4044–4049 Nucleic Acids Research, 1994, Vol. 22, No. 20

Bidirectional promoter of the mouse thymidylate synthase gene

Wen-Chieh Liao, John Ash and Lee F.Johnson*
Departments of Molecular Genetics and Biochemistry, The Ohio State University, Columbus, OH 43210, USA

Received August 11, 1994; Accepted August 29, 1994

ABSTRACT

The promoter of the mouse thymidylate synthase (TS) gene lacks both a TATAA box and an initiator element and directs transcriptional initiation at multiple sites over a 90 nucleotide initiation window. Earlier studies defined an essential region near the 5′ end of the initiation window that is required for promoter activity. The essential region contains possible binding sites for Sp1 and Ets transcription factors. In the present study we show that this essential region stimulates transcription with approximately equal strength in both directions. Transcription is initiated over a broad initiation window in the reverse direction. The same elements are important for the reverse promoter and for the normal TS promoter. Sequences upstream of the essential region partially suppress expression in the reverse direction. The TS 5′ flanking region, in either the normal or inverted orientation, directs S phase-specific expression of a TS minigene. This raises the possibility that an upstream gene and the TS gene may be coordinately induced at the G1/S phase boundary by a common set of control elements.

INTRODUCTION

Thymidylate synthase (TS) is the enzyme that catalyzes the *de novo* synthesis of thymidylic acid. Because TS is essential for DNA replication in proliferating cells, it is the target of a variety of chemotherapeutic drugs. TS enzyme and its mRNA are present at low levels in quiescent mammalian cells. When such cells are stimulated to proliferate, TS enzyme and mRNA increase more than 10-fold as cells progress from G1 phase through S phase (1–3).

The TS promoter shares many features with other housekeeping promoters in that it is GC-rich and lacks a TATAA box (4). The promoter directs transcriptional initiation over an unusually broad 90 nucleotide initiation window that partially overlaps the essential region. The diversity of start sites is due to the absence of an initiator element (5). The core promoter elements are contained within a 30 nucleotide 'essential region' that is located between 75 and 105 nucleotides upstream of the AUG start codon (5,6). The essential region contains a weak binding site for Sp1 and two GGAAG sequences that correspond to the core binding motif for members of the Ets family of transcription factors (7). Mutagenesis of any of these elements leads to diminished promoter activity (5,6,8).

Since the TS promoter appears to lack an element specifying transcriptional orientation, we explored the possibility that it might have bidirectional activity. In the present study, we show that the essential region of the TS promoter stimulates transcriptional initiation with equal strength over a broad initiation window in both directions. The TS promoter is also capable of directing S phase specific expression in either orientation.

MATERIALS AND METHODS

Construction of reporter plasmids

TS promoter fragments were amplified by the polymerase chain reaction (PCR) using primers that flank the regions of interest. A restriction site (usually an *Nhe*I site) was engineered at both ends of most of the promoter fragments to facilitate insertion of the fragments upstream of reporter genes. For making luciferase reporter constructs, the restricted fragments were cloned into the *Nhe*I site of the polycloning region of the GL2-basic plasmid (Promega). For making TS reporter constructs, the amplified promoter fragments were cloned into an *Nhe*I site that was created by site-directed mutagenesis between −10 and −5 (relative to the AUG codon) of a TS minigene (5). The TS minigene contains the entire TS coding region, as well as introns 5 and 6 at their normal positions to ensure a high level of expression (9). The TS 5′ flanking sequences upstream of the *Nhe*I site were removed during the construction. The orientation and sequence of each promoter was checked by DNA sequencing.

Cell culture and transient transfection assays

Mouse 3T6 fibroblasts (10), and TS-deficient Chinese hamster V79 cells (11) were maintained on plastic tissue culture dishes in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% NuSerum (Collaborative Research). The medium for the TS-deficient cells was also supplemented with 10 μM thymidine.

The reporter constructs were transiently transfected into the V79 cells using a calcium phosphate co-precipitation procedure

*To whom correspondence should be addressed

as described previously (6). Cells were harvested for RNA or enzyme analyses about 40 h after transfection. When analyzing the expression of luciferase reporter constructs, 10 μg of test plasmid was cotransfected with 1 μg of the control plasmid pCH110 (Pharmacia), which contains the *lacZ* gene driven by the SV40 early promoter. When analyzing TS reporter constructs for TS enzyme level, 40 μg of test plasmid was cotransfected with 1 μg of p153luc, a control plasmid which contains the luciferase coding region driven by the wild-type TS promoter. When analyzing minigenes for the production of TS mRNA, the test plasmid was cotransfected with 3 μg of the control plasmid $pTI_{56}G$, a TS minigene that is tagged with an internal *Bam*HI deletion (12) which allows discrimination between mRNA derived from the test and control minigenes. In all assays, the level of expression of the test minigene was normalized to that of the control minigene to correct for differences in transfection efficiency.

Growth-stimulation assays

Wild-type mouse 3T6 cells were stably transfected (by electroporation) with a TS minigene (that was tagged with a 57 nucleotide internal *Bam*HI deletion) and a neo gene as a selectable marker as described previously (12). The cells were allowed to rest in medium containing 0.5% calf serum for 6 days, then were stimulated to proliferate by replacing the medium with fresh medium containing 20% calf serum at time = 0. At various times following stimulation, total cytoplasmic RNA was prepared and analyzed for TS mRNA derived from both the endogenous TS gene and the TS minigene by S1 nuclease protection assays.

RNA analyses

Cytoplasmic RNA was isolated from cells by phenol–chloroform extraction (13). S1 nuclease protection assays were performed as described previously (6,14). When measuring mRNA content, the probe was derived from an intronless TS minigene, pTSMG2 (15), and was 5′ end-labeled at the *Bgl*II site in exon 5. When mapping the transcriptional start sites, the probe was derived from the same TS reporter plasmid that was transfected into the cells and was end-labeled at the *Bam*HI site in exon 3. The fragments that were resistant to S1 nuclease digestion were analyzed by electrophoresis on a denaturing, 6% polyacrylamide sequencing gel. The dried gel was analyzed by autoradiography, and radioactivity was quantitated by using a Betascope (Betagen).

Enzyme assays

TS enzyme level was determined by measuring the formation of the ternary covalent complex between TS, [^{3}H]fluorodeoxyuridylate, and N5, N10-methylene tetrahydrofolate (16). Luciferase assays were performed by using the luciferase assay kit as described by the manufacturer (Promega). The β-galactosidase assay was performed by using the AMPGM chemiluminescent substrate as described by the manufacturer (Tropix). Luminescence was determined in a Berthold luminometer.

RESULTS

The core TS promoter is bidirectional

To determine if the TS promoter elements stimulate transcription in both directions, the TS 5′ flanking region from −11 to −153 (relative to the A of the AUG start codon) was cloned in both orientations upstream of TS or luciferase indicator genes. Earlier studies indicated that all of the essential TS promoter elements are contained within this region (5). The results (Table 1) clearly demonstrate that the TS promoter directed transcriptional initiation in both directions. When comparing TS mRNA levels, the TS promoter had somewhat greater activity in the reverse orientation than in its normal orientation. In contrast, when measuring luciferase or TS enzyme levels, the inverted promoter appeared to be less active than the normal promoter.

There are two likely explanations for the discrepancy. First, the reverse promoter has a very broad initiation window (see below), and some of the transcriptional initiation sites for the inverted pomoter are downstream of the AUG codon. This results in an improperly translated mRNA and a lower than expected enzyme activity. Since the distance between the *Nhe*I cloning site and the AUG codon is greater for the luciferase indicator gene than for the TS indicator gene, the discrepancy is expected to

Table 1. Activity of normal and inverted TS promoter

Promoter[a]	Expression levels[b] Luciferase Minigene	TS minigene Enzyme	mRNA
Normal	100	100	100
Inverted	44	24	166
None	<0.3	n.d.	n.d.

[a]The TS 5′ flanking region between −153 and −11 was amplified by PCR and cloned upstream of the luciferase or TS coding regions in the normal or inverted orientation.

[b]The minigenes were transfected into TS(−) V79 cells. The cells were assayed 2 days later for luciferase activity, TS enzyme activity or TS mRNA level, depending on the minigene that was used. The values represent the means of three independent experiments. The standard deviations were all less than 15% of the mean value. To facilitate comparisons, the values for the inverted promoter were normalized to those for the wild type promoter, which was arbitrarily set at 100. n.d., not determined.

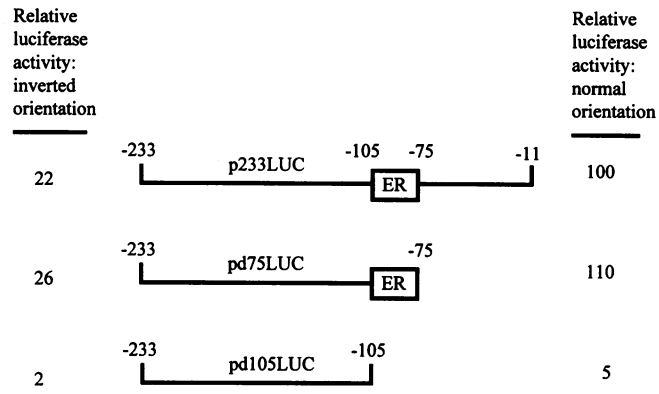

Figure 1. The essential region of the inverted promoter. The indicated regions of the TS promoter were amplified by PCR and cloned into the *Nhe*I site 48 nucleotides upstream of the luciferase coding region. The resulting minigenes were transiently transfected into TS(−) hamster V79 cells which were analyzed 2 days later for luciferase activity. Luciferase activities were all normalized to the value observed with p233LUC in the normal orientation, which was set at 100. The results represent the mean of at least 3 independent experiments. The standard deviations were less than 10% of the mean. The essential region (ER) of the TS promoter is indicated.

Table 2. Effect of mutations on promoter activity

Promoter[a]	Luciferase activity[b] Normal	Inverted
wild type (−118 to −11)	100	100
GC−80 (−118 to −11)	26	16
CIII (−118 to −11)	10	16
wild type (−233 to −11)	100	100
GC−130 (−233 to −11)	161	98

[a]The indicated TS 5′ flanking region that contained the wild type sequence or the indicated mutations was amplified by PCR and cloned upstream of the luciferase coding region in the normal or inverted orientation. The GC−80 mutation changes the Sp1 binding site at −80 from CCCGCCT to CCCGGCT. The CIII mutation changes the Ets binding site at −100 from ACTTCCGG to AGCTAGCG. The GC−130 mutation changes the Sp1 binding site at −130 from ACCCCGCCCC to ACCGCGGCCC.

[b]The minigenes were transfected into V79 cells, which were assayed 2 days later for luciferase activity. The values represent the means of three independent experiments. The standard deviations were all less than 20%. The values for the mutated promoters were normalized to those for the wild type promoter in each orientation, which was arbitrarily set at 100.

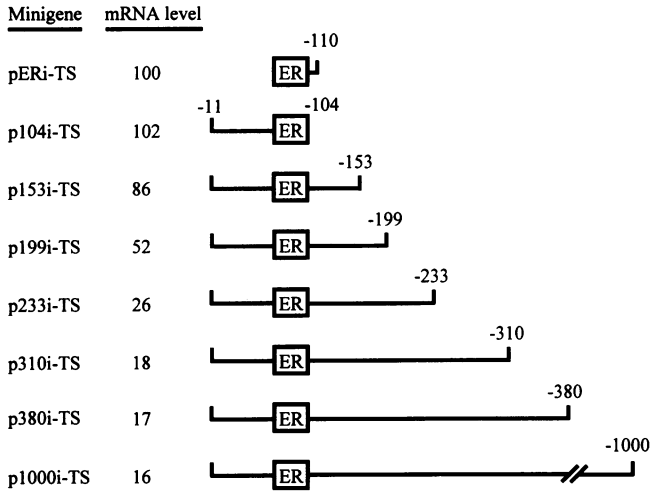

Figure 2. Inhibitory effect of upstream sequences. The indicated regions of the TS promoter were amplified by PCR as in Figure 1 and cloned in inverted orientation into an *NheI* site that was engineered 11 nucleotides upstream of the TS coding region. The resulting minigenes were transiently transfected into V79 cells. The cells were analyzed 2 days later for TS RNA content using an S1 nuclease protection assay. The results represent the mean of at least 3 independent experiments. TS mRNA levels were normalized to the value observed with pERi-TS, which was set at 100. The standard deviations were less than 20% of the mean. The essential region (ER) is indicated.

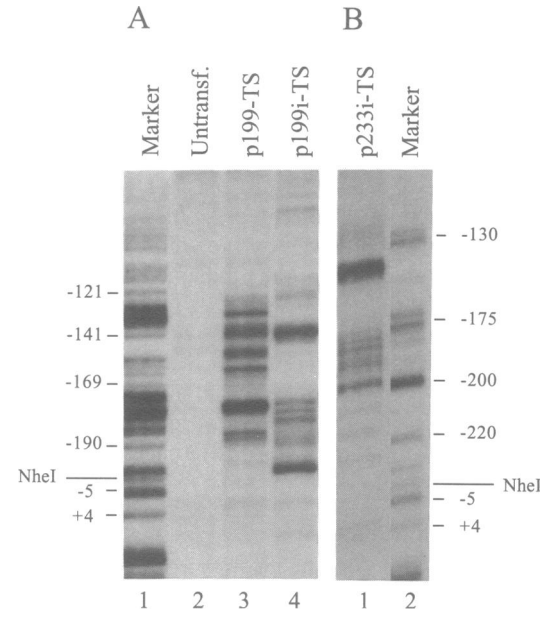

Figure 3. Initiation sites of the normal and inverted TS promoter. The TS promoter region between −11 and −199 (Panel **A**) or between −11 and −233 (Panel **B**) were linked to the TS coding region in the normal orientation to form p199-TS, or in the inverted orientation to form p199i-TS or p233i-TS, respectively. The minigenes were transiently transfected into TS(−) V79 cells. Total cytoplasmic RNA was isolated and analyzed by an S1 nuclease protection assay using 5′ end-labeled probes derived from the same minigene that was transfected into the cells. Panel A: Lane 1, Maxam−Gilbert sequencing ladder (the G reaction) of the inverted probe. Lane 2, RNA from untransfected cells analyzed with the inverted probe. Lane 3 and 4, RNA from cells transfected with the indicated minigenes. Panel B: Lane 1, RNA from cells transfected with p233i-TS. Lane 2, Maxam−Gilbert sequencing ladder (the G reaction) of the probe used in Lane 1. The nucleotide positions and the locations of the *NheI* site where the promoter fragments were inserted are indicated.

be less when using the luciferase indicator gene, as was observed. Second, the reverse promoter introduces a different 5′ untranslated region, which may lower the efficiency of translation.

To determine if the normal and reverse promoters have the same essential promoter region, the deleted promoters shown in Figure 1 were constructed and linked to the luciferase indicator gene in both orientations. The results (Fig. 1) show that the sequences between −75 and −105 were important for activity of the inverted as well as the normal promoter. The sequences between −75 and −11 were irrelevant for promoter activity in either orientation. To determine if the essential region was sufficient for bidirectional promoter activity, the TS promoter region between −118 and −75 was cloned in both orientations upstream of the luciferase reporter gene. The inverted core promoter was found to be approximately 1.8 times as active as the normal promoter in these analyses (data not shown).

To determine if the same elements within the core region are important in both directions, promoters with specific mutations were also analyzed. Table 2 shows that inactivation of the CIII (Ets) element at −100 led to a 6−10 fold reduction in promoter activity in both directions, whereas inactivation of the GC element at −80 led to a 4−6 fold reduction in both directions. Inactivation of the consensus Sp1 binding site at −130, which is outside of the core region, had little effect on promoter activity in either direction.

When comparing the data in Table I and Figure 1, we noted that the inclusion of additional 5′ flanking sequences led to a reduction in activity of the reverse (but not the normal) TS promoter. To investigate in more detail the inhibitory effects of sequences upstream of −105 on reverse promoter activity, a series of promoters with 5′ ends between −104 and −1 kb were linked in inverted orientation to the TS indicator gene. Promoter activity was determined by measuring TS mRNA levels. Figure 2 shows that the sequences between −104 and −153 (as well

```
          [ - - normal initiation window - -
AACGGAGCAGTCTGGCAGCAGTGTAGTCAGCGACAAAACCAGGACCTTCC   -50


- - - - - - - - - (Sp1 )(Ets) - ]     (Et
TGGCGCTCTTTTCCTCCAACACCAGCCCGCCTCTTCCTGCTGGGAAACTT   -100
                        [** Essential Region *****

s)                  [ - - - - - - - #- - -
CCGGCCGCCAGAATCCGTGGCAGACCCCGCCCCCACGTGACAGAAGTGGT   -150
****]                       (Sp1 ) (USF )

- - inverted initiation window - - - - - -
TACAGCTTGCAAGGCAAACACCTGCCCACTTTCTCAAATCTGTATTTACC   -200


- - - - - - - - - - ] (donor ?)
CTATTATGGCATTAAGAGCCTATAGGATCAGGGTGTGGGGATGCATGCCA   -250
                                        m h a i
    m a l r a y r i r v w g c m p

TTAAATCCCAGCTCCTGGGAGATTGAGTCGGCTAGAGGATCTGGAGTTTA   -300
  k s q l l g d .
l n p s s w e i e s a r g s g v .

                                    (donor ?)
AGGCAAGCCTTCACTTCATAGCAAGTTCAAGGCCAGCCTAGGTACTTGAG   -350

                                  (donor ?)
ACCCAGTCTCAAAAAGAGCCTTTGGCTTATACGATACTTGTACTTGGATC   -400

  (donor ?)       (donor ?)                 (do-
TTTATGTAGTTTGAGAACCACAGTAATTTTGGCTTCATAGAATGAATTGG   -450
   m .                                    m n w

nor ?)
GTAGAGGACCTTCTG                                      -465
v e d l l
```

Figure 4. The inverted TS promoter sequence. The reverse complement of the nucleotide sequence of the TS 5′ flanking region from −1 to −465 is shown. The sequence in the normal orientation has been published previously (4). The A of the AUG start codon (in the normal orientation) is designated +1. The approximate boundaries of the transcriptional initiation windows for the normal and inverted promoters are shown. The # symbol indicates the approximate location of the strongest start site for the inverted promoter. Binding sites for Sp1, Ets and USF *trans*-acting factors as well as potential splice donor sites (donor) are shown. AUG codons downstream of the inverted initiation window are underlined, and the amino acid sequences specified by the corresponding open reading frames are indicated.

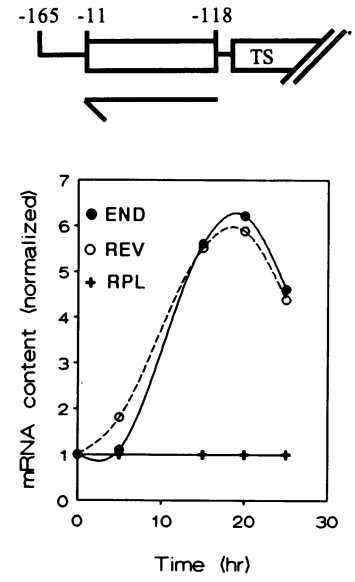

Figure 5. The inverted 5′ flanking region directs normal regulation. The structure of the minigene is shown at the top of the figure. The TS flanking region between −118 and −11 was amplified by PCR and cloned in the inverted orientation into a TS minigene at the *Xba*I and *Nhe*I sites engineered at −118 and −11, respectively. The TS coding region (TS) included intact TS intron 1 and an internally deleted intron 2 at their normal locations in the coding region. The minigene was tagged with a 57 nucleotide *Bam*HI deletion in exon 3. The minigene was stably transfected into mouse 3T6 fibroblasts. The transfected cells were allowed to rest in medium containing 0.5% serum, then serum stimulated at time = 0. At the indicated times, cultures were harvested and total cytoplasmic RNA was prepared. TS RNA derived from the endogenous TS gene (END, filled circles) or the TS minigene with the inverted TS promoter region (REV, open circles) was detected by using an S1 nuclease protection assay. A constitutively expressed mRNA, ribosomal protein L-32 mRNA (RPL, +), was also analyzed to correct for differences in RNA recovery. The mRNA levels were quantitated in a Betascope and normalized to the initial value, which was set at 1.0.

as between −75 and −11) had little effect on the reverse promoter activity. However, when the sequences between −153 and −310 were included, expression decreased about 5-fold. No further decrease in mRNA level was observed when sequences between −310 and −1 kb were included.

Preliminary RNase protection analyses have shown that an RNA species that corresponds to the reverse promoter is present at approximately 20% of the concentration of TS mRNA. The abundance of the upstream transcript is in agreement with the strength of the extended upstream promoter. The upstream transcript is cytoplasmic and polyadenylated, suggesting that it corresponds to a mRNA species (T.Xiang and L.F.Johnson, unpublished observations). Current efforts are directed toward a detailed characterization of the upstream transcript and gene.

Complex initiation pattern of the reverse promoter

The pattern of transcriptional initiation sites for the reverse TS promoter was determined by S1 protection assays. The reverse promoter, extending from −11 to −199, was linked to the TS indicator gene in these analyses. Figure 3A shows that the initiation pattern for the reverse promoter was of similar complexity to that of the normal TS promoter. The initiation pattern started at about −120 and extended into the indicator gene. Most of the start sites were between −140 and −199, and the strongest initiation site was near −142. To determine if the pattern would change with the inclusion of additional promoter sequences, a similar analysis was performed with an inverted TS promoter that extended from −11 to −233. Figure 3B shows that the pattern was very similar to that observed with the promoter that extended to −199; no additional strong initiation sites were observed.

Sequence of inverted TS promoter

The sequence of the inverted TS 5′ flanking region between −1 and −465 (relative to the A of the AUG start codon) is shown in Figure 4. The sequence between −390 and −465 corresponds to a portion of an inverted L1 repetitive element. The locations of the initiation window and the essential promoter region are indicated. The sequence downstream of the initiation window was examined for potential splicing and translation signals. Several possible splice donor sites were identified. Those at −341 and −405 have a 6 out of 9 match to the consensus splice donor consensus site, (C/A)AGGT(A/G)AGT (17), whereas the others have a 5 out of 9 match. Five ATG codons (underlined) are also present in this region. The context of the first ATG codon at

−206 (ATTATGG) matches the consensus translational initiation sequence (18). The contexts of the ATG codons at −241, −245, −404 and −442 do not match the consensus sequence. A search of the protein and DNA sequence databases (GenBank release 81) for the peptide or nucleotide sequences corresponding to the reverse transcript or the open reading frames shown in Figure 4 was performed at the NCBI using the BLAST network service (19). No strong homologies were detected (except for the L1 element upstream of −390).

The inverted TS promoter directs normal regulation

The TS 5′ flanking region is essential for normal S phase-specific regulation of stably transfected TS minigenes (20). All of the sequences that are required for normal regulation are downstream of −118 (J.Ash and L.F.Johnson, in preparation). To determine if the inverted TS promoter is capable of directing normal, S-phase specific expression, a minigene was constructed that consisted of the inverted TS promoter (between −118 and −11) linked to the TS coding region and polyadenylation signal (Figure 5). The coding region was tagged with a 57 nt *Bam*HI deletion to permit the simultaneous detection of mRNA derived from the endogenous TS gene and the transfected TS minigene. TS intron 1 (intact) and 2 (internally deleted) were also included at their normal locations in the coding region since these sequences are also important for normal regulation (12). The minigene was stably transfected into wild-type mouse 3T6 cells, and the expression of the minigene as well as the endogenous TS gene were measured in growth-stimulated cells as described previously (12,20). Figure 5 shows that mRNA derived from the minigene with the inverted TS promoter increased at the same time and to the same extent as wild-type TS mRNA, indicating that the inverted TS 5′ flanking region supported normal S-phase regulation.

DISCUSSION

Most mammalian genes are separated by thousands of nucleotides and are transcribed by unidirectional promoters. The directionality of such promoters is conferred by a TATAA box or an initiator element that specifies the site of transcriptional initiation as well as the direction of RNA synthesis. However, there are several examples of closely spaced, divergent mammalian genes that are transcribed by a common, bidirectional promoter. Many of the bidirectional promoters that have been studied so far are associated with genes for housekeeping proteins that are expressed in most cells. Examples include promoters of the genes for DHFR (21), TK (22), Surf (23), GPAT (24), histones H2A/H2B (25) and PCNA (26). Bidirectional promoters have also been found in several genes that are expressed at high levels in specific tissues, such as the promoters for certain collagen (27) and immunoglobulin (28) genes. Although some bidirectional promoters have TATAA boxes in both orientations (28), most lack TATAA boxes as well as initiator elements in either direction and stimulate transcriptional initiation at multiple sites over broad initiation windows.

Some bidirectional promoters are associated with divergent genes for proteins that have related functions. Examples include the divergent histone H2A and H2B genes (25), and the alpha 1(IV) and alpha 2(IV) collagen genes (27) as well as the divergent genes encoding two purine nucleotide synthetic enzymes (24). The divergent promoters may serve to coordinately regulate the expression of both genes in response to a common stimulus. However, other divergent genes encode proteins that bear no obvious relationship to each other or that may be expressed independently. For example, the protein encoded by the gene upstream of the DHFR gene bears homology to a bacterial mismatch repair enzyme (21). The TK promoter in its normal orientation is expressed at higher levels in proliferating cells than in quiescent cells, whereas the reverse TK promoter is not regulated in a growth-dependent manner (22). Finally, some promoters that exhibit bidirectionality when linked to indicator genes appear to lose this ability in their normal chromosomal context (29). This may be due to the presence of inhibitory sequences distal to the bidirectional core promoter region that block expression in the opposite direction. These inhibitory sequences may serve to prevent the synthesis of RNA species that do not correspond to functional molecules.

In the present study, we show that the TATAA-less mouse TS core promoter, which shares many of the properties of other bidirectional promoters, directs transcriptional initiation with approximately equal strength and over a large window in both directions. The same promoter elements are important for transcriptional activity in both directions. These include an Ets element and a weak Sp1 binding site, both of which are known to stimulate transcription independent of their orientation. The reverse TS promoter appears to direct the synthesis of a polyadenylated, cytoplasmic RNA species that is somewhat less abundant than TS mRNA.

Inclusion of additional sequences beyond the core elements led to a reduction in expression of genes linked to the reverse (but not the normal) TS promoter. The reductions may be the result of decreased transcription due to the presence of negative elements or terminators. Alternatively, the additional sequences may result in destabilization or inefficient translation of mRNA. Further analyses are required to distinguish between these possibilities.

Both the normal and the inverted TS promoters direct S phase-specific expression of a TS minigene. This demonstrates that the TS regulatory elements are not orientation dependent and raises the possibility that the upstream gene and the TS gene are coordinately regulated, in an S phase-specific manner, by a common set of control elements and factors. Current studies are focused on analyzing in more detail the mRNA and gene corresponding to the reverse promoter and determining if the upstream gene is regulated in the same manner and by the same regulatory mechanism as the TS gene.

ACKNOWLEDGEMENTS

These studies were supported by grants from the American Cancer Society (CB-71), the National Institute for General Medical Sciences (GM29356) and the National Cancer Institute (CA16058).

REFERENCES

1. Navalgund, L.G., Rossana, C., Muench, A.J. and Johnson, L.F. (1980) *J. Biol. Chem.*, **255**, 7386−7390.
2. Jenh, C.-H., Geyer, P.K. and Johnson, L.F. (1985) *Mol. Cell. Biol.*, **5**, 2527−2532.
3. Ayusawa, D., Shimizu, K., Koyama, H., Kaneda, S., Takeishi, K. and Seno, T. (1986) *J. Mol. Biol.*, **190**, 559−567.

4. Deng, T., Li, D., Jenh, C.-H. and Johnson, L.F. (1986) *J. Biol. Chem.*, **261**, 16000–16005.
5. Geng, Y. and Johnson, L.F. (1993) *Mol. Cell. Biol.*, **13**, 4894–4903.
6. Deng, T., Li, Y., Jolliff, K. and Johnson, L.F. (1989) *Mol. Cell. Biol.*, **9**, 4079–4082.
7. Wasylyk, B., Hahn, S.L. and Giovane, A. (1993) *Eur. J. Biochem.*, **211**, 7–18.
8. Jolliff, K., Li, Y. and Johnson, L.F. (1991) *Nucleic Acids Res.*, **19**, 2267–2274.
9. Deng, T., Li, Y. and Johnson, L.F. (1989) *Nucleic Acids Res.*, **17**, 645–658.
10. Todaro, G.J. and Green, H. (1963) *J. Cell Biol.*, **17**, 299–313.
11. Nussbaum, R.L., Walmsley, R.M., Lesko, J.G., Airhart, S.D. and Ledbetter, D.H. (1985) *Am. J. Hum. Genet.*, **37**, 1192–1205.
12. Ash, J., Ke, Y., Korb, M. and Johnson, L.F. (1993) *Mol. Cell. Biol.*, **13**, 1565–1571.
13. Johnson, L.F., Abelson, H.T., Green, H. and Penman, S. (1974) *Cell*, **1**, 95–100.
14. Favaloro, J., Treisman, R. and Kamen, R. (1980) *Meth. Enzymol.*, **65**, 718–749.
15. DeWille, J.W., Jenh, C.-H., Deng, T., Harendza, C.J. and Johnson, L.F. (1988) *J. Biol. Chem.*, **263**, 84–91.
16. Lockshin, A., Moran, R.G. and Danenberg, P.V. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 750–754.
17. Shapiro, M.B. and Senapathy, P. (1987) *Nucleic Acids Res.*, **15**, 7155–7174.
18. Kozak, M. (1986) *Cell*, **44**, 283–292.
19. Altschul, S.F., Gish, W., Miller, W. and Myers, E.W. (1990) *J. Mol. Biol.*, **215**, 403–410.
20. Li, Y., Li, D., Osborn, K. and Johnson, L.F. (1991) *Mol. Cell. Biol.*, **11**, 1023–1029.
21. Linton, J.P., Yen, J.-Y.J., Selby, E., Chen, Z., Chinsky, J.M., Liu, K., Kellems, R.E. and Crouse, G.F. (1989) *Mol. Cell. Biol.*, **9**, 3058–3072.
22. Weichselbraun, I., Ogris, E. and Wintersberger, E. (1990) *FEBS Lett.*, **275**, 49–52.
23. Lennard, A.C. and Fried, M. (1991) *Mol. Cell. Biol.*, **11**, 1281–1294.
24. Gavalas, A., Dixon, J.E., Brayton, K.A. and Zalkin, H. (1993) *Mol. Cell. Biol.*, **13**, 4784–4792.
25. Sturm, R.A., Dalton, S. and Wells, J.R.E. (1988) *Nucleic Acids Res.*, **16**, 8571–8586.
26. Rizzo, M.G., Ottavio, L., Travali, S., Chang, C.D., Kaminska, B. and Baserga, R. (1990) *Exp. Cell Res.*, **188**, 286–293.
27. Pollner, R., Fischer, G., Poschl, E. and Kuhn, K. (1990) *Ann. NY Acad. Sci.*, **580**, 44–54.
28. Doyen, N., Dreyfus, M. and Rougeon, F. (1989) *Nucleic Acids Res.*, **17**, 1977–1987.
29. Johnson, P. and Friedmann, T. (1990) *Gene*, **88**, 207–213.