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The Mouse Thymidylate Synthase Promoter: Essential Elements Are in Close Proximity to the Transcriptional Initiation Sites

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The promoter region of the mouse thymidylate synthase gene was analyzed by deletion and site-directed mutagenesis. Elimination of an upstream Sp1 element reduced expression threefold, whereas elimination of an adenovirus upstream stimulatory factor element had little effect. All of the upstream elements that are essential for promoter activity are located within 22 nucleotides of the first transcriptional initiation site.

Thymidylate synthase (TS) is the enzyme that catalyzes the de novo synthesis of thymidylic acid (5). TS enzyme and mRNA levels in a variety of eucaryotes are regulated during the cell cycle and following growth stimulation (3, 15, 18, 19, 25). We have shown that the mouse TS gene is 12 kilobases (kb) in length and that the 1-kb coding region is interrupted by six introns (6, 12, 21). The 5′-flanking region of the gene is shown in Fig. 1. The region lacks a TATAA box, and this lack is probably responsible for the fact that transcription is initiated at multiple sites (6). The region contains two potential Sp1 elements, which are known to be important for expression of a wide variety of cellular and viral genes (9–11). Adjacent to the upstream Sp1 site is a potential binding site for adenovirus upstream stimulatory factor (USF) (24) (or major late transcription factor [1]). This element is less common but has been shown to be important for expression of the adenovirus major late promoter as well some cellular promoters (2, 17).

To facilitate a detailed analysis of the TS regulatory sequences, we have constructed an intronless TS minigene (pTSMG2) (Fig. 2). This minigene consists of 1 kb of the normal 5′-flanking region and 0.25 kb of the normal 3′-flanking region of the TS gene linked to TS cDNA at common restriction sites in the first and last exons (8). Another minigene (pI56; Fig. 2) retains introns 5 and 6 at their normal locations in the coding region. When transfected into *ts*$^-$ hamster V79 cells (20), pI56 has a level of expression about eight times greater than that of pTSMG2 (7). The same transcriptional initiation and polyadenylation sites used for the minigenes are used for the wild-type gene in mouse cells (7, 8). In the present study, we have used derivatives of these TS minigenes to identify the sequences that are important for promoter activity.

Interaction of Sp1 and USF with the TS promoter. We showed previously that TS minigenes deleted to −150 were just as active as pTSMG2 but that those deleted to −54 were inactive (8). This suggested that the USF or Sp1 elements located between these deletion endpoints might be important for promoter activity. To determine whether these proteins interact with the TS promoter, DNase I footprint analyses were performed. Sp1 protected a 13-base-pair region from −123 to −136, whereas USF protected a 19-base-pair region from −125 to −144 (Fig. 3A and B). The sizes of the footprints for each protein were comparable to those observed in analyses of other promoters (11, 24). The USF and Sp1 footprints overlap to a major extent, indicating that the two proteins were unlikely to bind simultaneously to the TS promoter. There was no evidence for the binding of Sp1 at the downstream Sp1? site (Fig. 1).

Analysis of TS minigenes with altered promoters. To determine the importance of the Sp1 and USF elements for TS promoter activity, the elements were altered by site-directed mutagenesis. The intronless minigene was cloned into Bluescribe M13(+) (Stratagene), and specific nucleotide alterations were generated by the method of Zoller and Smith (26). The structure of the altered promoter was confirmed by DNA sequence analysis (23). The mutations prevented the interaction of the proteins with their binding sites, as revealed by gel retardation assays (data not shown). The mutated promoter was inserted into the pI56 minigene by replacing the *Xba*I-to-*Apa*I restriction fragment of pI56 with the corresponding fragment from the altered pTSMG2.

The altered TS minigenes (12 pmol, along with 4.5 μg of pSV2cat) were transfected into *ts*$^-$ hamster V79 cells by using a calcium phosphate protocol (7, 8). The cultures were harvested 48 h later, and the level of minigene expression was determined by measuring TS enzyme level with a ^{3}H-labeled 5-fluorodeoxyuridylic acid-binding assay (22). The content and 5′-terminal structure of TS mRNA produced from pI56 with the various promoter mutations were determined by 5′ S1 nuclease protection assays (6–8). These values were normalized to chloramphenicol acetyltransferase activity (7, 13) to correct for differences in transfection efficiency.

Inactivation of the USF element had little effect on TS promoter activity, whereas inactivation of the Sp1 site led to a threefold reduction in activity (Table 1). A promoter with alterations in both the USF and Sp1 sites had about the same activity as did the Sp1-deficient promoter. The content of TS mRNA (Fig. 4A) correlated well with the TS enzyme level. The pattern of S1-resistant fragments observed for promoters with mutations in the USF and Sp1 sites was indistinguishable from that observed for pTSMG2. Therefore, the Sp1 element appears to function as a positive element in the TS promoter but is not essential for gene transcription. However, in contrast to the situation in other genes, the

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FIG. 1. Important features of the promoter region. The sequence of the 5'-flanking region of the mouse TS gene (6) is shown in boldface letters. Numbering starts at the A of the AUG initiation codon. The approximate locations of the major (V) or minor (v) transcriptional start sites are indicated. The locations of the potential USF (CACGTG)-, Sp1 (GGGCGG)-, and Sp1? (AGGCGG)-binding sites are indicated. The *Alu*I (Alu), *Hin*fI (Hinf), and *Hae*II (Hae) restriction sites were used to generate probes in the footprint analyses. The nucleotide changes introduced by site-specific mutagenesis (*) and the deletion endpoints (:) are indicated. The sequence of the deleted minigene from the 5' end of the genomic sequence (uppercase letters) to the *Eco*RI site in the polycloning site of the pUC18 vector (lowercase letters) is shown.

USF-binding site does not appear to play a pivotal role in the mouse TS promoter.

Analysis of the promoter by deletion mutagenesis. To define the promoter region that was essential for TS gene expression, additional deletion mutations were generated by the method of Dale et al. (4) as described previously (8). The extent of each deletion was determined by DNA sequence analysis (23). The locations of the deletions and the sequences at the plasmid-promoter boundary are shown in Fig. 1. Deleted promoters were cloned back into the TS minigenes by replacing appropriate restriction fragments.

A promoter deleted to −113 (which eliminated both the Sp1 and the USF elements) had almost the same activity as that with the −150 deletion (Table 2). This confirms that the USF and Sp1 elements are not essential for promoter activity. Deletion to −105 led to a slight reduction in gene expression. However, when the sequences upstream of −85 were eliminated, the gene was almost completely inactivated.

S1 nuclease protection studies (Fig. 4B) show that, with the exception of an S1-resistant fragment that mapped to the deletion endpoint, there was no detectable change in the pattern of transcriptional start sites for deletions −150, −113, and −105. However, the normal pattern of start sites

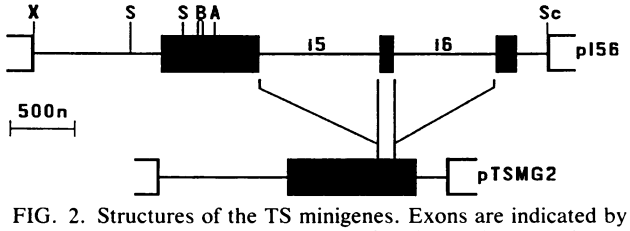

FIG. 2. Structures of the TS minigenes. Exons are indicated by the closed boxes. Thin lines represent flanking regions and intron sequences. The following restriction sites are indicated: A, *Apa*I; B, *Bam*HI; S, *Sph*I; Sc, *Sac*I; and X, *Xba*I. n, Nucleotides.

TABLE 1. Effects of promoter mutations on TS minigene expression[a]

Promoter mutation[b]	TS enzyme level[c] in:		TS mRNA level[d] in pI56
	pTSMG2	pI56	
None (normal)	1.00	1.00	1.00
−USF	1.86 ± 0.47	0.88 ± 0.30	0.82 ± 0.06
−Sp1	0.36 ± 0.08	0.40 ± 0.12	0.30 ± 0.16
−USF and Sp1	0.43 ± 0.02	0.32 ± 0.12	0.37 ± 0.08

[a] TS and chloramphenicol acetyltransferase enzyme levels were determined and normalized to the values obtained with the normal promoter. Data are the averages (± standard deviations) of at least three independent experiments.

[b] The 5'-flanking region of each minigene extends to the *Xba*I site 1 kb upstream of the AUG codon. The promoter mutations are shown in Fig. 1. −, Inactivated.

[c] The mutated promoters were linked to the intronless coding region (pTSMG2) or to the coding region containing introns 5 and 6 (pI56).

[d] RNA was quantitated by densitometric analysis of autoradiograms from S1 nuclease assays. The values were corrected for the level of chloramphenicol acetyltransferase gene expression and then normalized to the value observed for the normal promoter.

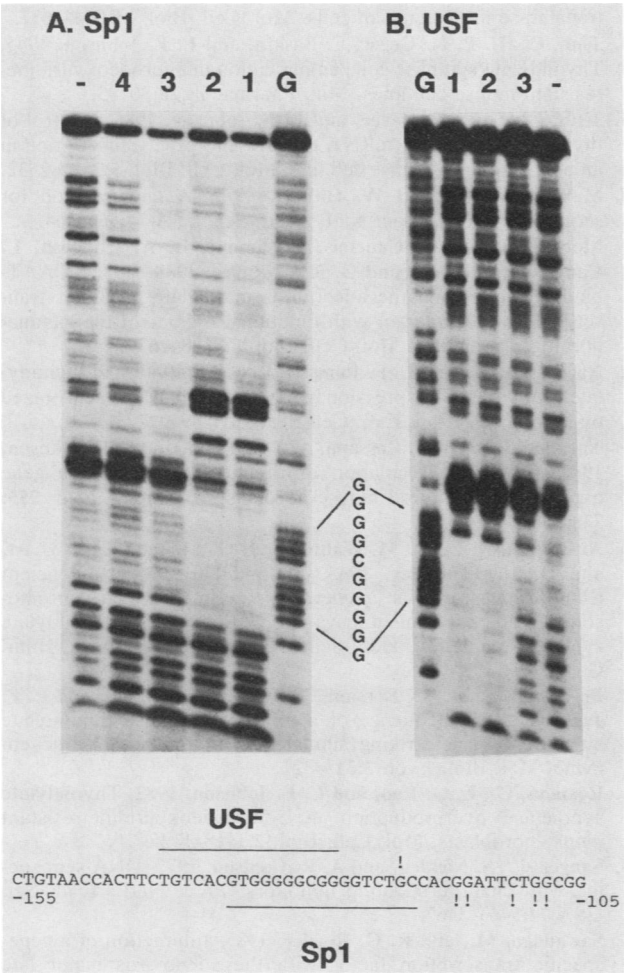

FIG. 3. Footprint analysis of the Sp1 and USF elements. The probe consisted of 102 base pairs of the TS promoter (the *Alu*I-to-*Hae*II restriction fragment shown in Fig. 1, which contains the Sp1 and USF elements) and 20 base pairs of polylinker sequence from the pUC18 vector sequence which was 5′ end labeled at the *Eco*RI site in the polylinker. The probe was subjected to the G-specific reactions in the Maxam and Gilbert sequencing protocol (16) (lane G) and serves as a position marker. Fragments obtained when the probe was digested with 10 ng of DNase I in the absence of proteins (−) are indicated. (A) Sp1 footprint. Highly purified Sp1 was generously provided by J. Kadonaga and R. Tjian. Reaction conditions were as described elsewhere (11). Lanes 1 through 4, Digestion products of the probe in the presence of 15, 10, 5, and 1 ng, respectively, of Sp1. (B) USF footprint. Partially purified USF was generously provided by M. Sawadogo and R. Roeder. Footprinting conditions were as described elsewhere (24). Lanes 1 through 3, Digestion products of the probe in the presence of 1.0, 0.5, and 0.25 μl of a solution containing partially purified USF. The summary of the DNase I footprint analyses (bottom) shows the DNA sequence of the coding strand from −155 to −105. Sequences protected from DNase I digestion by USF or Sp1 are bracketed. DNase I-hypersensitive sites (!) are indicated.

was completely lost with deletions −85 and −54. These observations (as well as those with the Sp1 and USF mutations) rule out the possibility that each start site is specified by a unique upstream promoter element. It is still possible that the complex pattern is the result of multiple protein-binding elements downstream of −100.

TABLE 2. Effects of promoter deletions on TS minigene expression[a]

Promoter deleted to[b]:	TS enzyme level[c] in:		TS mRNA level[d] in pI56
	pTSMG2	pI56	
−150	1.00	1.00	1.00
−113	1.10 ± 0.17	0.76, 0.74	1.2, 0.71
−105	0.36 ± 0.02	0.63, 0.51	0.78, 0.65
−85	0.05 ± 0.01	0.02, 0.05	0.0, 0.0
−54	0.03 ± 0.02	0.01, 0.01	0.0, 0.0

[a] Experiments were performed and analyzed as described in Table 1, footnotes *a* through *d*, except that the values were normalized to those of the −150 deletion, which is expressed at about the same level as that of pTSMG2 (8). The experiments with pI56 were performed twice, and both values are presented.

[b] The TS 5′-flanking region was deleted to the indicated positions (Fig. 1).

[c] Deleted promoters were linked to the intronless coding region (pTSMG2) or to the coding region that contained introns 5 and 6 (pI56).

[d] When the amount of TS mRNA was being determined, the band corresponding to the deletion endpoint (Fig. 4B) was ignored.

The deletion studies revealed that at least one essential promoter element is located in close proximity to the most distal transcriptional start site. We are currently attempting to identify the proteins that bind to the essential region and to determine the mechanism by which they stimulate the initiation of transcription at multiple sites. In addition, we are analyzing sequences that are located downstream of this region in order to determine if there are additional regulatory elements in the mouse TS promoter.

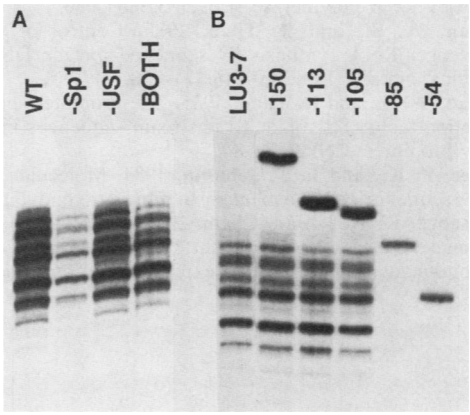

FIG. 4. Effect of mutations on the pattern of transcriptional start sites. Cytoplasmic poly(A)$^+$ mRNA was isolated from mouse LU3-7 fibroblasts (a cell line that overproduces TS by a factor of 50 [22] because of gene amplification [14]) or from *ts*$^-$ hamster V79 cells that were transiently transfected with the indicated TS minigenes. S1 nuclease protection assays were performed with 100 ng of mRNA from LU3-7 cells or with 4 μg of mRNA from cells transfected with TS minigenes containing the wild-type (WT) promoter or promoters with the indicated point mutations (A) or deletion mutations (B). The probe was derived from pTSMG-2 (Fig. 2). It was 5′ end labeled at the promoter-proximal *Bam*HI site in the coding region and extended to the *Xba*I site in the 5′-flanking region. Digestion products were analyzed on a 6% polyacrylamide sequencing gel and detected by autoradiography. (See reference 6 for a more detailed description of the pattern of fragments.)

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