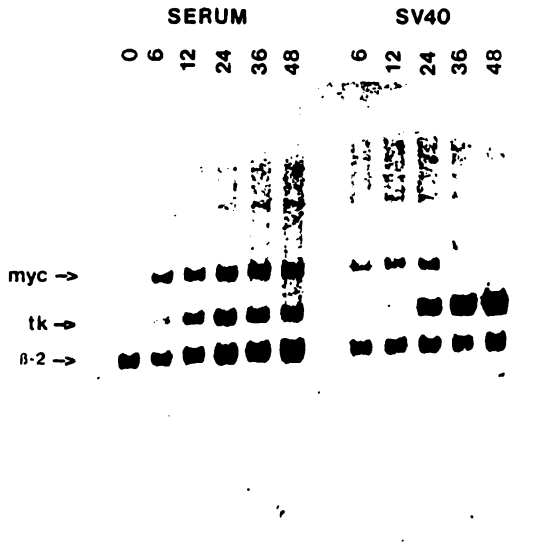

FIG. 6. Comparison of serum and SV40 induction of CV1 cells. Confluent CV1 cells were induced to reenter the cell cycle either by addition of fresh media containing 10% serum or by infection with SV40 (MOI = 5). At various times (6, 12, 24, 36, and 48 h) after treatment, poly(A$^+$) RNA was prepared,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

The level of c-*myc* mRNA appears to increase approximately threefold by 6 h after both serum stimulation and viral infection. In the case of serum-stimulated cells this level remains relatively constant, although it may decrease somewhat by the 48-h time point. In the case of the SV40-infected cells we see a dramatic disappearance of the c-*myc* mRNA at the 36- and 48-h time points.

DISCUSSION

We report the molecular cloning of the human TK gene and its use in monitoring TK mRNA levels in both serum-stimulated and virally infected simian CV1 cells. This gene has been isolated by others (4, 14, 17), and our mapping results are largely in agreement with theirs. Although the restriction maps of the 5′ ends of the gene(s) are virtually identical, considerable differences are seen within the large intron and at the 3′ end. Although three of four maps contain a *Bam*HI site at the 3′ end of the gene, the fourth map does not (4). These differences may reflect true polymorphisms in the gene or may be an artifact of the rounds of transfections used to isolate the genes. Our isolation of a functional cDNA clone has allowed us to determine the orientation of the gene, to estimate a minimum number of introns, and to localize the approximate positions of the 5′ and 3′ ends.

It has been reported that papovavirus infection or serum stimulation of contact-inhibited cells increases the levels of TK enzyme activity by approximately 20-fold (11, 12). Using

a subclone from within the TK mRNA coding region, we have examined the steady-state levels of TK mRNA in resting and stimulated simian CV1 cells. Our results show that the length of the simian TK mRNA is approximately 1.5 kb, the same size as has been reported for the human mRNA. In contact-inhibited CV1 cells, the level of the RNA is quite low, and in some experiments it is barely detectable. After infection with SV40 the levels of RNA increase, reaching a maximum of about 15- to 20-fold by 48 h postinfection. The first induction detected occurs at between 16 and 20 h postinfection and coincides with the onset of DNA synthesis. It is also interesting to note that the first accumulation of TK mRNA is not detectable until 8 to 12 h after the appearance of T antigen, which has been implicated as the viral protein necessary for induction. That this time interval is somewhat longer than that reported previously is due to our ability to detect T antigen earlier than previous investigators. In the long term, we would like to determine whether T antigen is acting directly to induce the synthesis of TK, or whether T antigen acts indirectly by initiating other events during those 8 to 12 h which in turn induce TK activity. In the case of serum-stimulated cells, induction of TK mRNA occurs by 12 h after treatment and again coincides with the onset of DNA synthesis. Thus it appears that the time interval between either serum or T-antigen stimulation of CV1 cells and the onset of DNA synthesis is approximately 8 to 12 h. The TK mRNA seen after induction seems to be identical in size to that seen in untreated cells within the limits of resolution of these gels. Of course, small changes in molecular weights or 5′ and 3′ ends would not be detected in these experiments.

These results indicate that at least most of the induction of TK enzyme activity can be accounted for by increases in the steady-state levels of TK mRNA. These changes in mRNA levels may be due to control at several steps during RNA synthesis, including transcription, processing, and RNA stability. Experiments studying cell cycle regulation of dihydrofolate reductase (DHFR) indicate that the increase in DHFR mRNA levels seen during S phase is due to differences in mRNA stability (20). Also interesting in this regard is the result that the DNA sequences required for DHFR regulation map to the 3′ end of the gene (14). In experiments studying regulation of the chicken TK gene in differentiating muscle cells, it has been shown that the sequences required for regulation are localized within the body of the gene itself (26, 27). Thus it is possible that cell cycle-controlled genes such as TK and DHFR are not regulated (at least exclusively) at the level of transcription. Experiments are currently in progress to measure the rates of TK transcription before and after SV40 infection and to map the DNA sequences required for cell cycle regulation of TK.

We have also presented preliminary evidence that c-*myc* mRNA levels increase two to threefold within 6 h of both serum stimulation and SV40 infection of resting CV1 cells. This result is surprising, since we have not been able to detect any early viral mRNA or protein synthesis by this time. Since the magnitude of induction is quite low, further experiments will be required to determine whether this effect occurs reproducibly, but if it does it implies that factors other than early viral gene expression are responsible for the increase in c-*myc* mRNA levels. This viral induction of c-*myc* is not sufficient to cause the cells to progress to S phase, since the synthesis of T antigen at ~8 h postinfection is necessary for induction of both TK and DNA synthesis (30). One marked difference between the serum- and virus-stimulated cells is the fact that c-*myc* mRNA is absent in virally infected cells at 36 and 48 h. We are currently investigating the mechanism of this shutoff.

In summary, we have shown that TK mRNA levels increase dramatically in both serum-stimulated and SV40-infected CV1 cells. Although this induction is delayed by approximately 8 to 12 h in the SV40-infected cells, we believe that this delay is due to the fact that it takes 8 to 12 h for the virus to infect cells and express its early proteins. In both cases, TK induction parallels the entry of cells into S phase, but the TK mRNA accumulates to a higher level in the virus-infected cells.

ACKNOWLEDGMENTS

We thank Michael Botchan, in whose lab this work was initiated, for helpful advice and discussions.

This work was supported by Public Health Service grant CA30490 to M. Botchan from the National Institutes of Health and by NRSA Fellowship CA06928-02 and Public Health Service grant CA37144-01 to S. Conrad from the National Institutes of Health.

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APPENDIX II

APPENDIX II

TK Transcriptional Regulation in SV40 Infected Cells

The final transcription experiment in Chapter 2 involved a nuclear run on analysis of RNA made just before and during DNA synthesis in SV40 infected CV-1 cells. Just prior to the time that this experiment was done, we had subcloned our human TK cDNA clone from an Sp64 vector (Pharmacia) into a T7/T3 vector (Bethesda Research Laboratories) for the purpose of saving money on the cost of the RNA polymerase required. The new construct transcribed well, and the experiment was performed with this construct and the previously used $\beta_2$microglobulin gene still in an Sp64 vector. Following publication of the chapter 2 manuscript, I was informed by Dr. David Shalloway at the Pennsylvania State University that a sequence had been found between the T7 promoter and its polylinker which specifically binds to 28s Ribosomal RNA. This sequence has not been found in Sp6 vectors, so I therefore have repeated the last experiment from Chapter 2 using my original constructs in the Sp6 vector. The experiment was performed with both high passage and low passage CV-1 cells, yet the

results were quite reproducible. Figure 1 shows an autoradiogram of the experiment from high passage cells, and figure 2 shows a graphic representation of both experiments. It can be plainly seen that there is a 3-3.5 fold transcriptional increase in SV40 infected cells by 14-16 hours post infection.

Figure 1 Nuclear transription assays performed at one hour intervals prior to and during the onset of DNA synthesis in SV40 infected CV-1 cells. Nuclei were prepared at the times indicated after viral infection and used for transcription assays as described in Chapter 2. 2.0×10^6 cpm of labelled RNA was added to each hybridization. This autoradiogram shows the results of hybridization to nitrocellulose filters containing cRNAs and cDNAs of interest.

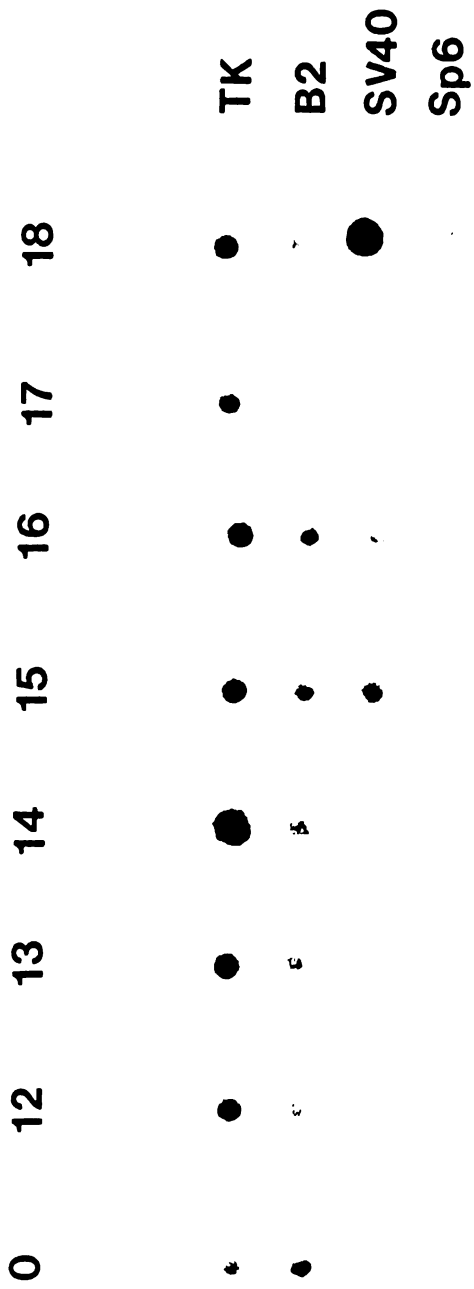

FIGURE 1

Figure 2 Graphic analysis of the results in figure 1, as well as the results of the repeat experiment. Plotted are values of TK/β-2 microglobulin (as described in Chapter 2).

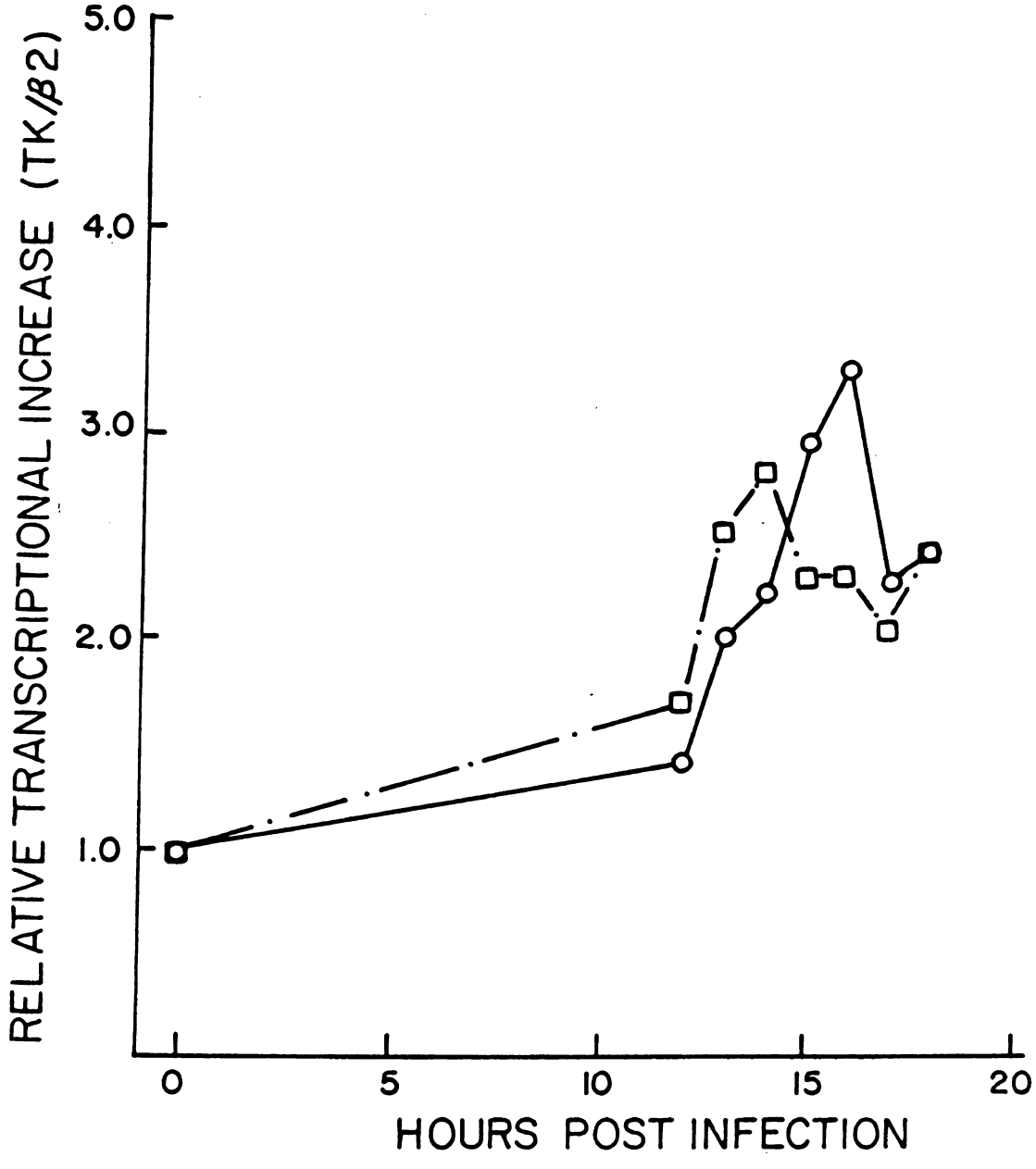
RELATIVE TRANSCRIPTIONAL INCREASE (TK/β2)
5.0
4.0
3.0
2.0
1.0
0
5
10
15
20
HOURS POST INFECTION

FIGURE 2