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Inactivation of MED-1 Elements in the TATA-less, Initiator-less Mouse Thymidylate Synthase Promoter Has No Effect on Promoter Strength or the Complex Pattern of Transcriptional Start Sites

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Abstract The mouse thymidylate synthase (TS) promoter is a member of a family of promoters that lack a TATA box as well as an initiator element and that initiate transcription at many sites over a broad initiation window. An element (MED-1) downstream of the initiation window of almost all promoters of this family has been proposed to be important for promoter activity, as well as for multiple start site utilization. Two consensus MED-1 elements are located downstream of the initiation window of the TS promoter. To determine the role of the MED-1 elements in the TS promoter, one or both elements were inactivated by site-directed mutagenesis and the effects on promoter function were determined. We found that inactivation of the MED-1 elements had no measurable effect on promoter strength, the boundaries of the initiation window, or the pattern of transcriptional start sites. Furthermore, inactivation of the elements did not affect the ability of the TS promoter to direct S phase-specific expression of the gene in growth-stimulated cells. We conclude that the MED-1 element does not play a significant role in TS promoter function and therefore is not an essential component of all TATA-less promoters with complex transcriptional initiation patterns. J. Cell. Biochem. 73:90–96, 1999.

Key words: gene transcription; gene transfection; cell cycle; cultured mammalian cells; transcriptional initiation; downstream promoter element

Most mammalian promoters initiate transcription at a single transcriptional start site defined either by an upstream TATA sequence or by an initiator element. The TATA sequence is located approximately 30 nucleotides (nt) upstream of the transcriptional initiation site and serves as the binding site for the transcription initiation factor TFIID [Goodrich et al., 1996; Roeder, 1996]. Many promoters that lack a TATA sequence but that retain an initiator element have a downstream promoter element (DPE) located approximately 30 nt downstream of the initiation site. The DPE sequence appears to serve a similar function as the TATA sequence in these promoters [Burke and Kadonaga, 1997]. However, a small number of promoters lack both a TATA box and an initiator element and initiate transcription at multiple sites over a broad initiation window [Geng and Johnson, 1993; Weis and Reinberg, 1992]. Many of these promoters are also bidirectional [Liao et al., 1994]. Little is known about the mechanism for transcriptional initiation of these unusual promoters.

In an attempt to identify common properties of the class of promoters that lack both a TATA box and an initiator element and that have complex patterns of transcriptional initiation, Ince and Scotto [1995a] compared the sequences of a panel of such promoters. These investigators identified a conserved, protein-binding sequence element [GCTCC(C/G)] located 20–45 nt downstream of the 3′ boundary of the initiation window in almost every promoter in this class. They named this element the *m*ultiple start site *e*lement *d*ownstream (MED-1) element. The striking conservation of this element suggested that it might be important for pro-

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moter function. To test this possibility, they examined the role of this element in the P-glycoprotein promoter, which uses either a single transcriptional start site (in wild-type cells) or multiple start sites (in cells that overproduce the enzyme in the absence of gene amplification) [Ince and Scotto, 1995b]. They found that when the MED-1 element of the P-glycoprotein promoter is inactivated, promoter strength is decreased by about fourfold. Analysis of the pattern of start sites showed that inactivation of the MED-1 element decreased the frequency of initiation from the downstream start sites, while initiation from the upstream start site was unaffected [Ince and Scotto, 1995a]. These investigators proposed that the presence of a downstream MED-1 element defines the class of promoters that use a complex pattern of transcriptional start sites. The fact that the element is downstream of almost all promoters with these properties is certainly consistent with this possibility. However, the importance of the MED-1 element has not been directly tested in any promoter besides the P-glycoprotein promoter.

One of the MED-1 class promoters identified by Ince and Scotto is the mouse thymidylate synthase (TS) promoter, which has two consensus MED-1 elements downstream of the initiation window. We have shown previously that the TS promoter, which lacks a TATA box as well as an initiator element, initiates transcription at many sites within a 90-nucleotide initiation window [Geng and Johnson, 1993] and has bidirectional activity [Liao et al., 1994]. The TS promoter also plays an essential role in the S phase-specific expression of the gene [Ash et al., 1995]. The goal of the present study was to determine whether the MED-1 elements played an important role in determining the strength of the mouse TS promoter, the pattern of transcriptional initiation sites, or the ability of the promoter to direct S phase-specific expression.

MATERIALS AND METHODS

Construction of MED-1 Mutations

The TS minigenes that were used in these studies contained the TS promoter region (extending 1 kb upstream of the AUG start codon), the TS coding region and the TS polyadenylation signal as well as introns 5 and 6 ($I_{5,6}$) or intron 1 and internally deleted intron 2 ($I_{1,2d}$) at their normal locations in the TS coding region. The minigenes were tagged by deleting a 57-nt *Bam*HI fragment from exon 3. Construction of the minigenes was described previously [Geng and Johnson, 1993; Ash et al., 1995]. Mutations in the MED-1 elements were created using polymerase chain reaction (PCR)-based strategies with primers that contained the desired nucleotide changes. The mutated regions were inserted into the TS minigenes by replacing appropriate restriction fragments. The mutated regions were sequenced to confirm the presence of the desired alterations and the absence of additional changes.

Cell Culture and Transfection

Mouse 3T6 fibroblasts [Todaro and Green, 1963] and *ts*- Chinese hamster V79 fibroblasts [Nussbaum et al., 1985] were maintained on plastic tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. The medium for the V79 cells also contained 10 mM thymidine. TS minigenes were transiently transfected into V79 cells using the calcium phosphate method [Wigler et al., 1979]. Cells were plated at 5×10^5 cells/100-mm dish and transfected 5 h later with 20 mg of the TS minigene and 5 mg of a control TS minigene, $SI_{5,6}S$, to correct for differences in transfection efficiency and RNA recovery [Lee and Johnson, 1998]. The control minigene was driven by the SV40 early promoter and retained the *Bam*HI fragment in exon 3. The medium was replaced 16 h after transfection and cytoplasmic RNA was isolated 40 h after transfection. To analyze S phase regulation, TS minigenes (80 mg) were stably transfected (via electroporation) into 3T6 cells along with a *neo* gene (1 mg) as a selectable marker [Ash et al., 1993]. After 2 days, selection medium containing 40 mg/ml G418 was added. After 12 days of selection, clones ($>$50) that were resistant to G418 were pooled and maintained as a mass culture. Quiescent cells were obtained by culturing the cells at confluent density in DMEM plus 0.5% calf serum for 7 days, feeding the cells with the same medium 2 and 4 days after seeding. Cells were then stimulated to proliferate by increasing the serum concentration to 10% [Johnson et al., 1974].

S1 Nuclease Protection Assays

Cytoplasmic RNA was purified by phenol-chloroform extraction [Johnson et al., 1974], digested with RNase-free DNase to remove any contaminating plasmid, and analyzed by an S1

nuclease protection assay as described previously [Favaloro et al., 1980] and in the figure legends. Protected fragments were separated on a 6% denaturing polyacrylamide gel and analyzed on a phosphorimager (Molecular Dynamics, Sunnyvale, CA) and by autoradiography.

RESULTS

In their original study, Ince and Scotto identified a MED-1 element in the mouse TS promoter beginning 14 nt downstream of the AUG translational start codon [Ince and Scotto, 1995a]. We identified an additional consensus MED-1 element that begins 8 nt upstream of the AUG codon, as well as two potential MED-1 elements that represent a 5 out of 6 match to the consensus sequence (Fig. 1). The presence of these elements in the TS promoter region raised the possibility that they may play a role in determining the strength of the TS promoter or the pattern of transcriptional start sites, as was observed previously in the P-glycoprotein promoter. To explore the role(s) of the MED-1 elements in TS promoter function, mutations were introduced that would inactivate one or both of the consensus MED-1 elements as well as the two potential MED-1 elements. The sequence alterations that were introduced are summarized in Fig. 1.

Effect of MED-1 Mutations on Promoter Strength

To determine whether the MED-1 elements were important in determining TS promoter strength, TS minigenes that contained promoters with either the wild-type sequence or with mutations in the MED-1 elements were transiently transfected into *ts*- hamster V79 cells. The amount of mRNA corresponding to the minigenes was determined two days later using an S1 nuclease protection assay. Figure 2 shows that inactivation of either the upstream or the downstream MED-1 elements had no effect on the level of expression of the TS minigene. Inactivation of all MED-1 elements had no effect on the expression of the TS minigene that contained introns 1 and 2d, although we did observe a slight (about twofold) decrease in expression of the minigene that contained introns 5 and 6. The reason for this difference (which was observed in several independent experiments that used different preparations of each plasmid) is unknown.

Effect of MED-1 Mutations on the Pattern of Transcriptional Start Sites

Inactivation of the MED-1 element in the P-glycoprotein promoter led to a profound change in the pattern of transcriptional start sites [Ince and Scotto, 1995a]. To determine whether this was true for the mouse TS promoter as well, we analyzed the transcriptional start site pattern of transiently transfected TS minigenes that contained the wild-type promoter or promoters with mutations in the upstream, downstream, or all the MED-1 elements. The pattern of start sites was determined by an S1 nuclease protection assay using probes that were derived from the same minigenes that were transfected into the cells. Figure 3 shows that the same complex pattern of transcriptional start sites was observed when the upstream, downstream, or all of the MED-1 elements of the TS promoter region were inactivated. In particular, there was no evidence for diminished utilization of the downstream start sites, as was observed for the P-glycoprotein promoter [Ince and Scotto, 1995a]. The same results were observed when analyzing minigenes that contained introns 1 and 2d or 5 and 6 (data not shown).

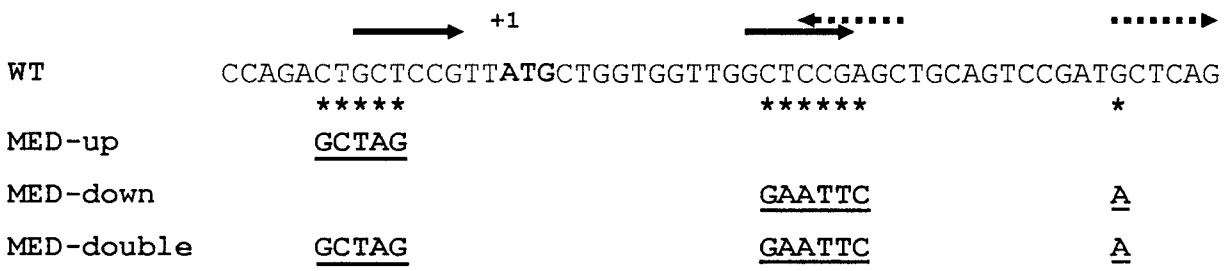

Fig. 1. Mutations in the MED-1 elements. The wild-type TS gene sequence from −15 to +39 is shown. The +1 position corresponds to the A of the ATG start codon. Asterisks, nucleotides that were mutated; underlined letters, changes that were introduced. Solid arrows, perfect match to the consensus MED-1 element, GCTCC(C/G). Broken arrows, 5/6 match. The direction of the arrow indicates the orientation of the element.

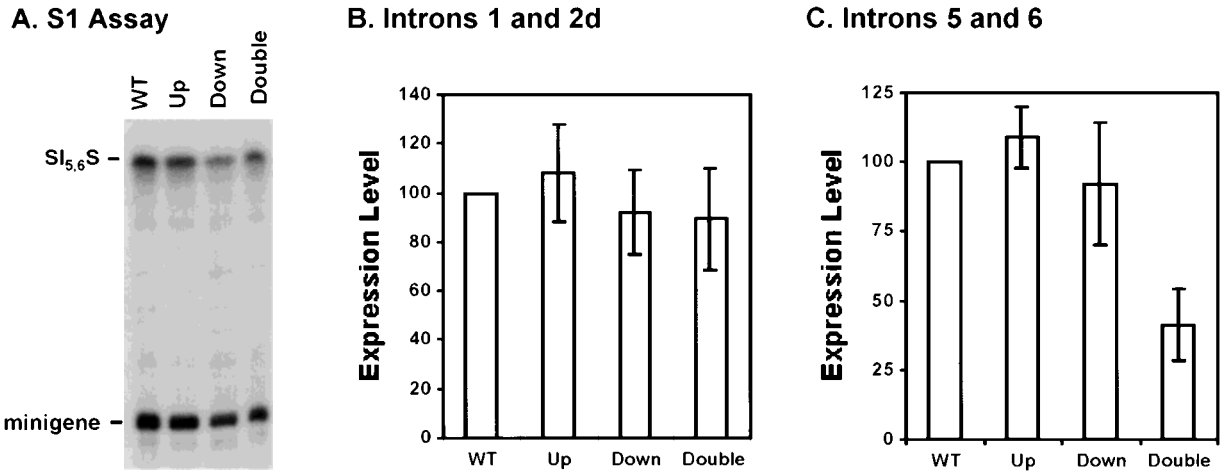

Fig. 2. Effect of MED-1 mutations on promoter strength. V79 cells were transiently transfected with test minigenes that contained either the wild-type (WT) promoter sequence or the indicated mutations in the MED-1 elements. The minigenes were tagged by deleting a 57-nt *Bam*HI fragment from exon 3. A control minigene, $SI_{5,6}S$, which retained the *Bam*HI fragment, was co-transfected along with the test minigene to permit corrections for differences in transfection efficiency and RNA recovery. Cytoplasmic RNA was isolated 40 h later and analyzed by an S1 nuclease protection assay. The probe was derived from the $SI_{5,6}S$ minigene and was 5′ end-labeled with ^{32}P at the *Bgl*II site located in exon 5. The signal for the test minigene (218 nt fragment) was normalized to that for $SI_{5,6}S$ (560nt fragment) to correct for differences in transfection efficiency and RNA recovery. The activity of the wild-type minigenes was set at 100. Error bars represent the standard deviation from at least four independent experiments. **A:** Autoradiogram of a typical S1 nuclease protection assay. **B:** Summary of the results obtained with minigenes that contain introns 1 and 2d. **(C)** Summary of results obtained with minigenes that contain introns 5 and 6.

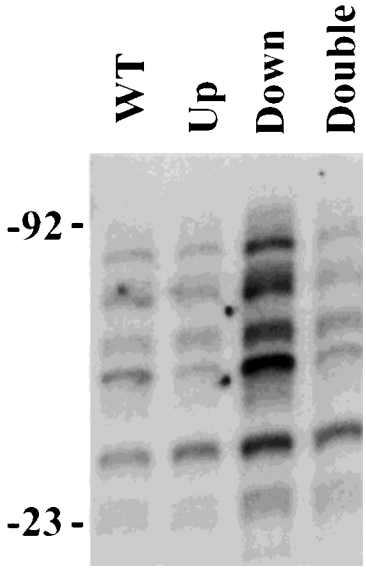

Fig. 3. Inactivation of the MED-1 elements has no effect on the transcriptional start site pattern. V79 cells were transiently transfected with TS minigenes containing the indicated mutations in the MED-1 elements. Cytoplasmic RNA was isolated 40 h later and analyzed by an S1 nuclease protection assay. The probe, which was derived from the TS minigene that was analyzed in each experiment, was 5′ end-labeled with ^{32}P at the *Bam*HI site in exon 3. The autoradiogram of the protected fragments is shown. The approximate locations of the fragments relative to the AUG codon are indicated.

Effect of MED-1 Mutations on S Phase Regulation

The TS promoter region plays an important role in directing S phase-specific expression of the mouse TS gene in growth-stimulated cells [Ash et al., 1995]. We explored the possibility that the MED-1 elements may be important for controlling expression of the TS gene during the G1 to S phase transition. These analyses were performed with TS minigenes that contained either the wild-type TS promoter region or mutations in the MED-1 elements. The minigenes also contained TS introns 1 and 2d, since they are necessary for proper S phase regulation of the transfected minigene [Ash et al., 1993, 1995]. The minigenes were stably transfected into mouse 3T6 cells. The cells were synchronized in the quiescent state by maintaining the cells in 0.5% serum for 7 days and then stimulated to proliferate by increasing the serum concentration to 10%. The cells enter S phase approximately 12 h after serum-stimulation, which coincides with a major (approximately 15-fold) increase in TS gene expression [Ash et al., 1995]. The amount of TS mRNA corresponding to the endogenous TS gene and the transfected TS minigene was determined by an S1 nuclease protection assay. Figure 4A

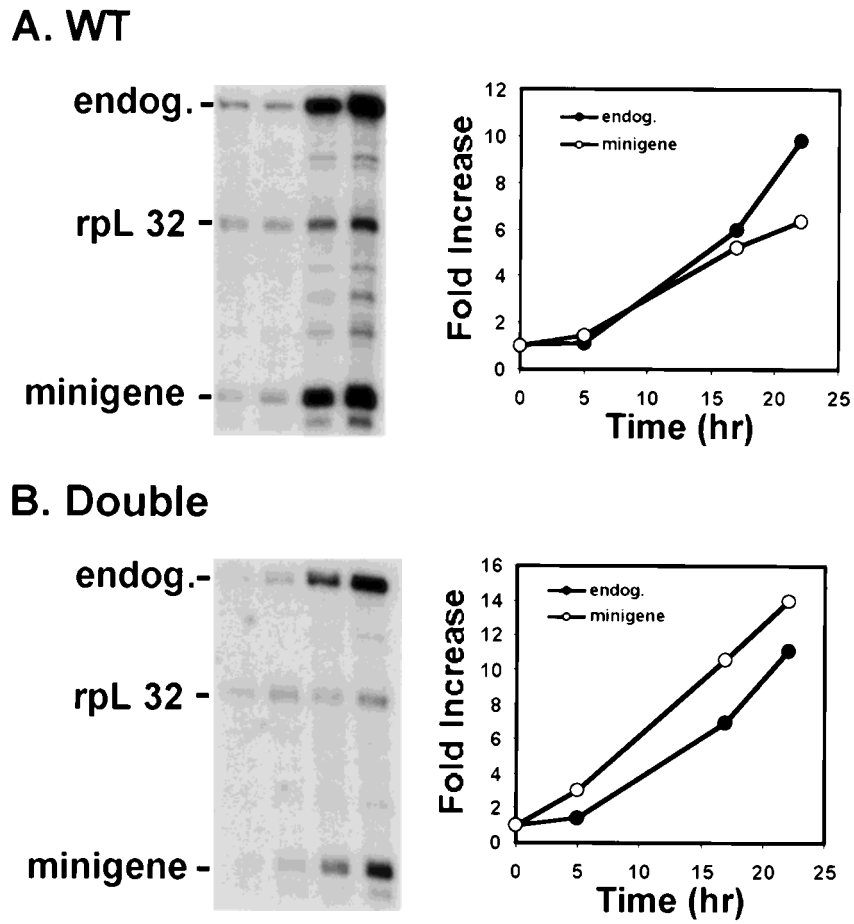

Fig. 4. Inactivation of the MED-1 elements does not affect S phase regulation. Mouse 3T6 cells were stably transfected with the wild-type (WT) TS minigene **(A)** or with a minigene in which all of the MED-1 elements (MED double) were inactivated **(B)**. The cells were growth-arrested for 7 days and then serum stimulated at time = 0. At the indicated times after stimulation, total cytoplasmic RNA was isolated. S1 nuclease protection assays were performed with 25 mg of RNA for each time point. The TS probe was the same as the one described in Fig. 2. This probe detects mRNA from the test minigene (218-nt fragment) as well as the endogenous (endog.) wild-type TS gene (547-nt fragment). The probe for the control ribosomal protein rpL32 mRNA (350-nt fragment) was derived from an intronless version of the rpL32 gene [Chung and Perry, 1989] and was 5′ end-labeled with ^{32}P at the *Dra*III site in exon 4 [Dudov and Perry, 1984]. The autoradiograms of the protected fragments are shown at the left of each panel. Radioactivity corresponding to the endogenous TS gene and the TS minigene was quantitated by a phosphorimager and normalized to the amount of rpL32 mRNA to correct for differences in RNA recovery. The values were then normalized to the amount of radioactivity at time = 0 to facilitate comparison. The results of these analyses were plotted as a function of time after serum stimulation and are shown to the right of the autoradiograms.

shows that the TS minigene that retained the wild-type promoter region was regulated in the same manner as the endogenous TS gene, consistent with our earlier studies [Ash et al., 1995]. Figure 4B shows that the TS minigene in which all of the MED-1 elements had been inactivated was also regulated in the same manner as the endogenous TS gene (Fig. 4B). We also found that inactivation of either the upstream or the downstream MED-1 elements had no effect on S phase regulation (data not shown). Thus, it appears that the MED-1 elements are not important for regulation of the TS gene during the G0 to S phase transition.

DISCUSSION

The MED-1 element was originally identified as a highly conserved nucleotide sequence located slightly downstream of the 3′ boundary of the initiation window of promoters that lack a TATA box and initiator element and that display a complex pattern of transcriptional start sites [Ince and Scotto, 1995a]. The fact that the element was so highly conserved raised the

possibility that it played an important role in the function of such promoters. Direct analysis of the effects of inactivation of the MED-1 element in the P-glycoprotein promoter showed that the element was an important determinant of both promoter strength and the pattern of transcriptional start sites for this promoter [Ince and Scotto, 1995a]. This raised the possibility that the element would have similar properties in other promoters in this family.

The results of the present study show that the MED-1 elements located downstream of the transcriptional initiation window of the mouse TS promoter do not play a significant role in the function of this promoter. Inactivation of the MED-1 elements had no observable effect on the boundaries of the initiation window, the relative utilization of the various start sites within the window, the activity of the promoter, or the ability of the promoter to direct S phase-specific expression. Thus, in spite of the striking conservation of the element, it does not appear to play an important role in TS promoter function. It will be important to examine the role of this element in other promoters in this family to gain additional insight into the possible significance of the MED-1 element in other contexts.

We also examined the TS promoter region and initiation window for the presence of DPE-like elements that have been shown to be important for TATA-less promoters that retain an initiator element [Burke and Kadonaga, 1997]. However, we were unable to identify any sequences that resembled DPE elements, so this downstream sequence also does not appear to be important for mouse TS promoter function.

Although the MED-1 element does not appear to affect the pattern of transcriptional start sites of the mouse TS promoter, we found previously that other protein-binding elements in the TS promoter region did have such an effect. For example, inactivation of the Sp1 motif in the TS essential promoter region led to an increase in the utilization of upstream start sites. In addition, the creation of a spurious, unidentified protein-binding site after mutagenesis of a sequence within the initiation window led to a striking shift in the pattern to downstream sequences. The mechanism responsible for this shift in the initiation window has not been investigated. We also found that insertion of a strong initiator element within the initiation window led to a major shift in the pattern of start sites, with most of the initiation occurring at the site specified by the initiator element [Geng and Johnson, 1993].

Our earlier studies [Geng and Johnson, 1993; Deng et al., 1989; Jolliff et al., 1991] showed that transcription of the mouse TS gene is directed by sequences within a 30-nt essential promoter region that overlaps the 5′ boundary of the initiation window. There was no evidence for the presence of any sequences that were important for transcription downstream of the essential region, in line with the results of the present study. The essential promoter region contains potential binding sites for Ets and Sp1 transcription factors and appears to determine promoter strength as well as the boundaries of the initiation window. The diversity of start sites appears to be due to the absence of a strong initiator sequence within the initiation window. The pattern of start sites may reflect preferences of the transcriptional initiation complex for specific nucleotide sequences within the window rather than the presence of multiple initiator elements or the presence of a downstream element.

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