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The 5′-Flanking Region of the Mouse Thymidylate Synthase Gene Is Necessary But Not Sufficient for Normal Regulation in Growth-Stimulated Cells

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The thymidylate synthase (TS) gene is a housekeeping gene that is expressed at much higher levels in proliferating cells than in quiescent cells. We have studied the role of the TS 5′-flanking sequences in regulating the level of expression of the mouse TS gene. A variety of chimeric TS minigenes that contain different promoters linked either to the TS coding region (with or without introns) or to the chloramphenicol acetyltransferase (CAT) coding region were constructed. The activities of the minigenes were determined by transfecting them into cultured cells and measuring the levels of mRNA or enzyme derived from the chimeric genes. We found that the mouse TS promoter had about the same strength as the simian virus 40 early promoter but was significantly stronger than the herpes simplex virus thymidine kinase promoter. Stable transfection studies revealed that minigenes consisting of the normal TS promoter (extending to −1 kb), coding region, and polyadenylation signal were regulated normally in response to growth stimulation. When the TS promoter was replaced by the simian virus 40 early promoter or by a TS promoter that retained only 60 nucleotides upstream of the first transcriptional start site, the minigene was expressed constitutively. A minigene consisting of the TS promoter (extending to −1 kb) linked to the CAT coding region was also expressed constitutively. These observations indicate that sequences upstream of the transcriptional start sites of the TS gene are necessary, although not sufficient, for normal growth-regulated expression of the mouse TS gene.

Thymidylate synthase (TS) is a housekeeping enzyme that is responsible for the formation of thymidylic acid in the de novo biosynthetic pathway. The enzyme is present at a much higher level in proliferating cells that are engaged in DNA replication than in quiescent cells (4, 26). We have been studying the mechanisms that are responsible for regulating expression of the mouse TS gene. We have cloned and analyzed the sequences of the cDNA (29) and gene (5) for this enzyme. The gene is 12 kb in length and has a 1-kb coding region interrupted by six introns. The G+C-rich promoter region lacks a TATAA box and initiates transcription at multiple sites over a 60-nucleotide region (5, 8). Mouse TS mRNA is highly unusual in that the predominant species lacks a 3′ untranslated region (18). The upstream polyadenylation signal is AUUAAA and is located within the coding region (15).

The amount of TS mRNA increases at least 20-fold during a serum-induced transition from the quiescent (G0) phase to the S phase of the cell cycle (8, 19). However, nuclear run-on analyses have shown that the rate of transcription of the TS gene increases only about threefold during this interval (19). This suggests that the mouse TS mRNA level is controlled at the posttranscriptional level and, to a lesser extent, at the transcriptional level in growth-stimulated cells. Posttranscriptional regulation of TS mRNA content was also observed in serum-stimulated human diploid fibroblasts (1). Combinations of transcriptional and posttranscriptional regulation have been observed previously with other housekeeping genes. A particularly relevant example is the mammalian thymidine kinase (TK) gene, which appears to be regulated by several different mechanisms under different physiological conditions (12, 13, 21, 33, 37).

Transient transfection assays with TS minigenes have shown that normal levels of gene transcription and the normal pattern of transcriptional start sites are observed with TS 5′-flanking regions that retain as few as 15 nucleotides upstream of the first transcriptional initiation site (or 105 nucleotides upstream of the ATG start codon) (7). Introns are also important for efficient expression of the gene; addition of introns 5 and 6 to an intronless minigene increases the level of expression about eightfold (6). Similar stimulatory effects have been observed previously in other genes (2, 3, 14). However, addition of intron 4 did not lead to an increase in expression, indicating that the stimulatory effect is not universal for all TS introns (6).

In this study, we analyzed the role of the 5′-flanking region in regulating mouse TS gene expression. We constructed a series of chimeric minigenes consisting of different promoters linked to the normal TS coding region and polyadenylation signal. The minigenes were transfected into cultured cells, and the level of expression was measured. Our results indicate that sequences upstream of the essential promoter element(s) are required for normal regulation of TS gene expression in growth-stimulated cells. However, the 5′-flanking sequences are not sufficient for normal regulation.

MATERIALS AND METHODS

Construction of minigenes. The structures of the minigenes used in these analyses are summarized in Fig. 1. The construction of pTTT (also named pTSMG2 [9]) and $pTI_{56}T$ (also named pI56 [6]) has been described. Both of these minigenes have 1 kb of the normal 5′-flanking region and 0.25 kb of the normal 3′-flanking region of the mouse TS gene

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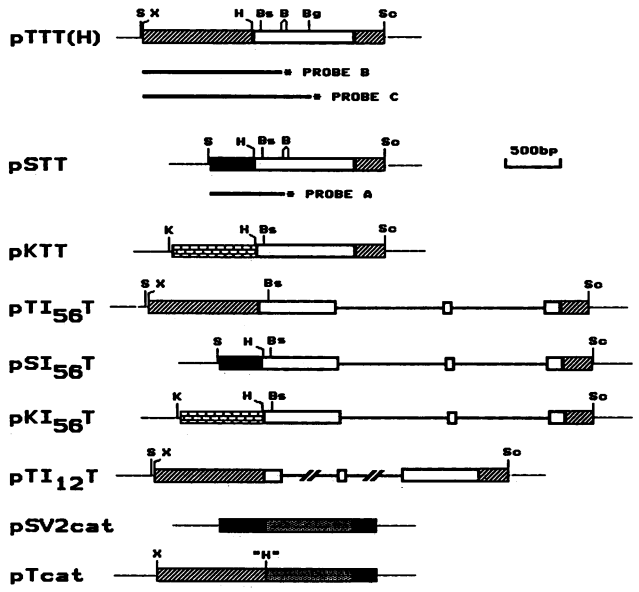

FIG. 1. Structures of the TS minigenes. Minigenes were constructed as summarized in Materials and Methods. The probes used for S1 nuclease protection assays were derived from the minigenes and are also shown. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bsu*36I; H, *Hin*dIII; "H," filled-in *Hin*dIII site; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sc, *Sac*I; X, *Xba*I. Introns 1 and 2 are not drawn to scale, as indicated by //. Symbols: ▨, TS promoter or polyadenylation signal; ■, SV40 early promoter or polyadenylation signal; ▦, HSV TK promoter; ▥, CAT coding region; □, TS coding region; —— TS intron sequence; – – –, vector sequence.

linked to the TS coding region at restriction sites in the first and last exons. The minigenes are cloned into pUC18. pTTT lacks introns, whereas $pTI_{56}T$ retains intact introns 5 and 6 at their normal locations in the coding region. Construction of $pTI_{56}T(-150)$, in which the promoter was deleted to −150 (relative to the ATG start codon), has also been described (7). $pTI_{12}T$, which contains intact introns 1 and 2 at their normal locations in the coding region, was constructed by cloning the *Xba*I fragment of phage clone TSB-9, which contains 1 kb of 5′-flanking region, exon 1, intron 1, exon 2, and part of intron 2 of the mouse TS gene (5), into the *Xba*I site of pBluescript M13+ (Stratagene) to create construct I. The *Hin*dIII-*Apa*I fragment of pTTT was replaced by the *Hin*dIII-*Apa*I fragment of pTSPPH1, which contains most of intron 2 and part of exon 3 of the TS gene (5), to form construct II. The *Hin*dIII fragment of construct I that contained the 5′ portion of the TS gene was then inserted into the *Hin*dIII site of construct II to generate $pT_{12}T$. In some cases, the TS minigenes described above were tagged with a 57-nucleotide deletion between the adjacent *Bam*HI sites in the coding region. The deletion was created by digesting the plasmid with *Bam*HI and recircularizing the plasmid.

To facilitate the construction of minigenes with alternative promoters, pTTT was modified in the following manner. The sequence CTGCTCCGTT (starting 10 nucleotides upstream of the ATG initiation codon) was changed to AAGCTTCG TT by site-directed mutagenesis (22), thereby creating a *Hin*dIII site upstream of the coding region. The resulting minigene was designated pTTT(H). To create the minigene pSTT, the *Sal*I-*Hin*dIII fragment of pTTT(H), which contains the TS promoter, was replaced by the 368-nucleotide *Sal*I-*Hin*dIII fragment of the DSP-1 vector (3) (kindly provided by M. Reff). This fragment contains the *Pvu*II-*Hin*dIII fragment of the simian virus (SV40) early region which includes the promoter-enhancer region and transcriptional start sites but lacks the translational start codon. To construct $pSI_{56}T$, the *Sal*I-*Bsu*36I fragment of $pTI_{56}T$ was substituted for the *Sal*I-*Bsu*36I fragment of pSTT.

To construct pKTT, the 0.7-kb *Bam*HI-*Bgl*II fragment of the herpes simplex virus (HSV) TK promoter (25) was inserted into the *Bam*HI site of pUC18. This fragment contains the promoter elements and transcriptional start sites but lacks the translational start codon for the HSV TK gene (24). To eliminate the ATG sequence in the *Sph*I site in the polylinker region of pUC18 (which might compete for translational initiation), the plasmid was linearized at the *Sph*I site, the protruding 3′ ends were removed with T4 DNA polymerase, and the blunt ends were ligated. Elimination of the ATG sequence was confirmed by DNA sequence analysis. The *Kpn*I-*Hin*dIII fragment of this plasmid was then inserted into the *Kpn*I-*Hin*dIII sites of pBluescript M13+. The TS coding region and 3′-flanking region derived from the *Hin*dIII-*Sac*I fragment of pTTT(H) were then inserted to form pKTT. $pKI_{56}T$ was constructed by substituting the *Bsu*36I-*Sac*I fragment of pKTT with the *Bsu*36I-*Sac*I fragment of $pTI_{56}T$. In control experiments, we determined that the level of expression of pKTT cloned into Bluescript M13+ was the same as that observed with pKTT that was cloned into pUC18.

pTcat was constructed in the following manner. The mouse TS promoter region extending from the *Xba*I site in the 5′-flanking region to the *Pst*I site in the first exon was cloned into pBluescript M13+. The exon sequences downstream of the A of the ATG start codon were removed by exonuclease III digestion. The deleted TS promoter was excised by digesting the plasmid with *Sac*I and *Kpn*I. The ends of the fragment were made blunt by T4 DNA polymerase, and the fragment was inserted into the promoterless pSV0cat gene (11) at the *Hin*dIII site (filled in by T4 DNA polymerase) that is located immediately upstream of the chloramphenicol acetyltransferase (CAT) coding region. A plasmid that contained the deleted TS promoter in the correct orientation was identified by restriction analysis and was designated pTcat. Plasmid pSV0cat includes the small t-antigen intron as well as the SV40 early polyadenylation signal downstream of the open reading frame. pSV2cat is identical to pSV0cat except that it contains the SV40 early promoter (from the *Pvu*II to the *Hin*dIII site) upstream of the CAT coding region (11).

Cell culture and transfections. Mouse 3T6 fibroblasts (36) were maintained on plastic petri dishes in Dulbecco modified Eagle medium supplemented with 10% calf serum. The thymidylate synthase-negative (*ts*$^-$) hamster V79 cells (28) were maintained in medium supplemented with 10% NuSerum (Collaborative Research) and 10 μM thymidine. Cells were transfected by the calcium phosphate procedure as described previously (7, 9). Stably transfected 3T6 cells were selected by cotransfecting a plasmid that confers resistance to the antibiotic G418 along with the test minigene in a ratio of about 1:40 (9). Clones of cells that were resistant to 400 μg of G418 (GIBCO) per ml were pooled and maintained as a mass culture. Transient transfection experiments were performed with the *ts*$^-$ V79 cells. Cultures were transfected with 8.5 pmol of TS minigene along with 0.8 pmol (5 μg) of pSV2cat (control plasmid) or with 3.0 pmol of pTcat along with 2.1 pmol (10 μg) of $pTI_{56}T$ (control plasmid). Cultures were harvested 2 days after transfection.

TABLE 1. Activities of TS and CAT minigenes[a]

Minigene	TS Level[b]	CAT activity[c]
Intronless		
pTTT	1.0	
pSTT	1.3 ± 0.5	
pKTT	0.44 ± 0.15	
Intron containing		
$pTI_{56}T$	11.4 ± 1.3	
$pSI_{56}T$	7.7 ± 2.3	
$pKI_{56}T$	1.6 ± 0.6	
$pTI_{12}T$	20.8 ± 4.4	
pTcat		1.0
pSV2cat		0.87 ± 0.10

[a] The indicated TS or CAT minigenes were transfected into *ts*⁻ V79 cells along with a control gene (pSV2cat or $pTI_{56}T$, respectively) to correct for differences in transfection efficiency. Extracts were prepared 2 days later and assayed for TS level and CAT enzyme activity. The results are averages (± standard deviations) of 3 to 10 independent experiments.

[b] Normalized to CAT activity. The value obtained for pTTT was set to 1.0 to facilitate comparisons.

[c] Normalized to TS enzyme level. The value obtained for pTcat was set to 1.0 to facilitate comparisons.

Enzyme assays. TS enzyme level was determined by measuring the formation of the covalent inhibitory ternary complex between TS, 5,10-methylenetetrahydrofolate, and [^{3}H]fluoro-dUMP (31) and expressed as counts per minute of [^{3}H]fluoro-dUMP bound per microgram of protein. CAT enzyme activity was determined by using the thin-layer chromatography assay (6, 11) and expressed as the percentage of substrate converted to product per 100 μg of protein.

S1 nuclease protection assays. Cytoplasmic poly(A)$^+$ mRNA was isolated from cells by phenol-chloroform extraction followed by oligo(dT)-cellulose column chromatography (20). S1 nuclease protection assays were performed as described previously (6, 7), using the 5′-$^{32}PO_4$-end-labeled probes described in Fig. 1.

RESULTS

Analysis of promoter strength. TS represents only about 0.01% of total cell protein in proliferating cells, which is typical for the product of a housekeeping gene. To determine whether this low level of expression is due to the fact that the gene has an exceptionally weak promoter, we compared the strength of the mouse TS promoter with that of two other well-characterized promoters, the HSV TK promoter and the SV40 early promoter. The TS promoter used in most of these studies extended from the *Xba*I site 1 kb upstream of the open reading frame to the A of the ATG start codon. The various promoters were linked to the intronless TS coding region and polyadenylation signal (Fig. 1). The minigenes were transfected into *ts*⁻ hamster V79 cells (28) and assayed for TS enzyme level 2 days later. The strength of the TS promoter was approximately the same as that of the SV40 early promoter but about two times greater than that of the HSV TK promoter (Table 1).

Inclusion of introns 5 and 6 led to a significant (4- to 11-fold) increase in the level of expression regardless of which promoter was used (Table 1), indicating that the stimulatory effect was not specific for the TS promoter. The TS and SV40 early promoters still had approximately equivalent activities in the intron-containing minigenes. We also found that inclusion of introns 1 and 2 at their normal locations in the coding region led to a 20-fold increase in gene expression (Table 1), indicating that the stimulatory effect is not highly intron specific.

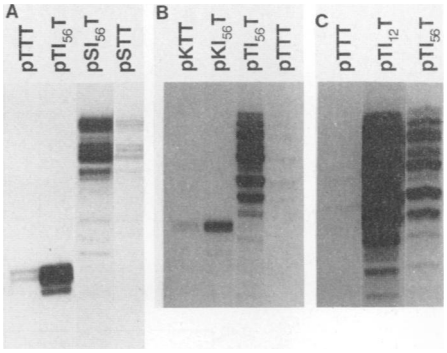

FIG. 2. S1 nuclease protection assays of TS mRNA isolated from cells transiently transfected with TS minigenes. (A) Comparison of TS mRNA levels derived from minigenes with the TS or SV40 early promoter. Cytoplasmic poly(A)$^+$ mRNA from transfected cells was isolated, and 4 μg of mRNA from each sample was hybridized with probe A (Fig. 1) derived from pSTT. The probe was labeled at the 5′ end with ^{32}P at a *Bam*HI site in the TS coding region (256 nucleotides downstream of the AUG start codon) and extended to the *Sal*I site 377 nucleotides upstream of the AUG codon. (B and C) Comparison of TS mRNA levels derived from minigenes with the TS or HSV TK promoter (B) or from $pTI_{12}T$ and $pTI_{56}T$ minigenes (C). The studies were performed as described above except that probe B (derived from pTTT) was used, and 8 μg of mRNA was analyzed.

To determine whether the same relative promoter strength would be observed with a different coding region and polyadenylation signal, the TS promoter was linked to the CAT coding region and SV40 early polyadenylation signal of the promoterless pSV0cat (11) to form pTcat (Fig. 1). The activity of pTcat was compared with that of pSV2cat (11), which has the SV40 early promoter in place of the TS promoter but is otherwise identical to pTcat. Approximately the same amount of CAT activity was observed with both minigenes (Table 1), again indicating that the TS and SV40 early promoters had approximately the same strength. These results also demonstrate that all of the essential TS promoter elements are located upstream of the ATG start codon.

To be certain that the differences in enzyme levels were due to differences in mRNA levels, cytoplasmic poly(A)$^+$ mRNA was isolated from the transfected cells and analyzed by S1 nuclease protection assays. The probe used in these analyses (probe A) corresponded to the TS coding region linked to the SV40 early promoter. Therefore, mRNA derived from minigenes with the SV40 promoter protected a DNA fragment extending from the TS coding region to the transcriptional start sites of the SV40 promoter, whereas mRNA derived from minigenes with the TS promoter protected shorter fragments that extended to the point of insertion of the SV40 sequences. There was good agreement between the relative amount of mRNA and the relative amount of enzyme derived from each minigene (Fig. 2). Densitometric scanning of the autoradiograms revealed that approximately the same amount of mRNA was produced by minigenes with the SV40 promoter as by minigenes with the TS promoter. Minigenes with the HSV TK promoter produced about two to three times less mRNA than did minigenes with the TS promoter; $pTI_{12}T$ produced at least twice as much mRNA as did $pTI_{56}T$.

Regulation of minigene expression in growth-stimulated cells. To characterize the sequences and mechanisms that

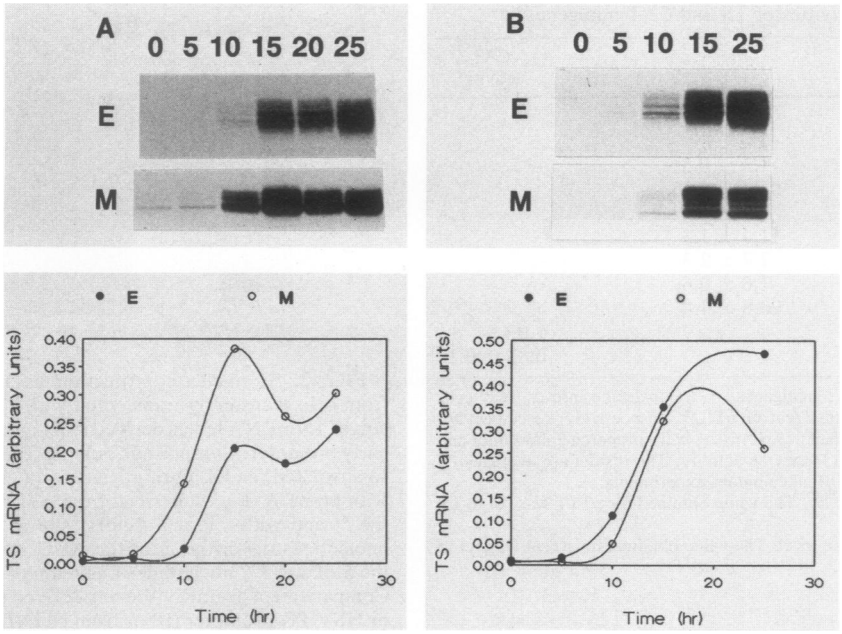

FIG. 3. Expression of pTI56T(d) and $pTI_{12}T(d)$ in growth-stimulated cells. 3T6 cells that were stably transfected with $pTI_{56}T(d)$ (A) or $pTI_{12}T(d)$ (B) were allowed to rest in 0.5% calf serum for 7 days and then induced to reenter the cell cycle by feeding them with fresh medium containing 10% serum at time 0. At the indicated times, cytoplasmic poly(A)$^+$ mRNA was isolated and equal amounts of mRNA (approximately 1 μg) were analyzed by S1 nuclease protection assays. Probe C (derived from pTTT) was used for this experiment. The probe was 5′ end labeled at the *Bgl*II site in the TS coding region 531 nucleotides downstream of the AUG initiation codon and extended to the *Xba*I site 1 kb upstream of the AUG codon. Fragments labeled E and M correspond to the fragments protected by TS mRNA derived from the endogenous TS gene and the transfected TS minigene, respectively. The autoradiograms were scanned with a densitometer, and the results are plotted below each autoradiogram.

are important for regulating TS gene expression, it is first necessary to demonstrate that the regulatory sequences are present and functional in the minigene. Our strategy was to stably transfect the minigenes into wild-type mouse 3T6 fibroblasts and to study the expression of each minigene in parallel with that of the endogenous TS gene in growth-stimulated cells. A plasmid that confers resistance to the antibiotic G418 was cotransfected with the TS minigene to permit selection of cells that had stably incorporated the minigenes into their genome. To avoid possible artifacts that may result from the site of integration of the minigenes into the host cell genome, the regulation studies were performed by using pools of cells representing at least 100 independent G418-resistant clones rather than single clones.

Since the 3T6 cells were wild type, it was not possible to measure minigene expression by monitoring TS enzyme levels. However, it was possible to distinguish TS mRNA derived from the endogenous TS gene from that derived from the transfected TS minigenes by using 5′ S1 nuclease protection assays. For studies of minigenes in which the TS promoter was linked to the TS coding region, the minigene was tagged by deleting a 57-nucleotide fragment from the middle of the coding region (between two *Bam*HI sites). Minigenes that contained the deletion were designated by the suffix (d). The S1 probe did not contain the deletion and extended to the *Bgl*II site 218 nucleotides 3′ of the deletion. Thus, RNA derived from the minigene protected the probe up to the site of the deletion, whereas RNA derived from the endogenous TS gene protected the probe to the sites of transcriptional initiation.

The regulation of $pTI_{56}T(d)$ was very similar to that of the endogenous TS gene in serum-stimulated mouse 3T6 cells (Fig. 3A). TS mRNA levels from the minigene and from the endogenous TS gene remained low until about 10 h after serum stimulation and then increased dramatically. Densitometric analysis revealed that the amount of TS mRNA increased about 20-fold between 5 and 15 h both for the endogenous TS gene and for the minigene. Therefore, all of the sequences that are necessary and sufficient for normal growth regulation appear to be contained within this minigene. $pTI_{12}T(d)$ was also regulated normally (Fig. 3B). Again, there was at least a 20-fold increase in TS mRNA derived from the endogenous and transfected minigenes between 5 and 15 h after stimulation. These experiments were repeated several times with two different stably transfected cell lines, and similar observations were made each time.

Role of the TS 5′-flanking region in growth-regulated expression. To determine whether sequences upstream of the ATG start codon are important for regulating TS gene expression, the SV40 early promoter was substituted for the TS promoter in the $pTI_{56}T$ minigene to create $pSI_{56}T$ (Fig. 1). Previous studies have shown that the SV40 early promoter is constitutively expressed in serum-stimulated cells (35). The $pSI_{56}T$ minigene was stably transfected into 3T6 cells as described above. Transcripts derived from the minigenes were distinguished from those derived from the endogenous TS gene by S1 nuclease protection assays using probe A (Fig. 1). The amount of mRNA derived from the minigene increased only two- to threefold following serum stimulation, whereas the endogenous gene was regulated normally (Fig. 4A).

We showed previously in transient transfection experiments that wild-type promoter activity (including the entire

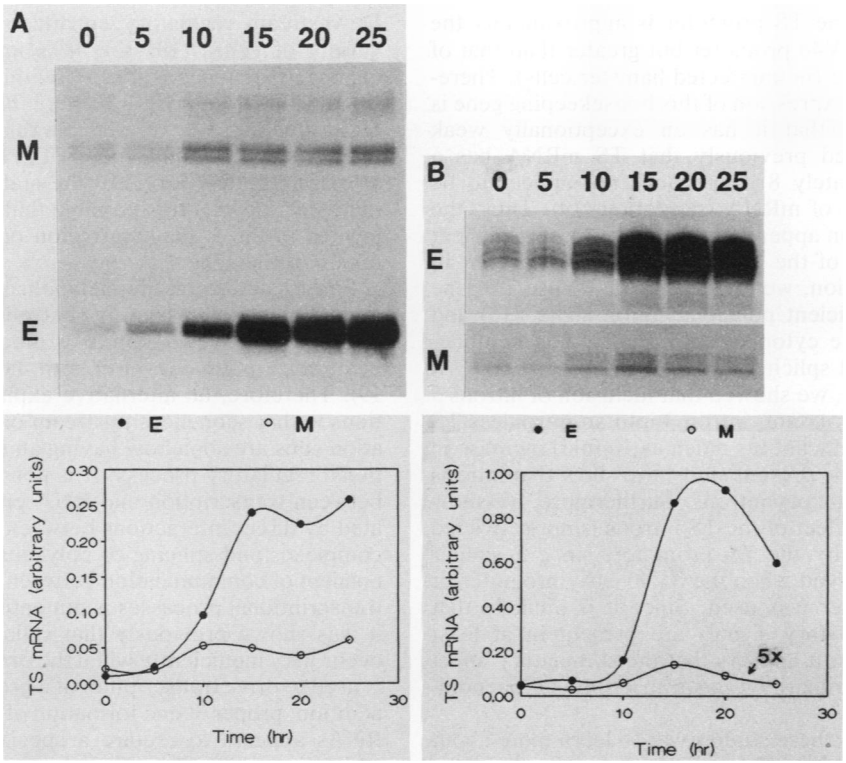

FIG. 4. Expression of $pSI_{56}T$ and $pTI_{56}T(d)(-150)$ in growth-stimulated cells. The experiment was performed as described for Fig. 3 except that the cells were stably transfected with $pSI_{56}T$ and probe A was used for the S1 protection assays (A) or the cells were stably transfected with $pTI_{56}T(d)(-150)$ and probe C was used for the S1 protection assays (B). Densitometric analyses are plotted below each autoradiogram. The values for the minigene in panel B were multiplied by 5 so that they could be more readily compared with the values for the endogenous gene.

spectrum of transcriptional start sites) was observed with $pTI_{56}T(-150)$, in which 5′-flanking sequences upstream of −150 relative to the ATG start codon (or −60 relative to the first transcriptional start site) were deleted (7). To determine whether the regulatory elements were also located downstream of −150, this minigene (with the internal *Bam*HI deletion) was stably transfected into 3T6 cells. This minigene was constitutively expressed in serum-stimulated cells (Fig. 4B).

These studies indicated that sequences in the 5′-flanking region of the TS gene are necessary for normal regulation. To determine whether this region is sufficient for normal regulation, we analyzed the expression of pTcat (Fig. 1) in serum-stimulated 3T6 cells. Because CAT activity is not present in mammalian cells, we were able to measure gene expression at the protein level instead of the mRNA level in this experiment. TS enzyme level was also determined to be certain that the endogenous TS gene was regulated normally in the transfected cell line. Although the TS enzyme level increased about sixfold between 10 and 25 h after stimulation, there was no significant change in CAT enzyme activity (Fig. 5). Similar results were obtained when about 7 kb of 5′-flanking DNA from the normal TS gene was linked to the CAT coding region (data not shown). Therefore, it appears that the 5′-flanking region alone is not sufficient for normal regulation in response to growth stimulation.

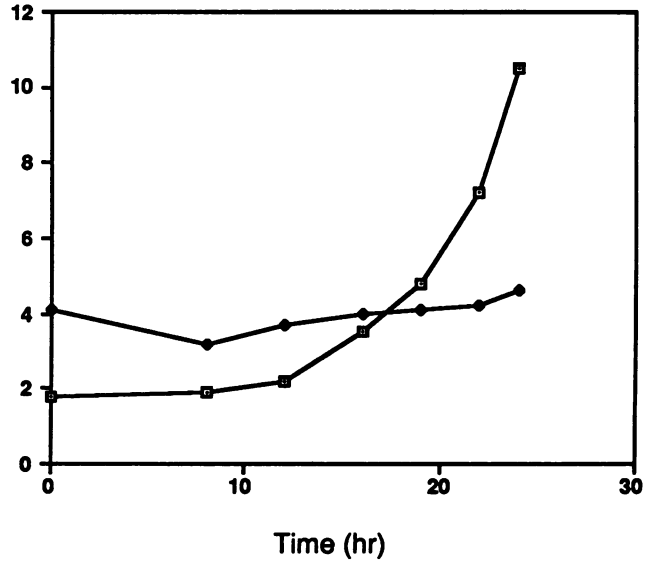

FIG. 5. Expression of pTcat in growth-stimulated cells. Serum-arrested 3T6 cells that were stably transfected with pTcat were serum-stimulated as described in the legend to Fig. 3. At the indicated times, cells were harvested and CAT activity (percent conversion per 100 μg of protein; ◆) and TS enzyme level (counts per minute per microgram of protein; ⊡) were determined. The data represent the averages of two independent experiments.

DISCUSSION

We have analyzed the role of the 5′-flanking region in regulating the expression of the mouse TS gene. We show

that the strength of the TS promoter is approximately the same as that of the SV40 promoter but greater than that of the HSV TK promoter (in uninfected hamster cells). Therefore, the low level of expression of this housekeeping gene is not due to the fact that it has an exceptionally weak promoter. We showed previously that TS mRNA has a half-life of approximately 8 h and does not appear to be regulated at the level of mRNA translation (19). Thus, the low level of expression appears to be the result of inefficient processing or export of the transcript to the cytoplasm. In line with this conclusion, we found that the mouse TS gene has a relatively inefficient polyadenylation signal (15) and that about 10% of the cytoplasmic TS mRNA is nonfunctional due to aberrant splicing of intron 5 (23).

In a previous study, we showed that inclusion of introns 5 and 6 and, to a lesser extent, intron 3 into an intronless TS minigene led to a significant (as much as 10-fold) increase in gene expression. In the present study, we show that introns 1 and 2 are also stimulatory introns. Furthermore, we show that the stimulatory effect of the TS introns is not restricted to minigenes driven by the TS promoter, since a similar stimulation was observed when the SV40 early promoter or the HSV TK promoter was used. Since it is unlikely that transcriptional stimulatory signals are present in at least three different introns, it appears that the stimulatory effect of the TS introns is primarily a posttranscriptional phenomenon.

Our primary goal in these studies was to learn more about the sequences and mechanisms that are responsible for regulating TS gene expression in growth-stimulated cells. Minigenes that included 1 kb of the TS 5′-flanking region, the TS coding region (with introns 5 and 6 or 1 and 2 to increase the level of expression), and 0.25 kb of the TS 3′-flanking region were regulated in growth-stimulated cells in a manner that was almost identical to that of the endogenous TS gene. Therefore, sequences that are both necessary and sufficient for proper regulation of the mouse TS gene are contained within these minigenes. Since these analyses were performed with mass cultures that consisted of at least 100 independent clones of stably transfected cells, it is unlikely that proper regulation is highly dependent on the site of chromosomal integration of the transfected genes.

Substitution of the SV40 early promoter for the TS promoter led to constitutive expression in growth-stimulated cells. This finding suggests that important regulatory sequences are located within the 5′-flanking region of the TS gene. Deletion of sequences upstream of −60 (relative to the first transcriptional start site) also resulted in constitutive expression. Since all of the elements that are sufficient for normal transcriptional initiation are located downstream of −60 (7), at least some of the regulatory sequences are located upstream of the essential promoter elements.

Even though the upstream sequences are necessary, they do not appear to be sufficient for proper regulation, since a minigene consisting of the 1-kb mouse TS promoter linked to the CAT coding region was also expressed constitutively in growth-stimulated cells. This observation suggests that proper regulation depends on the presence of multiple regulatory elements that are located in more than one region of the gene. One possible explanation for our observations is that TS gene transcription is regulated by a downstream enhancer sequence that is necessary (but not sufficient) for growth phase-specific transcriptional regulation. This downstream control sequence would presumably function in cooperation with control sequences upstream of the essential promoter elements to bring about proper regulation. Downstream regulatory enhancers have been found in a variety of genes (10, 32). It is unlikely that the putative enhancer sequence is located within one of the TS introns, since $pTI_{56}T$ and $pTI_{12}T$ were both regulated normally. Unfortunately, we were unable to analyze the regulation of the intronless TS minigene, pTTT, because it was expressed at extremely low levels in the stably transfected cell lines (data not shown). It is possible that the putative enhancer is located in the 3′-flanking region or even within the coding region of the gene.

Earlier studies on the metabolism of TS mRNA indicated that TS gene expression is controlled to some extent at the transcriptional level but to a much greater extent at the posttranscriptional level in both mouse and human cells (1, 19). Therefore, an alternative explanation for our observations is that sequences upstream of the transcriptional initiation sites are somehow having an effect on a posttranscriptional regulatory process. It is possible that communication between transcription and RNA processing could be mediated by direct interactions between transcriptional initiation complexes and splicing or polyadenylation complexes. The concept of communication between transcriptional and posttranscriptional processes is not entirely novel. For example, it was shown previously that splicing and polyadenylation occur very inefficiently when the promoter for an rRNA gene is used to drive transcription of a protein-coding gene (34). In addition, proper 3′ end formation of U1 and U2 small nuclear RNAs appears to require a special transcription complex which is specified by sequences in the 5′-flanking region of the small nuclear RNA gene. Substitution of other RNA polymerase II promoters results in incorrect 3′ end formation (16, 17, 27). Identification of the precise sequences that are required for proper regulation of TS gene expression as well as the *trans*-acting factors with which they interact should lead to a greater understanding of the complex regulatory mechanisms that are used to control the expression of this gene.

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