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Regulation of the Cellular Thymidine Kinase Gene Promoter in Simian Virus 40-Infected Cells

HOLGER H. ROEHL,† MICHELLE M. ANDERSON, CHRISTINE S. MEHIGH,‡ AND SUSAN E. CONRAD*

Department of Microbiology, Michigan State University, East Lansing, Michigan 48824-1101

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We examined the regulation of the cellular thymidine kinase (TK) gene promoter in simian virus 40 (SV40)-infected simian CV1 cells. Nuclear run-on transcription assays demonstrated a three- to fourfold increase in the rate of transcription of the endogenous gene at 14 to 16 h following viral infection. In addition, hybrid genes containing the human TK promoter linked to the bacterial neomycin resistance gene were induced by SV40 in stably transfected cells, indicating that promoter sequences are sufficient to confer viral regulation. Analysis of human TK promoter deletion mutants indicated that sequences localized between −67 and +30 bp relative to the transcriptional initiation site are sufficient to confer regulation on SV40-infected cells. These sequence elements are distinct from those required for serum induction, which were previously localized to the region between −135 and −67. These results suggest that SV40 activates novel cellular pathways that are not activated by serum stimulation of quiescent cells.

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Regulation of proliferation in mammalian cells is complex and occurs at several points in the cell cycle. One critical control point, termed the restriction or R point, occurs late in G_1 (34). Once cells pass R, they are committed to proceed to the S phase. To investigate the events that regulate the transition from G_1 to S, we and others have studied the expression of genes that are induced as cells leave G_1 and enter the S phase. This class of G_1-S-phase-regulated genes includes several that code for enzymes involved in DNA synthesis, including those for thymidine kinase (TK) (4, 17), thymidylate synthase (32), and dihydrofolate reductase (16). Of these, TK is one of the most highly induced and has been widely used as a model G_1-S-regulated gene. When quiescent, serum-starved cells are induced to re-enter the cell cycle by mitogenic treatments, such as addition of fresh serum or infection with simian virus 40 (SV40), TK enzyme levels are low throughout G_1, increase sharply at the G_1-S boundary, and remain elevated throughout S and G_2 (4, 17, 23). Enzyme levels are also highly regulated in continuously cycling cells, being low in G_1, increasing at G_1-S, and remaining high during G_2 and into M (39). The molecular basis for the regulation of TK enzyme levels is complex and involves both transcriptional and posttranscriptional events. In the case of mitogenically stimulated cells, the amount of enzyme is determined largely by the mRNA level, which is extremely low in resting cells and highly induced at the G_1-S interface (43). In continuously cycling cells, however, TK mRNA levels fluctuate only 2- to 3-fold during the cell cycle, while enzyme levels fluctuate 10- to 20-fold (39). Regulation of the enzyme is therefore controlled primarily by posttranscriptional events in cycling cells.

Several lines of evidence indicate that the increase in TK mRNA at G_1-S in serum-stimulated cells is due to transcriptional regulation. By using nuclear run-on transcription assays, we demonstrated that the rate of TK gene transcription increases six- to sevenfold at the G_1-S boundary in serum-stimulated CV1 cells (42). This result has been confirmed by others with different cell types (5, 28). In addition, we and others have shown that a human TK promoter fragment containing 444 bp upstream and 30 bp downstream of the transcriptional start site is sufficient to confer regulation on a heterologous reporter gene during serum stimulation (22, 29, 37, 44). The *cis*-acting DNA sequences required for human TK promoter activity and regulation by serum have also been investigated by using hybrid genes. By analyzing a series of 5′ promoter deletion mutants, we demonstrated that sequences between −135 and −67 bp are required for G_1-S-phase regulation of the promoter during serum stimulation (37). This result was confirmed by Kim et al. (20, 22), who also reported that sequences from −135 to −67 bp are sufficient to confer G_1-S-phase regulation on a heterologous promoter. Thus, this region is both necessary and sufficient for regulation in serum-stimulated cells.

The precise sequence elements involved in regulation of the human TK promoter in serum-stimulated cells are unknown. Analysis of the DNA sequence between −135 and −67 reveals several potential regulatory motifs, including one inverted CCAAT sequence, an Sp1-binding site, and several elements with homology to E2F-binding sites (Fig. 1). Mutational analyses have shown that at least one of the two CCAAT elements within the complete promoter is required for maximal levels of expression (2), and in one report a cell cycle-specific interaction between nuclear proteins and a CCAAT element was detected (24). However, the role of the CCAAT elements in G_1-S regulation has not been established by mutational analyses. Protein binding to the Sp1 site at −116 has also been demonstrated (1a), but the role of this or other putative Sp1-binding sites in promoter function has not been tested. Recently, it was reported that replacement of sequences from −88 to −113 with a random DNA sequence abolishes G_1-S-phase regulation without affecting basal expression (21). This result is intriguing, since the region contains two potential binding sites for transcription factor E2F, which has been proposed to be involved in the induction of genes at G_1-S (33). It also contains two regions of homology to factor Yi, which has been implicated in regulation of the murine TK promoter and may be related to E2F (7).

* Corresponding author.
† Present address: Department of Microbiology, University of California at Irvine, Irvine, CA 92717.
‡ Present address: Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824.

A

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

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

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

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

B

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

FIG. 1. (A) Sequence of the human TK promoter from −135 to +30 bp relative to the major transcription initiation site (25). (B) Positions and sequences of a number of putative transcription factor-binding sites (7, 9, 36). Nucleotides that do not match the consensus sequence are in lowercase. Rev indicates that the consensus sequence is on the strand opposite the one shown.

Although transcription of the gene for TK clearly increases when quiescent cells are mitogenically stimulated by serum addition, the situation in SV40-infected cells is less clear. Infection of serum-starved cells with SV40 induces them to re-enter the cell cycle and progress to the S phase; thus, SV40 acts as a potent mitogen (12). The viral protein responsible for both induction of cell DNA synthesis and induction of TK activity is large T antigen (35). Large T antigen is a multifunctional protein that possesses a number of biochemical activities that might be related to its mitogenic function (10). These include the ability to bind to and functionally inactivate cellular proteins pRb (6) and p53 (27), the ability to *trans*-activate promoters (19, 48), and the ability to bind DNA (18). We previously demonstrated that endogenous TK mRNA levels are more highly induced in SV40-infected than in serum-stimulated CV1 cells (43) but were unable to detect induction of the gene by nuclear run-on transcription assays (42). In the present study, we re-examined regulation of the TK promoter in SV40-infected cells and compared it with regulation during serum stimulation. By nuclear run-on assays with CV1 cells, we detected a modest (three- to fourfold) increase in transcription of the endogenous TK gene. We also examined the activity of a series of hybrid genes containing the bacterial neomycin resistance gene (*neo*) linked to human TK promoter fragments in stably transfected cells. These experiments demonstrated that both a promoter fragment containing only 67 bp of upstream sequences (plus 30 bp of downstream sequences) and one containing an internal deletion from −138 to −67 are regulated in SV40-infected cells but not in serum-stimulated cells. This indicates that sequences between −135 and −67, previously characterized as being required for serum regulation, are not required for SV40 induction and suggests that the two mitogenic agents activate the promoter via different, although possibly overlapping, mechanisms.

MATERIALS AND METHODS

Cell culture, serum stimulation, and SV40 infection. CV1 cells were cultured at 37°C in Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 5% calf serum (HyClone Laboratories, Logan, Utah). Stably transfected cells containing *neo* genes were maintained in medium containing 100 μg of G418 per liter. Except where noted, cells were grown in medium without G418 during serum stimulation and SV40 infection experiments. For synchronization of cells in G_0, they were grown to confluence and then serum starved for 24 h in medium containing 0.1% fetal calf serum. For serum stimulation, the low-serum medium was removed at time zero and replaced with fresh medium containing 10% serum. For SV40 infection, the low-serum medium was removed at time zero and saved. Cells were then infected with SV40 at a multiplicity of infection of 15. The virus used was purified by equilibrium centrifugation in a cesium chloride gradient. Infections were done for 1 h at 37°C in 1 ml of low-serum medium. At the end of the infection, the low-serum medium that had been removed from the cells at time zero was added to the plates.

DNA transfections. $CaPO_4$ transfections were done as described by Wigler et al. (45). Stable transfectants were selected in medium containing 400 μg of G418 per ml. After resistant colonies were clearly visible, 10 to 50 colonies per construct were pooled and expanded for analysis or single colonies were picked and propagated as clonal cell lines. After the initial selection, transfected cells were maintained in medium containing 100 μg of G418 per ml.

Plasmid constructions. Plasmids 444-Neo, 135-Neo, and 67-Neo were described previously (37). Plasmid *dl*−138/67-Neo, which contains the 444-bp TK promoter with an internal deletion of sequences from −138 to −67, was constructed via the oligonucleotide-mediated mutagenesis procedure described by Kunkel (26). A 30-base oligonucleotide containing 15-bp matches on either side of the sequence to be mutated was used in the mutagenesis. The presence of the desired deletion was confirmed by DNA sequencing.

Preparation of total RNA. Total RNA was prepared by a modification of the method of Favaloro et al. (11). Cells were washed once with phosphate-buffered saline without calcium and magnesium and lysed in 1 ml of lysis buffer (100 mM Tris-HCl [pH 7.5], 12 mM EDTA, 150 mM NaCl, 1% sodium dodecyl sulfate, 200 μg of proteinase K per ml) per 10-cm-diameter plate. Cell lysates were scraped off the plate, and cellular DNA was sheared by several passages through a 22-gauge needle. The lysate was incubated at 37°C for 45 to 60 min, and nucleic acids were extracted twice with phenol-chloroform and ethanol precipitated. The precipitate was resuspended in 400 μl of TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) containing 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 U of RNasin, and 10 U of RNase-free DNase. The samples were incubated at 37°C for 45 to 60 min, extracted

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twice with phenol-chloroform, and ethanol precipitated. The precipitated RNA was resuspended in TE, and its concentration was determined by reading the A_{260}.

Northern (RNA) blot analysis and nucleic acid hybridizations. Equal amounts of total RNA (20 μg) from all of the time points were electrophoresed in 1% agarose-formaldehyde gels containing 500 ng of ethidium bromide per ml. After electrophoresis, gels were photographed to ascertain that the same amount of RNA was loaded in each lane. RNA was transferred to nitrocellulose filters by blotting overnight. Prehybridization, hybridization, and washes after hybridization were done as described previously (37). To determine the amounts of *neo* mRNA on blots, several different film exposures were scanned on an AMBIS Image Acquisition and Analysis system. To calculate induction values, the amount of mRNA at 24 h was divided by the amount at time zero.

A ^{32}P-radiolabeled probe was prepared with the Boehringer Mannheim Random Primed DNA Labeling Kit in accordance with manufacturer specifications. The following fragments were used to probe for expression of the relevant gene: *neo*, a 916-bp *Hin*dIII-*Nco*I fragment from within the *neo* gene; SV40 early transcripts, a 1,169-bp *Hin*dIII fragment from the early region of the viral genome; TK, a 1.2-kb *Sma*I-*Bam*HI fragment from within the human TK cDNA; β-2 microglobulin, a 600-bp *Pst*I fragment from the human cDNA.

Primer extension assays. Primer extension assays were performed essentially as described by Eisenberg et al. (8). The primer was a 25-base oligonucleotide (5′CGGACTG GCTTTCTACGTGTTCCGC3′) located from +72 to +47 bp downstream of the *Hin*dIII site at the 5′ end of the *neo* gene. It was purchased from the Macromolecular Structure Facility, Michigan State University. A 100-ng sample of the primer was labeled at 37°C for 30 min by using 2 μl of [γ-^{32}P]ATP (6,000 Ci/mmol, 150 mCi/ml) and 10 U of T4 polynucleotide kinase.

Hybridization and extension were performed as follows. A 20-μg portion of total RNA was precipitated and resuspended in 10 μl of H_2O. A 5-μl volume of hybridization solution (3.5 μl or approximately 10,000 cpm of primer and 1.5 μl of 10× HB, which contains 1.5 M KCl, 100 mM Tris-HCl [pH 8.3], and 10 mM EDTA) was added, and the mixture was incubated at 65°C for 1.5 h and then gradually cooled to room temperature. A 30-μl volume of an extension reaction mixture (20 mM Tris-HCl [pH 8.3], 10 mM $MgCl_2$, 6 mM dithiothreitol, each deoxynucleoside triphosphate at 0.3 mM, 150 μg of actinomycin D per ml, 10 U of avian myeloblastosis virus reverse transcriptase [Life Sciences Inc.]) was added to the reaction, and the mixture was incubated at 37°C for 60 min. DNase-free RNase A was added to a final concentration of 30 μg/ml, and incubation was continued for 15 min at 37°C. Nucleic acids were extracted once with phenol-chloroform and ethanol precipitated. The pellet was resuspended in 4 μl of sequencing gel loading buffer, heated to 90°C for 3 min, and electrophoresed on a 9% polyacrylamide–8 M urea gel in 1× TBE.

Nuclear transcription assays. Nuclear run-on transcription assays were performed as described previously (42). The following probes were bound to filters for detection of specific transcripts: SV40, double-stranded plasmid DNA (pJY1) containing the entire SV40 genome cloned in pBR322; TK, cRNA made from a construct containing an internal *Sma*I-*Bam*HI fragment from the human TK cDNA cloned in pSp64; β-2, a 0.6-kb *Pst*I fragment from the human β-2 microglobulin cDNA cloned in pSp64; pSp64, RNA transcribed from the pSp64 vector. In all cases, 5 μg of nucleic acid was bound to the filter as a probe. After development of the autoradiograms, each spot was cut out and quantitated by scintillation counting.

Preparation of ^{32}P-labeled cRNA probes. Plasmid pT7/T3-18 (Bethesda Research Laboratories) was digested with either *Eco*RI or *Hin*dIII. The *Eco*RI site is located 62 bp downstream of the T3 promoter, and the *Hin*dIII site is located 58 bp downstream of the T7 promoter. The digested DNAs (0.5 μg) were then used as templates for in vitro transcription reactions with either T3 (*Eco*RI digest) or T7 (*Hin*dIII digest) polymerase. Reactions were done for 1 h at 40°C in a final volume of 20 μl in a buffer containing 40 mM Tris-HCl (pH 7.5); 6 mM $MgCl_2$; 2 mM spermidine; 10 mM NaCl; 10 mM dithiothreitol; 20 U of RNasin; ATP, GTP, and CTP at 0.5 mM each; 12.5 μM UTP; 5 μl of [α-^{32}P]UTP (10 mCi/ml); and 10 U of T3 or T7 RNA polymerase. Following the transcription reaction, 1 U of RNase-free DNase was added and incubation was continued for 15 min at 37°C. The products were then purified by phenol-chloroform extraction and precipitated with ethanol. Incorporation was monitored by precipitation with trichloroacetic acid, and 2×10^7 cpm of each probe was used per Northern blot hybridization.

RESULTS

Nuclear run-on transcription assays in SV40-infected CV1 cells. In a previous report, we demonstrated a five- to sevenfold increase in the rate of TK gene transcription at the G_1-S boundary in serum-stimulated CV1 cells but were unable to demonstrate an equivalent increase during SV40 infection (42). This result was surprising, since TK mRNA levels are induced to a greater extent in SV40-infected cells than in serum-stimulated cells (43). The probes used to detect labeled nuclear transcripts in the run-on experiments were TK cRNAs synthesized in vitro from bacteriophage promoters. One difference between the serum stimulation and SV40 infection experiments was that the cRNA probe used to measure TK transcript levels in the SV40 infections was synthesized from a TK insert in a T3-T7 vector by using T3 polymerase, while the cRNA probe used in serum stimulation experiments was synthesized from a TK insert in pSp64 by using Sp6 polymerase. Subsequent to these experiments, we noted that RNA probes synthesized from the T3 promoter in T3–T7-18 showed significant hybridization to CV1 cell 28S rRNA on Northern blots. This hybridization was not due to TK sequences, since it also occurred when vector sequences alone were transcribed. In the experiment whose results are shown in Fig. 2, ^{32}P-labeled RNA synthesized from either the T3 or the T7 promoter in T3–T7-18 was hybridized to a Northern blot containing total CV1 cell RNA. RNA synthesized from the T3 promoter showed significant hybridization to 28S RNA, while that synthesized from the T7 promoter did not. Thus, it seemed likely that some or all of the TK signal observed in SV40-infected cells in our previous nuclear run-on experiments was due to cross-hybridization with 28S rRNA.

After obtaining these results, we re-examined TK gene transcription in SV40-infected CV1 cells by using a TK cRNA probe transcribed from pSp64. Nuclei were prepared from cells at various times after SV40 infection, and ^{32}P-labeled RNAs synthesized in these nuclei were hybridized to cRNA or DNA probes bound to nitrocellulose filters. The results of two such experiments, shown in Fig. 3, demonstrated a reproducible three- to fourfold increase in TK transcription at 14 to 15 h following SV40 infection. This coincides with the timing of TK mRNA induction in SV40-infected CV1 cells (42).

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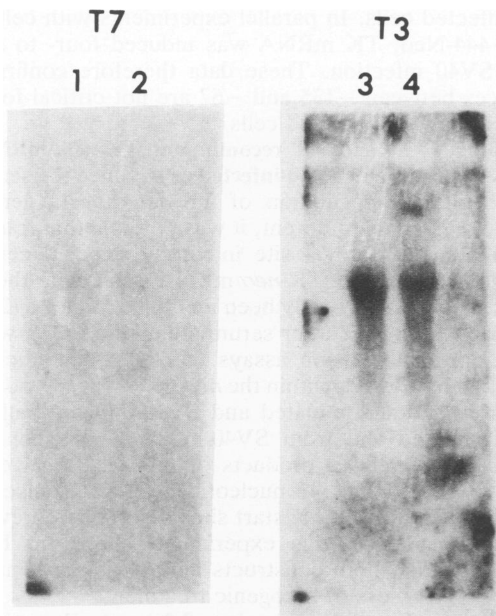

FIG. 2. Hybridization of cRNA probes to CV1 cell RNA. The vector pT3–T7-18 was digested with either *Hin*dIII or *Eco*RI, which cleave at opposite ends of the polylinker. Digested DNA (0.5 μg) was transcribed with either T3 (*Eco*RI digest) or T7 (*Hin*dIII digest) polymerase as described in Materials and Methods. The resulting ^{32}P-labeled transcripts (2×10^7 cpm) were then used as probes to hybridize to Northern blots containing total CV1 cell RNA. Each blot contained RNA from cells at 0 (lanes 1 and 3) or 18 (lanes 2 and 4) h after SV40 infection.

Regulation of human TK promoter-*neo* hybrid genes in SV40-infected cells. To characterize the TK sequences required for regulation during SV40 infection, we examined expression of TK promoter-*neo* hybrid genes. We had previously demonstrated that such constructs are induced by serum in stably transfected Rat3 cells and that sequences required for this induction are located between −135 and −67 bp relative to the transcription start site (37). To assay for activation of the TK promoter in SV40-infected cells, we initially infected the pools of stably transfected Rat3 cells with SV40. No induction of the TK-*neo* hybrid genes was observed under these conditions. When the Northern blots were reprobed with an SV40 early region fragment, however, no SV40 transcripts were detected (data not shown). We therefore conclude that in serum-starved Rat3 cells, SV40 either fails to efficiently infect or detectably express its early genes.

To continue this study, we examined the regulation of hybrid genes in simian CV1 cells. These cells are efficiently infected by SV40, and we previously demonstrated that endogenous TK mRNA is highly induced after serum starvation and viral infection (43). Three TK-*neo* constructs were studied initially: 444-Neo, which contains a 444-bp promoter fragment that is fully active and regulated by serum in Rat3 cells; 135-Neo, which contains the smallest TK promoter fragment (135 bp) that was regulated by serum in Rat3 cells; and 67-Neo, which contains a 67-bp promoter fragment that was not inducible in Rat3 cells. These constructs were transfected into CV1 cells, and G418-resistant colonies were selected. In all cases, transfection with 67-Neo was slightly (two- to fivefold) less efficient than with the other two constructs. Pools of 10 to 50 independent colonies from all three constructs were expanded for analysis. In the

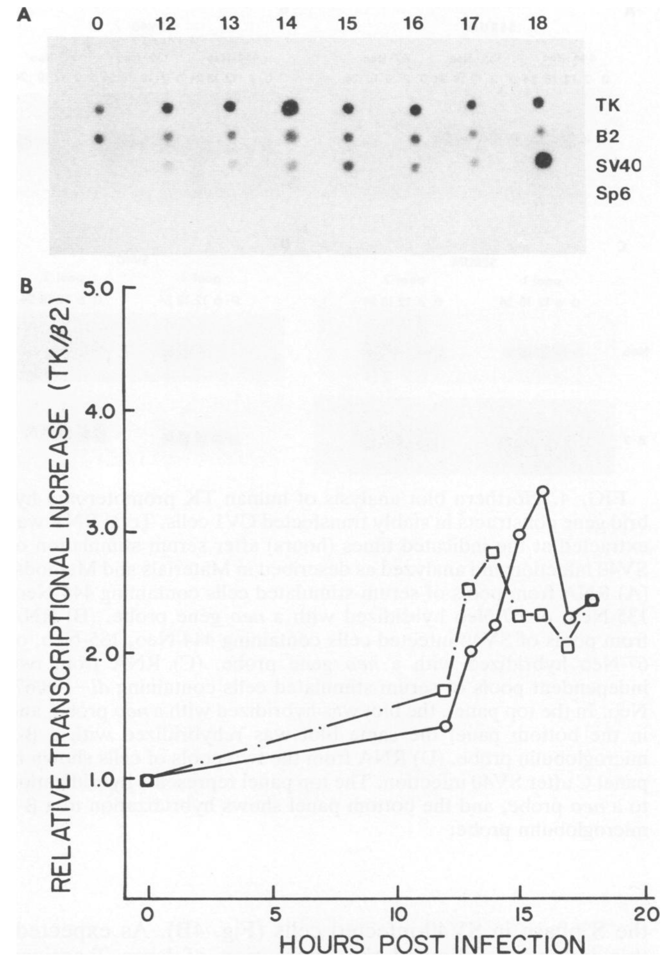

FIG. 3. Nuclear transcription analysis in SV40-infected CV1 cells. Nuclei were prepared at the times indicated (in hours) after viral infection and used for transcription assays as described in Materials and Methods. Equal numbers of trichloroacetic acid-precipitable counts (2.0×10^6 cpm) from all of the time points were hybridized to filters containing cRNA or DNA probes. The probes bound to the filters were as follows: TK, cRNA transcribed from human TK cDNA; β-2, cRNA transcribed from human β-2 microglobulin cDNA; SV40, linearized plasmid DNA containing the entire SV40 genome; Sp6, cRNA transcript of the pSp64 vector (details of the probes are given in Materials and Methods). (A) Autogradiogram of hybridizations from one experiment. (B) The dots shown in panel A were cut out of the filters and counted directly in a scintillation counter. The values were plotted as the TK/β-2 ratio. Values from both the experiment depicted in panel A (□) and one duplicate experiment (○) were plotted.

case of 67-Neo, single colonies were also expanded into cell lines.

Since the endogenous CV1 cell TK gene is induced between 8 and 10 h after serum stimulation and between 14 and 18 h after SV40 infection (43), we initially examined *neo* mRNA levels in transfected cells at 0, 2, 12, 18, and 24 h following either serum stimulation or SV40 infection. In serum-stimulated cells (Fig. 4A), the results were very similar to those seen previously in Rat3 cells. Both 444-Neo and 135-Neo were induced between G_0 and the S phase, while 67-Neo was expressed at low levels and failed to be induced as cells entered the S phase. In contrast, all three constructs, including 67-Neo, were induced between G_1 and

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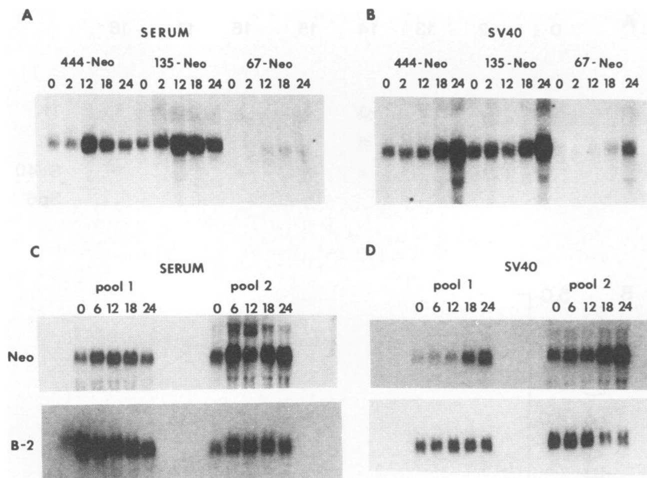

FIG. 4. Northern blot analysis of human TK promoter-*neo* hybrid gene constructs in stably transfected CV1 cells. Total RNA was extracted at the indicated times (hours) after serum stimulation or SV40 infection and analyzed as described in Materials and Methods. (A) RNA from pools of serum-stimulated cells containing 444-Neo, 135-Neo, or 67-Neo hybridized with a *neo* gene probe. (B) RNA from pools of SV40-infected cells containing 444-Neo, 135-Neo, or 67-Neo hybridized with a *neo* gene probe. (C) RNA from two independent pools of serum-stimulated cells containing *dl*−138/67-Neo. In the top panel, the blot was hybridized with a *neo* probe, and in the bottom panel the same blot was rehybridized with a β-2 microglobulin probe. (D) RNA from the two pools of cells shown in panel C after SV40 infection. The top panel represents hybridization to a *neo* probe, and the bottom panel shows hybridization to a β-2 microglobulin probe.

the S phase in SV40-infected cells (Fig. 4B). As expected, this induction followed the expression of large T-antigen mRNA, which appeared between 12 and 18 h (data not shown). The induction of 67-Neo between 18 and 24 h was surprising and was confirmed by using two cell lines derived from independent single colonies. In both single colonies, 67-Neo was regulated and clearly induced between G_1 and the S phase (data not shown). We were unable to quantitate the induction of 67-Neo, since the mRNA expressed from this construct was undetectable in G_0 and G_1-phase cells.

The results presented above suggested that TK promoter sequences located between −135 and −67, which are required for serum induction, are not required for induction by SV40. 67-Neo contains a highly truncated promoter, however, and might give rise to misleading results. We therefore confirmed this conclusion by constructing a mutant (*dl*−138/67) which contains an internal deletion of TK sequences from −138 to −67 in a 444-bp promoter background (see Materials and Methods for details of mutant construction). Since sequences between −135 and −444 contribute approximately 40% of promoter activity in transient assays (2), we predicted that higher basal levels of expression would occur with this internal deletion mutant than with the truncated 67-bp promoter. The *dl*−138/67 mutant promoter was linked to the *neo* gene and stably transfected into CV1 cells, and its activity was examined following serum stimulation and SV40 infection. Figure 4C and D show the results obtained with two independent pools of transfectants. Expression from this promoter increased slightly between 0 and 6 h after serum stimulation, but no further induction was seen between 6 and 12 h as the cells progressed from G_1 to S. In contrast, expression was induced six- to eightfold at G_1-S in SV40-infected cells. In parallel experiments with cells containing 444-Neo, TK mRNA was induced four- to sixfold during SV40 infection. These data therefore confirm that sequences between −135 and −67 are not critical for G_1-S induction in SV40-infected cells.

Mapping of 5′ ends of recombinant TK-*neo* mRNAs in serum-stimulated and SV40-infected cells. Since the sequence requirements for regulation of TK-*neo* hybrid genes by serum and SV40 are different, it was possible that transcription initiated at a novel site in virally infected cells. To determine whether the TK-*neo* mRNA initiated at the same cap site that had previously been identified in HeLa (25) and transfected Rat3 cells after serum stimulation (37), we performed primer extension assays. A 25-bp oligonucleotide primer that hybridizes within the *neo* gene was hybridized to RNA from serum-stimulated and SV40-infected cells containing 444-Neo and from SV40-infected cells containing 67-Neo. The extended products of this reaction were expected to be 101 and 95 nucleotides long if transcription initiated at both of the TK start sites determined previously (25). The results of this experiment, shown in Fig. 5, demonstrate that both constructs initiated at authentic TK cap sites, regardless of mitogenic treatment.

Regulation of *neo* mRNA expressed from the Rous sarcoma virus (RSV) long terminal repeat in SV40-infected cells. The fact that both 67-Neo and *dl*−138/67-Neo are regulated by SV40 and not by serum suggests that the virus mediates its effect via TK sequences between −67 and +30. An alternative explanation is that induction is mediated by sequences within the *neo* gene itself, either by changing the activity of the linked promoter or by changing the stability of the *neo* mRNA. To eliminate this possibility, we transfected CV1 cells with RSV-*neo*, in which expression of the *neo* mRNA is driven by the RSV promoter. CV1 cells containing RSV-*neo* were serum starved and then mitogenically stimulated by either addition of fresh serum or infection with SV40. RNA was prepared at various times after treatment, and the level of *neo* mRNA was determined by Northern blot analysis. The results of this experiment, shown in Fig. 6A, demonstrated that *neo* mRNA levels were constant throughout the time course when expressed from the RSV promoter. Rehybridization of the blot shown in Fig. 6A with a TK probe to detect the endogenous CV1 cell TK mRNA showed induction by both serum and SV40 (Fig. 6B), indicating that the cells were efficiently arrested and stimulated in this experiment.

Determination of TK-*neo* mRNA half-life in serum-stimulated and SV40-infected cells. Since the mRNAs expressed from the TK-*neo* constructs used in these studies are hybrid molecules containing 30 bp of human TK sequences linked to the *neo* sequences, it is conceivable that the TK sequences in 67-Neo and *dl*−138/67-Neo mediate viral induction at the posttranscriptional level. Since the constructs contain no introns, the most likely effect would be on the stability of the transcript. In fact, regulation of TK mRNA stability during the cell cycle has been reported (5). To address this possibility, we measured the half-life of the hybrid TK-*neo* mRNA in serum-stimulated cells, where the mRNA is not induced, and in SV40-infected cells, where it is induced. Quiescent CV1 cells containing 444-Neo were stimulated with either serum or SV40, and actinomycin D (5 μg/ml) was added to the culture at the indicated time after treatment (13 h for serum stimulation and 18 h for SV40 infection). Control experiments showed that this level of actinomycin D inhibited total RNA synthesis by more than 99% (data not shown). Total cell RNA was prepared at various times after actinomycin D addition and analyzed for

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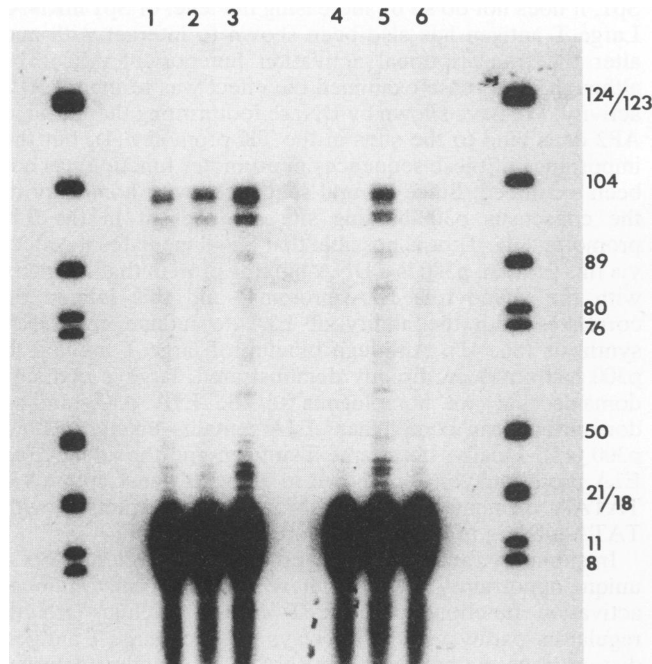

FIG. 5. Mapping of the transcriptional start site in human TK promoter-*neo* gene constructs by primer extension assays. Total RNA from pools of stably transfected CV1 cells containing 444-Neo or 67-Neo was extracted at various times after serum stimulation or SV40 infection. A 20-μg sample of RNA was analyzed in each extension reaction as described in Materials and Methods. Lanes: 1 to 3, RNA from cells containing 444-Neo; 1, serum starved; 2, 14 h after serum stimulation; 3, 24 h after SV40 infection; 4 to 6, RNA from cells transfected with 67-Neo; 4, serum starved; 5, 24 h after SV40 infection; 6, 36 h after SV40 infection.

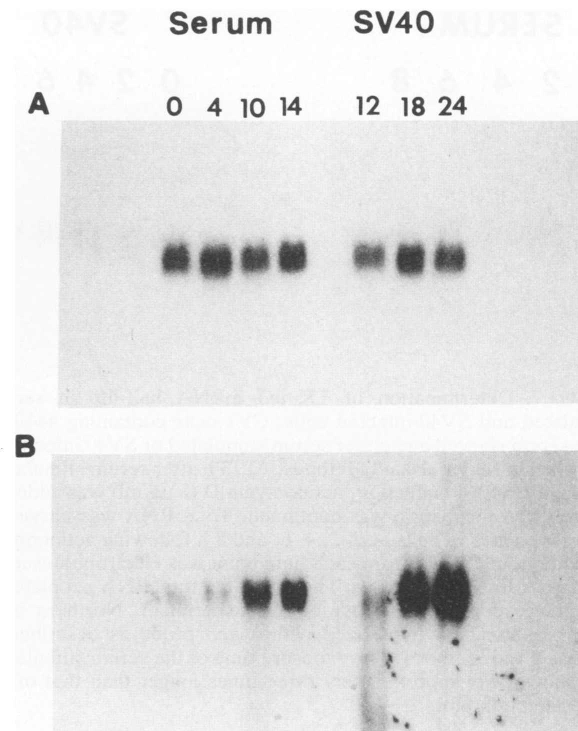

FIG. 6. Northern blot analysis of RSV-*neo* constructs in transfected CV1 cells. A pool of CV1 cells containing RSV-*neo* was serum starved for 24 h and then mitogenically stimulated by either serum addition or SV40 infection. Total cellular RNA was prepared poststimulation at the times indicated above the lanes in hours, and 20 μg of RNA from each time point was analyzed by Northern blotting. Panels: A, *neo* probe; B, TK probe.

the level of *neo* mRNA by Northern blotting (Fig. 7). Several autoradiograms (with different exposure times) were scanned by densitometry, and the values obtained were used to calculate the mRNA half-lives, which were approximately 8.5 h in serum-stimulated cells and 7.5 h in SV40-infected cells. Since these half-lives are not significantly different, the fact that 67-Neo and *dl*−138/67-Neo are induced by SV40 but not by serum is not due to increased stabilization of the mRNA in virally infected cells.

DISCUSSION

The data presented establish that the cellular TK gene is transcriptionally induced when quiescent CV1 cells are infected with SV40. A three- to fourfold induction of the endogenous CV1 cell TK gene was detected at 14 to 18 h after viral infection by nuclear run-on transcription assays. These results differ from previous work, in which we were unable to demonstrate transcriptional induction using similar assays. We have now established that the earlier negative results were due to the fact that hybridization between cRNA synthesized from the T3 promoter and 28S rRNA obscured the true TK signal. We have also demonstrated that human TK promoter-*neo* hybrid genes are induced in stably transfected CV1 cells infected with SV40. Several lines of evidence indicate that this induction is mediated via TK promoter sequences and occurs at the transcriptional level. Regulation is totally dependent on the TK sequences, since *neo* mRNA levels are constitutive when the gene is expressed from the RSV long terminal repeat. Because the TK-*neo* constructs used give rise to a hybrid mRNA containing 30 nucleotides of TK sequences, the possibility that regulation occurs via a change in mRNA stability was considered. We measured the stability of TK-*neo* mRNA in both serum-stimulated and SV40-infected cells and detected no significant difference. We therefore conclude that the increase in *neo* mRNA in SV40-infected cells is likely due to transcriptional events.

By using a series of deletion mutants, *cis*-acting DNA sequences sufficient for regulation of the human TK promoter in SV40-infected cells have been localized to between −67 and +30 relative to the major transcriptional initiation site. These promoter elements are different from those required for induction by serum, which are located between −135 and −67. Thus, the human TK promoter appears to contain at least two separable domains that are involved in its regulation during mitogenic stimulation: an upstream domain required for regulation during serum stimulation and a downstream domain sufficient for regulation during SV40 infection. Although the precise sequences within each domain that are required for regulation are unknown, several interesting possibilities exist. As indicated previously, the upstream region contains several elements with homology to binding sites for transcription factor E2F, which has been implicated in the control of G_1-S-phase-regulated genes. Although a role for this factor in human TK gene regulation has not been directly demonstrated, mutation of a 26-bp sequence encompassing the E2F sites abolishes serum regulation (21). In addition, potential E2F sites are present in a number of other G_1-S-regulated promoters, including dihy-

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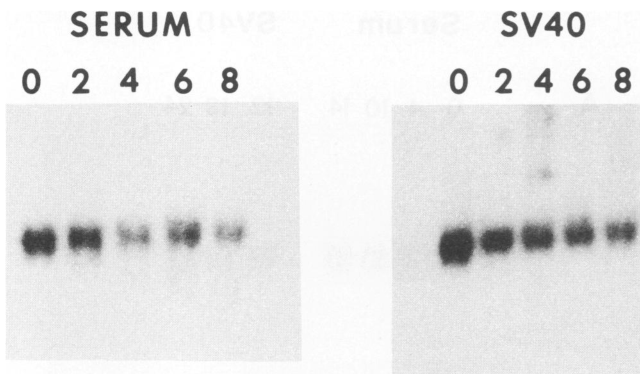

FIG. 7. Determination of TK-*neo* mRNA half-life in serum-stimulated and SV40-infected cells. CV1 cells containing 444-Neo were serum starved and either serum stimulated or SV40 infected as described in Materials and Methods. At 13 h after serum stimulation or 18 h after SV40 infection, actinomycin D (5 μg/ml) was added to cultures and incubation was continued. Total RNA was harvested from two plates of cells at 0, 2, 4, 6, and 8 h following actinomycin D addition, and 20 μg from each time point was electrophoresed on an agarose-formaldehyde gel. The amount of total RNA per plate did not change during the course of this experiment. Northern blots were prepared and hybridized with a *neo* probe as described in Materials and Methods. The exposure time of the serum stimulation blot shown was approximately three times longer than that of the SV40 infection blot.

drofolate reductase, thymidylate synthase, DNA polymerase α, and proliferating cell nuclear antigen (33). In the case of dihydrofolate reductase, mutation of these sites abolishes serum regulation (3, 14, 30). If, in fact, the E2F sites in the TK promoter are responsible for induction during serum stimulation, we predict that they would also function in SV40-infected cells, since large T antigen is able to release E2F from complexes with pRb and related proteins and, presumably, increase its transcriptional activation functions. Our experiments do not establish whether large T antigen can activate the TK promoter via the upstream element but do indicate that this element is not required for viral activation of the promoter. To determine whether large T antigen is capable of activating the upstream element, it will be necessary to assay its activity in the absence of the downstream sequences.

Activation of the −67/+30 TK promoter fragment in SV40-infected cells could be due either to a direct interaction of large T antigen with the promoter or to a change in the level or activity of cellular transcription factors. No interaction of large T antigen with the TK promoter was detected by gel shift or filter-binding assays (data not shown). We therefore propose that SV40 infection induces or alters the activity of a cellular transcription factor(s) that interacts with TK promoter elements. Analysis of the TK downstream sequence from −67 and +30 reveals several potential protein-binding sites that might be involved in regulation by SV40. These include a TATAA element, a CCAAT element, and elements with homology to AP2, Sp1, E2F, and p300-binding sites (Fig. 1). Because SV40 large T antigen was previously reported to cause increases in Sp1 RNA and protein levels (38), we considered the possibility that TK induction was mediated by increases in the level of Sp1. We measured Sp1 mRNA levels during both serum stimulation and SV40 infection and detected no increase following either mitogenic treatment (data not shown). Thus, if SV40 mediates its effect on the −67 to +30 bp promoter fragment via Sp1, it does not do so by increasing the level of Sp1 mRNA. Large T antigen has also been shown to interact with and alter the transcriptional activation function of AP2 (31), although in the case examined the effect was to inhibit AP2 activity. We have shown by DNase footprinting that purified AP2 does bind to the sites in the TK promoter (1), but the importance of these sequences in promoter function has not been examined. Since several sequences with homology to the consensus p300-binding site are present in the TK promoter (Fig. 1), it is possible that SV40 mediates its effect via this protein. p300 is a DNA-binding protein that interacts with the adenovirus E1A proteins, and this interaction correlates with the ability of E1A to induce cell DNA synthesis (36, 41). Although binding of large T antigen to p300 has not been directly demonstrated, large T contains domains that are homologous to the E1A p300-binding domain and can complement E1A mutants unable to bind p300 (47). Finally, both large T antigen and the adenovirus E1A protein have been shown to activate transcription via TATAA elements, possibly through an interaction with TATA-binding protein (13, 15, 40, 46).

In summary, analysis of TK promoter regulation offers a unique opportunity to coordinately study the transcriptional activation functions of large T antigen, cellular growth regulatory pathways, and the ways in which large T antigen disrupts these pathways. TK mRNA is highly induced during viral infection, and this induction occurs at the G_1-S interface, an important control point in the cell cycle. The TK promoter is therefore a downstream target of both large T antigen and cellular molecules, such as E2F and pRb, that are involved in the regulation of cell proliferation. Consequently, by investigating the DNA sequences and proteins that regulate the TK promoter and the ways in which large T interacts with these molecules, we will increase our understanding of the functions of both large T and the cellular molecules and pathways that regulate the passage from G_1 to the S phase.

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