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Thymidylate synthase gene expression is stimulated by some (but not all) introns

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ABSTRACT

We previously described the construction of an intronless mouse thymidylate synthase (TS) minigene that has the normal 5' and 3' flanking regions of the gene linked to full length TS cDNA. Transfection of the minigene into ts⁻ hamster V79 cells led to low level expression of normal mouse TS mRNA and protein. In the present study we analyzed the effect of introns on the expression of the TS minigene in transient transfection assays. Inclusion of introns 5 and 6 at their normal locations in the coding region led to an 8-9-fold stimulation of the level of TS and TS mRNA. Almost all of introns 5 and 6 could be deleted without diminishing the stimulatory effect. Inclusion of intron 3 also stimulated the expression of the minigene, although to a lesser extent than introns 5 and 6. However, inclusion of intron 4 had no stimulatory effect. Analysis of minigenes that contained various combinations of introns revealed that the stimulatory effects of the introns were not additive.

INTRODUCTION

Thymidylate synthase (TS) is a key enzyme in the de novo biosynthesis of thymidylic acid. The amount of enzyme is regulated over a wide range during the normal cell cycle and following growth stimulation in a variety of organisms (1-4). We have shown that in serum-stimulated mouse 3T6 cells, the increase in TS gene expression is the result of regulation at the transcriptional as well as the posttranscriptional levels (5).

We have cloned and analyzed full-length mouse TS cDNA as well as the gene for the enzyme. The cDNA is about 1 kb in length and contains a 921 nucleotide open reading frame. The predicted amino acid sequence of mouse TS shows a remarkable similarity to TS sequences from both procaryotes and eucaryotes (6). The mouse TS gene is 12 kb in length and contains 6 introns (7). Although the internal structure of the gene (including the splice junctions) does not appear unusual, there are unusual features at both ends of the gene. At the 3' end, we found that the translational termination codon (UAA) of the predominant TS mRNA is followed immediately by the poly(A) tail (8). At the

5' end, we found that transcription of the gene is initiated at several sites over a 60 nucleotide region. This is probably due to the fact that the promoter lacks a TATAA consensus region (7).

To facilitate our analysis of sequences that are important for regulating the expression of the TS gene, we constructed a minigene that consists of the normal 5' and 3' flanking regions of the gene linked to full length TS cDNA at common restriction sites in the first and last exons (9). The minigene is expressed when transiently or stably transfected into ts$^-$ hamster V79 cells. Analysis of the 5' and 3' ends of TS mRNA in the transfected cells revealed that they were indistinguishable from those of normal mouse TS mRNA, indicating that the hamster cells properly recognize the normal mouse transcription and polyadenylation signals. In addition, the TS minigene appears to be regulated following growth stimulation. Therefore the TS minigenes should be a useful model system for studying the sequences that are important for TS gene regulation.

The level of expression of the intronless mouse TS minigenes in the transiently or stably transfected cells was always about 5-times lower than that of the normal TS gene in the wild type hamster or mouse cells, even for minigenes having 4.5 kb of 5' flanking DNA. This raised the possibility that one or more of the introns contained sequences that were important for stimulating the expression of the TS gene. Earlier studies showed that elimination of all introns resulted in a drastic reduction in the expression of some genes (10-12).

In the present study, we have constructed TS minigenes that contain normal and internally deleted TS introns at their natural positions in the coding region. We show that inclusion of certain introns greatly stimulated the level of expression of the TS minigene, whereas inclusion of others had no stimulatory effect.

MATERIALS AND METHODS

Construction of TS minigenes.

The construction strategies are described in the figures. Briefly, plasmids or phage that contained appropriate regions of the mouse TS gene were subjected to partial or complete digestion with appropriate restriction enzymes. Specific restriction fragments were isolated by preparative agarose gel electrophoresis and substituted for the corresponding fragments in the intronless minigene. The structure of each minigene was confirmed by restriction analysis. The structure of pI4 was also confirmed by DNA sequence analysis in the regions of the intron-exon boundaries. Large amounts of the

minigenes were grown in E. coli HB101 and purified by two cycles of equilibrium centrifugation in CsCl gradients followed by extensive dialysis.

Cell culture and transfection.

The ts⁻ hamster V79 cell line (F88.13) (13) was a gift from Dr. Robert Nussbaum (University of Pennsylvania). Cells were cultured on plastic dishes (Falcon) in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% NuSerum (Collaborative Research) and 10^{-5} M thymidine. The ts⁻ cells were transiently transfected with TS minigenes using a calcium phosphate coprecipitation protocol as described previously (9). Briefly, 1 ml of solution containing the precipitated DNA (usually 12 pmoles of the TS minigene and 4.5 ug of a control plasmid, pSV2cat) was added to a rapidly growing culture of cells. In some experiments cells were transfected with the TS minigene alone. The medium was replaced 18 hr later and the cultures were harvested 48 hr after addition of DNA.

Analysis of minigene expression.

Cell extracts were assayed either for TS enzyme level or TS mRNA content. Enzyme level was determined using the [^{3}H]FdUMP binding assay (14) which measures the formation of the covalent ternary complex between TS, FdUMP, and 5,10-methylenetetrahydrofolic acid. Chloramphenicol acetyl- transferase (CAT) activity (% conversion of [^{14}C]chloramphenicol to the acetylated derivatives per mg protein) was determined as described by Gorman et al. (15) except that the cell extracts were heated to 60 degrees for 10 min prior to the assay to increase the sensitivity (16). The reaction time was 30 min and the percent conversion was never greater than 25% of input chloramphenicol. TS enzyme level was normalized either to total protein (17) or to CAT activity.

To measure TS mRNA content, cytoplasmic extracts were harvested and a small sample was assayed for CAT activity. Cytoplasmic poly(A)+ mRNA was purified from the remainder of the extract. The amount of TS mRNA as well as its 5' terminal structure were determined by an S1 nuclease protection assay as described previously (9). TS mRNA levels were quantitated by scanning the autoradiograms with a densitometer. The values were normalized to CAT activity.

DNA sequence determination.

Restriction fragments to be sequenced were cloned into M13 (mp18, mp19 or um20) and grown in E. coli NM522. Single stranded DNA was isolated and sequenced using the Sanger dideoxy sequencing protocol (18). Large fragments were sequenced using the deletion subcloning strategy of Dale et al. (19).

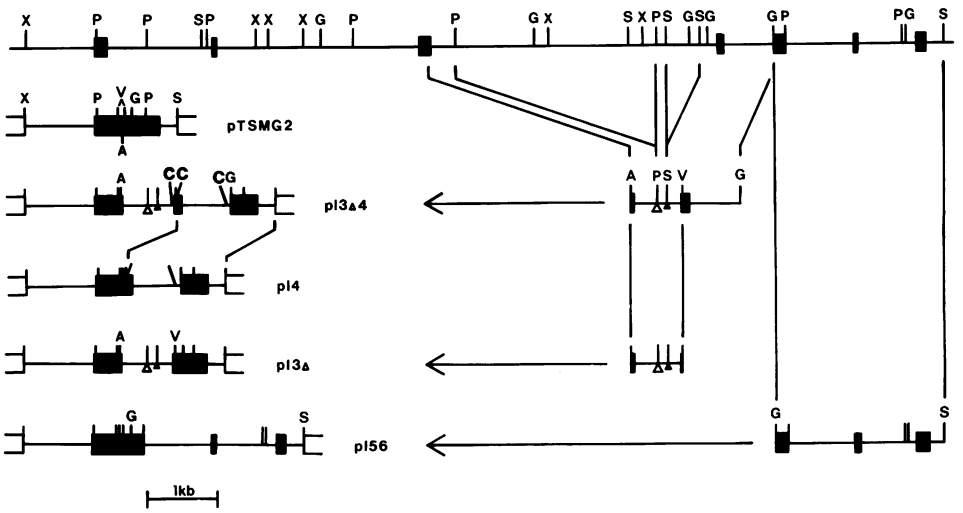


Figure 1. Construction of TS minigenes. Construction of the intronless TS minigene, pTSMG2 was described previously (9). Intron-containing TS minigenes were assembled by substituting regions of the mouse TS gene (top line) for regions of the intronless minigene as summarized in the diagram. All of the minigenes were cloned into the polycloning site of plasmid pUC18 (open box). Thin lines indicate intron or flanking sequences; thick lines indicate exons. Triangles indicate the locations of the deletions in intron 3. The following restriction sites are shown: A, ApaI; G, BglII; P, PstI; S, SacI; AvaI; X, XbaI;

RESULTS

Expression of the TS minigenes is stimulated by introns 5 and 6.

To determine if the presence of introns would affect the expression of the TS minigene, we constructed several minigenes that included full length or partially deleted introns from the normal mouse TS gene at their normal locations. Each minigene contained the same 5' and 3' flanking regions from the mouse TS gene. Therefore the structure of the spliced cytoplasmic TS mRNA that is produced from each minigene should be identical to that found in normal mouse cells. We first assembled a minigene (pI56) that contained introns 5 and 6, the final two introns of the gene. These introns were selected since they were relatively small and since convenient restriction sites were present in exon 5 and in the 3' flanking region of the gene. The strategy for constructing the pI56 minigene is summarized in Figure 1.

The activity of the minigene was determined by inserting it into ts$^-$ hamster V79 cells using the calcium phosphate transient transfection procedure (9). To correct for possible variations in transfection efficiency the cells

Table 1. Stimulation of TS Minigene Expression by Introns 5 and 6

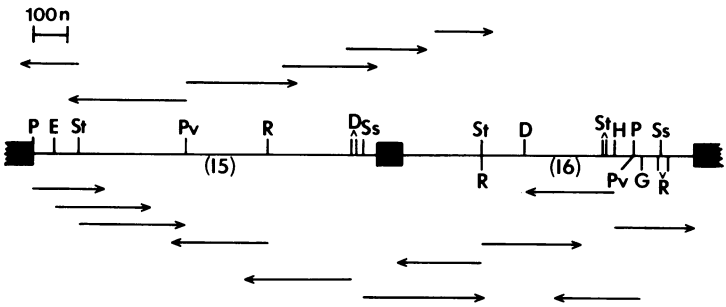
Expt.	Minigene	Enzyme level TS	CAT	TS/CAT
1	pTSMG2	1580 (1.0)	7.32 (1.0)	215 (1.0)
	pI56	12300 (7.8)	7.22 (0.99)	1704 (7.9)
2	pTSMG2	1870 (1.0)	8.24 (1.0)	227 (1.0)
	pI56	22800 (12.2)	10.1 (1.23)	2260 (9.9)
3	pTSMG2	1830 (1.0)	3.13 (1.0)	585 (1.0)
	pI56	20600 (11.3)	4.83 (1.54)	4270 (7.3)

Twelve picomoles of pTSMG2 (40 ug) or pI56 (56 ug) along with 4.5 ug of pSV2cat were cotransfected into ts^- hamster cells and harvested 2 days later. TS enzyme levels (cpm of [^{3}H]FdUMP bound/mg protein) and CAT enzyme levels (percent conversion of [^{14}C]chloramphenicol to the acetylated derivatives/mg protein) were determined. The values shown in parentheses have been normalized to the values obtained for pTSMG2. The results of 3 experiments are presented. Each value shown is the mean of 2 independent determinations in each experiment. Individual values usually differed by less than 20%.

were simultaneously transfected with a constant amount of control gene, pSV2cat (15). In control experiments we determined that cotransfection with pSV2cat did not affect the level of expression of the TS minigene (data not shown). Cultures were harvested 2 days later and TS enzyme level was determined by the sensitive [^{3}H]FdUMP-binding assay (14). TS level was then normalized to protein content or CAT activity. The TS enzyme level observed with the intron-containing construct was compared with that observed with pTSMG2, a minigene that lacks introns but is otherwise identical to pI56. Table 1 shows that the presence of introns 5 and 6 greatly stimulated the production of TS in the transfected cells. Although there was variation from experiment to experiment, the degree of stimulation was generally about 8-fold.

Analysis of the sequence of introns 5 and 6.

Our previous analysis of the structure of the mouse TS gene included the sequences of the 5' and 3' flanking regions as well as each intron-exon boundary but did not include the complete sequence of the introns (7). The sizes of introns 5 and 6 had to be roughly estimated in the earlier analysis due to the absence of any useful restriction sites within the small (72 nucleotides) exon 6. This fact also prevented us from constructing minigenes that contained only intron 5 or intron 6. To eliminate the uncertainty in the sizes of introns 5 and 6 and to permit a more detailed analysis of the stimu-

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                                                                  exon 5 >
                                                          ...GGCCTGCAG
                                                             EcoRI
gtgagtttctttcggaaggcatagttagagccaatgcaacacattgacctcatagcagggaattcagtct    70
                                                              StyI
gattagtattagaatttttatacatgtaaataagacatgaattggattaccttataatcctccttggagct   140

ttctggcctcttgttgcctatgaaaactaacattcacataggttgcgtgtgtgtgtgtgtgtgtgtgtct   210

gtagggggggagagtttttgttttttgttttgagacatggtctcaaagtctacattagctctaacttatt   280

gtcacccagtccaagaatgaagacctaagatttctaagtgcttgtgaaaaaacaagagtcttgtgacttt   350

agtttttttcaaaaagagaattgttcttggaatgtgtgttcatgctcctctcatgtgtagcagataggaga   420

gagtttacttcagctgttatttatataccttttatggaaaaacatgtttttgccatatgcagcttgacttt   490

gattatatactttactcacgtgactcttcttaacgctcagagaatagagtgtctgatgctctggatagag   560

ctggctattgcatgagactttagtccacctcatttttaggccctccctgctcttctaggttcacaatgcc   630

aacgagagcagtaaatatgtgtgtgagccatcgtgtacaacttaaaaaaacttttgtaaattagctcttc   700

tcaaaagccacactgataaaattcctgctttgggacgatttgaagtatttgcatgttatatagtgtgtta   770

ttgtgtatgtgggggcacacatattccattgtatgtgtgtagagatcagaggacagtggctccccaggttg   840
                                                                  DraI
tcagtcttggtgacaaatggctttactcactgagccatctcacctgcccttaatctgttttaaaatgatg   910
    DraI                                                          <----
tgctttaaaaagaattgaaacgaaaatattaactgttgtgtttttttcctgtgagacatttttagCCAGG   980
----------- exon 6 ----------------------------------------------->
TGATTTTGTCCACACTTTGGGAGATGCACATATTTACCTGAATCATATAGAGCCGCTGAAAATTCAGgta  1050

agaaatatacctgttaggtctctttttgagcttggtgccttctcttgacaacagattggatggcagggca  1120

tcctttgtctcacagtcactctggttgatgctgtcttatatggaagtgctccaactgtagggcttttaat  1190

aacggggtgagcagggatgagaccataaccagccttagcaaccataagcactgaaaatcgtaaggtgtta  1260
          StyI
cataccagtaccaaggagaaaagcccagagatatgtcattttaactcataatttttacttgaagaatcaaa  1330

gtataaatgtttgactttagtttgatgcttaagtccagtaagcagttaggtctgagctttaaaaaggctg  1400

tctgttttaatccctataacacagtagccatcaaatgaatggagatggcagtaagagtgagtgtctacgt  1470

ttcgtggcccttaagggaaaaaactagactggcatgagtaaacaactgtgtccctaagtttagtatattt  1540

atcaggagttatctggtgcttcagccgatgatcacagctagttaagagactcttggagtaggagcattcc  1610
StyI     StyI
aaggcttgcctaggctacagactgggctcaacataagcttagataactgaccaacaccctgtctcacaat  1680
               PstI                    BglII
aagaagctgggctgcagctgaatcgtagattacttcgtagatctcgcaaggcccaaggtgcagtccctaa  1750

tgttgcagaaagaaaagaagtacaatattgcactcctgtaaaatagtaccctgtggtaaaatactgtatt  1820
                                                          < exon 7
ggtttcatgaggagacacaggaatgaatgtaaaataatgccatgattttgttttttagCTACAGCGA...  1887
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latory sequences within these introns, the complete sequence of this region was determined using the dideoxynucleotide method of Sanger (18). The sequencing strategy and the nucleotide sequence are shown in Figure 2. Intron 5 was found to be 975 nucleotides and intron 6 was found to be 831 nucleotides in length, rather than 0.6 and 1.2 kb, respectively, as estimated previously (7). The corrected structure of this region of the gene is shown in Figure 2 and that of the entire mouse TS gene is shown in Figure 1.

A preliminary analysis of the intron sequences revealed the presence of several interesting features. The sequence between 184 and 208 is a repeating sequence of purines and pyrimidines with the potential to form Z DNA, which is frequently associated with transcriptional enhancers (20). Although there were no perfect matches of the enhancer core element GTGG(A/T)(A/T)(A/T)G there were many examples of imperfect matches (one or two differences) with this and a variety of other enhancer elements (21). The introns also contain sequences frequently associated with promoters. For example, the octanucleotide sequence ATTTGCAT found between 747-754 is an important promoter element for a variety of genes (21). In addition, there were many TATAA and CCAAT elements in both orientations although no GGGCGG elements.

Deletion of most of introns 5 and 6 does not diminish the stimulatory effect.

If the stimulatory effect of introns 5 and 6 is due to the presence of a strong positive-acting element (such as a transcriptional enhancer sequence) within one or both of the introns, elimination of the element should abolish the stimulatory effect. The nucleotide sequence of introns 5 and 6 revealed the presence of several convenient restriction sites that could be used to construct intron deletions. Figure 3 shows the construction of three minigenes with deletions in introns 6 (pI56Δ1) or in introns 5 and 6 (pI56Δ2) or in intron 5 (pI56Δ3). These deletions were designed to eliminate large regions in the interior of the introns while maintaining at least 60 nucleotides of normal intron sequence next to each exon to permit proper splicing. The activities of these minigenes were determined following transfection into the ts$^-$ hamster cells. The results (summarized in Table 2) show that none of

Figure 2. Nucleotide sequence of introns 5 and 6. The sequencing strategy shows the regions that were sequenced and the direction of reading. Thin lines represent intron 5 (I5) or intron 6 (I6); thick lines represent exons. Restriction sites are abbreviated as in Figure 1 or as follows: D, DraI; E, EcoRI; H, HindIII; Pv, PvuII; R, RsaI; Ss, SspI; St, StyI. The numbering of the nucleotide sequence begins at the first nucleotide of intron 5. Exons are in capital letters, introns are in lower case letters. The locations of several restriction sites are indicated. Sequences in the vicinity of each exon-intron junction were reported previously (7).

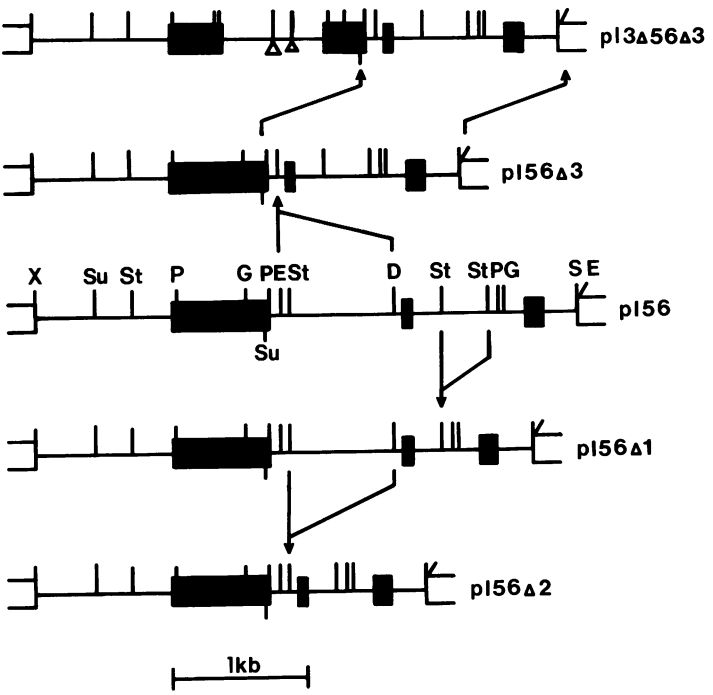

Figure 3. Construction of minigenes with deletions in introns 5 and 6. Minigenes were constructed by digesting (partially or completely) pI56 with the indicated restriction enzymes. DNA fragments of the desired size were isolated, blunt-ended by incubating with the Klenow fragment of DNA polymerase in the presence of all 4 deoxynucleoside triphosphates and ligated. Restriction sites are abbreviated as in Figures 1 and 2. pI3Δ56Δ3 was constructed by substituting the StuI (Su) to EcoRI fragment from the 3' end of pI56Δ3 for that found in pI3Δ.

these deletions resulted in a significant decrease in the stimulatory effect of the introns. Therefore, if the stimulation is due to the presence of an enhancer sequence, it must be located in close proximity to an intron-exon boundary. Since the intron 5 deletions eliminate the Z DNA stretch as well as the ATTTGCAT element, it appears that neither of these plays an important role in the stimulatory effect.

Effects of introns 3 and 4 on TS minigene expression.

It was also possible that a specific stimulatory sequence did not exist but rather that the stimulation was simply due to the presence of an intron within the minigene. To distinguish between these possibilities, we wanted to determine if other introns would also stimulate TS minigene expression. Minigenes that contained intron 3 (pI3Δ) or intron 4 (pI4) at their normal loca-

Table 2. Activity of TS Minigenes

Minigene	TS enzyme/CAT	TS mRNA/CAT
pTSMG2	1.0	1.0
pI56	8.1 ± 1.4 (10)	9.0 ± 0.6 (5)
pI56Δ1	9.6 ± 2.3 (3)	
pI56Δ2	6.5 ± 2.1 (3)	
pI56Δ3	10.6 ± 4.0 (3)	
pI3Δ	3.4 ± 1.9 (6)	
pI3Δ4	2.4 ± 0.3 (7)	5.1 ± 1.7 (3)
pI4	0.4 ± 0.1 (7)	1.0 ± 0.3 (3)
pI3Δ56Δ3	7.8 ± 1.6 (3)	

Transfections and enzyme assays were performed as described in Table 1. TS mRNA content was quantitated by densitometric analysis of autoradiograms from S1 nuclease assays. All values were normalized to those observed with the cultures transfected with pTSMG2. The mean and standard deviation of the results for each minigene are shown. The number of determinations of each value is shown in parentheses.

tions were constructed as summarized in Figure 1. Some of the interior of the large intron 3 was deleted to facilitate the construction of pI3Δ. Intron 4 was not altered in the construction of pI4. The activities of these minigenes were tested in transient transfection assays and the results are summarized in Table 2.

The level of TS enzyme was about 3-4 times higher with minigene pI3Δ than with the intronless minigene. The fact that a different intron also stimulated TS minigene expression raised the possibility that high level expression of the mouse TS minigene simply required the presence of an intron in the minigene, but that any intron might be able to fulfill this function. However, when we determined the activity of the minigene that contained intron 4, we were surprised to find that this intron did not stimulate TS minigene expression. In fact, the TS enzyme level was usually somewhat lower than that observed with the intronless TS minigene. To be certain that the structure of pI4 had not undergone any unanticipated alterations during construction, we determined the sequence of pI4 in the regions of the intron-exon boundaries (not shown). The sequence was identical to that of the gene (7). Thus it appears that the presence of an intron in the TS minigene does not necessarily lead to the stimulation of TS gene expression.

To test for possible synergistic or additive effects of intron sequences, minigenes that contained combinations of introns were constructed. When partially deleted intron 3 as well as intact intron 4 were both included in

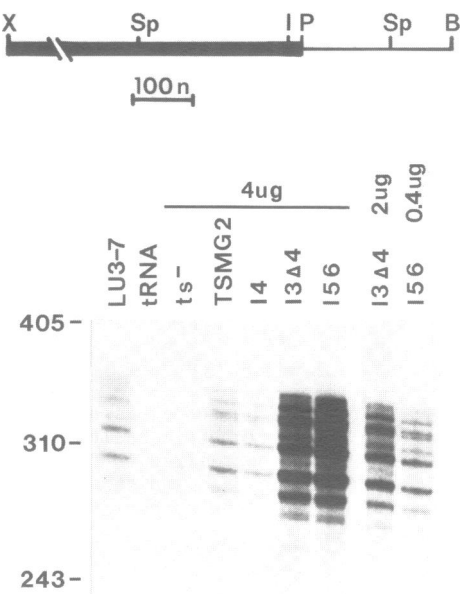

Figure 4. S1 protection analysis of TS mRNA isolated from transfected cells. Transfections were conducted as in Figure 1. Cultures were harvested 2 days later and the amount of TS mRNA as well as the structures of the 5' termini of the mRNA were determined by S1 nuclease protection analysis. The probe (top of the figure) was derived from pTSMG2 and included TS cDNA (thin line) and the 5' end of the TS gene (thick line) linked at a common PstI (P) site in the first exon. It was labeled at the 5' end with $^{32}PO_4$ at a BamHI site (B) 266 nucleotides downstream of AUG initiation codon (I) and extended to the XbaI site (X) 1 kb upstream of the AUG codon. The locations of two SphI sites (Sp) are also shown. S1 analyses were performed with: lane 1, 1 ug of total cytoplasmic RNA from mouse LU3-7 fibroblasts, a TS overproducing cell line (5); lane 2, 20 ug of yeast tRNA. Analyses in lanes 3-9 were performed with the indicated amounts of cytoplasmic poly(A)+ mRNA isolated from untransfected ts$^-$ cells (lane 3) or cells that had been transfected with the minigenes as shown. Size markers (in nucleotides) are indicated. For more information on the structure of the 5' end of TS mRNA, see references 7 and 9.

the minigene (pI3Δ4, see Figure 1), the activity was about 2-3 times as high as when the cells were transfected with the intronless minigene (Table 2). This indicates that the stimulatory effect of intron 3 is not blocked by the presence of intron 4. The highest level of TS minigene expression was observed with pI56Δ3. To determine if the addition of intron 3 to pI56Δ3 would lead to a further increase in the level of minigene expression, a minigene (pI3Δ56Δ3) was constructed that contained all of these introns at their natural locations (see Figure 3). Table 2 shows that inclusion of intron 3 did not lead to any significant increase in minigene expression.

The presence of introns leads to an increased content of TS mRNA.

We next wanted to determine if the increased amount of TS enzyme was the result of a corresponding increase in content of TS mRNA. Minigenes pI56, pI3Δ4, pI4 and the intronless minigene pTSMG2 were transiently transfected into the ts⁻ cell line. Cytoplasmic poly(A)+ mRNA was isolated 2 days later and analyzed using a 5' S1 nuclease protection assay. This analysis would enable us to measure the content of TS mRNA and to determine if there were any changes in the structure of the 5' end of TS mRNA. The probe used for these studies was derived from pTSMG2. It was 5' end-labeled at a BamHI site in the coding region and extended about 1 kb upstream of the AUG codon.

The results of this analysis are shown in Figure 4. The pattern of 5' termini of TS mRNA isolated from cells transfected with the intron-containing and intronless minigenes was the same as that observed in mouse fibroblasts. Densitometric scans of the patterns shown in this Figure and in other such experiments are summarized in Table 2. There was good agreement between the increase in enzyme level and mRNA content for pI56. However, the content of TS mRNA was somewhat higher than expected for the other two minigenes. The reason for this discrepancy is not known. It may simply reflect the rather high degree of variability that is inherent in transfection experiments. This analysis again showed that intron 4 had no stimulatory effect on TS minigene expression.

DISCUSSION

These results clearly demonstrate that the addition of some of the introns from the mouse TS gene to an intronless TS minigene had significant effects on the level of expression of the minigene. Inclusion of partially deleted intron 3 or intact introns 5 and 6 led to a significant stimulation in TS enzyme and mRNA levels when compared to that observed with the intronless minigene. However, inclusion of intact intron 4 did not stimulate minigene expression. It will be interesting to determine if inclusion of introns 1 and 2 will also stimulate TS gene expression (in progress).

The 5' and 3' flanking regions of the TS minigenes were from the normal mouse TS gene and were the same for all of the minigenes. Therefore the structure of the mature mRNA produced in each transfected cell line should be the same as that found in normal mouse fibroblasts. S1 nuclease protection analysis confirmed that the structure of the 5' end was indistinguishable from that of TS mRNA isolated from LU3-7 cells. Therefore the stimulatory effects were not the result of differential promoter utilization or changes in the structure of the 5' untranslated region of TS mRNA. We have also examined the

structure of the 3' end of TS mRNA in cells transfected with pI56 and found that it was the same as that of normal TS mRNA (C. Harendza and L. Johnson, unpublished observation).

Previous studies have shown that the effects of introns on gene expression vary considerably with the different genes that have been examined. Several early studies showed that introns were essential for the production of stable cytoplasmic mRNA from certain viral or cellular genes (10-12). Recent studies have shown that at least one intron is essential for the production of ribosomal protein mRNA in mammalian cells (S. Chung and R. Perry, personal communication). However, this observation is certainly not universal. Several mammalian genes that are expressed at high levels lack introns. Examples include many histone (22), heat shock (23) and interferon genes (24). In addition, introns are not essential for the expression of a variety of recombinant genes (e.g. 25, 26). In recent analyses, Gross et al. (27) suggested that introns had no significant effect on the expression of the chicken thymidine kinase gene. Brinster et al. (28) found that introns had little effect on the expression of several chimeric genes in transfected cell lines. Curiously, when the expression of these genes was examined in transgenic mice, 10 to 100 times more mRNA was produced from minigenes that contained introns than from those that lacked introns.

The mechanisms by which introns stimulate gene expression have been identified for only a few genes. Because introns are not present in mature cytoplasmic mRNA, it is likely that their primary effect is on processes that occur in the cell nucleus. Introns have been shown to stimulate the transcription of several genes due to the presence of transcriptional enhancer sequences, usually within the first intron. For example, a tissue-specific enhancer has been identified in the first intron of the rearranged immunoglobulin heavy chain gene (29). The human alpha 1(I) collagen gene also contains a tissue-specific enhancer as well as negative- acting elements in the first intron (30-32). If the stimulatory effects observed in the present study are the result of enhancer sequences, it is unusual that they are located in introns that are more distal to the promoter.

Introns may affect gene expression at a variety of levels in addition to transcriptional initiation. For example, some introns contain sequences that appear to function as transcriptional terminators (33). Sequences present in certain introns may stimulate or retard the rate of degradation of an hnRNA molecule or the rate of splicing of the introns (34, 35). Hamer and Leder (10) suggested that if splicing and nuclear export were coupled, the presence

of an intron might greatly facilitate the production of cytoplasmic mRNA. Finally, it is possible that the presence of introns could have an effect on the polyadenylation reaction.

Elucidation of the mechanisms by which the introns bring about their stimulatory and inhibitory effects on TS gene expression will involve the identification of the critical sequences that are responsible for the effect as well as the trans-acting factors that may be involved. Our initial attempt to identify the stimulatory sequences in introns 5 and 6 involved the construction of TS minigenes that contained only small portions of these introns. Unfortunately, none of the deletions reduced the stimulatory effect of these introns on TS minigene expression. Further studies are required to determine the identity of the stimulatory sequences, their mechanism of action, and their role in the regulation of TS gene expression during the cell cycle.

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