Regulation of mouse thymidylate synthase gene expression in growth-stimulated cells: upstream S phase control elements are indistinguishable from the essential promoter elements

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ABSTRACT

Expression of the mammalian thymidylate synthase (TS) gene in growth-stimulated cells is closely coordinated with entry into S phase. Previous studies with transfected TS minigenes have shown that sequences upstream of the coding region as well as an intron in the transcribed region are both necessary for proper regulation of TS mRNA content in growth-stimulated cells. The goal of the present study was to identify the upstream regulatory elements. Minigenes consisting of TS 5' flanking sequences linked to the TS coding region (interrupted by introns 1 and 2) were stably transfected into mouse 3T6 cells. Deletion and site-directed mutagenesis of the 5' flanking region revealed that there is a close correspondence between the upstream sequences that are necessary for S phase regulation and the 30 nucleotide region that is essential for promoter activity. These observations raised the possibility that regulation of the TS gene occurs at the transcriptional level. However, nuclear run-on assays showed that the rate of transcription of the TS gene changed very little during the G1–S phase transition. Furthermore, when the TS promoter was linked to an intron-less luciferase indicator gene, there was no change in expression following growth-stimulation. Therefore it appears that the TS gene is controlled primarily at the posttranscriptional level, and that the TS essential promoter region is necessary (although not sufficient) for proper S phase regulation.

INTRODUCTION

Thymidylate synthase (TS) is an essential enzyme that catalyzes the *de novo* synthesis of thymidylic acid. Expression of the TS gene increases greatly at the G1/S phase boundary in yeast and mammalian cells (1–3). In yeast, TS gene expression is controlled primarily at the transcriptional level (4). However, in mammalian cells, the regulatory mechanisms are more complex. In growth-stimulated human or mouse cells, the steady-state level of TS mRNA increases 10–20-fold as cells progress from G0 through

S phase. However, nuclear run-on assays have shown that the rate of TS gene transcription increases very little during this interval (5,6), suggesting that the content of mammalian TS mRNA is regulated primarily at the posttranscriptional level. In addition, the synthesis of human TS enzyme has been shown to be regulated by an autogenous translational control mechanism (7).

To identify the *cis*-acting regulatory elements that are responsible for directing S phase regulation of mouse TS mRNA content, we have analyzed the regulation of TS minigenes in stably transfected mouse 3T6 fibroblasts. We found that proper regulation requires the presence of the TS 5' flanking region as well as an intron within the coding region (8,9). The mechanism by which the upstream regulatory sequences cooperate with introns to bring about S phase specific regulation is not known.

The promoter of the mouse TS gene (Fig. 1) is G/C-rich and lacks both a TATA box and an initiator element. The promoter is also bidirectional and initiates transcription at many sites over large initiation windows in both directions (10,11). The sequences of the TS promoter that are essential for efficient expression are contained within a 30 nucleotide (nt) region between –104 and –75 (relative to the AUG codon), which has been designated the 'essential region' (12,13). The essential region contains GGAAG motifs centered at –100 and –85 that resemble binding sites for members of the Ets family of transcription factors (14). In addition, it contains a G/C box centered at –80 that resembles the recognition motif for Sp1. Inactivation of any of these elements leads to a significant reduction in promoter activity (12,13,15). There are also binding sites for USF and Sp1 as well as a sequence that resembles an E2F element upstream of the essential promoter region (Fig. 1). However, elimination of these sites has little effect on promoter activity (12,13).

The goal of the present study was to identify the sequences in the 5' flanking region of the TS gene that are necessary for proper regulation of the TS gene in growth-stimulated cells. Our results show that the upstream regulatory sequences are very similar, if not identical to the essential promoter elements. We have also re-examined the rate of TS gene transcription in growth-stimulated cells using nuclear run-on assays as well as by determining the expression of the luciferase indicator gene driven by the TS promoter. Both analyses confirm our previous conclusion that the

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The m115 mutation was generated by a two reaction PCR approach. A wild-type template was amplified using the m115

oligo as the 5' primer and the m10 oligo as the 3' primer. The product of the reaction was mixed with a wild-type promoter template that spanned the TS promoter sequence from -165 to -20, and was reamplified using the m150 oligo as the 5' primer and the m10 oligo as the 3' primer. The final PCR fragment extended from -156 to -10 and contained the m115 mutation. This fragment was cloned into a TI12dT minigene in the same manner as described above.

To create the -119 and -112 deletions at the 5' end of the promoter region, minigenes that extended to -156 and that contained either the m124 or m115 mutation as well as the m150 mutation were digested at the *Xba*I site created by the downstream mutation and at the *Sal*I site created by the m150 mutation. Overhangs were filled in using T4 DNA polymerase and the blunt ends were ligated using T4 DNA ligase (USB).

The -104 deletion was created by digesting the m110 mutation site with *Nhe*I and the m155 mutation site with *Spe*I followed by ligation of the compatible overhangs. To generate minigenes with the promoters -131 to -11, -131 to -75 or -131 to -89, the TS promoter was amplified using the m124 primer (which contains the m124 mutation and extends to -131) as the 5' primer, and the m10, m75 or the m85 oligo as the 3' primer, respectively. The PCR products were digested at the *Nhe*I site created by the 3' primer and cloned into a TI12dT minigene which contains an *Nhe*I site at m10 and a blunted *Sal*I site located in the vector. All promoter modifications were confirmed by DNA sequence analysis (19).

Nuclear run-on assays

The nuclei from cultured 3T6 or LU3-7 cells were isolated and incubated for *in vitro* transcription as described previously (20,21) with minor modifications. It was necessary to use 35 strokes with the Dounce homogenizer (B pestle) to ensure complete cell lysis. The nuclei were collected by ultracentrifugation through a 1 M sucrose cushion, resuspended at a concentration of $\sim 10^8$ nuclei/ml and frozen at -70°C . Nuclei were transcribed *in vitro* at 26°C in the presence of [α - ^{32}P]UTP. Labeled RNA was isolated and partially fragmented by a 20 min incubation with 0.2 M NaOH at 0°C to facilitate hybridization.

The following hybridization probes were used. pTSKS- contains mouse TS cDNA sequences from the 59th nt of exon 3 to the end of exon 7 as well as TS introns 5 and 6 at their normal positions. The plasmid was created by insertion of a *Bam*HI/*Sst*I fragment from TI56G (9) into the *Bam*HI and *Sst*I sites of Bluescript KS-. The same fragment was also cloned in Bluescript KS+ to create pTSKS+. The plasmids were maintained in XL-1 blue. Single-stranded sense or antisense probes were recovered using M13K07 helper phage. pL32Sma contains the mouse ribosomal protein L32 gene from the middle of intron 1 to the middle of intron 3. pS16Sac contains ribosomal protein S16 gene from the middle of exon 1 to 110 nt 3' of exon 5. The ribosomal protein probes were provided by Dr Robert Perry. The pL32Sma and pS16Sac plasmids were linearized by *Sac*I.

Hybridization filters were prepared in the following manner. Single-stranded or linearized double-stranded DNA was incubated in 0.1 M NaOH at room temperature for 30 min. The samples were neutralized with 10 vol 6 $\times$ SSC, cooled on ice, and applied to 0.2 μM Nitran membrane (Schleicher & Schuell) using a dot blot apparatus (Schleicher & Schuell). Each dot contained 5 μg of DNA. The DNA was linked to the membrane by a UV

crosslinker (BioRad). Hybridization, washing and incubation of the filters with RNase A was performed as described (22). The amount of radioactivity hybridized to each dot was quantitated by a Betagen Blot Analyzer and visualized by autoradiography.

Enzyme assays

Cells were analyzed for luciferase activity using a kit obtained from Promega. β -galactosidase activity was measured using a luminescence assay kit obtained from Tropix. Luminescence was measured in a Berthold Lumat luminometer.

RESULTS

Structure and analysis of minigenes

The minigenes and probes used in these analyses are described in Figure 2. The minigenes are named in the following manner. The first letter indicates the promoter and 5' flanking region: S indicates SV40 early promoter; T indicates the TS promoter. The last letter indicates the polyadenylation signal and 3' flanking region: T indicates the TS signal; G indicates the human β -globin signal. The middle symbols refer to the coding region: T indicates the intron-less TS coding region; I12 indicates the TS coding region with intact TS introns 1 and 2, L indicates the coding region for the luciferase indicator gene. The minigenes with TS coding regions were tagged with a 57 nt deletion in the middle of the coding region to permit distinction between mRNA derived from the TS minigenes and the endogenous TS gene. The minigenes were stably transfected into wild-type mouse 3T6 fibroblasts, and TS mRNA content was quantitated in growth-stimulation experiments by S1 nuclease protection assays. Each minigene was analyzed in at least two independent cell lines, and multiple analysis were usually performed on each cell line. Similar results were obtained with each repeat analysis.

The TS 5' flanking region directs S phase regulation

Figure 3A shows that a stably transfected TS minigene that contains intact introns 1 and 2 at their normal locations in the coding region (minigene TI12T) can reproduce the normal S phase regulation of the endogenous TS gene. Similar results were obtained in earlier studies (8,9). Unfortunately, the 3 kb TS intron 2 contains many inconvenient restriction sites which would hinder the construction of minigenes that had alterations in the 5' end. To eliminate this difficulty and to reduce the size of the minigene, the interior of intron 2 was deleted to create TI12dT (Fig. 2). Figure 3B shows that the TI12dT minigene was regulated in the same manner as the endogenous TS gene, indicating that no essential regulatory elements had been deleted. Therefore, the TI12dT minigene was used for further analyses of the 5' regulatory sequences. To be certain that regulation depended on the presence of TS 5' flanking sequences, the TS 5' flanking region of TI12dT was replaced by the SV40 early promoter to create SI12dT (Fig. 2). Figure 3C shows that SI12dT was constitutively expressed in growth-stimulated cells.

The 5' and 3' boundaries of the regulatory region

Preliminary analyses of the upstream regulatory region suggested that multiple regulatory elements may be present and that elimination of some of the elements led to partial loss of regulation. To improve our ability to detect subtle differences in the patterns

Figure 2. TS minigenes. The nomenclature used to describe the minigenes is given in Results. The minigenes TTT and STT have been described previously (8). Both minigenes contain the intact, intron-less TS coding region and the TS polyadenylation signal (shaded boxes). The minigene TTT contains a 1 kb TS 5' genomic flanking region including the TS promoter (open box), while the minigene STT contains the SV40 early promoter (filled box) in place of the TS promoter/flanking region. The TI12T minigene, which retains intact TS introns 1 and 2 at their normal locations in the coding region, has been described previously (8). The TI12dT minigene was created by deleting 2.7 kb of intron 2, between the *Hind*III and *Eco*RI sites. The deleted intron 2 retains 100 bp at the 5' end and 75 bp at the 3' end of the intron. The SI12dT minigene was created by substituting the SV40 early promoter for the TS 5' flanking region of TI12dT. The TLG intron-less minigene consists of the TS promoter region from -1 to -11 Kb (relative to the AUG codon) linked to the luciferase coding region of pGL2-basic (Promega). The SV40 polyadenylation signal/small T intron of pGL2-basic was replaced with the polyadenylation signal from the human β -globin gene (0.8 kb *Eco*RI to *Pst*I fragment). Probes for S1 nuclease protection assays were made from the minigenes TTT and STT as indicated by the heavy lines below these minigenes. The probes were labeled at the 5' end with 32 PO $_4$ (filled circle). The probe for ribosomal protein L32 (rpL32) mRNA (not shown) was derived from an intron-less derivative of the mouse rpL32 gene (43); it was end-labeled at the *Dra*III site in exon 4 (44) and extended to a *Hind*III site in the pUC18 vector. Abbreviations: H, *Hind*III; 'H', engineered *Hind*III sites; B, *Bam*HI; Bg, *Bgl*II; C, *Cl*aI; E, *Eco*RI; L, *Sal*I; N, *Nde*I; Nh, *Nhe*I; S, *Sac*I; SI, *Sal*I; St, *Stu*I; X, *Xba*I.

Figure 3. The 5' flanking region directs S phase expression. 3T6 cells containing stably integrated minigenes TI12T (A), TI12dT (B) or SI12dT (C) were growth-arrested in 0.5% calf serum and then serum stimulated to re-enter the cell cycle. Total cytoplasmic RNA was isolated at the indicated times after stimulation. Messenger RNA content was analyzed by performing S1 nuclease protection assays. A constant amount of RNA (~20 μ g) and 1×10^5 c.p.m. of TTT or STT probe (Fig. 2) were used for each time point. Relevant regions of the autoradiograms from the S1 assays are shown above the graphs. mRNA derived from the minigene (M) protects a 216 nt fragment due to the fact that the minigenes lack a 57 bp *Bam*HI fragment exon 3. mRNA from the endogenous TS gene (E) protects a 560 nt fragment. The radioactivity in each band was quantified in a Betagen Blot Analyzer. The fold increase for each mRNA was calculated by normalizing the radioactivity at each time point by the radioactivity at time = 0, which was set at 1.

of minigene regulation, we also monitored the expression of the ribosomal protein L32 (rpL32) gene since it is present at a high and relatively constant level in growth-stimulated cells (23). To correct the data, the amount of probe protected by TS mRNA derived from the endogenous TS gene and the TS minigene was normalized to the amount of probe protected by rpL32 mRNA. This correction resulted in a significant increase in the experimental reproducibility and was used in all subsequent analyses.

The upstream boundary of the regulatory element(s) was determined by analyzing the regulation of minigenes with deletions in the 5' flanking region. The deletions were made by eliminating the nucleotide sequences upstream or downstream of restriction sites that were engineered into the TS 5' flanking region (Fig. 1). Elimination of sequences upstream of -250, -165 (data not shown), -119 (Fig. 4A) or -112 (Fig. 4B) had no significant effect on TS regulation. This shows that sequences upstream of the essential promoter region, including the USF element (E box) centered at -135 and the Sp1 element centered at -130, do not play important roles in the S phase regulation of the mouse TS gene.

The observation that the -119 and -112 deletions maintained proper regulation was unexpected in view of our earlier study which showed that deletion of TS sequences upstream of -150 led to a significant loss of regulation (8). In the previous study, this minigene was expressed at a very low level and was difficult to quantitate by densitometric scans of autoradiograms. Improvements in our methods of analysis have greatly reduced such difficulties.

We next analyzed a minigene in which sequences upstream of -104 were deleted. This deletion removed a potential E2F binding

Figure 4. Defining the regulatory region. 3T6 cell lines were stably transfected with T112dT-based minigenes that contained modified TS promoters, and the expression of each minigene was analyzed in growth stimulation assays as described in Figure 3. TS probes derived from the STT or TTT minigenes (Fig. 2) and an rpl32 probe were used simultaneously to detect mRNA derived from the endogenous TS gene (E), TS minigenes (M) and the rpl32 gene (R). The specific activity of the rpl32 probe was much less than that of the TS probe so that the intensity of the rpl32 signal would be comparable with that of the TS signals. For each time point, the signals from the endogenous TS gene and the minigene were normalized to the signal derived from the rpl32 gene to correct for differences in RNA recovery. The fold stimulation was calculated from the normalized data as described in Figure 3. The minigenes contain the regions of the TS 5' flanking region and the mutations that are indicated at the top of each panel. The nucleotide sequence alterations introduced by each mutation are shown in Figure 1.

site that is immediately adjacent to the essential promoter region (Fig. 1). This minigene also showed growth-regulated expression, as shown in the autoradiogram of Figure 4C. However, quantitative analysis of the radioactivity (as shown in the graph of the data) revealed that the fold increase in expression of the minigene was significantly less than that of the endogenous TS gene. In addition, minigene expression appeared to increase somewhat earlier than that of the endogenous TS gene.

We also examined the regulation of minigenes with specific point mutations upstream of the essential promoter region. In these experiments, the TS 5' flanking region extended from -165 to -11.

The sequence alterations introduced by the m130, m124 and m115 mutations had no effect on regulation (data not shown). Note that the m130 mutation inactivates the consensus Sp1 element at -130. Figure 4D shows that minigenes with the m110 mutation, which inactivates the potential E2F element, still displayed S phase regulation. However, the fold increase was somewhat reduced relative to that observed for the wild-type minigene. The m100 mutation inactivated the GGAAG box that is located at the 5' end of the essential promoter region. Minigenes with the m100 mutation were expressed at a relatively low level due to the inactivation of this important promoter element. The autoradiogram (Fig. 4E) shows that the gene is still growth-regulated. However, the magnitude of the increase was significantly less than that of the endogenous TS gene.

To determine if there were any S phase regulatory elements between the essential promoter region and the AUG codon, promoters with internal deletions were created. In these experiments, the 5' boundary of the TS promoter was at -119. Deletion of sequences between -74 and -5 had very little effect on regulation (Fig. 4F) indicating that all of the upstream S phase regulatory elements were located between -119 and -75. Deletion of sequences downstream of -89 led to inactivation of the promoter (data not shown) due to the removal of the downstream GGAAG and Sp1 elements from the essential promoter region.

Rate of TS gene transcription

Previous nuclear run-on transcription analyses indicated that there was little change in the rate of TS gene transcription in growth-stimulated mouse and human cells (5,6). However, the observation that the upstream S-phase regulatory elements of the mouse TS gene coincided with the core promoter elements is consistent with the idea that the gene might be controlled at the transcriptional level. For this reason, it was important to re-examine this issue.

In our previous nuclear run-on analyses, the probe was double-stranded TS cDNA that extended from the beginning of exon 1 to the end of exon 7 (5). The results obtained in these analyses might be incorrect if the TS promoter directed the synthesis of a high, constitutive level of transcripts that are prematurely paused or terminated within the first exon (24). In addition, the double-stranded probes used previously might give unreliable results if there were significant transcription of the gene in the antisense orientation.

To avoid these potential difficulties, nuclear run-on assays of the TS gene were repeated using single-stranded probes for sense and antisense transcripts of the TS gene. The probes extended from the middle of exon 3 to the end of exon 7. Introns 5 and 6 (which lack any known repetitive elements) were also included to increase the signal strength. TS transcription rates were compared with those of the ribosomal protein rpl32 and rpS16 genes or with total transcription.

Figure 5A (right panel) shows the results of run-on assays performed with 3T6 cells. The amount of radioactivity associated with the TS sense probe relative to that associated with the rpl32 or rpS16 probes did not change significantly when comparing G1 with S phase nuclei (data not shown). However, the amount of radioactivity associated with the TS sense probe was similar to that associated with the antisense probe. This might indicate that the sense and antisense signals were due primarily to nonspecific binding of labeled RNA to the probes. Alternatively, it might indicate that the TS gene is transcribed equally in both directions.

To distinguish between these possibilities and to increase the strength of the hybridization signal, the run-on assays were

Figure 5. Nuclear run-on analyses. Nuclei were isolated from serum-stimulated 3T6 or LU3-7 cells and used for nuclear run-on analyses. The amount of labeled RNA corresponding to the indicated genes was analyzed by DNA-excess hybridization. The resulting autoradiograms are shown in (A). The amount of radioactivity was quantitated on a Betagen Blot Analyzer. The ratio of labeled TS RNA was normalized either to total labeled RNA or to the amount of labeled ribosomal protein mRNA (rpL32 + rpS16 RNA) and plotted in (B) and (C), respectively. The results of several analyses are shown.

repeated using serum-stimulated LU3-7 cells. LU3-7 cells are a fluorodeoxyuridine-resistant derivative of 3T6 cells in which TS mRNA and protein are increased ~50-fold due to amplification of the TS gene (17). We showed previously that TS gene expression is regulated in the same manner in LU3-7 cells as in 3T6 cells (5,25). Figure 5A (left panel) shows that the signal corresponding to TS sense transcripts was much stronger with the LU3-7 cells than with 3T6 cells, as expected. However, the amount of radioactivity associated with the TS antisense probe was about the same

Figure 6. Expression of the intron-less TS-luciferase minigene. The TLG minigene and a control minigene consisting of the β -galactosidase coding region driven by the SV40 early promoter were stably transfected into 3T6 cells. Growth stimulation experiments were performed as in Figure 3. Cell extracts were assayed for luciferase and β -galactosidase activity at the indicated times following serum stimulation. Luciferase activity (usually between 50 000 and 100 000 light units) was normalized to β -galactosidase activity to correct for differences in recovery. The resulting values were normalized to that observed at time = 0. The results of three experiments are shown.

(relative to rpL32 or rpS16) with both cell lines. Therefore it appears that the radioactivity associated with the antisense probe corresponds to nonspecific hybridization rather than antisense TS transcripts; the latter would be expected to increase in parallel with TS sense transcripts in the amplified cell line. Subsequent analyses were performed with LU3-7 cells to increase the reliability of the data.

The results of several analyses show that the rate of TS gene transcription remains relatively constant during the G0-S phase transition. This is true regardless of whether labeled TS sense RNA was normalized to labeled ribosomal protein transcripts (Fig. 5B) or to total labeled RNA (Fig. 5C). These observations are in agreement with the results of our previous study.

The ratio of labeled ribosomal protein RNA to total labeled RNA also remained relatively constant following growth stimulation (data not shown). This is as expected since the amount of ribosomal protein mRNA changes very little following growth-stimulation (23). Since the promoters of the rpL32 and rpS16 genes have approximately equal strength (21), the amount radioactivity associated with the rpL32 probe relative to the rpS16 probe should be in the ratio of 1.26:1 (after correcting for the number of U residues detected by each probe). We found that the ratio remained relatively constant at ~1.6 in all experiments, in reasonable agreement with the expected value.

Regulation of the TS-luciferase minigene

Another independent approach to detect the ability of a promoter to direct transcriptional regulation is to monitor the expression of an indicator gene that is linked to the promoter. We have constructed and analyzed the expression of the TLG minigene which consists of 1 kb of the 5' flanking region of the TS gene linked to an intronless luciferase minigene (Fig. 2). The minigene was stably transfected into 3T6 cells which were analyzed for luciferase activity following growth-stimulation. Figure 6 shows that luciferase expression remained relatively constant following growth stimulation.

DISCUSSION

In the present study, we have identified the sequences in the 5' flanking region of the TS gene that are necessary for S phase-specific regulation. We have compared the regulation of TS minigenes that were modified by deletions or point mutations in the 5' flanking region with the regulation of the endogenous TS gene as well as a constitutively expressed gene. All of the upstream regulatory sequences of the TS gene that were necessary for proper regulation were between -119 and -75 relative to the AUG codon. Minigenes that contained these upstream sequences (as well as spliceable introns in the transcribed region) were regulated in a manner that was nearly identical to that of the endogenous TS gene. Thus the upstream S phase regulatory region of the TS gene is very similar, if not identical, to the essential promoter region, which is between -104 and -75 (13).

Protein binding elements in the regulatory region

The TS promoter/regulatory region contains several potential binding sites for *trans*-acting factors. First, the region contains two GGAAG motifs that are potential binding sites for members of the Ets family of transcription factors. The GGAAG element at -100, which is not conserved in the human TS promoter (26), appears to play an important role in expression as well as regulation of the TS gene. Inactivation of the element leads to a 7-10-fold reduction in promoter activity (13). In addition, minigenes with this mutation are regulated to a lesser extent than the endogenous TS gene (Fig. 4E). This element is not sufficient for expression since a minigene that contains wild-type TS sequences from -119 to -89 (which includes the GGAAG element) was expressed at an extremely low level. We showed in earlier analyses that a factor (complex III) interacts with the GGAAG box at -100 (15). The identity of the factor has not been determined although it is presumably a member of the Ets family.

Another potential Ets binding site is located at -85. Although we have not been able to detect a factor interacting with this element, transient transfection assays have shown that alteration of the GGAAG box at -85 leads to a 3-fold reduction in promoter activity (13). This suggests that this region is important for expression. There are several other GGAAG or GGAA sequences downstream of -75. However, deletion of these sequences has no effect on promoter activity (13) or regulation (Fig. 4F), so the GGAA elements downstream of -75 appear to be irrelevant.

The promoter/regulatory region also contains a binding site for Sp1 at -80, immediately adjacent to the GGAAG motif at -85. An identical Ets/Sp1 element is also found in the human TS promoter (26), which is consistent with the possibility that this region plays an important role in expression or regulation. In line with this, mutation of the Sp1 binding site leads to a 3-fold decrease in promoter activity in transient transfection assays (13). Purified Sp1 binds to the G/C-rich region at -80 although with relatively low affinity (15). It is possible that additional factors that have not yet been identified also bind to this region.

Sp1 and Ets binding sites are present in a wide variety of mammalian promoters that are not regulated in a cell cycle-dependent manner. For example, Sp1 elements are present in the SV40 early promoter, which does not direct S phase-specific expression of TS minigenes (Fig. 3C). The sequence GCCGGAAGT (at -100 of the TS promoter) is present in the 5' flanking region of the constitutively expressed rpl32 gene (21). Therefore, the participation of Sp1 and Ets factors in growth-

regulated gene expression is dependent on their context and probably requires the interactions of additional factors. Studies are currently underway to determine which Ets factors bind to the TS 5' essential region and to understand how they influence TS expression in a cell cycle-specific manner.

The role of E2F in the TS promoter

E2F elements are found in the promoters of several S phase genes, including the genes for E2F-1, ribonucleotide reductase, HSP70, thymidine kinase and dihydrofolate reductase, and have been shown to be important for S phase-specific expression of some of these genes (27). The TS gene has also been viewed as a target gene for E2F (e.g. 28), although there is little direct evidence to support this view. For this reason, we have investigated the role of E2F in controlling mouse TS gene expression.

A potential E2F binding element is present in the TS 5' flanking region at -110, immediately upstream of the essential region. In addition, weak homologies to E2F binding motifs can be identified downstream of the essential promoter/regulatory region. We found previously that elimination of the potential E2F element at -110 or deletion of the downstream E2F motifs had no significant effect on TS promoter activity (12,13). Figure 4C and D shows that elimination of the E2F motif at -110 did not eliminate growth-regulated expression of the TS gene. However, inactivation of the motif did reduce the magnitude of the increase, raising the possibility that E2F might play a minor role in S phase regulation of the mouse TS gene. Since these E2F alterations are immediately adjacent to the important Ets motif -100, it is possible that the changes at -110 are affecting regulation by perturbing the interaction of an Ets protein at -100 rather than by preventing the binding of an E2F transcription factor.

Li *et al.* (29) also showed in co-transfection experiments that expression of mouse E2F1 in quiescent mouse 3T3 cells led to only a minor increase in TS promoter activity, in line with our observations. In contrast, DeGregori *et al.* (30) have recently shown that TS mRNA is elevated in quiescent rat fibroblasts that are infected with an adenovirus that expresses E2F1, suggesting that E2F1 may play a major role in controlling TS gene expression. The promoter and regulatory elements of the rat TS gene have not been defined. However, there is at least one consensus E2F element ~35 nt upstream of the AUG start codon of the rat TS gene (31). This element is not present in the mouse TS 5' flanking region. Thus it is possible that E2F plays a more important role in the rat TS promoter than in the mouse TS promoter. An alternative explanation is that the E2F stimulation observed by DeGregori *et al.* is indirect; E2F might increase the expression of a gene that in turn stimulates TS expression.

Upstream elements affect post-transcriptional processes?

The observation that the TS regulatory and promoter elements coincide are consistent with the possibility that the gene is controlled at the transcriptional level. However, nuclear run-on assays revealed that the rate of transcription of the TS gene did not change significantly during the G0-S phase transition. Furthermore, the luciferase indicator gene driven by the TS promoter was expressed at a constant level in growth-stimulated cells. Therefore, it appears that the TS gene is transcribed at a constant rate and that regulation is exerted primarily at the posttranscriptional level. In contrast, nuclear run-on assays have shown that other S phase genes (such as thymidine kinase, dihydrofolate reductase

and DNA polymerase- α) are regulated, at least in part, at the transcriptional level. Furthermore, when the promoters of these genes are linked to the CAT or luciferase indicator genes, the genes are expressed in an S phase-specific manner (e.g. 32–34). Thus the biochemical mechanism(s) that are important for TS gene regulation are different from those that control the expression of several other S phase genes.

The TS 5' flanking region is capable of conferring S phase specific expression to the TS coding region if a spliceable intron is retained within the transcribed region. However, if the introns are eliminated, the minigenes are expressed at a low, constant level during the G1–S phase transition (9). We have recently shown that the intron requirement is directly related to the splicing reaction rather than to the presence of a regulatory sequence in the interior of the intron. When splicing of the intron in a TS minigene is prevented by inactivating the splicing signals, or by inverting the intron, the minigene is no longer S phase regulated. We also found that when an intron is included in the intronless TS-luciferase construct described in the present study, luciferase expression increases at the G1–S phase boundary. In addition, the TS promoter is able to cooperate with introns from foreign genes that are not S phase regulated to bring about S phase-specific expression (35).

The dual requirement for upstream sequences as well as introns for proper regulation suggests some form of communication between the two regions. It appears unlikely that the introns are influencing TS gene transcription. It also appears unlikely that the introns are simply leading to a general stimulation in gene expression since in this case the stimulatory effect appears to be cell cycle specific and depends on the nature of the promoter. One possibility that we have previously suggested (8,9,35,36) is that the processing of introns is somehow influenced by sequences in the TS promoter region such that splicing is more efficient during S phase than during G0/G1. Although this mechanism is unusual, several earlier studies have also suggested communication between promoters and RNA processing reactions in other genes (37–42). Other mechanisms are also possible. Clearly, additional studies will be required to understand why the TS promoter and introns are both necessary for proper S phase regulation of the TS gene.

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REFERENCES

- Conrad, A.H. (1971) *J. Biol. Chem.* **246**, 1318–1323.
- Navalgund, L.G., Rossana, C., Muench, A.J. and Johnson, L.F. (1980) *J. Biol. Chem.* **255**, 7386–7390.
- Storms, R.K., Ord, R.W., Greenwood, M.T., Mirdamadi, B., Chu, F.K. and Belfort, M. (1984) *Mol. Cell. Biol.* **4**, 2858–2864.
- McIntosh, E.M., Ord, R.W. and Storms, R.K. (1988) *Mol. Cell. Biol.* **8**, 4616–4624.
- Jenh, C.-H., Geyer, P.K. and Johnson, L.F. (1985) *Mol. Cell. Biol.* **5**, 2527–2532.
- Ayusawa, D., Shimizu, K., Koyama, H., Kaneda, S., Takeishi, K. and Seno, T. (1986) *J. Mol. Biol.* **190**, 559–567.
- Chu, E., Koeller, D.M., Casey, J.L., Drake, J.C., Chabner, B.A., Elwood, P.C., Zinn, S. and Allegra, C.J. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8977–8981.
- Li, Y., Li, D., Osborn, K. and Johnson, L.F. (1991) *Mol. Cell. Biol.* **11**, 1023–1029.
- Ash, J., Ke, Y., Korb, M. and Johnson, L.F. (1993) *Mol. Cell. Biol.* **13**, 1565–1571.
- Deng, T., Li, D., Jenh, C.-H. and Johnson, L.F. (1986) *J. Biol. Chem.* **261**, 16000–16005.
- Liao, W.-C., Ash, J. and Johnson, L.F. (1994) *Nucleic Acids Res.* **22**, 4044–4049.
- Deng, T., Li, Y., Jolliff, K. and Johnson, L.F. (1989) *Mol. Cell. Biol.* **9**, 4079–4082.
- Geng, Y. and Johnson, L.F. (1993) *Mol. Cell. Biol.* **13**, 4894–4903.
- Wasylyk, B., Hahn, S.L. and Giovane, A. (1993) *Eur. J. Biochem.* **211**, 7–18.
- Jolliff, K., Li, Y. and Johnson, L.F. (1991) *Nucleic Acids Res.* **19**, 2267–2274.
- Todaro, G.J. and Green, H. (1963) *J. Cell Biol.* **17**, 299–313.
- Rossana, C., Rao, L.G. and Johnson, L.F. (1982) *Mol. Cell. Biol.* **2**, 1118–1125.
- Jenh, C.-H., Geyer, P.K., Baskin, F. and Johnson, L.F. (1985) *Mol. Pharmacol.* **28**, 80–85.
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
- Kelley, D.E. and Perry, R.P. (1986) *Nucleic Acids Res.* **14**, 5431–5447.
- Hariharan, N., Kelley, D.E. and Perry, R.P. (1989) *Genes Dev.* **3**, 1789–1800.
- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1989) Greene Publishing, New York.
- Geyer, P.K., Meyuhas, O., Perry, R.P. and Johnson, L.F. (1982) *Mol. Cell. Biol.* **2**, 685–693.
- Schilling, L.J. and Farnham, P.J. (1994) *Nucleic Acids Res.* **22**, 3061–3068.
- Jenh, C.-H., Rao, L.G. and Johnson, L.F. (1985) *J. Cell. Physiol.* **122**, 149–154.
- Takeishi, K., Kaneda, S., Ayusawa, D., Shimizu, K., Gotoh, O. and Seno, T. (1989) *J. Biochem.* **106**, 575–583.
- Farnham, P.J., Slansky, J.E. and Kollmar, R. (1993) *Biochim. Biophys. Acta Rev. Cancer* **1155**, 125–131.
- Almasan, A., Yin, Y., Kelly, R.E., Lee, E.Y.-H.P., Bradley, A., Li, W., Bertino, J.R. and Wahl, G.M. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 5436–5440.
- Li, Y., Slansky, J.E., Myers, D.J., Drinkwater, N.R., Kaelin, W.G. and Farnham, P.J. (1994) *Mol. Cell. Biol.* **14**, 1861–1869.
- DeGregori, J., Kowalik, T. and Nevins, J.R. (1995) *Mol. Cell. Biol.* **15**, 4215–4224.
- Ciesla, J., Weiner, K.X.B., Weiner, R.S., Reston, J.T., Maley, G.F. and Maley, F. (1995) *Biochim. Biophys. Acta Gene Struct. Expression* **1261**, 233–242.
- Kim, Y.K., Wells, S., Lau, Y.-F.C. and Lee, A.S. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5894–5898.
- Means, A.L., Slansky, J.E., McMahon, S.L., Knuth, M.W. and Farnham, P.J. (1992) *Mol. Cell. Biol.* **12**, 1054–1063.
- Pearson, B.E., Nasheuer, H.-P. and Wang, T.S.-F. (1991) *Mol. Cell. Biol.* **11**, 2081–2095.
- Ke, Y., Ash, J. and Johnson, L.F. (1995) *Mol. Cell. Biol.* in press.
- Johnson, L.F. (1994) In Hu, V.W. (ed.) *The Cell Cycle: Regulators, Targets, and Clinical Applications*. Plenum Press, New York, pp. 141–147.
- Hernandez, N. and Lucito, R. (1988) *EMBO J.* **7**, 3125–3134.
- Hernandez, N. and Weiner, A.M. (1986) *Cell* **47**, 249–258.
- Neuman de Vegvar, H.E., Lund, E. and Dahlberg, J.E. (1988) *Cell* **47**, 259–266.
- Sisodia, S.S., Sollner-Webb, B. and Cleveland, D.W. (1987) *Mol. Cell. Biol.* **7**, 3602–3612.
- Smale, S.T. and Tjian, R. (1985) *Mol. Cell. Biol.* **5**, 352–362.
- Neuberger, M.S. and Williams, G.T. (1988) *Nucleic Acids Res.* **16**, 6713–6724.
- Chung, S. and Perry, R.P. (1989) *Mol. Cell. Biol.* **9**, 2075–2082.
- Dudov, K.P. and Perry, R.P. (1984) *Cell* **37**, 457–468.