Introduction of an Initiator Element in the Mouse Thymidylate Synthase Promoter Alters S Phase Regulation but has no Effect on Promoter Bidirectionality

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Abstract   The promoter of the mouse thymidylate synthase (*TS*) gene lacks a TATAA box and an initiator element, is bidirectional and initiates transcription at multiple start sites across broad initiation windows upstream and downstream of the 30 nt essential promoter region. The TS promoter also plays an essential role in the post-transcription regulation of *TS* gene expression during the G_1–S phase transition. The goal of this study was to determine if the addition of a TATAA box or an initiator element would have a significant effect on start-site pattern, promoter bidirectionality and S phase regulation of the *TS* gene. A TATAA box and/or an initiator element were inserted downstream of the TS essential promoter region, and the modified promoters were used to drive expression of indicator genes. The engineered genes were transfected into cultured mammalian cells, and the effects of the mutations were determined. Addition of the TATAA box and especially the initiator element had a significant effect on the transcription start site pattern, indicating that the elements were functional. Unexpectedly, addition of one or both of these elements had no effect on promoter bidirectionality. However, inclusion of the initiator element led to a significant reduction in S phase regulation of TS mRNA levels, indicating that changes in promoter architecture can perturb normal S phase regulation of *TS* gene expression. J. Cell. Biochem. 97: 599–608, 2006.   © 2005 Wiley-Liss, Inc.

Key words: post-transcriptional regulation; S phase gene expression; cell cycle; bidirectional promoter; TATAA box; transcription start sites

The promoters for protein-coding genes frequently include common motifs that are important for basal promoter activity, including the TATA box, the initiator (Inr) element, TFIIB recognition element (BRE) and the downstream promoter element (DPE) [Butler and Kadonaga, 2002; Ohler et al., 2002]. These *cis*-acting elements are important for directing the formation of the RNA polymerase II transcription initiation complex, determining the transcription start site and initiating transcription.

Additionally, these promoters contain proximal or distal binding sites for *trans*-acting factors that further affect transcription initiation.

The TATA box was the first *cis*-acting promoter element identified in eukaryotic protein-coding genes and serves as the binding site for TFIID [Smale and Kadonaga, 2003]. The TATA box was shown to define transcription directionality in simple, synthetic core promoters [Xu et al., 1991; O'Shea-Greenfield and Smale, 1992] and contribute to promoter strength and start site selection. However, approximately 2/3 of all human promoters lack a well-defined TATAA box [Smale and Kadonaga, 2003], so the element is certainly not essential for promoter activity.

The initiator element is found in TATA-containing as well as TATA-less promoters. In promoters containing an initiator element, transcription initiates at a single nucleotide (usually A at the +1 position) within the initiator, whereas in the absence of an initiator

element, transcription usually initiates at multiple start sites [Smale and Baltimore, 1989; Smale et al., 1990]. The initiator element also contributes to promoter strength in TATA-containing promoters. When separated by a distance of 25–30 nts, the TATA box and initiator element function synergistically [O'Shea-Greenfield and Smale, 1992].

Initially it was thought that promoters were unidirectional and that the TATA box and/or initiator element were responsible for conferring directionality to the promoters. However, many bidirectional promoters have now been characterized. Genome-wide analyses have identified possible bidirectional promoters in more than 10% of all human genes [Adachi and Lieber, 2002; Trinklein et al., 2004]. Bidirectional promoters are generally GC-rich, with an average GC content of 66%. Only 8% of the bidirectional promoters have a TATA box on either strand. In many cases, bidirectional promoters are used to drive the expression of divergent genes encoding proteins that need to be expressed in a coordinated manner. However, little is known about the function and regulation of GC-rich bidirectional promoters.

The promoter of the mouse thymidylate synthase (*TS*) gene (Fig. 1a) is an example of a GC-rich bidirectional promoter. It lacks a TATA box and initiator element and initiates transcription at many sites over broad initiation windows upstream and downstream of a 30 nt essential promoter region [Geng and Johnson, 1993; Liao et al., 1994]. These unusual features have been conserved in the rat and human TS promoters as well [Dong et al., 2000; Lee and Johnson, 2000]. The essential region of the mouse TS promoter contains binding sites for LSF, Sp1, and GABP transcription factors [Jolliff et al., 1991; Powell et al., 2000; Rudge and Johnson, 2002]. Insertion of an initiator element within the transcription initiation window of the mouse TS promoter (downstream of the essential region) results in transcription originating at a strong site within the initiator element and loss of the surrounding start sites. Creation of a TATAA box near the 5′ end of the initiation window eliminated transcription start sites upstream of the TATAA box although there was little effect on downstream start sites [Geng and Johnson, 1993].

TS gene expression is tightly regulated in growth-stimulated cells, in line with its important role in the synthesis of a DNA precursor.

The amount of TS mRNA increases at least 10-fold during the G_1–S phase transition [Jenh et al., 1985; Ash et al., 1995]. However, nuclear run-on assays showed that the rate of *TS* gene transcription remained constant during this interval [Ash et al., 1995], indicating that *TS* gene expression is regulated at the post-transcriptional level. Analyses performed with stably transfected TS minigenes revealed that the essential promoter region as well as a spliceable intron in the transcribed region are required for proper S phase regulation of the TS mRNA content [Ash et al., 1993, 1995; Ke et al., 1996]. This suggests that some form of communication between the promoter and the RNA processing machinery is necessary for proper regulation of mature TS mRNA production during the G_1–S phase transition.

Our model is consistent with many recent studies that have demonstrated a high level of coordination between transcription, mRNA processing, and nuclear export [Proudfoot et al., 2002; Reed, 2003]. This coordination is mediated in many situations by the phosphorylated C-terminal domain of the large subunit of RNA polymerase II, which serves as a landing pad for mRNA processing factors [Greenleaf, 1993; McCracken et al., 1997; Bentley, 2002]. In some cases these factors are initially bound to the promoter and subsequently transferred to the RNA molecule via the C-terminal domain [Calvo and Manley, 2003]. Furthermore, the promoter of a gene has been shown to influence alternative splicing [Cramer et al., 1997, 1999] as well as 3′ end formation [Hernandez and Weiner, 1986] of its transcript.

In the present study, we have investigated whether the insertion of a TATA box or an initiator element would influence TS promoter bidirectionality or S phase regulation of *TS* gene expression. We found that introduction of these elements had no effect on promoter bidirectionality. However, introduction of the initiator element led to a significant decrease in S phase regulation of the TS mRNA levels.

MATERIALS AND METHODS

Cell Culture

Mouse 3T6 fibroblasts [Todaro and Green, 1963] and TS-deficient (ts−) V79 Chinese hamster fibroblasts [Nussbaum et al., 1985] were maintained in Dulbecco's modification of Eagle's medium (DMEM, Invitrogen, Carlsbad,

a

-104   Essential promoter region

ACGTGGGGGCGGGGTCTGCCACGGATTCTGGCGGCCGGAAGTTTCCCAGCAGGAAGAGGCG

-75

GGCTGGTGTTGGAGGAAAAGAGCGCCAGGAAGGTCCTGGTTTTGTCGCTGACTACACTGCTG

CCAGACTGCTCCGTT**ATG**
+1

b

-104   Essential promoter region

ACGTGGGGGCGGGGTCTGCCACGGATTCTGGCGGCCGGAAGTTTCCCAGCAGGAAGAGGCG

-75

GGCTGGTG<u>TATAA</u>GGAAAAGAGCGCCAGGAAGGTCCTGGTTTTGTCGCTGACTACACTGCTG
-69

CCAGACTGCTCCGTT**ATG**
+1

c

-125   Essential promoter region

ACGTGGGGGCGGGGTCTGCCACGGATTCTGGCGGCCGGAAGTTTCCCAGCAGGAAGAGGCG

-96

GGCTGGTGTTGGAGGAAAAGAGCGCCAGGAA<u>GCTAGCCCTCATTCTGGAGACGCTAGCGGTT</u>
                                  *
-67   -57   -43

TTGTCGCTGACTACACTGCTGCCAGACTGCTCCGTT**ATG**
+1

d

-125   Essential promoter region

ACGTGGGGGCGGGGTCTGCCACGGATTCTGGCGGCCGGAAGTTTCCCAGCAGGAAGAGGCG

-96

GGCTGGTG<u>TATAA</u>GGAAAAGAGCGCCAGGAA<u>GCTAGCCCTCATTCTGGAGACGCTAGCGGTT</u>
-90   *
-67   -57   -43

TTGTCGCTGACTACACTGCTGCCAGACTGCTCCGTT**ATG**
+1

Fig. 1. Mouse thymidylate synthase (TS) promoter sequence. **a**: Wild-type promoter and flanking regions. **b**: Promoter containing the TATAA box mutation at −69. **c**: Promoter containing the terminal deoxynucleotidyltransferase (TdT) initiator element inserted at −43. **d**: Promoter containing the TATAA box and the initiator element. Nucleotide positions and the essential promoter region are indicated. "*" Designates the adenosine at which transcription initiates within the initiator sequence.

CA) supplemented with 10% calf serum (Colorado Serum). The medium for the V79 (ts−) cells was also supplemented with 10 μM thymidine.

Analysis of Transcription Start Sites

Analyses of the transcription start site pattern were performed using the $TI_{5,6}T$ minigene. This minigene contains the wild-type TS promoter and 5′ flanking region beginning at the *Xba*I site at −985 (relative to the AUG start codon), TS coding region, TS introns 5 and 6 at their normal locations and the TS polyadenylation signal. Promoter alterations that introduced an initiator element from the terminal nucleotidyl transferase gene or a TATAA box were described previously [Geng and Johnson, 1993]. The TS minigene that contains both the TATAA and initiator elements in the promoter was constructed by introducing the TATAA box into the minigene that contained the

initiator element using the Quick Change Site-Directed Mutagenesis kit (Stratagene, Carlsbad, CA). The primers used for mutagenesis were (forward) 5′GGCGGGCTGGTG-TATAAGGAAAAGAGCGCC3′ and (reverse) 3′CCGCCCGACCACATATTCCTTTTCTCG-CGG5′. The structure of the mutated promoter was confirmed by sequence analysis. The sequences of the wild-type and mutant promoter regions are shown in Figure 1. The minigenes (0.6 µg) were transiently transfected into V79 (ts−) cells using Lipofectamine (Invitrogen) as described by the manufacturer. Cytoplasmic RNA was harvested 40–48 h post-transfection using the RNeasy miniprep kit (Qiagen, Valencia, CA). The pattern of transcription start sites was determined using an S1 nuclease protection assay [Favaloro et al., 1980] as described previously [Geng and Johnson, 1993].

Dual-Luc Minigenes

The promoter-less Dual-Luc minigene, which has the firefly luciferase gene and the renilla luciferase gene in a head-to-head orientation, was constructed in the following manner. pRL-CMV (Promega, Madison, WI), which contains the renilla luciferase coding region, was used as the vector backbone. The CMV immediate early enhancer-promoter, T7 promoter and intron were removed from pRL-CMV following digestion with BglII and NheI. The firefly luciferase gene linked to the SV40 polyadenylation signal was recovered from the pGL3 basic vector (Promega) following digestion with NheI and BamHI and ligated into the digested pRL-CMV vector backbone to create the promoter-less Dual-Luc vector.

The mutant TS promoter regions containing the initiator element +/− the TATAA box and extending from an engineered BglII site at −8 to an engineered XbaI site at −233 were amplified by PCR and ligated into the NheI and BglII sites between the two luciferase genes in the Dual-Luc vector. In these constructs, the TS promoter faced the firefly luciferase gene. The wild-type TS promoter and the TS promoter containing the TATAA box were PCR amplified using primers that inserted an XbaI site at −233 and an NheI site at −11 and ligated in both orientations into the NheI site of the Dual-Luc vector. The CMV promoter-enhancer region was isolated by PCR from the pRL-CMV vector and ligated into the Dual-Luc vector as a unidirectional control promoter. Forward and reverse PCR primers each included an NheI site to allow ligation of the promoter into the NheI site of the Dual-Luc vector in both orientations. The structures of all constructs were verified by sequence analysis.

V79 (ts−) cells were transiently co-transfected with 40 ng of the Dual-Luc construct and 10 ng of control SV40-βgal vector using Lipofectamine (Invitrogen) as described by the manufacturer. The total amount of DNA transfected was brought to 1 µg using the carrier plasmid pUC19. Cell lysates were harvested 40–48 h post-transfection in 200 µl 1× passive lysis buffer (Promega). Ten microliters of cell lysate was assayed for firefly and renilla luciferase activity using the Dual Luciferase Assay Kit (Promega). Luminescence was measured in a Lumat LB 9507 luminometer (Berthold, Bad Wilbad, Germany). Expression was normalized to β-galactosidase activity in the same cell extract using the Galactolight chemiluminescence kit (Tropix, Foster City, CA).

Analysis of TS mRNA Levels in Growth-Stimulated Cells

Analyses of TS minigene regulation were performed with $TI_{1,2d}T$ minigenes [Ash et al., 1995]. The minigene was tagged by deleting a 57 nt BamHI fragment from exon 3. It also contains an engineered NheI site at −11 to facilitate promoter exchange. The 1 kb promoter and 5′ flanking region of $TI_{1,2d}T$ was removed by digesting the minigene with XbaI and NheI. A 221 bp fragment containing the wild-type or mutant TS promoter region was isolated by PCR from the Dual-Luc vector containing the desired promoter using primers that introduced XbaI sites at −233 and at −11. The amplified fragment was digested with XbaI and inserted into the XbaI and NheI sites of the $TI_{1,2d}T$ minigene. 3T6 cells were stably co-transfected with 10–80 µg $TI_{1,2d}T$ minigene and 1 µg pSV$_2$neo (Clontech, Mountain View, CA) by electroporation at 960 uF and 250 V. The transfected cells were grown for 3 weeks in culture medium containing 400 µg/ml G418 to select for stably transfected cells. The G418-resistant clones (at least 25) were pooled together and maintained as a mixed population in this selective medium.

3T6 cells stably transfected with the $TI_{1,2d}T$ minigenes were growth-arrested in DMEM containing 0.5% calf serum and 400 µg/ml

G418 for 7 days. The medium was replaced 2 days and 4 days after seeding. After 7 days, the cells were stimulated to re-enter the cell cycle by the addition of DMEM supplemented with 10% calf serum and 400 µg/ml G418. Cytoplasmic RNA was harvested using the RNeasy miniprep kit (Qiagen). cDNA was synthesized using the Superscript II first strand cDNA synthesis kit (Invitrogen). Control reactions were incubated under the same conditions except for the omission of reverse transcriptase.

Primers for real time PCR were designed to distinguish between mRNA derived from the TS minigene and the endogenous *TS* gene. The forward primer for endogenous TS mRNA contained sequences between the two *Bam*HI sites that were deleted from the TS minigene. The forward primer for the minigene-derived mRNA corresponded to sequences that flanked the *Bam*HI deleted region. The reverse primer for the minigene spanned the junction of exons 3 and 4 to prevent amplification of any contaminating genomic DNA. The minigene and endogenous primers were designed such that the PCR product obtained would be approximately the same size (~120 bp). Ribosomal protein L4 (rpL4) mRNA was used as the internal control. Primers used for endogenous TS mRNA detection were: (forward) 5′GCTAAAGAAT-TGTCCTCAAAG3′ and (reverse) 5′GGAAAC-CATAAACTGGGC3′. Primers used for mingene mRNA detection were: (forward) 5′GTTTAT-CAAGGGATCCCG3′ and (reverse) 5′CCGAGT-AATCTGAATCCAT3′. Primers used for rpL4 mRNA detection were: (forward) 5′CCTTTGG-AAATATGTGTCGTGG3′ and (reverse) 5′TTT-AGACATCACCAAAGCTGG3′. Primer efficiencies for all primer pairs were 90%–95%. Hot-StarTaq PCR master mix (Qiagen) with Mg^{++} concentration brought to 3 µM was used for PCR amplification. SyBr Green (Molecular Probes, Carlsbad, CA) was used at a final concentration of 1:125,000 for product detection. Primers were used at a final concentration of 0.25 µM. Realtime PCR was performed in 96-well plates using the BioRad iCycler iQ Real-Time Detection System.

RESULTS

Effects of Initiator Element and TATAA Box on Transcription Start Sites

The TATA-less and initiator-less mouse TS promoter initiates transcription at multiple sites from −92 to −14 (relative to the A of the AUG start codon) [Geng and Johnson, 1993]. The absence of a TATA box and/or initiator element might be responsible for these multiple start sites. To address this possibility, V79 (ts−) cells were transiently transfected with the $TI_{5,6}T$ minigenes containing either the wild-type TS promoter or the TS promoter with a TATAA box at −69 and/or an initiator element from the terminal deoxynucleotidyltransferase (*TdT*) gene at −43 (Fig. 1). Cytoplasmic RNA was analyzed by an S1-nuclease protection assay to determine the transcription start sites. The mRNA derived from the transfected TS minigene driven by the wild-type TS promoter (Fig. 2b) has the same complex pattern of start sites as observed for TS mRNA derived from cultured mouse 3T6 cells (Fig. 2c). Insertion of the TATAA box eliminated start sites upstream of it and introduced new start sites near the TATAA box insertion site as well as 30 nt downstream (indicated by arrowheads in Fig. 2b) but otherwise had little effect on the complex pattern of start sites. In contrast, insertion of the initiator element condensed the complex pattern of start sites into a single strong site, although a few weak sites were still observed. The double mutant showed a combination of these effects. These results indicate that the initiator element plays a key role in start site determination in these modified TS promoters.

Construction of the Dual-Luc Vector

In order to study the effects of the TATA and initiator elements on the bidirectionality of the TS promoter we constructed the Dual-Luc vector, which contains the firefly and renilla luciferase genes in a head-to-head orientation (Fig. 3a). This vector allowed us to simultaneously analyze promoter activity in both directions by cloning the promoter into the *Bgl*II and/or *Nhe*I site between the two genes. Since, more than 10% of all human genes have been identified to contain bidirectional promoters [Trinklein et al., 2004], this construct may prove to be a valuable tool for the analysis of this class of promoters.

TATAA Box and Initiator Element do not Affect Bidirectionality

The mouse TS promoter is a bidirectional promoter exhibiting approximately equal strength

in both directions [Liao et al., 1994]. It is possible that the TS promoter might be bidirectional due to the absence of a TATAA box and/or initiator element, which are thought to be responsible for imparting promoter directionality. For this reason, we investigated the
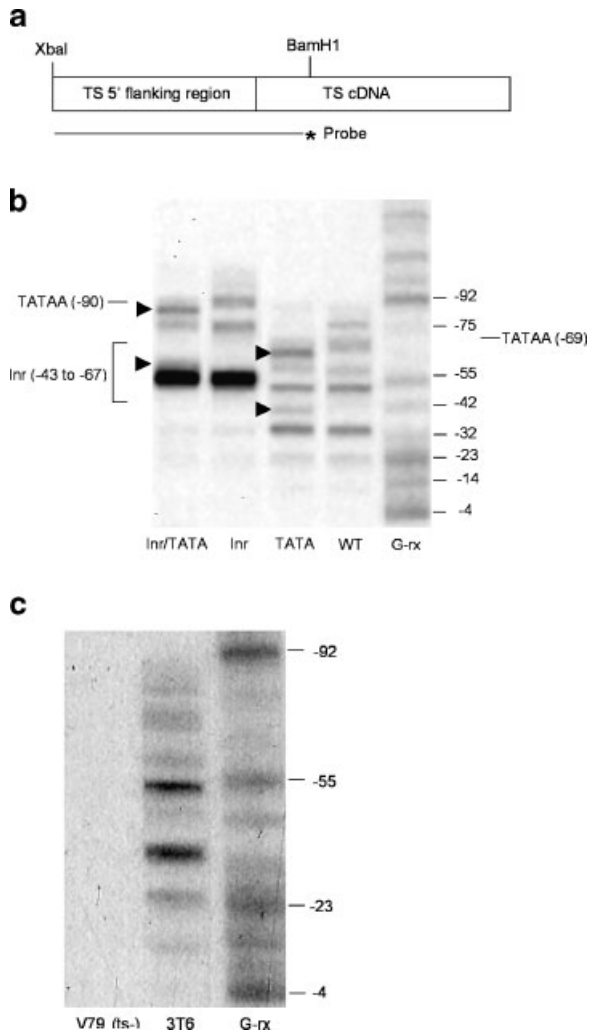

effects of inserting an initiator element and/or a TATAA box on TS promoter bidirectionality.

The wild-type or mutant TS promoters were inserted into the *Bgl*II and/or *Nhe*I sites of the Dual-Luc vector. Constructs containing the TS promoter oriented towards the firefly gene were denoted as "F" and constructs containing the promoter oriented towards the renilla gene were denoted as "R." These constructs were transiently transfected into V79 (ts−) cells and firefly and renilla luciferase activities were measured 2 days later. Our expectation was that the introduction of a TATAA box or initiator element or both might render directionality to the TS promoter. Surprisingly, as shown in Figure 3b, neither the TATAA box nor the initiator element nor both together had any effect on promoter bidirectionality.

To be sure that the Dual-Luc vector provided a valid indication of promoter directionality, the unidirectional CMV promoter-enhancer region was cloned into the Dual-Luc vector in place of the TS promoter. Figure 3c shows that these constructs displayed unidirectional promoter activity regardless of the orientation of the promoter in the Dual-Luc vector.

Initiator Element Decreases S Phase Stimulation of the *TS* Gene

Our model for the post-transcriptional regulation of *TS* gene expression predicts some form of communication between the TS promoter and the RNA processing machinery that results in an increase in the efficiency of TS RNA processing and nuclear export during the G_1–S transition [Ash et al., 1993; Johnson, 1994]. We reasoned that if transcription and processing are interdependent, altering the basic architecture of the TS promoter following the introduction of a TATAA box and/or initiator element might alter the recruitment of RNA processing factors to the TS promoter, thereby affecting S phase regulation of *TS* gene expression.

To test this hypothesis, $TI_{1,2d}T$ minigenes containing the wild-type TS promoter or the TS promoter containing TATAA box and/or initiator element were stably transfected into cultured mouse 3T6 cells. Stable cell lines were synchronized in G_0 by serum deprivation and then serum-stimulated to re-enter the cell cycle. Cytoplasmic RNA was isolated at subsequent times and the amount of mRNA derived from the endogenous *TS* gene and the transfected TS

Fig. 2. Effect of the initiator element and TATAA box on start site pattern. V79 (ts−) cells were transiently transfected with $TI_{5,6}T$ minigenes driven by the wild-type (WT) TS promoter or the TS promoter with the indicated mutations. Cytoplasmic RNA was analyzed by an S1 nuclease protection assay. The probe (**panel a**), which was derived from the minigene used for transfection, was 5′ ^{32}P end-labeled at the *Bam*HI site in exon 3 and extended to the engineered *Xba*I site at −233. The Maxam–Gilbert G-reaction (G-rx) was performed with the wild-type TS probe to indicate the positions of G-residues. **Panel b**: Effect of the TATA box and/or initiator element on transcription initiation patterns. The positions of the initiator and TATAA sequences are shown. Arrowheads indicate the new start sites that were created due to the insertion of the TATAA box. **Panel c**: Cytoplasmic RNA isolated from V79 (ts−) cells (negative control) and mouse 3T6 cells (positive control) was analyzed in the same manner as in panel b.

minigene were analyzed using quantitative real-time PCR. TS minigenes driven by the wild-type TS promoter were regulated in the same manner as the endogenous *TS* gene (Fig. 4b). In agreement with our hypothesis, minigenes containing the initiator element (alone or in combination with the TATAA box) showed a threefold decrease in S phase stimulation of TS mRNA derived from the TS minigene as compared to mRNA derived from the endogenous *TS* gene (Fig. 4c,d). Introduction of the TATAA box alone had little effect on S phase regulation of the TS minigene (data not shown).

DISCUSSION

In the present study, we have explored the effects of changing the architecture of the mouse TS promoter region on the directionality of the promoter as well as the ability of the promoter to direct S phase regulation of the gene. We have engineered a TATAA box and/or an initiator element downstream of the 30 nucleotide TS essential promoter in a region that was shown previously to be irrelevant for promoter activity [Geng and Johnson, 1993] or *TS* gene regulation [Ash et al., 1995]. Both of these inserted sequences appear to be functional as judged by their effects on the pattern of transcription start sites. This is especially true for the initiator element, which collapsed the broad pattern of start sites observed with the wild-type promoter into a single major start site from within the initiator sequence. The effects of the TATAA box were less significant but still discernable. The TATAA box may be located at a suboptimal distance from a critical element in the TS essential promoter region, leading to a relatively weak effect.

Even though the TATAA box and the initiator element influenced the start site patterns, they had no detectable effect on the bidirectionality of the TS promoter. This was surprising in view of earlier studies indicating that these elements had a major influence on the directionality of an artificial GC-rich promoter [O'Shea-Greenfield and Smale, 1992]. It is possible that the TATAA box and initiator element were placed at inappropriate positions relative to key elements within the essential promoter to influence promoter directionality. However, a more likely explanation is that the TS essential promoter region itself (rather than the absence of the
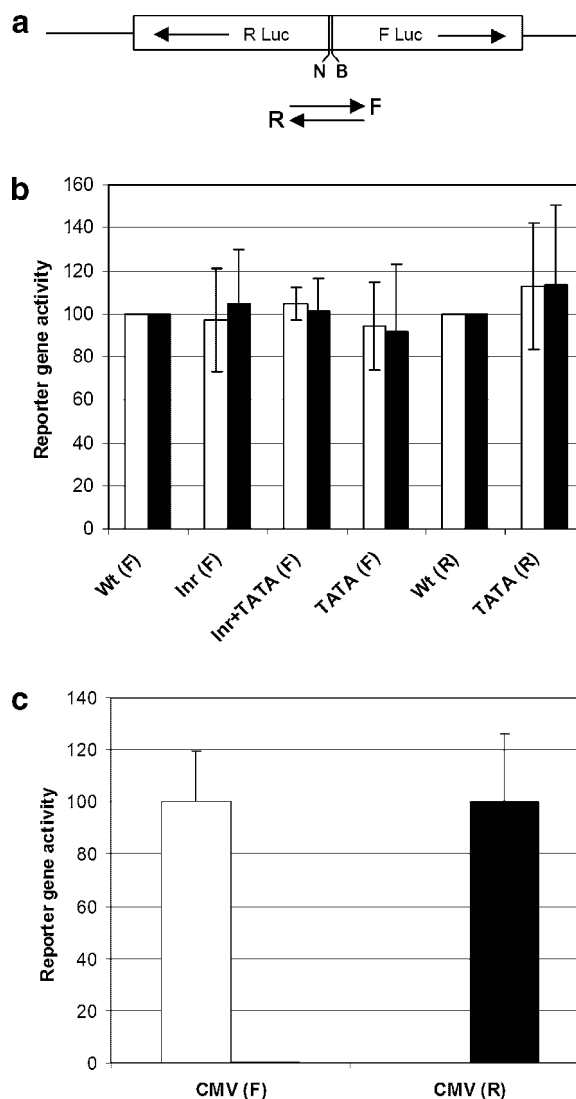

Fig. 3. Analysis of promoter directionality. **Panel a**: Configuration of the Dual-Luc indicator genes. The promoter-less firefly luciferase (F Luc) and renilla luciferase (R Luc) genes were cloned in a head-to-head configuration. Promoter regions were inserted at the *Bgl*II (B) and/or *Nhe*I (N) sites between the two reporter genes. **Panel b**: V79 (ts−) cells were transiently transfected with the Dual-Luc-TS vectors, which contained either the wild-type TS promoter region or TS promoter containing the TATAA box and/or initiator element, along with a control vector that expressed β-galactosidase. Promoter constructs facing the firefly gene are denoted as ''F.'' Promoter constructs facing the renilla gene are denoted as ''R.'' Cell lysates were harvested 24 h post-transfection and analyzed for firefly (white bars) and renilla (black bars) luciferase expression using the Dual-Luciferase assay kit. Data were normalized to β-galactosidase expression to correct for differences in transfection efficiency. **Panel c**: V79 (ts−) cells were transfected with Dual-Luc vectors that contained the CMV promoter-enhancer region oriented toward either the firefly luciferase gene or the renilla luciferase gene. Transfections and analyses were performed as in panel b.
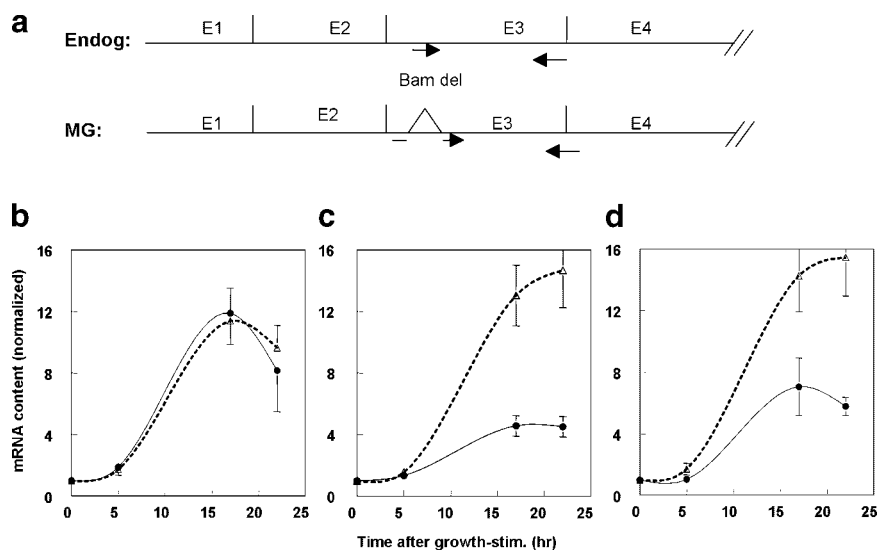
Fig. 4. Effect of initiator element on S phase regulation of TS mRNA. **Panel a**: Primers used for real time PCR. The upstream primer used for detecting mRNA derived from the endogenous *TS* gene (Endog.) corresponded to sequences within the *Bam*HI fragment that was deleted from the TS minigene. The upstream primer used for detecting mRNA derived from the TS minigene (MG) spans the *Bam*HI deletion (Bam del). The downstream primer for mRNA derived from the minigene spans exons 3 and 4 whereas the downstream primer for the endogenous mRNA is in exon 3. The exons are not drawn to scale. 3T6 cells were stably transfected with TI$_{1,2d}$T minigenes containing either the wild-type TS promoter (**panel b**) or TS promoter containing the initiator element (**panel c**) or the initiator element plus the TATAA box (**panel d**). Cells were synchronized in G$_0$ and then serum-stimulated to enter the cell cycle. Cytoplasmic RNA was harvested at the indicated time points and analyzed for mRNA derived from the TS minigene (solid line) and the endogenous *TS* gene (dashed line). Data were normalized to rpL4 mRNA levels to correct for recovery differences. At least two different stable cell lines were created with each minigene, and each cell line was analyzed at least three times. Representative results from a single cell line are shown. Standard deviations from the repeat analyses are indicated.

TATAA and initiator elements) is the key determinant of bidirectionality. This is consistent with the observation that an Sp1 element in the TS essential promoter region is important for promoter activity [Deng et al., 1989; Jolliff et al., 1991], and that Sp1 elements are known to promote bidirectional transcription [O'Shea-Greenfield and Smale, 1992].

Although the initiator element had no effect on TS promoter bidirectionality, it had a clear inhibitory effect on S phase regulation of TS mRNA levels (Fig. 4). Our model for *TS* gene regulation suggests that some form of communication between the promoter and the RNA processing machinery is necessary for proper post-transcriptional control of TS mRNA production during the G$_1$–S phase transition. We have speculated that the TS promoter may interact with factors that affect the efficiency of processing of TS mRNA in a cell cycle-dependent manner. These factors may be transferred to the RNA molecule via the C-terminal domain of RNA polymerase where they influence either the efficiency of processing or the export of the mature mRNA from the nucleus to the cyto-

plasm [Ash et al., 1993, 1995; Johnson, 1994; Ke et al., 1996].

Introducing an initiator element into the TS promoter region significantly alters the transcription initiation complex that is assembled on the promoter. Earlier studies have shown that several different proteins are able to interact specifically with the initiator element, including TFII-I, YY-1 as well as TFIID [Smale and Kadonaga, 2003]. Furthermore, TFIID is a dynamic protein complex consisting of TATAA-binding protein (TBP) in association with different TBP-associated factors (TAFs), depending on the promoter structure. For these reasons, the protein composition and/or three-dimensional structure of the initiation complex varies widely in different mammalian promoters [Smale and Kadonaga, 2003; Muller and Tora, 2004]. Since the TS promoter inherently lacks a TATAA box and an initiator element, introduction of these elements into the TS promoter is likely to alter the protein composition of TFIID or other factors that are recruited to the promoter, including factors that may be important for regulating TS mRNA production

or export. A detailed analysis of proteins associated with the wild-type and mutated TS promoters will be required to determine the nature of the TFIID complexes and other proteins associated with the TS promoter and the effects of these proteins on the regulation of TS mRNA production during the G_1−S phase transition. Furthermore, since many other S phase genes are driven by promoters that lack initiator elements, it will be interesting to determine if adding an initiator element to these promoters also leads to a reduction in S phase regulation of these genes.

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