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Transcriptional Control Elements and Complex Initiation Pattern of the TATA-less Bidirectional Human Thymidylate Synthase Promoter

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Abstract The nucleotide sequences that are important for transcription of the human thymidylate synthase gene were analyzed by deletion and site-directed mutagenesis of the promoter region. Deletion analyses from the 5′ and 3′ ends indicated the presence of multiple positive and negative elements. The promoter had approximately the same strength in the normal or inverted orientation. The region between 161 and 141 nt upstream of the translational start codon was found to be both necessary and sufficient for high-level promoter activity in both directions and was designated the essential promoter region. This region, which is highly conserved in human, mouse and rat TS promoters, contains potential binding sites for Ets, Sp1, and LSF transcription factors. Site directed mutagenesis of each of these elements led to large decreases in promoter strength. However, inactivation of potential Sp1 and E2F elements adjacent to the essential promoter region led to increases in promoter strength. The transcriptional start site pattern was analyzed by S1 nuclease protection assays of mRNA isolated from cells transiently transfected with TS minigenes. Multiple start sites were detected, most of which were between 160 and 120 nt upstream of the AUG codon. J. Cell. Biochem. 77:50–64, 2000. © 2000 Wiley-Liss, Inc.

Key words: mammalian gene expression; gene transfection; housekeeping gene; cell cycle; cancer chemotherapeutic target enzyme

Thymidylate synthase (TS) catalyzes the reductive methylation of dUMP to form dTMP in the de novo biosynthetic pathway. TS is an essential enzyme in proliferating cells and an important target of a variety of chemotherapeutic drugs [Carreras and Santi, 1995]. The TS gene is expressed at a much higher level in cells that are undergoing DNA replication than in quiescent cells [Conrad, 1971; Navalgund et al., 1980; Jenh et al., 1985; Ayusawa et al., 1986]. In contrast to the situation with many other mammalian S phase genes, TS gene expression is controlled primarily at the post-transcriptional level, rather than the transcriptional level [Johnson, 1994; Ayusawa et al., 1986]. Analyses of stably transfected TS minigenes have shown that sequences in the promoter region as well as an intron in the transcribed region are both necessary (but neither is sufficient) for proper S phase accumulation of TS mRNA [Takayanagi et al., 1992; Ash et al., 1993, 1995; Ke et al., 1996]. This finding suggests that some form of communication between the promoter and RNA processing machinery is necessary for proper regulation of TS mRNA production during the G1 to S phase transition. In addition, the translation of TS mRNA is negatively regulated by an inhibitory interaction between TS enzyme and its mRNA. This interaction is disrupted by the presence of excess TS substrates or inhibitors, leading to increased synthesis of TS enzyme without a change in TS mRNA content [Chu et al., 1991; Keyomarsi et al., 1993]. Finally, the stability of the TS enzyme has been found to increase significantly in the presence of TS inhibitors, which leads to an increase in enzyme level in the absence of changes in TS mRNA content or translation [Kitchens et al., 1999].

The TS gene has been cloned and analyzed from mouse [Deng et al., 1986] and human

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[Kaneda et al., 1990] cells. The structures of the two genes are similar. Both are interrupted by 6 introns at the same locations in the coding regions, and the nucleotide sequences of the open reading frames are highly conserved [Perryman et al., 1986]. However, there are major differences between the two genes in the sequences upstream and downstream of the coding regions, as well as in the introns. For example, mouse TS mRNA is highly unusual in that it lacks a 3′ untranslated region; the UAA termination codon is followed immediately by the poly(A) tail [Perryman et al., 1986]. In contrast, the human TS mRNA contains a long 3′ untranslated region [Ayusawa et al., 1984].

The mouse TS promoter lacks a TATA box and an initiator element and initiates transcription at many sites between 92 and 14 nt upstream of the AUG codon [Geng and Johnson, 1993]. The promoter is also bidirectional [Liao et al., 1994]. Sequences that are sufficient for wild-type promoter activity are located between 105 and 75 nt upstream of the AUG codon [Geng and Johnson, 1993]. Motifs within this region that are important for promoter activity have been identified. First, there are two potential binding sites for members of the Ets family of transcription factors [Wasylyk et al., 1993]. Second, there is a weak Sp1 binding site immediately adjacent to the downstream Ets element [Jolliff et al., 1991]. Third, there is an LSF binding site that overlaps the downstream Ets/Sp1 motif [Powell et al., 1999]. This LSF element is necessary for the S phase-specific expression of the gene in growth-stimulated cells.

Comparison of the human and mouse TS promoter regions revealed several similarities. For example, both promoters are G/C-rich and lack a TATA box. Furthermore, the downstream Ets/Sp1/LSF motif within the mouse essential promoter region is conserved in the human TS promoter [Geng and Johnson, 1993; Takeishi et al., 1989], where it appears to play an important role in determining promoter strength [Horie and Takeishi, 1997]. However, the human promoter is different from the mouse promoter in several respects. For example, the human promoter lacks the upstream Ets element but contains potential E2F as well as additional Sp1 motifs in the vicinity of the Ets/Sp1/LSF elements. E2F motifs play an important role in regulating the expression of a variety of genes during the G1 to S phase transition [Farnham et al., 1993; DeGregori et al., 1995]. In addition, the human (but not the mouse) TS promoter has three tandem repeats of a 28-nt G/C-rich sequence in the first 100 nt upstream of the AUG codon, as well as one inverted copy of the repeat starting at −139 [Kaneda et al., 1990]. The repeated sequences reduce the efficiency of translation of human TS mRNA [Kaneda et al., 1987], presumably due to the formation of secondary structure in the 5′ untranslated region. The repeated sequences have also been reported to be necessary for efficient expression of reporter genes driven by the human TS promoter [Horie et al., 1995].

In the present study, we have determined the sequences that are important for the activity of the human TS promoter by analyzing the effects of 5′ and 3′ promoter deletions as well as site-directed alterations of potential promoter elements. We have identified a 20-nt region that is both necessary and sufficient for high-level promoter activity and have shown that this region also has bidirectional promoter activity. Finally, we have used an improved strategy to determine the pattern of transcriptional start sites as well as the effects of promoter mutations on the start site pattern.

MATERIALS AND METHODS

Cell Culture

HeLa cells were maintained on plastic petri dishes in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% calf serum (Colorado Serum). TS-deficient hamster V79 cells [Nussbaum et al., 1985] were grown in medium that was supplemented with 10 μM thymidine and 10% NuSerum (Collaborative Biomedical Products). The Hep-2/500 cell line, a human cell line in which the TS gene is amplified about 100-fold [Berger et al., 1985], was cultured in medium supplemented with 10% fetal bovine serum (FBS).

Construction of Minigenes

Various human TS promoter regions between −484 and +108 (relative to the translational initiation codon) were amplified by polymerase chain reaction (PCR) from human genomic DNA isolated from Hep-2/500 cells using 5′ (Forward) and 3′ (Reverse) primers that are described in Table I. A *Hin*dIII site was included in the Forward primer and an *Nhe*I site was

TABLE I. Oligos Used in PCR Amplification for Deletion and Site-Directed Mutagenesis*

5′-end Deletion			
Reverse Primer	−98	S7	CTAGCTAGCTGGCAGGACCCCTTCCGCGCGCCT
Forward Primer	−484	S17	CCCAAGCTTACCACCACTTGCTTCGGTT
	−243	S15	CTCAAGCTTACGTAGGGCTCCGTTCTGTGCCA
	−208	S12	CTCAAGCTTTCCCCCTGGCGCA
	−172	S22	ATAAAGCTTCCGCGACCCCGCCGAG
	−161	S29	AATAAGCTTCCGAGCAGGAAGAGG
	−141	S41	AATAAGCTTGCGGGACGGCCGCGGGAAAA
3′-end Deletion			
Forward Primer	−243	S15	CTCAAGCTTACGTAGGGCTCCGTTCTGTGCCA
Reverse Primer	+108	S14	GAGGCTAGCAAGATCTGCCCCAGGTACT
	+35	S16	AATGCTAGCGGCCGGCGCGGCAGCTCCGAGCCGGCCA
	+2	S9	TTAGCTAGCTATGGCGCGGCGGGCGGGGGACGGA
	−98	S7	CTAGCTAGCTGGCAGGACCCCTTCCGCGCGCCT
	−141	S13	AATGCTAGCGCTCCGCCTCTTCCTGCT
	−148	S30	AATGCTAGCTCTTCCTGCTCGGC
	−173	S42	AATGCTAGCGGCCCCCGCTCTAGAGA
Mutagenesis			
Forward Primer	Sp1nr	S51	TCTCTAGAGCGGGGACGTCCGCGACCCCGCCGA
Reverse Primer	Med	S47	AATGCTAGCGGCCGGCGCGGCATACGTAAGCCGGCCACAGGCAT
Reverse Primer	Sp1+30	S63	AATGCTAGCCTTAAGCGCGGCAGCTCCGA
Inversion			
Forward Primer	inv-243	S8	CTAGCTAGCCCGGGCTCCGTTCTGTGCCA

*The primers are shown in the 5′ to 3′ direction. The underlined nucleotides indicate the locations of the mutations.

included in the reverse primer. The amplified fragments were digested by *Hin*dIII and *Nhe*I and cloned into indicator genes. For minigenes to be used for RNA analyses, the fragments were inserted between the *Hin*dIII site in the polycloning region of the vector and an engineered *Nhe*I site 11 nt upstream of the AUG translational start codon of a mouse TS indicator gene, TI1G-d1.0 [Ke et al., 1996]. For luciferase analyses, the fragment was inserted at the *Nhe*I site in the polylinker region 54 nt upstream of the open reading frame of the luciferase indicator gene (GL3, Promega, Madison, WI). (The polycloning region of the GL3 plasmid was modified by deleting 13 nt between the *Bgl*II and *Hin*dIII sites.) The minigene containing the inverted −243−98 promoter region was constructed by amplifying the TS promoter region using the inv-243 forward primer and the −98 reverse primer (Table I), digesting the fragment with *Nhe*I and inserting the digested fragment between the engineered *Nhe*I site in the TS indicator gene and an *Xba*I site in the polycloning region. The minigene containing the inverted −161/−141 promoter was constructed by inserting a synthetic oligo between the *Nhe*I and *Hin*dIII sites. Some of the promoter deletions were made by eliminating DNA upstream or downstream of restriction sites in the promoter region or by inserting synthetic double-stranded oligos downstream of the BssHII site.

Site-specific mutations between −182 and −135 were usually generated by substituting synthetic oligos that contained the desired nucleotide changes for the *Xba*I/*Eag*I fragment of the promoter region (Fig. 1). The Sp1nr mutation was generated by PCR, using primers S51 and S7 (Table I) and inserted between the *Xba*I and *Eag*I sites. Mutations of the potential E2F sites between −128 and −98 were generated by substituting a synthetic DNA fragment that contained the desired mutation for the EagI/*Nhe*I fragment. Site-specific mutants between +1 and +35 were generated by PCR mutagenesis, using reverse primers that contained the desired mutations and Forward primer S15 (Table I). The sequence of each synthetic or PCR-amplified DNA fragment was analyzed after cloning the fragment into the minigene of interest.

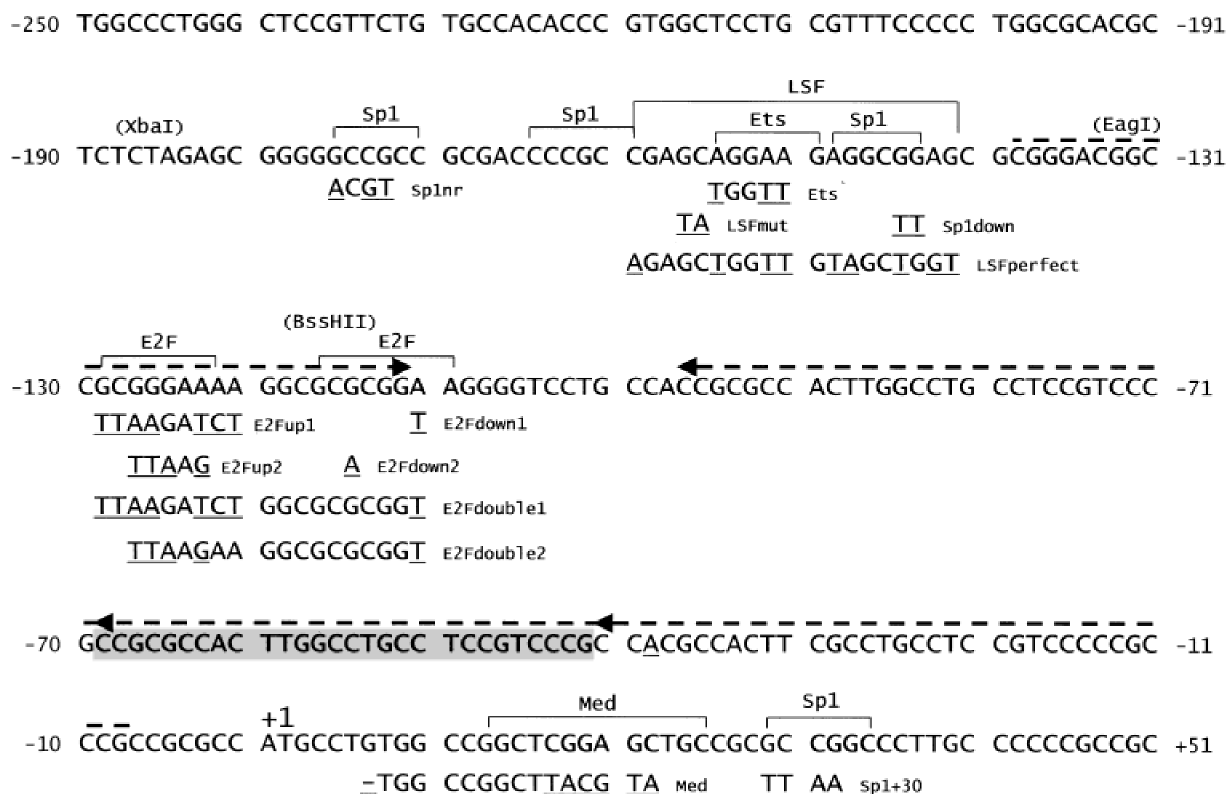

Fig. 1. The human TS promoter region. The sequence of the human TS gene [Takeishi et al., 1989] between −250 and +51 (relative to the A of the AUG translational start codon) is shown. The direct and inverted repeat sequences are indicated by dashed lines. The sequence of the TS promoter region analyzed in the present study is identical to the published sequence except that the shaded direct repeat sequence is missing in the promoter we analyzed and the underlined A at position −39 is a G in the published sequence. The locations of important restriction sites are shown in parenthesis. Potential binding sites for the indicated transcription factors are shown above the sequence. The nucleotide changes (underlined) that were introduced to inactivate these binding sites are shown below the promoter sequence. Underlined dash, a nucleotide that was inadvertently deleted during the mutagenesis procedure.

Transient Transfection

Large-scale preparations of plasmids were purified twice on CsCl density gradients [Maniatis et al., 1982]. Transient transfections were performed by the calcium phosphate method [DeWille et al., 1988]. At least two independent plasmid preparations were analyzed for each promoter construct, and each preparation was analyzed at least twice. Cultured cells were split 6 h before transfection. For RNA assays, 20 μg of the test minigene were cotransfected with 1 μg of the SI5,6S minigene [Lee and Johnson, 1998a] into subconfluent HeLa cells on 100-mm culture dishes. For luciferase assays, 1.2 μg of test minigene and 0.3 μg of an SV-40-driven β-galactosidase expression vector were cotransfected into subconfluent HeLa cells on 35-mm culture dishes. The medium was replaced with fresh medium 16 h later, and cells were harvested for RNA or luciferase analyses 40–60 h after addition of DNA.

RNA Quantitation

Total cytoplasmic mRNA was prepared by phenol-chloroform extraction, followed by ethanol precipitation [Maniatis et al., 1982]. S1 nuclease protection assays were performed as described previously [Deng et al., 1989b] using 20–50 μg of total cytoplasmic RNA and 10^5 cpm of probe in each reaction. The probe was 5′ end-labeled at the *Bgl*II site in exon 5. The test minigenes were tagged with a 57-nt internal *Bam*HI deletion in exon 3 so that RNA derived from the test minigene could be distinguished from that derived from the SI5,6S minigene, which retains the 57 nt *Bam*HI fragment. The protected DNA fragments were resolved on a 6% polyacrylamide sequencing gel, detected by

autoradiography, and quantitated using a PhosphorImager (Molecular Dynamics). The amount of radioactivity corresponding to mRNA derived from the test minigene was normalized to that corresponding to the SI5, 6S minigene to correct for differences in transfection efficiency and RNA recovery.

Luciferase Assays

Cells were harvested and assayed for luciferase activity using a kit obtained from Promega and for β-galactosidase activity using a luminescence assay kit obtained from Tropix. Luminescence was measured in a Berthold Lumat Luminometer. The values obtained with the luciferase assay were normalized to the values obtained for the β-galactosidase assay to correct for differences in transfection efficiency and protein recovery.

Transcription Start-Site Pattern

Total cytoplasmic RNA (25–100 µg) was isolated from untransfected cells or cells transfected with a TS minigene and analyzed by an S1 nuclease protection assay. The probe, which was derived from the transfected minigene, was 5′ end-labeled at the *Bsu*36I site in exon1 (85 nt downstream of the AUG translational start codon) of the mouse coding region and extended to an *Ssp*I site in the plasmid vector, approximately 600 nt upstream of the promoter region. For analysis of endogenous HeLa TS mRNA, the probe was 5′ end-labeled at +111 at an engineered *Bgl*II site in human TS exon 1 and extended to the *Sst*I site in the plasmid vector, about 2.7 kb upstream of the promoter region.

RESULTS AND DISCUSSION

Promoter Isolation

Various regions of the human TS promoter were amplified by PCR, using synthetic oligos corresponding to the 5′ and 3′ boundaries of the regions to be amplified as primers. We focused our analyses on sequences downstream of −500 because earlier studies indicated that this region was sufficient for efficient expression [Kaneda et al., 1990] and because there are multiple alu repetitive elements upstream of −500 [Takeishi et al., 1989; Kaneda et al., 1990]. The template was genomic DNA isolated from Hep-2/500 cells, in which the TS gene is amplified approximately 100-fold. The sequence of the human TS promoter region [Takeishi et al., 1989] is shown in Figure 1. The PCR-amplified TS promoter region was found to have only two of the direct repeats and an A substituted for a G at position −39, but otherwise was the same as the published sequence. Variations in the number of direct repeats have also been observed previously [Horie et al., 1995]. To avoid confusion, the numbering system used to describe the DNA analyzed in our experiments is the same as the published numbering system (i.e., we ignored the missing 28-nt repeat sequence). All numbering is relative to the A of the AUG translational start codon. The amplified DNA regions were linked to a TS indicator gene that consisted of the mouse TS coding region flanked at the 3′ end by the polyadenylation signal of the human βglobin gene, as shown in Figure 2A. Since the expression of the mouse TS minigene is intron dependent [Deng et al., 1989a], an internally deleted version of mouse TS intron 1 was included at its normal location in the coding region to increase the level of expression.

To determine the strength of the promoter region, the minigenes were transiently transfected into HeLa cells; the amount of mRNA corresponding to the minigene was determined by an S1 nuclease protection assay. At least two different preparations of each minigene were isolated, and each preparation was analyzed at least twice. The strategy of the S1 nuclease protection assay is shown in Figure 2A. We chose to measure mRNA content rather than the activity of an indicator protein (e.g., CAT or luciferase) so that our results would not be affected by differences in the efficiency of translation of the mRNA that may result from differences in the 5′ untranslated region of the mRNA. In all our analyses, we assume that changes in mRNA content are due to differences in promoter strength. However, we cannot rule out the possibility that changes in mRNA stability may also occur for those promoter alterations that affect the transcribed region.

Promoter 3′ Deletion Analyses

To determine the locations of important regulatory regions, promoter deletion analyses were performed. Our first goals were to establish the 3′ boundary of the TS essential promoter region and to further explore the roles of the direct and inverted repeats in TS promoter activity. In these analyses, the 5′ boundary of the promoter

region was kept at position −243 while the 3′ boundary was varied between +108 and −187. The results of a typical S1 nuclease protection assay are shown in Figure 2B, and the results of multiple independent experiments are summarized in Figure 3A. We found that the promoter extending to +108 had high activity. Deletion of the sequences between +108 and +35 had little effect on promoter activity. However, deletion of sequences downstream of +2 led to a 3-fold reduction in promoter activity, suggesting that a positive-acting promoter element (or an element that enhances mRNA stability) is located between +2 and +35. Promoter activity remained constant when we deleted sequences downstream of −70 (which removes all but one of the direct repeats) or −98 (which removes all of the direct repeats, but not the inverted repeat). However, deletion of sequences downstream of −141 led to a 2.5-fold increase in promoter activity, indicating the presence of a negative acting element between −141 and −98. An earlier study also suggested the presence of a weak negative element in this region [Horie et al., 1995]. Deletion of an additional 7 nucleotides (to −148) led to a 4-fold decrease in promoter activity, indicating that a strong positive-acting element(s) had been deleted. Deletion of sequences downstream of −173 had little effect, but deletion to −187 led to inactivation of the promoter. The small amount of residual activity that was observed with this promoter was the same as that observed with the promoter-less construct (control) and presumably represents a small amount of transcription that is directed by sequences within the plasmid vector.

These analyses showed clearly that sequences within the direct or inverted repeat regions were not essential for promoter activity. These observations are contradictory to those of earlier studies that reported that at least one of the direct repeats and the inverted repeat were necessary for efficient expression [Horie et al., 1993, 1995]. In the earlier studies, the human

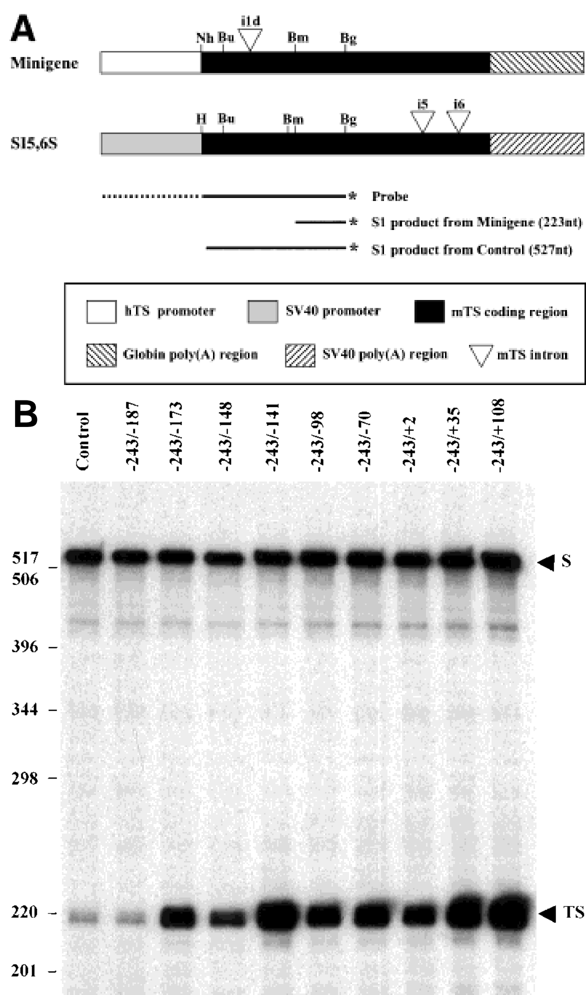

Fig. 2. Structure and analysis of TS minigenes. **A:** Structures of the TS minigene and the SI5,6S gene that were used for the RNA assays. The diagrams are not drawn to scale. The TS minigene consists of the human TS promoter (various regions) linked to a mouse TS indicator gene [Ke et al., 1996] consisting of mouse TS cDNA interrupted by mouse TS intron 1 with a 1-kb internal deletion (i1d) at its normal location in the transcribed region. The polyadenylation signal was from the human β-globin gene. The SI5,6S gene consists of the mouse TS cDNA (interrupted by mouse TS introns 5 and 6 (i5 and i6) at their normal locations) driven by the SV40 early promoter. The polyadenylation signal was also derived from SV40. The approximate locations of the following restriction sites are indicated: Bg, *Bgl*II; Bm, *Bam*HI; Bu, *Bsu*36I; H, *Hin*dIII; Nh, *Nhe*I. A 57 nt *Bam*HI fragment was deleted from the TS minigene, but not from the SI5, 6S gene, to permit unambiguous identification of the mRNA derived from the two transfected genes. The strategy for the S1 nuclease protection assays that were used to quantitate TS mRNA is also shown. The probe, which corresponds to the transcribed region of the SI5,6S gene, was 5′ end-labeled at the *Bgl*II site in exon 5 and extends into the vector region (dashed line). **B:** The results of a typical S1 nuclease protection assay are shown. Cells were transiently co-transfected with the SI5,6S gene and a TS minigene. Two days later, RNA was isolated and analyzed using the S1 nuclease protection assay. The TS minigenes that were transfected either lacked a promoter region (control) or contained the wild-type human TS promoter region, the 5′ and 3′ boundaries of which are indicated at the top of each lane. The fragments corresponding to TS mRNA derived from the SI5,6S gene (S) and the TS minigene (TS) are indicated at the right side of the autoradiogram. The positions of molecular-weight markers that were electrophoresed in an adjacent lane are shown at the left side of the autoradiogram.

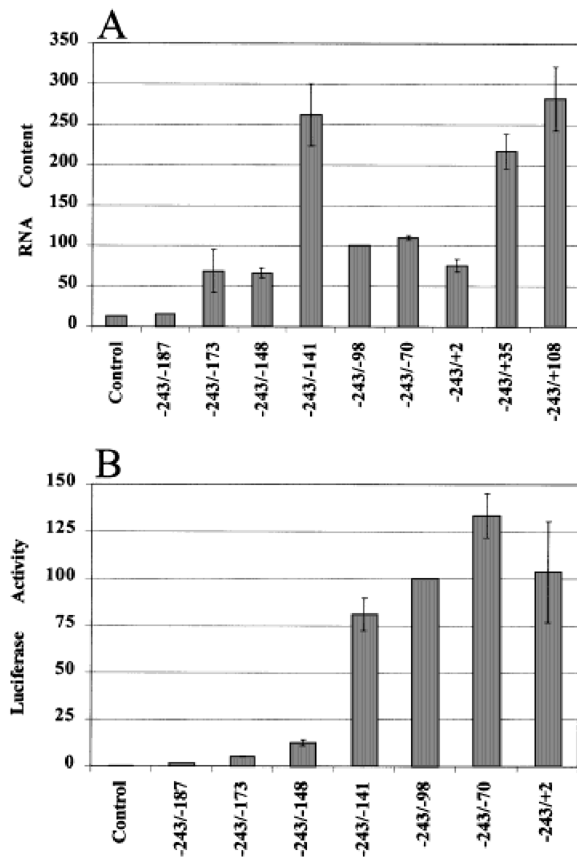

Fig. 3. Promoter 3′ deletion analyses. **A:** RNA content. The results of multiple S1 nuclease protection analyses similar to those shown in Fig. 2B are summarized. In each experiment, the amount of radioactivity corresponding to the test minigene was normalized to that corresponding to the SI5,6S minigene. Each of these values was then normalized to the value for the −243/−98 promoter, which was set at 100. Error bars indicate the standard deviation of multiple experiments. **B:** Luciferase activity. The results of multiple analyses of luciferase indicator genes driven by the indicated TS promoter regions are shown. The values observed for each minigene were normalized to that observed for the minigene driven by the −243/−98 promoter, which was set at 100.

TS promoter was linked to the CAT coding region, and expression was determined by measuring CAT enzyme activity. However, CAT activity is a reflection of the content as well as the efficiency of translation of the mRNA. It is known that sequences in the 5′ untranslated region of human TS mRNA can have a significant effect on the efficiency of translation of the TS mRNA [Kaneda et al., 1987; Chu et al., 1991]. Therefore, it was possible that the discrepancy between our results (based on measurements of mRNA content) and the earlier results may be due to differences in the efficiency of translation of the mRNA.

To clarify this issue, we repeated some of our 3′ deletion analyses by linking the deleted promoters to the luciferase coding region and determining the levels of expression of this indicator gene in transient transfection assays. The results of these analyses (Fig. 3B) were similar to those obtained in the RNA analyses, except that the promoters that extended from −243 to −141, −148 and −173 had lower than expected luciferase levels (as compared with the mRNA levels shown in Fig. 3A). The reason for this discrepancy is unknown, but it may be related to the fact that the 3′ deletions upstream of −100 remove portions of the normal transcriptional initiation window of the human TS promoter (see below). Transcription may now initiate, at least in part, in the 54-nt region between the polycloning site and the luciferase open reading frame. Truncation and/or changes in the nucleotide composition or secondary structure of the 5′ untranslated regions may reduce the efficiency of translation of the mRNAs derived from these minigenes. However, we again found no evidence that the direct or inverted repeat sequences were necessary for promoter activity. In particular, the promoter that extended to −141 had as much activity as the promoters that extended to −98, −70 or +2. The reason for the difference between our results and those presented in the earlier study is unknown. In all subsequent analyses, promoter strength was determined by measuring mRNA content, rather than luciferase activity, to avoid complications that may be caused by differences in translation efficiency.

Promoter 5′ Deletion Analyses

We then performed a 5′ deletion analysis of the TS promoter region to establish the 5′ boundary of the essential promoter region. In this case, the 3′ boundary was kept at −98 so that the promoter retains the inverted repeat, in which many of the transcriptional initiation sites occur (see below), but not the direct repeat sequences. The 5′ boundary of the promoter region varied between −484 and −141. Figure 4A shows that the level of expression of the minigene driven by the promoter that extended to −484 was only slightly above that observed for the promoter-less construct, in agreement with earlier observations [Horie and Takeishi, 1997]. The fact that the longer promoter regions have low activity implies that there must

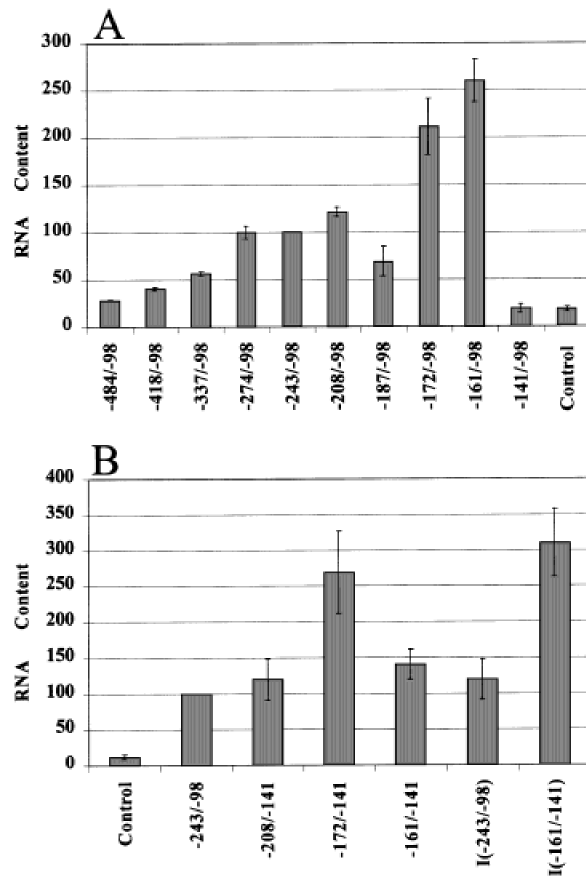

Fig. 4. Promoter 5′ deletion assays. **A:** Analysis of promoters extending to −98. **B:** Analysis of promoters extending to −141. The 5′ and 3′ end points of the promoters that were analyzed in the normal or inverted (I) orientations are indicated. Control minigenes lacked a promoter region. The results were analyzed as described in Fig. 3A. The values observed for each minigene were normalized to that observed for the minigene driven by the −243/−98 promoter, set at 100.

be positive-acting elements elsewhere in the gene that serve to counteract these inhibitory elements. An enhancer-like sequence near the 5′ end of the first intron of the human TS gene may serve this function [Kaneda et al., 1992].

Promoter strength increased gradually as sequences between −484 and −274 were deleted, indicating the presence of multiple negative-acting sequences in the distal region. Deletion of sequences upstream of −208 had little effect, but deletion to −187 led to a slight decrease in promoter activity, suggesting the presence of a positive element between −187 and −208, as observed previously [Horie and Takeishi, 1997].

The sequences between −187 and −147 were shown previously to contain sequences that were essential for TS promoter activity [Horie and Takeishi, 1997]. However, a detailed analysis of this region was not performed. For this reason, we focused particular attention on this important region. Deletion to −172 led to a major increase in promoter activity, indicating the presence of a negative element(s) between −187 and −172. Promoter strength remained approximately the same when sequences upstream of −161 were deleted, but was reduced to the control level when sequences upstream of −141 were deleted, indicating the presence of essential promoter elements between −161 and −141.

To define the minimal region that retained efficient promoter activity, a 5′ deletion analysis was also performed keeping the 3′ end at −141. Figure 4B shows that the promoter region between −172 and −141 had somewhat higher activity than the promoter region between −161 and −141. Nevertheless, the 20-nt region between −161 and −141 was sufficient for high-level promoter activity and has been designated as the essential promoter region of the human TS promoter. This region is highly conserved in the mouse TS promoter (see below) where it has also been shown to be essential for promoter activity [Geng and Johnson, 1993].

Bidirectional Activity of the Human TS Essential Promoter Region

An earlier study showed that the region of the human TS gene extending from −2 kb to the AUG start codon has bidirectional promoter activity [Kaneda et al., 1990]. To determine whether the essential region of the human TS promoter was responsible for this bidirectional activity, the −243 to −98 and the −161 to −141 regions were cloned upstream of the TS indicator gene in reverse orientation [I(−243/−98) and I (−161/−141), respectively]. Figure 4B shows that these regions stimulated the production of higher levels of mRNA when they were cloned in the inverted orientation than in the normal orientation. Therefore, the essential region of the human TS promoter has bidirectional activity.

Previous studies have shown that many housekeeping promoters are bidirectional. In some cases, the upstream transcripts encode functional proteins [Linton et al., 1989; Sturm et al., 1988]. We have shown that the mouse TS promoter region has bidirectional activity [Liao et al., 1994]. Cytoplasmic RNA molecules that correspond to the upstream region and that are

directed by the TS promoter could be detected. However, analysis of these upstream transcripts showed that they extended into an L1 repetitive element and thus were unlikely to correspond to a functional mRNA [Lee and Johnson, 1998b]. It will be interesting to determine whether the human TS promoter directs the synthesis of an upstream mRNA molecule that encodes a protein.

Site-Directed Mutagenesis of Potential Promoter Elements

We then analyzed the sequences in the vicinity of the essential promoter region by site-directed mutagenesis to identify more precisely the regulatory elements in this region. The nucleotide alterations that were analyzed are shown in Figure 1. All the mutations were made in the TS promoter region extending from −243 to −98.

An earlier study showed that a mutation that inactivated both the Ets and Sp1 elements near −150 of the human TS promoter led to inactivation of the promoter [Horie and Takeishi, 1997]. To study the role of each element, we created mutations that would inactivate either the Sp1 or the Ets element without affecting the adjacent element (Sp1down and Ets mutations, respectively). Figure 5A shows that both mutations led to major (4- to 5-fold) reductions in promoter activity, suggesting that both of these elements are important for promoter activity.

The Sp1/Ets sequence of the mouse TS promoter, which is conserved in the human TS promoter (see below), also contains a binding site for the LSF transcription factor (consensus sequence: WNRRCTGGKTNKRGCYNGY, where W = A or T, R = A or G, K = G or T, Y = T or C, and N = any nucleotide). LSF was recently shown to play an important role in S phase-specific expression of the mouse TS gene [Powell et al., 1999]. The Sp1 and Ets mutations in the essential region of the human TS promoter also affect the overlapping LSF element. To determine the role of LSF more directly, we analyzed the effect of a mutation (LSFmut) that prevents LSF binding without affecting the binding of Ets or Sp1 factors [Powell et al., 1999]. Figure 5A shows that this LSF mutation also led to a significant (at least 3-fold) decrease in promoter activity, although the decrease was not as great as that observed with the Sp1down or Ets mutations. We also introduced a mutation (LSFperfect) that inactivated

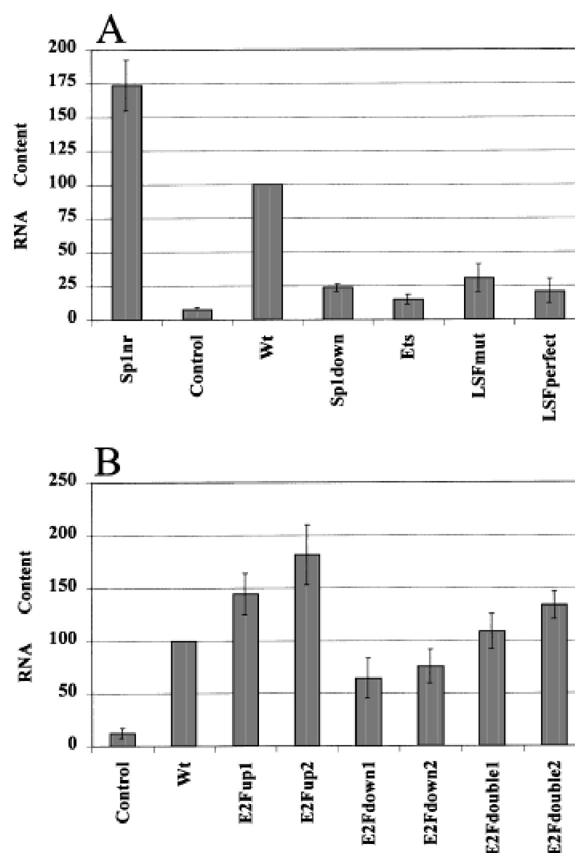

Fig. 5. Site-directed mutagenesis of promoter elements. **A:** Mutations of positive and negative elements in the vicinity of the essential promoter region. **B:** Mutations in potential E2F elements. Each promoter region extends from −243 to −98 and contains the indicated mutations. The sequence alterations that were introduced are described in Fig. 1. Control minigenes lacked a promoter region. The results were analyzed as described in Fig. 3A. The value observed for each minigene was normalized to that observed for the minigene driven by the wild-type (Wt) promoter, which was set at 100.

the Ets and Sp1 elements, while creating a consensus LSF binding site. This promoter was also far less active than the wild-type promoter region. Therefore, it appears that the full activity of the promoter region may require the presence of all three of these elements.

The region immediately upstream of the essential promoter region is highly G/C-rich and contains potential binding sites for Sp1 (Fig. 1). Figure 5A shows that disruption of the G/C-rich region at −175 (Sp1nr mutation) led to a 70% increase in promoter activity. This is consistent with the results of the 5′ deletion assays (Fig. 4A), which indicated the presence of a negative element in the region between −187 and −172. Sp1 is generally considered to be a positive-acting factor. Therefore, it is possible that an-

other factor (besides Sp1) that inhibits promoter activity binds to this motif. Additional studies will be required to identify this negative-acting factor.

There is another potential Sp1 motif beginning at −165. However, deletion of the region between −172 and −161, which leads to the inactivation of the Sp1 element at −165, did not have a major effect on promoter activity (Fig. 4A,B). Therefore, the Sp1 element at −165 is not essential for promoter activity.

The human TS promoter region includes at least two potential E2F binding sites within the inverted repeat region (Fig. 1). E2F elements frequently function as negative elements in many S phase promoters and are important for proper regulation of gene expression during the G1 to S phase transition [Weintraub et al., 1992; Zhang et al., 1999]. Our 3′ deletion analyses (Fig. 3A) showed that there is a negative promoter element(s) between −98 and −141. This raised the possibility that the E2F motifs in the inverted repeat region may correspond to the negative promoter element(s). Inactivation of the consensus upstream E2F motif at −125 by two different mutations (E2Fup1 and E2Fup2) led to a 50% to 75% increase in promoter activity. By contrast, inactivation of the weak E2F motif at −115 by two different mutations (E2Fdown1 and E2Fdown2) led to a slight decrease in promoter activity. When both E2F elements were inactivated (E2Fdouble1 and E2Fdouble2), promoter strength was only slightly higher than that of the wild-type promoter. Therefore, it appears that the upstream E2F motif may represent a relatively weak negative element, at least when assayed by transient transfection in unsynchronized cells. Additional studies are required to determine whether the E2F element(s) plays a more significant role in S phase-specific expression of the TS gene.

The 3′ deletion analyses also indicated the presence of a positive element between +2 and +35 (Fig. 3A). The region near +20 contains a MED-1 motif (GCTCCS, where S = G or C) in the inverted orientation as well as two similar sequences in the normal orientation. The MED-1 motif has been observed in many promoters (including the mouse TS promoter) that lack a TATA box and an initiator element and has been shown to play a role in determining promoter strength as well as the pattern of transcriptional start sites of the P-glycoprotein promoter [Ince and Scotto, 1995]. However, inactivation of all three of these MED-1 motifs in the human TS promoter (Med mutation, Fig. 1) had no significant effect on TS promoter activity (data not shown). Similar observations were also made with the mouse TS promoter [Rudge and Johnson, 1999]. The +2 to +35 region also contains a highly G/C-rich region between +24 and +36 that may correspond to an Sp1 motif. However, when this potential Sp1 motif was disrupted (Sp1+30 mutation, Fig. 1), there was no change in promoter activity (data not shown).

Transcriptional Start Sites

The mouse TS promoter directs transcriptional initiation over a broad range beginning at −92 (in the essential promoter region) and extending to −14 [Geng and Johnson, 1993]. The same pattern of start sites was observed using either S1 nuclease protection assays or primer extension assays. The absence of a TATA box and an initiator element appears to be responsible for the diversity of start sites.

The transcriptional start site pattern for the human TS promoter has been difficult to determine due to the presence of the G/C-rich direct and inverted repeats in the 5′ untranslated region of the mRNA. S1 nuclease protection assays of human TS mRNA demonstrated a complex pattern of start sites extending from −173 to approximately −10 relative to the AUG codon [Takeishi et al., 1989]. Subsequent primer extension analyses suggested that the transcriptional start sites extended from −179 to −160 [Kaneda et al., 1990]. However, the primer that was used corresponded to the region between −120 and −101. Therefore, transcripts that initiate within a short distance upstream of the primer or downstream of the primer would not have been detected by this approach. Attempts to use primers farther downstream proved unsuccessful, presumably because of the presence of the direct repeats [Kaneda et al., 1990].

We have analyzed the transcriptional start site pattern of the human TS promoter by using S1 nuclease protection assays. In our initial analyses, the probe included the human TS promoter region from −243 and extended to +111 nt in the open reading frame. Figure 6A shows that when RNA isolated from HeLa cells was analyzed by this approach, a complex pattern of protected fragments was detected that extended from approximately −166 to −30. The bands between approximately −100 and −80,

which is within the region corresponding to the first direct repeat (Fig. 1) were particularly intense.

It was possible that some of the bands could be attributed to a difference in the number of direct repeats in the probe than in the TS mRNA isolated from HeLa cells. For this reason, the analysis was also performed using RNA derived from hamster V79 cells that were transfected with a TS minigene driven by the human TS promoter that extends from −243 to +111. In this case, the sequence of the TS promoter region is identical to that of the probe. Figure 6A shows that the same complex pattern of bands was observed, except that the band near +100 is absent. The patterns we observed are reminiscent of those observed by Takeishi et al. [1989], who used an S1 probe that had three direct repeats. They suggested that many of the bands whose 5′ ends mapped to the repeat regions of the TS promoter could be due to S1 artifacts resulting from the formation of secondary structure.

To circumvent these difficulties, we devised a new strategy for determining the transcriptional start site pattern of the human TS promoter. This involved analyzing the 5′ termini of TS mRNA derived from a transfected TS minigene consisting of the human TS promoter region linked to the mouse TS coding region. We have shown previously, in analyses of the mouse TS promoter, that the same pattern of start sites is observed with transfected minigenes as with TS mRNA isolated from mouse cells [DeWille et al., 1988; Deng et al., 1989b]. The human TS promoter region that was analyzed

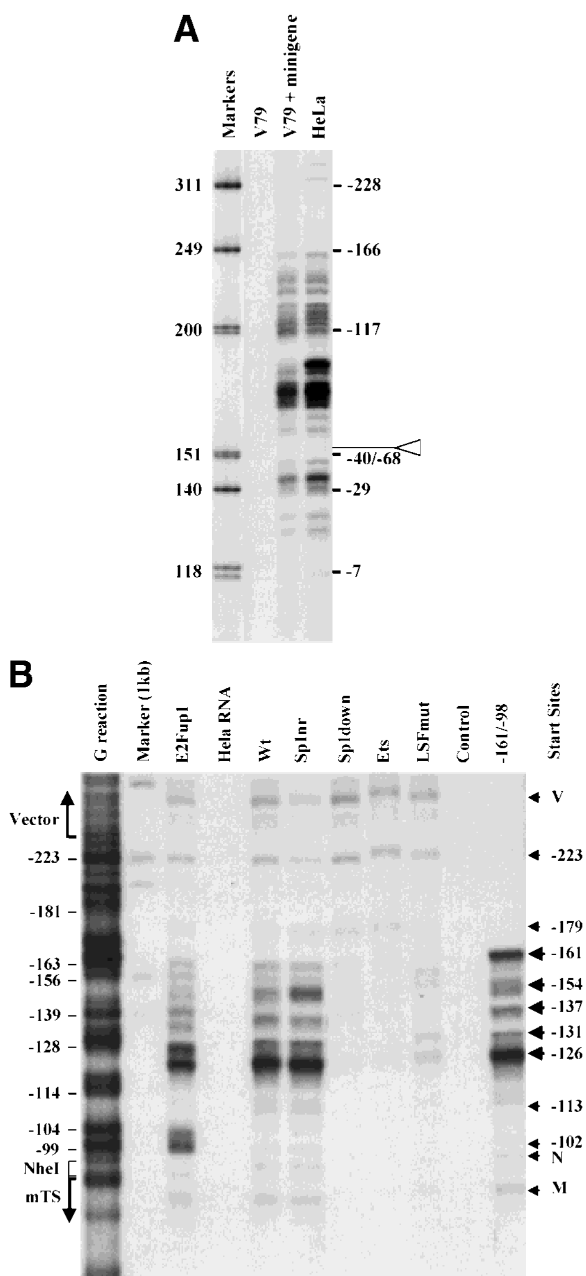

Fig. 6. Transcriptional initiation pattern. **A:** Analysis of the intact promoter region. RNA was isolated from untransfected HeLa cells (HeLa) or hamster V79 cells (V79) or from V79 cells transiently transfected with the TS indicator gene driven by the human TS promoter region between −243 and +111 (V79 + minigene). RNA was analyzed by an S1 nuclease protection assay. The probe was derived from the human TS promoter region that was included in the minigene and was 5′ end-labeled at +111. The sizes (nt) of the molecular-weight markers are indicated on the left, and their corresponding locations in the human TS promoter region are indicated on the right. The triangle on the right indicates the position of the missing 28-nt direct repeat sequence. **B:** Analysis of the truncated promoter region. HeLa cells were transiently transfected with TS minigenes that were driven by the −243/−98 human TS promoter region. The promoters had the wild-type (Wt) sequence or the indicated mutations (described in Fig. 1). The control minigene lacked a promoter region. RNA was isolated from the cells and analyzed by an S1 nuclease protection assay. The probe was derived from the minigene that was used for transfection except for the −161/-98 minigene, which was analyzed using the probe derived from the wild-type promoter. RNA isolated from untransfected HeLa cells (HeLa RNA) was also analyzed using the probe derived from the wild-type promoter. Each probe was 5′ end-labeled at the *Bsu*36I site in exon 1 (Fig. 2A). The wild-type probe was subjected to the Maxam and Gilbert G reaction to provide a partial sequencing ladder that would correspond to the positions of C residues of the promoter (Fig. 1), some of which are shown to the left of the autoradiogram. The approximate locations of the major transcriptional start sites in the TS promoter region are indicated to the right of the autoradiogram. V indicates a start site that is in the vector sequence. N indicates a start site that occurs in the engineered *Nhe*I site that links the promoter region to the indicator gene. M indicates a start site that is in the minigene sequence.

in these studies extended from −243 to −98. It included the inverted repeat sequence, but none of the direct repeats, so as to avoid artifacts that might be caused by secondary structure. The minigene was transiently transfected into HeLa cells and the pattern of start sites was determined by an S1 nuclease protection strategy using a probe derived from the minigene. The probe was 5′ end-labeled at +85 (at the *Bsu*36I site) and extended to the SspI site in the plasmid vector, 0.6 kb upstream of the inserted promoter region. Since there is considerable sequence divergence between the mouse and human TS mRNA sequences near the 5′ end of the transcribed region, the probe should be specific for TS mRNA derived from the transfected minigene and should not detect the endogenous TS mRNA from the HeLa cells.

The results of a typical analysis are shown in Figure 6B. No protected fragments were observed when analyzing RNA derived from untransfected HeLa cells (HeLa RNA), as expected. When the analysis was performed on RNA isolated from cells transfected with the TS minigene driven by the wild-type human TS promoter (Wt), a complex pattern of fragments was observed. The 5′ termini of most of the fragments mapped between −160 and −120. The most intense band was at approximately −126. Some faint bands were also observed upstream of −160 and downstream of −120. All the bands upstream of −243 (e.g., the band labeled "V") correspond to transcripts initiating within the plasmid vector and may represent RNA molecules that are synthesized in response to cryptic promoter elements within the plasmid. The faint bands labeled N and M corresponds to RNA molecules that are initiated within the engineered *Nhe*I site and within mouse TS indicator gene, respectively, and probably do not correspond to legitimate transcriptional start sites. However, all other start sites mapped within the human TS promoter region and are thus likely to represent legitimate start sites. The same pattern was observed with RNA isolated from hamster V79 cells transfected with the TS minigene (data not shown). Therefore, assuming that sequences downstream of −98 do not affect the pattern of start sites, it appears that the transcriptional initiation window of the human TS promoter region extends from approximately −161 to −120, with some additional minor start sites upstream and downstream of this region.

The same pattern of start sites was also observed with the promoter that was deleted at the 5′ end to −161 (−161/−98). This shows that all the elements that are important for determining the normal pattern of transcriptional start sites are located within or downstream of the human TS essential promoter region, as was observed previously for the mouse TS promoter [Geng and Johnson, 1993]. The increased intensity of the band at −161 is due to the divergence between the minigene and the probe at that location; all the transcripts that initiate in the plasmid sequences upstream of that point result in a signal at −161.

To determine whether any of the mutations in the promoter region would affect the pattern of start sites, similar analyses were performed with minigenes driven by mutated promoters. In these analyses, the S1 probes were derived from the mutated minigenes. Inactivation of the Sp1 element at −147 (Sp1down), the adjacent Ets element at −154 (Ets) or the LSF motif (LSFmut) greatly reduced the intensities of the protected fragments between −160 and −98, in line with the effects of these mutations on TS promoter activity (Fig. 5). However, these mutations had no effect on the faint fragments upstream of −160, which is consistent with the possibility that these bands correspond to RNA molecules that are synthesized in response to cryptic promoter elements in the plasmid vector.

Inactivation of the Sp1 element at −175 (Sp1nr mutation, Fig. 1), which led to a significant increase in promoter activity (Fig. 5A), resulted in an increase in the intensity of a band that was 20 nt downstream of the site of the mutation. Finally, inactivation of the E2F motif at −129 (E2Fup1, Fig. 1), which resulted in a 50% increase in promoter activity (Fig. 5B), led to subtle changes in the band intensities between −161 and −131, an increase in the intensity of a band near −128 (which coincides with the mutated region) as well as a major increase in the intensity of the band at −102. These observations show that the increase in promoter strength that results from the inactivation of these negative-acting elements is due, at least in part, to the increased use of specific transcriptional initiation sites rather than an increased use of the entire spectrum of start sites.

CONCLUSIONS

Our studies have shown that the region between 141 and 161 nt upstream of the AUG codon of the human TS gene is both necessary and sufficient to drive efficient expression of an indicator gene. Figure 7 shows that this 20-nt region (the essential promoter region) is nearly identical to a region of the mouse TS promoter that is also essential for promoter activity. Within this conserved region are binding motifs for Ets, Sp1, and LSF transcription factors. Inactivation of any of these motifs leads to a major reduction in human TS promoter activity, as observed previously for the mouse TS promoter [Geng and Johnson, 1993]. The human TS promoter lacks a TATA box, has a broad transcriptional initiation window and has bidirectional promoter activity, as also observed for the mouse TS promoter [Deng et al., 1986; Geng and Johnson, 1993; Liao et al., 1994]. Thus these fundamental features of the two promoters have been conserved during evolution.

However, several major differences between the human and mouse TS promoters were also uncovered. First, the upstream Ets element at −100 of the mouse TS promoter, which is important for high-level promoter activity [Deng et al., 1989b; Geng and Johnson, 1993] is absent in the human TS promoter. Second, the human (but not the mouse) TS promoter has a motif that resembles an Sp1 element 15 nt upstream of the essential region that functions as a negative promoter element. Third, there is a consensus E2F element 13 nt downstream of the essential region of the human TS promoter that also functions as a negative element. This element is not present in the mouse TS promoter. Fourth, a potential E2F element that is 8 nt upstream of the mouse essential region is not present in the human TS promoter. Fifth, the human TS promoter region includes tandem repeats, as well as an inverted repeat sequence, which are absent in the mouse TS promoter. Although these repeated sequences appear to affect the efficiency of translation of the mRNA [Kaneda et al., 1987], we found that they do not play an important role in TS promoter activity. Finally, the region upstream of the human essential promoter region contains multiple negative elements, as observed previously [Horie and Takeishi, 1997]. By contrast, the sequences up to 1 kb upstream of the mouse essential promoter region have little effect on promoter activity [Deng et al., 1989b]. The fact that the longer human promoter regions have negligible activity implies that there must be positive-acting elements elsewhere in the gene that serve to counteract these inhibitory elements. Additional studies will be required to determine the physiological significance of the differences between the mouse and human TS promoters.

We have shown previously that sequences within the essential region of the mouse TS promoter as well as a spliceable intron in the

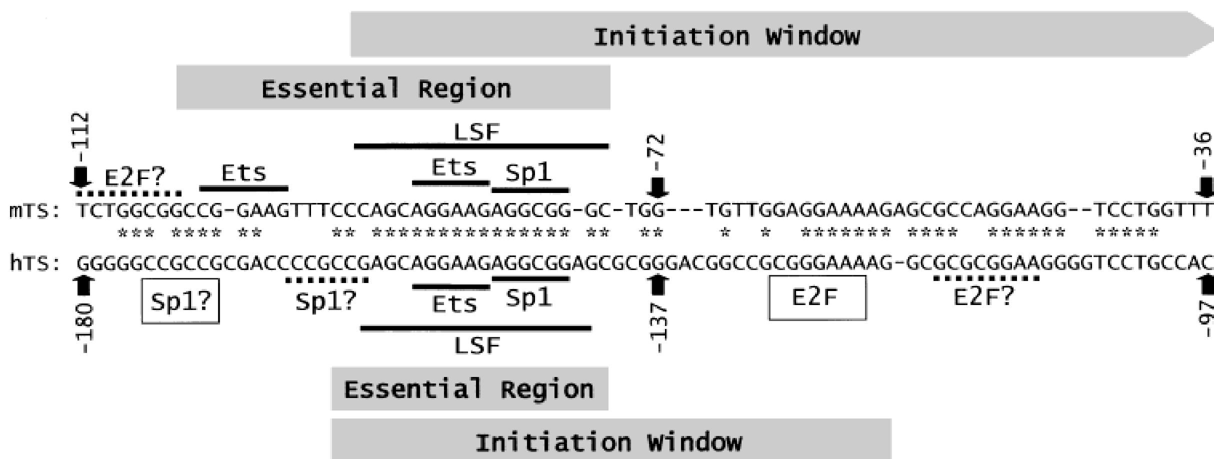

Fig. 7. Comparison of mouse and human TS promoter regions. The sequences of the mouse TS gene (mTS) from −112 to −36 and the human TS gene (hTS) from −180 to −97 (relative to the A of the AUG translational start codon) are shown. *, nucleotide identities. The essential promoter regions and the boundaries of the transcriptional initiation windows for the major transcriptional start sites are indicated. Arrow, mouse initiation window extends to −14. The locations of potential binding sites for various transcription factors are indicated. Black lines, positive-acting sites; boxes, negative-acting sites; dashed lines, potential binding sites for transcription factors that, when inactivated, had little effect on promoter activity.

transcribed region are both necessary (though neither is sufficient) for S phase-specific expression [Ash et al., 1993; Ash et al., 1995; Ke et al., 1996]. It will be important to determine whether this is also true for the human TS gene. Further-more, it will be interesting to explore the possible role of the E2F element(s) in regulating the expression of the TS gene during the G1 to S phase transition. E2F has been shown to play a major role in regulating the transcription of a variety of genes whose expression increases during this interval [Farnham et al., 1993; DeGregori et al., 1995], although it does not appear to play an important role in the mouse TS gene [Ash et al., 1995]. Instead, the LSF factor that binds to the essential promoter region has been shown to be important for S phase regulation of the mouse TS gene [Powell et al., 1999]. Since the LSF binding site is conserved in the human TS gene, it will be interesting to determine whether this factor is important for S phase regulation of the human TS gene.

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