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Induction of Cellular Thymidine Kinase Occurs at the mRNA Level

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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

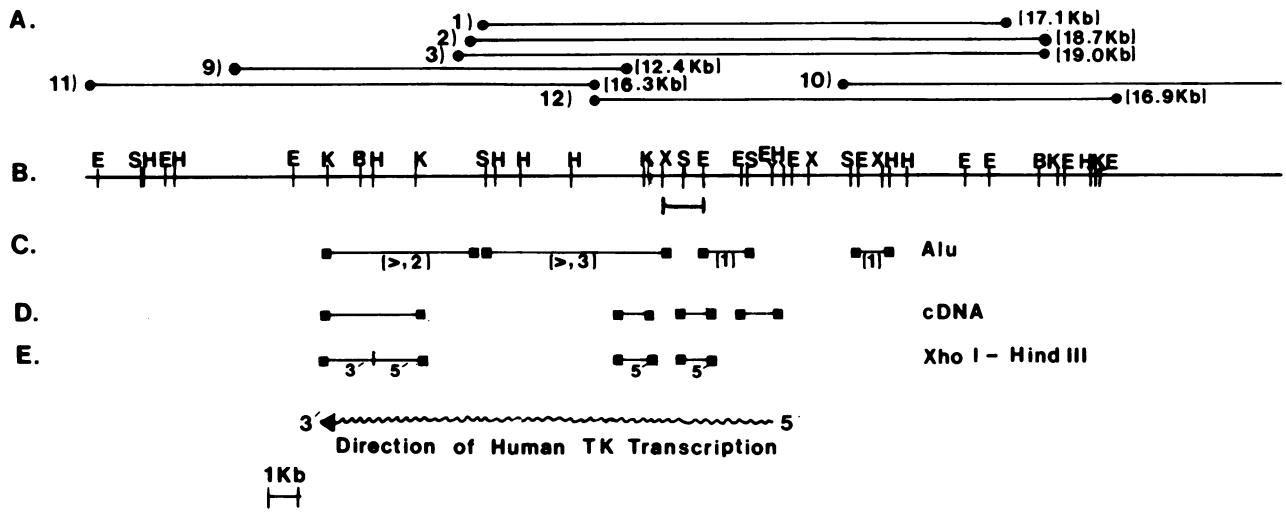

FIG. 1. Restriction map of the human TK locus. (A) Seven overlapping clones from the human TK locus were mapped by restriction enzyme/blot hybridization analysis. The relative positions of these seven clones are shown. Cotransfection of clones 1 + 11, 1 + 9, and 11 + 12 gives rise to TK^+ colonies. (B) 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.

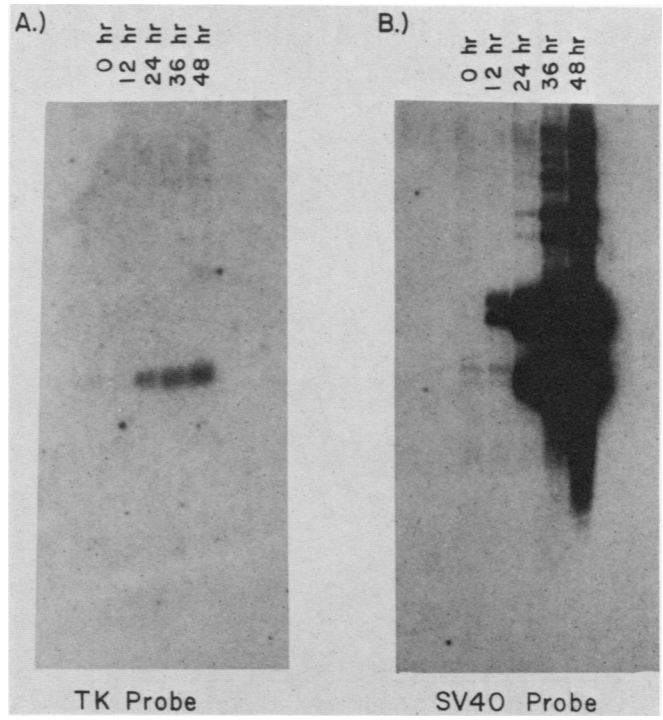

FIG. 2. Northern blot analysis of SV40-infected CV1 cells. Confluent plates of CV1 cells were infected with wild-type SV40 at an MOI of 5 at zero time. Poly(A^+) mRNA was isolated from cells at 12-h intervals after infection. RNA from equal numbers of cells was electrophoresed on 1.2% formaldehyde gels 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

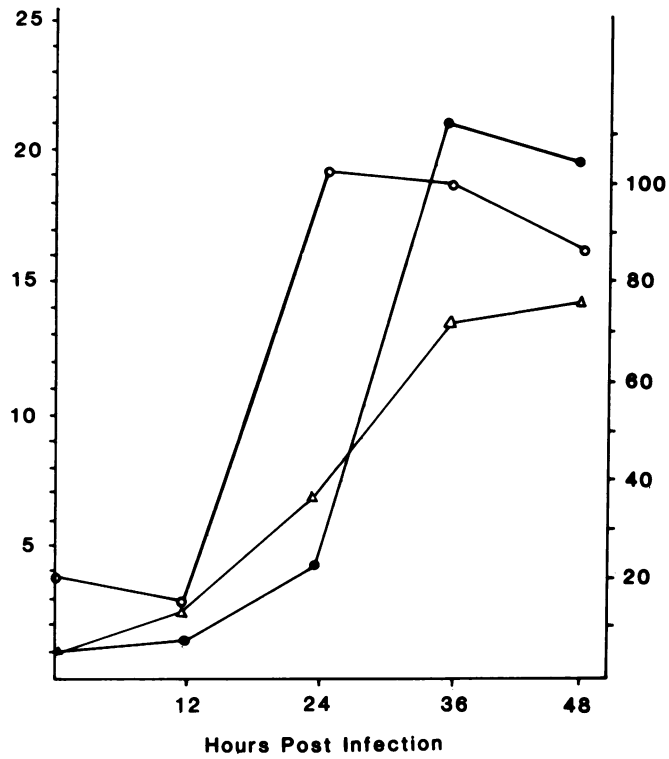

FIG. 3. SV40 induction of TK in simian CV1 cells. Confluent plates of CV1 cells were infected with SV40 at an MOI of 5 at zero time. At 12-h intervals after infection, samples were taken for TK enzyme assays, TK mRNA analysis, and pulse-labeling of total DNA. TK enzyme and mRNA analyses are plotted using 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

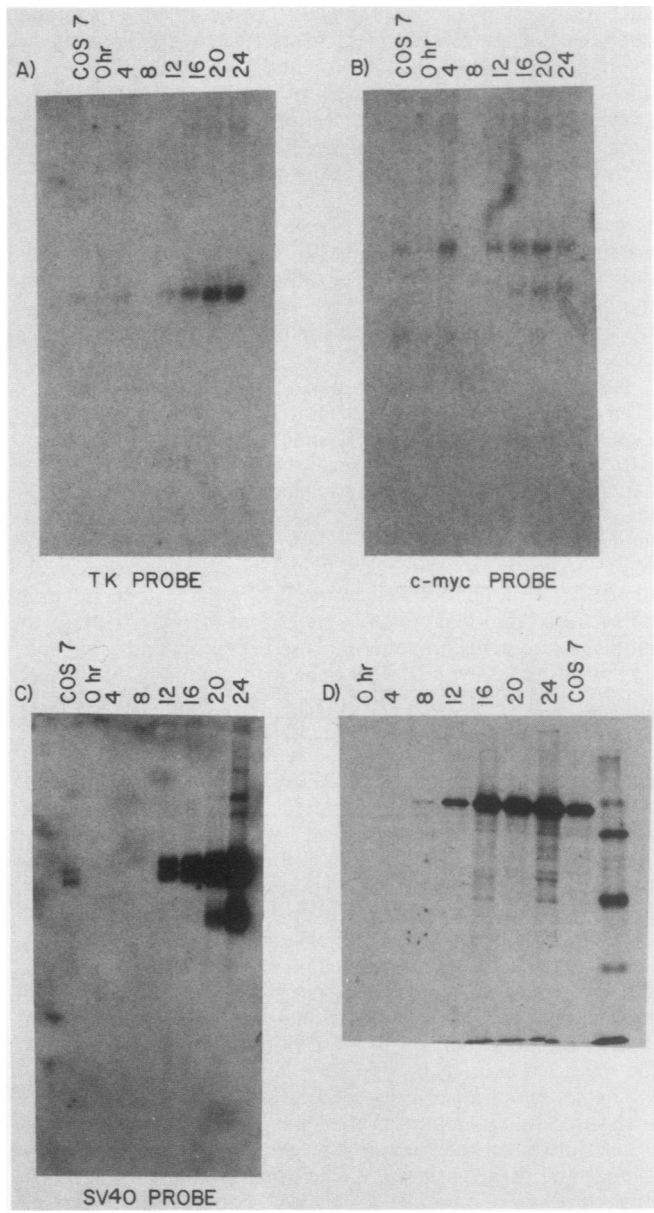

FIG. 4. Twenty-four-hour time course of SV40-infected CV1 cells. Confluent CV1 cells were infected with SV40 as described in the legend to Fig. 3. Plates were harvested at 4-h intervals for mRNA and T-antigen analyses. Also included as a control are RNA and protein from COS7 cells, which constitutively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

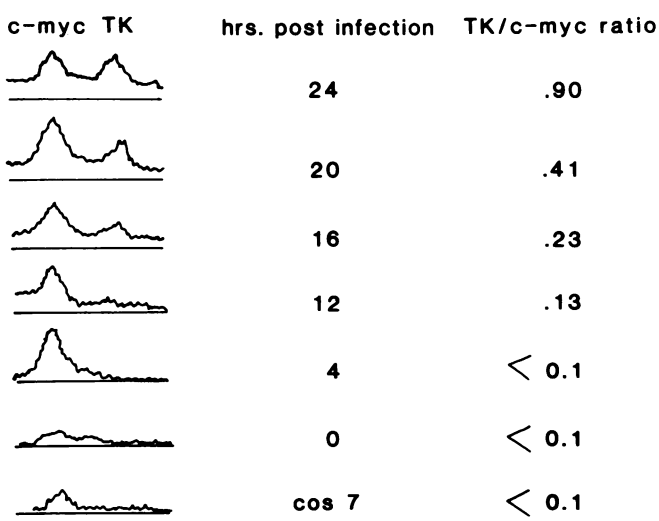

FIG. 5. Densitometer tracing of autoradiogram in Fig. 4B. The ratio of TK/c-*myc* RNA is shown.

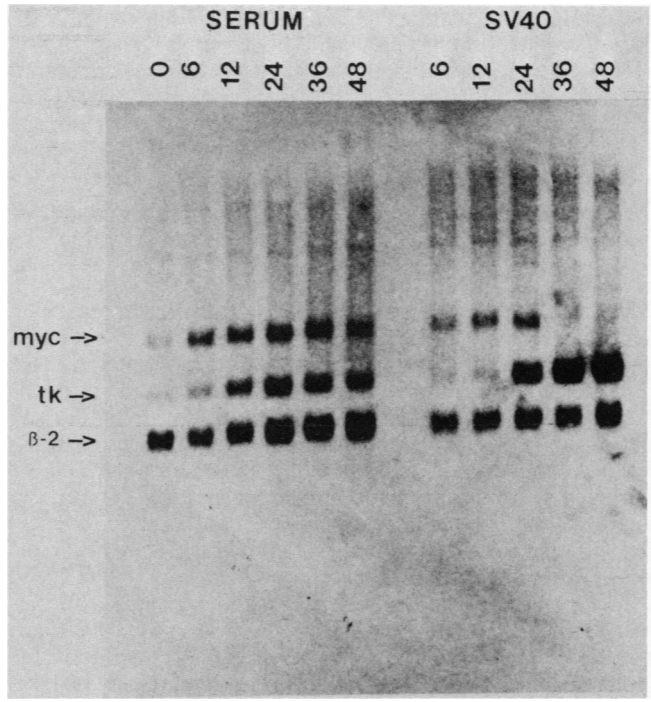

FIG. 6. Comparison of serum and SV40 induction of CV1 cells. Confluent CV1 cells were induced to reenter the cell cycle either by addition of fresh media containing 10% serum or by infection with SV40 (MOI = 5). At various times (6, 12, 24, 36, and 48 h) after treatment, poly(A$^+$) RNA was prepared, 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.

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