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

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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, G0-phase cells, but increase dramatically (10- to 20-fold) during the S phase in both serum-stimulated and simian virus 40-infected cells. To determine whether 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 simian virus 40 infection of growth-arrested simian CV1 cells. When assays were performed at 12-h intervals, a small (two- to threefold) but reproducible increase in TK transcription was detected during the S phase. When time points were chosen to span the G1-S interface a larger (six- to sevenfold) increase in transcriptional activity was observed in serum-stimulated cells but not in simian virus 40-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. 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.

An understanding of the regulation of genes encoding products involved in DNA replication is an important step toward identifying the elements that are involved in, and possibly necessary for, the control of the eucaryotic 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 simian virus 40 (SV40) (12, 19, 24); 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 the TK gene and the relationship between the ability of SV40 to affect the expression of these genes and to immortalize 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 CV1 cells is paralleled by an increase in the steady-state levels of TK mRNA, and that in both cases the increase occurs during the 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 CV1 cells. In addition, we have used several hybrid TK cDNA 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 gene, has been examined; 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 G1-S boundary, but that the increased transcriptional level is not maintained throughout the S phase (2). A study of thymidylate synthase gene expression has reported the existence of both transcriptional and post-transcriptional controls (8).

Experiments with both 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 TK cDNA 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 six- to sevenfold increase in TK gene transcription at the G1-S interface in serum-stimulated CV1 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 CV1 African Green Monkey kidney cell line was grown in Dulbecco modified Eagle medium supplemented with 5% calf serum and 5% fetal bovine serum (both from Hyclone).

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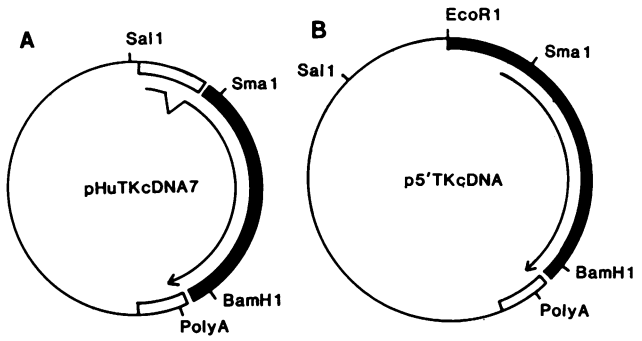

FIG. 1. Structures of cDNA clones capable of expressing TK after transfection into TK⁻ cells. pHuTKcDNA7 expresses the human TK message from the SV40 early promoter and also contains an SV40 splice donor and acceptor and polyadenylation signal. p5'TKcDNA expresses the TK mRNA from the human genomic TK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

Rat-3 TK⁻ cells (25) were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum. To obtain synchronous populations of cells, cells were plated in medium plus 10% serum so that they would reach confluence in 4 to 5 days, and they were held at confluence for an additional 48 h 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 1 h. 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 blotting, DNA pulse-labeling, and TK enzyme extraction and assays. Northern blotting, DNA pulse-labeling, and TK extraction 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 eucaryotic cells, are diagrammed in Fig. 1. pSP64 Bam-Sma TK contains the 1.2-kilobase *Bam*HI-*Sma*I fragment from within the TK cDNA in pHuTKcDNA7 subcloned into the polylinker in pSp64. The human β2 microglobulin cDNA clone was a gift of Hsiu-Ching Chang and contained the β2 cDNA cloned in the *Pst*I site of pBR322. pSpβ2 contained this *Pst*I fragment subcloned into pSp64. A plasmid containing a genomic *Bam*HI fragment from within the human c-*myc* locus was a gift of Hsing-Jien Kung. A *Sal*I-*Eco*RI 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 Jerry Dodgson. All fragments subcloned into Sp6 vectors were inserted in an orientation relative to the Sp6 promoter that allows for transcription of the complementary (antisense) message.

Sp6 vector transcription. Complementary RNA transcripts were made by using fragments subcloned into either pSp64 or pSp65 (ProMega Biotech). Reactions were 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 U of Sp6 polymerase (ProMega) was added per 100-μl 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 with a dot-blot manifold (Bethesda Research Laboratories, Inc.). For each dot, 5 μg of RNA was suspended in 15 μl of diethyl pyrocarbonate-treated, distilled H_2O. Formaldehyde (5 μl) was added, the RNA was heated to 65°C for 10 min, 130 μl of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) 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 suspension in 25 μl of distilled H_2O, 5 μl of 2 M NH_4OH was added, and the sample was boiled for 3 min at 90°C and quickly quenched on ice. Next, 20 μl 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 2× 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 CV1 cells were either serum stimulated or infected with SV40 virus at an MOI of 15 at various times before harvesting. Upon harvesting, cells were washed once in phosphate-buffered saline, once in hypotonic buffer (20 mM Tris [pH 8.0], 5 mM $MgCl_2$, 6 mM $CaCl_2$, 0.5 mM dithiothreitol), placed on ice for 5 min, and lysed on plates with Nonidet P-40 lysing buffer (0.6 M sucrose, 0.2% Nonidet P-40, 0.5 mM dithiothreitol). The lysate was scraped off the plates with a rubber policeman, and nuclei were pelleted at 2,000 rpm in an IEC model CL centrifuge. The nuclei were washed once in hypotonic buffer and then incubated at 26°C for 45 min in a reaction mixture containing 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (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 ribonucleoside triphosphates (except UTP), and 100 μCi of [α-^{32}P]UTP (3,000 Ci/mmol; New England Nuclear Corp.). The newly labeled transcripts were treated with sodium dodecyl sulfate-proteinase K solution (0.3% sodium dodecyl sulfate, 100 mg of yeast tRNA per ml, and 100 μg of proteinase K per ml in 10 mM Tris–1 mM EDTA containing 100 U of RNasin (ProMega) per ml for 20 min at 37°C, extracted with H_2O-saturated phenol, and ethanol precipitated. Prehybridization, hybridization, and posthybridization washes were performed by the method of Groudine et al. (6). Equal numbers of incorporated counts from each time point were hybridized to filter-immobilized probes for 36 h. Blots were exposed to X-ray film with screens at −70°C.

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

RESULTS

TK mRNA induction in CV1 cells. We previously reported (24) that TK mRNA levels increase 10- to 20-fold after either serum stimulation or SV40 infection (MOI of 5) of CV1 cells, with the levels of RNA peaking at 12 h after serum stimulation or 24 h after viral infection. In our current experiments

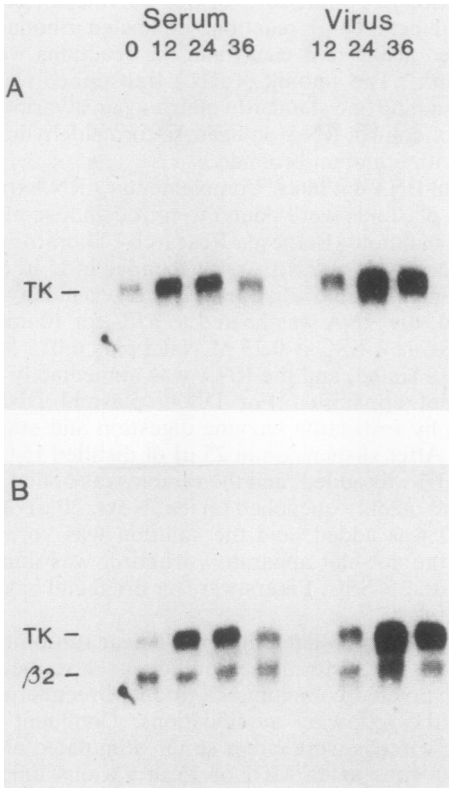

FIG. 2. Northern blot analysis of TK mRNA levels after serum stimulation or SV40 infection of CV1 cells. Confluent, growth-arrested CV1 cells were induced to reenter the cell cycle by the addition of fresh serum or by infection with SV40 as described in Materials and Methods. Poly(A)$^+$ RNA was prepared from two plates of cells at 0, 12, 24, and 36 h 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-kilobase internal to the TK cDNA. (B) Rehybridization of the filter in (A) with a human β2 microglobulin probe.

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 24 h 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 h after treatment and analyzed by Northern blot analysis as described in Materials and Methods. The results of this experiment (Fig. 2) are similar to those described previously (24). TK mRNA levels are low in resting cells and peak at 12 h after serum stimulation or 24 h after SV40 infection (Fig. 2A). In addition, the TK mRNA accumulates to a higher level in the virally infected cells than in the serum-stimulated cells. As an internal control for the amount of RNA in each lane, the filter in Fig. 2A was hybridized with a human β2 microglobulin probe, since it has been shown that the level of β2 mRNA is relatively constant throughout the cell cycle (11, 17). The results of this experiment (Fig. 2B), confirm that approximately equal amounts of mRNA were present in each lane.

Measurements of transcription rates in nuclei isolated from CV1 cells. To determine whether the increased levels of TK mRNA seen in serum-stimulated and SV40-infected CV1 cells are due to increased rates of transcription of the gene, we performed nuclear run-on transcription assays as described in Materials and Methods. Figure 3 shows the results from two sets of assays, one with serum-stimulated cells and one with SV40-infected cells. Nuclei were prepared at 0, 12, 24, and 36 h after treatment. RNA was transcribed, purified, and hybridized to filters containing cRNA to 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 RNA on the filters greatly increases the sensitivity of these assays and allows us to measure TK transcription in CV1 cells. In the case of SV40-infected cells, plasmid pJY1 DNA, which contains a linear copy of the SV40 genome inserted at the *Bam*HI site of pBR3322, was bound to the filters and used as a positive control. After hybridization and washing the filters were exposed to X-ray film (Fig. 3). To quantitate the amount of RNA bound, each dot was cut out after autoradiography and counted for ^{32}P. These values, given as counts per minute per dot, are also shown in Fig. 3.

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

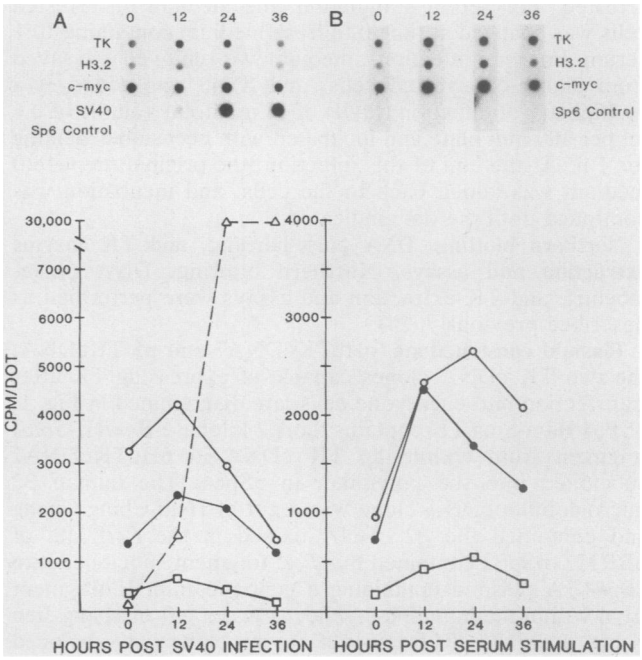

FIG. 3. Nuclear run-on transcription assays at 12-h intervals after serum stimulation or viral induction of CV1 cells. Growth-arrested CV1 cells were induced to reenter the cell cycle by the addition of fresh serum or infection with SV40 as described in Materials and Methods. Nuclei were prepared at 12-h 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 counts per minute hybridized per dot. Symbols: (●) TK, (□) H3.2, (○) c-*myc*, (△) SV40. (A) Results of hybridizations with RNA prepared from SV40-infected cells; 3.0×10^6 cpm of labelled RNA from each time point was 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 was added to each hybridization.

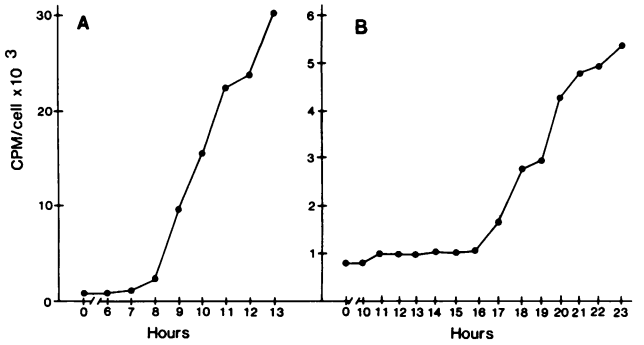

FIG. 4. Measurements of DNA synthesis in serum-stimulated (A) and SV40-infected (B) cells. CV1 cells were arrested and then serum stimulated or infected with SV40 (MOI of 15) as described in Materials and Methods. At the indicated times after treatment, they were labeled with [^{3}H]thymidine for 1 h, and the counts per minute incorporated per cell were determined by trichloroacetic acid precipitation of a given number of cells.

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 threefold increase at 12 and 24 h. No hybridization to the Sp6 control was seen in this or any other experiment. The patterns of transcription in the SV40-infected cells were similar to that seen in serum-stimulated cells, with small increases at 12 and 24 h (Fig. 3A). The obvious exception is the PJY1 control, which shows that SV40 transcription begins by 12 h and reaches very high levels at 24 and 36 h 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 CV1 cells. In fact, the rate of transcription appears relatively constant throughout the cell cycle in these experiments.

Transcription at the G1-S interface. Although the experiments reported above 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 G1-S phase interface, where a sevenfold increase over the level found in resting cells was seen. This increase occurs just before 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 G1-S interface in serum-stimulated and SV40-infected CV1 cells. We have previously shown that DNA synthesis is induced by 12 h after serum stimulation and 24 h after SV40 infection of CV1 cells (24). To determine the exact onset of DNA synthesis, cells were pulse-labeled for 1 h at 1-h intervals surrounding the approximate initiation of S phase, and incorporation was monitored as described in Materials and Methods. DNA synthesis began between 8 and 9 h after serum stimulation and between 17 and 18 h after SV40 infection (Fig. 4).

Nuclear run-on transcription assays were performed at 0, 6, 7, 8, 9, 10, 11, and 12 h after serum stimulation to span the G1-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 cell cycle. There was a sharp increase in TK transcription at 9 h after serum stimulation, and the level fell by 11 and 12 h (Fig. 5). We quantitated these results by comparing the levels of TK transcription to the β2 microglobulin levels at each time point and determined that there is a six- to sevenfold increase in TK transcription at the 9-h time point (Fig. 5B and Table 1). The H3.2 histone gene again showed a small increase at the 9- to 12-h time points, and the β2 microglobulin control was relatively constant throughout the time course. This experiment has been repeated, and each time the TK gene showed approximately a sixfold increase in transcription at the G1-S boundary (Table 1). 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 the S phase.

We next examined TK transcription at the G1-S boundary in SV40-infected cells. CV1 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 h after infection. The results of this experiment (Fig. 6 and Table 1) were somewhat surpris-

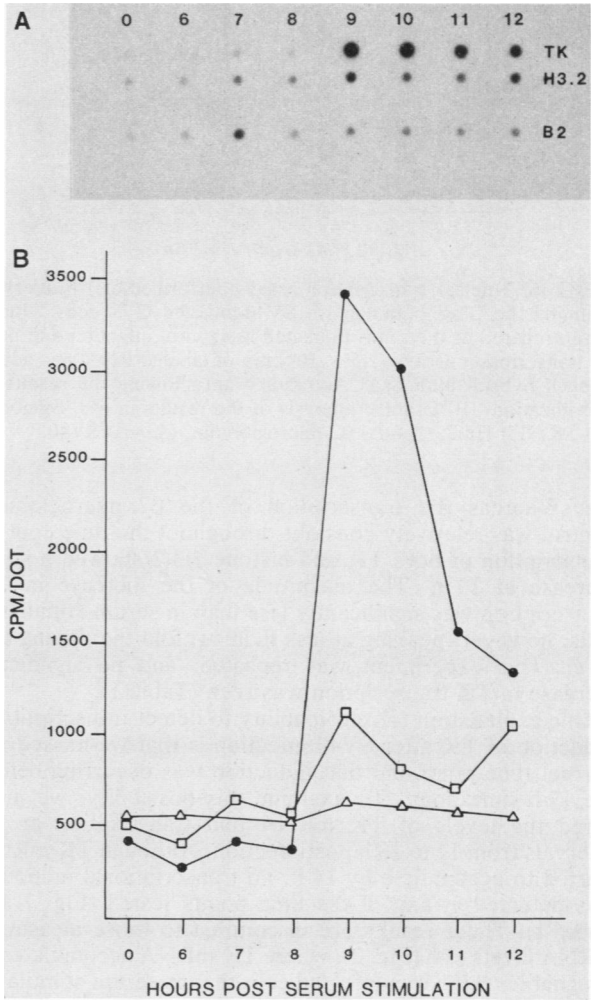

FIG. 5. Nuclear transcription assays performed at 1-h intervals spanning the G1-S boundary in serum-stimulated CV1 cells. Nuclei were prepared at the times indicated after serum stimulation and used for transcription assays; 2.0×10^6 cpm of labeled RNA was added to each hybridization. (A) Autoradiogram showing results of hybridization. (B) Graphic analysis of the results in (A). Symbols: (●) TK, (□) H3.2, (△) β2 microglobulin.

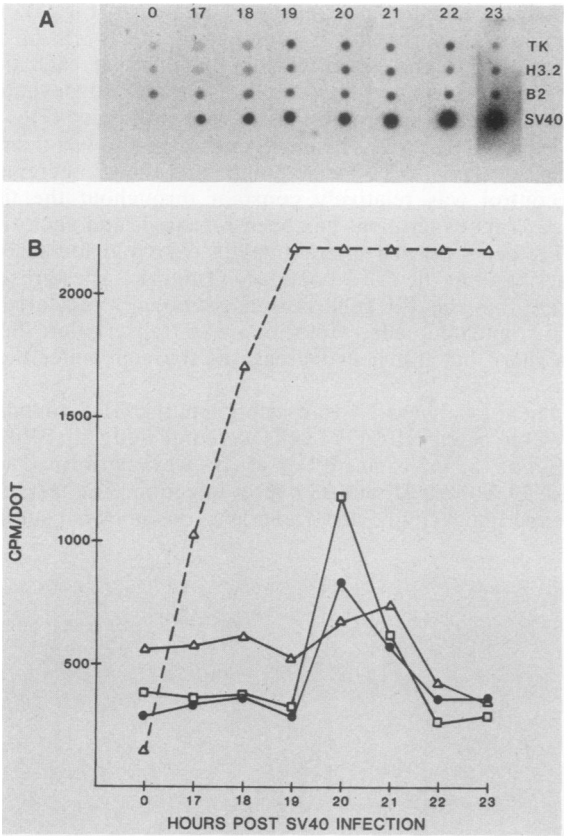

FIG. 6. Nuclear transcription assays performed at 1-h intervals spanning the G1-S boundary in SV40-infected CV1 cells. Nuclei were prepared at the times indicated after viral infection and used for transcription assays; 2.5×10^6 cpm of labeled RNA was added to each hybridization. (A) Autoradiogram showing the results of hybridization. (B) Graphic analysis of the results in (A). Symbols: (●) TK, (□) H3.2, (△—△) β2 microglobulin, (△---△) SV40.

ing. Whereas the transcription of the β2 microglobulin control was relatively constant throughout the time course, transcription of both TK and histone H3.2 showed a small increase at 20 h. The magnitude of the increase in TK transcription was significantly less than in serum-stimulated cells, however, peaking at less than twofold the resting cell level. This experiment was repeated, and no significant increase in TK transcription was seen (Table 1).

One explanation for our inability to detect transcriptional induction of TK after SV40 infection is that we missed the correct time point and that induction was occurring before the 17-h time point. To examine this possibility, we measured the levels of TK mRNA and transcription at 1-h intervals from 12 to 18 h postinfection. Although TK mRNA started to accumulate by 14 h, no transcriptional induction was detected at any of the time points tested (Fig. 7 and Table 1). These results are in contrast to those measuring mRNA levels (24) (Fig. 2), where TK mRNA accumulates to a higher level in virus-infected cells than in serum-stimulated cells, and suggest that SV40 is utilizing an alternate mechanism for inducing high levels of TK mRNA.

TK induction in cell lines transfected with human TK cDNA constructs. To identify the DNA sequences required in *cis* for the regulation of TK gene expression, we have isolated several different TK cDNA constructs and studied their expression after transfection into Rat-3 TK^- cells. These

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

Time (h) after serum stimulation	TK gene transcription in serum expt:			Time (h) after SV40 infection	TK gene transcription in SV40 expt:			
	1	2	3		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		

[a] Transcription assays were performed as described in Materials and Methods. To quantitate the level of TK transcription, dots containing ^{32}P-labeled RNA hybridized to either TK or β2 microglobulin cRNA were cut out and counted in a scintillation counter. The ratio of counts per minute hybridized to TK to those hybridized to β2 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.

plasmids (Fig. 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 *Sma*I site within the first exon of the gene. These two plasmids were transfected into Rat-3 TK^- cells, and TK^+ transformants were selected and propagated both as pools of approximately 50 colonies each and as clonal cell lines derived from single colonies.

The resulting cell lines were tested for their ability to

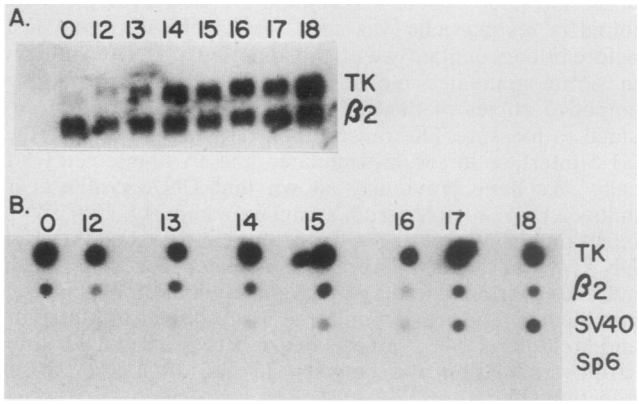

FIG. 7. Measurements of TK poly(A)$^+$ RNA levels and transcription rates in SV40-infected cells. Resting CV1 cells were infected with SV40 (MOI of 15) at time 0, and at the times indicated plates were harvested for either the preparation of poly(A)$^+$ RNA or nuclear transcription assays. (A) Northern gel analysis of poly(A)$^+$ RNA hybridized to TK and β2 microglobulin probes. (B) Hybridization of [^{32}P]RNA synthesized in isolated nuclei to dot blots containing 5 μg of TK, β2, and Sp6 cRNA and 5 μg of PJY1 (cloned SV40) DNA.

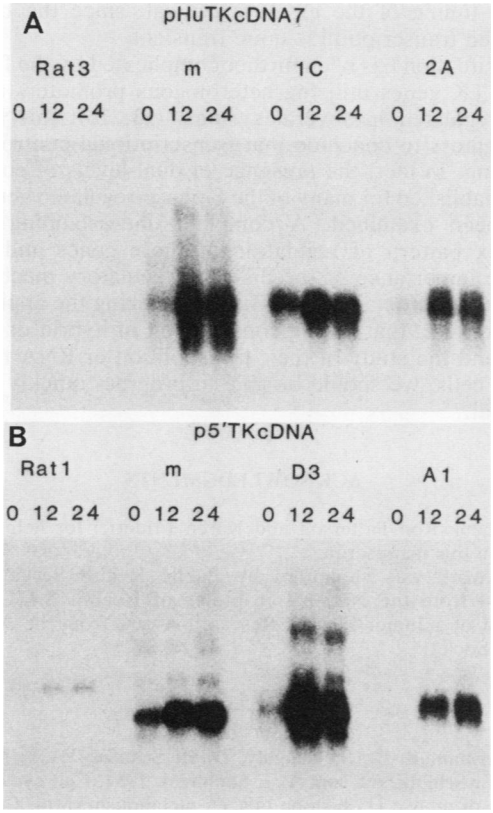

FIG. 8. Northern blot analysis of TK poly(A)$^+$ RNA in cell lines transfected with TK cDNA constructs. Rat-3 TK$^-$ cells were transfected with either pHuTKcDNA7 or p5'TKcDNA, and HAT^r colonies were selected. Transfected cell lines were growth arrested and serum stimulated as described in Materials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

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 h after serum stimulation and quantitating this RNA by Northern blot analysis as described previously (24). In Figure 8A three cell lines containing pHuTKcDNA7 were examined; cell lines 1C and 2A were derived from single colonies, and cell line m was derived from a pool of colonies. In Figure 8B the results are shown from cell lines containing p5'TKcDNA; D3 and A1 were derived from single colonies, and m was derived from pooled colonies. All of these cell lines showed increased levels of TK mRNA by 12 h 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 2. These results are in agreement with the mRNA data, with peak levels of TK enzyme activity at either 12 or 24 h after 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 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 N. Heintz have indicated that this promoter is not activated during the transition of cells from G1 into the S phase (N. Heintz, personal communication).

DISCUSSION

The results presented here suggest that the TK 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 G1 after serum stimulation. As the cells enter S phase, which is at approximately 8 to 9 h after serum stimulation, there is a six- to sevenfold increase in the rate of TK gene transcription. Transcription drops to threefold the level in resting cells by 12 h after serum addition and continues to decrease at 24 h. These results are similar to those reported for the DHFR gene in mouse cells, where there is a sevenfold increase in transcription at the G1-S boundary (2). In contrast, no sharp increase in TK transcription was detected in SV40-infected CV1 cells in these experiments, although the TK mRNA accumulates to higher levels in virally infected cells than in serum-stimulated cells. Thus the mechanisms of induction by these two mitogenic agents appear to differ in some or all aspects. This was also suggested by earlier experiments, where it was shown that induction of DHFR by polyomavirus 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 in serum-stimulated CV1 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 h, whereas the mRNA levels increase 10- to 20-fold and remain high for at least 12 h. 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

TABLE 2. TK enzyme assays in cell lines transfected with HuTK cDNA constructs[a]

Time (h) after serum stimulation	TK units ($\times 10^4$)							
	Rat-3	Rat-1	pHuTKcDNA7			p5TKcDNA		
			Mass	1C	2A	Mass	D3	A1
0	0.003	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		9.2	3.9	7.2	4.6	12.6	4.1	3.7
36		11.8	2.7	1.2	0.9	0.7	0.8	0.9

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

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 laboratories with both the chicken (16) and hamster (13) chromosomal TK genes, where it was shown that hybrid genes expressed from the herpesvirus 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 it 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 herpesvirus TK promoter, or the SV40 early promoter. This seems unlikely, however, since even in the case of CV1 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 the expression of the TK gene during S phase.

We cannot unambiguously determine from our results whether 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. 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 laboratories, 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 have been demonstrated for some time (7, 21, 22, 23). In the case of other S phase-regulated genes such as the DHFR, thymidylate synthase, and TK genes 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 thymidylate synthase 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); 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 or RNA metabolism in cells, we should be able to progress quickly toward that goal.

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