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Pre-mRNA Processing Enhancer (PPE) Element Increases the Expression of an Intronless Thymidylate Synthase Gene But Does Not Affect Intron-Dependent S Phase Regulation

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Abstract The pre-mRNA processing enhancer (PPE) element is an RNA sequence element derived from the intronless HSV-TK gene. Insertion of the element into the highly intron-dependent human β-globin gene leads to efficient expression in the absence of splicing. We have analyzed the effect of the PPE element on the expression of mouse thymidylate synthase (TS) minigenes. We have previously shown that the expression of intronless TS minigenes is moderately (up to 20-fold) stimulated by the inclusion of introns. Furthermore, S phase-specific expression of TS minigenes in growth-stimulated cells depends on the presence of a spliceable intron as well as the TS promoter. The goal of our study was to determine if the PPE element would overcome the dependence on introns for efficient expression and for S phase-specific expression of transfected TS minigenes. We found that insertion of the PPE element into an intronless TS minigene partially overcame intron dependence. However, the increase in expression was much less than that observed for the intronless β-globin gene. We also found that intronless TS or HSV-TK genes that contained the PPE element and that were driven by the TS promoter were expressed at a constant level in serum-stimulated cells. However, when an intron was included in these genes, they were expressed in an S phase-specific manner. Thus the PPE element was not able to overcome the dependence on introns for S phase-specific expression of TS minigenes. J. Cell. Biochem. 69:104–116, 1998.

Key words: mRNA export; cell cycle; gene transfection; cultured mammalian cells; hnRNP L; nuclear transport

Thymidylate synthase (TS) is an essential enzyme that catalyzes the de novo biosynthesis of thymidylic acid. TS gene expression is tightly regulated in response to the proliferative state of the cell [Conrad and Ruddle, 1972; Maley and Maley, 1960; Navalgund et al., 1980]. TS enzyme and mRNA levels increase 10- to 20-fold in cultured mammalian cells as they progress from the G1 through S phases of the cell cycle following growth-stimulation [Navalgund et al., 1980; Jenh et al., 1985]. Nuclear run-on assays have shown that there is very little change in the rate of TS gene transcription during this interval [Jenh et al., 1985; Ayusawa et al., 1986; Ash et al., 1995]. Furthermore, cytoplasmic TS mRNA has a half-life of approximately 8 h in both quiescent and rapidly proliferating cells [Jenh et al., 1985]. Therefore, the production of TS mRNA is controlled primarily at the posttranscriptional level by a process that occurs in the nuclear compartment.

To analyze the mechanism for S phase regulation of the mouse TS gene, we have constructed a variety of TS minigenes and examined their regulation in stably transfected cells. TS minigenes that contain both the TS essential promoter region as well as a spliceable intron display S phase regulation. However, minigenes that lack an intron or that include the SV40 early promoter in place of the TS promoter are expressed at a constant level during the G1 to S phase transition [Li et al., 1991; Ash et al., 1993, 1995; Ke et al., 1996]. Our observations are consistent with the possibility that S

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phase regulation of the TS gene requires some form of communication between the TS promoter and the splicing machinery that leads to more efficient processing of nuclear TS pre-mRNA into cytoplasmic TS mRNA during S phase than during G1 phase of the cell cycle. The mechanism by which this might occur is not known.

It has long been recognized that the efficient expression of many mammalian, plant, and viral genes is highly dependent on the presence of introns within the gene [e.g., Buckman and Berg, 1988; Chung and Perry, 1989; Neuberger and Williams, 1988; Callis et al., 1987]. For example, the level of expression of a transfected human β-globin gene decreases approximately 100-fold if the introns are removed from the gene [Hamer and Leder, 1979; Liu and Mertz, 1995]. We have shown that the expression of an intronless mouse TS gene is moderately stimulated by the inclusion of one or more efficiently spliced TS introns. Expression increases between 3- and 20-fold, depending on which introns are included [Deng et al., 1989; Korb et al., 1993; Li et al., 1991]. In contrast, some genes are expressed at functional levels in the absence of introns. Examples of naturally intronless genes include HSV thymidine kinase (HSV-TK) [McKnight, 1980], interferon [Lengyel, 1982], heat shock protein [Hunt and Morimoto, 1985], and histone [Hentschel and Birnstiel, 1981] genes. Furthermore, elimination of the introns from some genes has little effect on their level of expression [Gross et al., 1987]. These observations raise the possibility that these intron-independent genes contain sequence elements that permit efficient production of cytoplasmic mRNA in the absence of splicing. Such sequences have recently been identified in both viral and cellular transcripts [Liu and Mertz, 1995; Huang and Carmichael, 1997].

Liu and Mertz [1995] have identified a 119-nt RNA element (the pre-mRNA processing enhancer or PPE element) between 361 and 479 nt downstream of the transcriptional start site of the HSV-TK gene that enables intron-independent production of cytoplasmic mRNA. When this element is included in the 5′ untranslated region of an intronless β-globin gene, the level of expression increases greater than 20-fold (although expression is still about 5-fold less than that of the intron-containing gene). Inclusion of the element within an intron-containing β-globin gene has no detectable effect. The PPE element contains a binding site for hnRNP L, an abundant 68-kD protein whose function was previously unknown. These observations raise the possibility that hnRNP L may facilitate the production of cytoplasmic mRNA from intronless precursors. The mechanism by which hnRNP L carries out this function has not been determined. The PPE element may prove to be generally useful for increasing the expression of cDNA versions of genes that normally require introns for efficient expression in cultured cells or in transgenic animals [Brinster et al., 1988; Choi et al., 1991; Palmiter et al., 1991].

The goals of the present study were to explore the effects of the PPE element on the expression and S phase-specific regulation of the mouse TS gene. Specifically, we wished to determine if the PPE element would stimulate the expression of an intronless version of the TS gene to the same extent as the human β-globin gene. We also wished to determine if the presence of the element would overcome the dependence on introns for S phase-specific expression of the TS gene.

MATERIALS AND METHODS

Cell Culture

Mouse 3T6 fibroblasts and COS (monkey kidney) cells were maintained on plastic culture dishes (Falcon, Lincoln Park, NJ) in Dulbecco's modification of Eagle's medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% calf serum (Colorado Serum). TS-deficient hamster V79 fibroblasts [Nussbaum et al., 1985] were maintained in the same medium that was supplemented with 10 μM thymidine. 3T6 cells were growth-arrested in medium containing 0.5% calf serum and fed 3 days later with the same medium. Seven days after seeding, the cells were stimulated to reenter the cell cycle by feeding them with fresh medium containing 10% calf serum as described previously [Ke et al., 1996].

Construction of TS Minigenes

$TI_{1d1.4}G$ and TTG were described previously [Ash et al., 1993; Ke et al., 1996]. TS minigenes containing the "PPE(orf)" element were constructed in the following manner. The nucleotide sequence between +361 and +480 (relative to the transcriptional start site) of the wild

type HSV-TK gene was amplified by PCR using primers that included a *Bsu*36I site. The 5′ primer was TACTTCCTGAGGATGACTTACTGGCAGGT and the 3′ primer was TATTTCCTCAGGGCGCTTGTCATTACCAC. The amplified DNA was digested with *Bsu*36I and cloned into the *Bsu*36I site in the first exon of the TS minigenes.

TS minigenes that contained the PPE element in the 5′ untranslated region were constructed in a similar manner except that the region between +363 and +478 of the HSV-TK gene was PCR amplified and the primers included an *Nhe*I site. The PPE element was inserted at an *Nhe*I site that was engineered between -5 and -10 relative to the ATG start codon, as described previously [Geng and Johnson, 1993]. For the "PPE(utr)" insert, the 5′ primer was TACTTGCTAGCTGACTTACTGGCAGGT and the 3′ primer was TATTTGCTAGCTTAGCTTGTCATTACCA. For the "PPE(utr-atg)" insert, in which all of the AUG codons in the PPE element were eliminated, the 5′ primer was the same as for the "PPE(utr)" insert, and the 3′ primer was TATTTGCTAGCGCTTGTGATTACCA. The sequences of the PPE elements in the TS minigenes were confirmed by DNA sequence analysis.

TS-TK was constructed by PCR amplifying a 1.5-kb DNA fragment of the HSV-TK gene that contains 41 bp of 5′ flanking region, the coding region, and 314 bp of 3′ flanking region. The 5′ primer was GGTGGCTAGCAACTCCCGCACCTCTT and the 3′ primer was GAATTCCACGAACCATAAACCAT. The amplified DNA was digested with *Nhe*I and *Eco*RI (which cut in the primer regions) and the resulting fragment was used to replace the TS coding and 3′ flanking region of a TS minigene that had the *Nhe*I site engineered between -5 and -10 relative to the ATG start codon and an *Eco*RI site in the polycloning sequence at the 3′ end of the gene. TS-TK:$I_{1d1.0}$ was constructed by isolating TS intron 1 (with a 1.0-kb internal deletion) from a minigene that was constructed previously [TGG:I1-d1.0(WT)] [Ke et al., 1996] by digesting the minigene with *Nco*I, isolating the appropriate fragment and blunting the ends by incubating with DNA polymerase (Klenow fragment). TS-TK was linearized by digestion with *Nhe*I and the ends were also blunted. The fragments were then ligated, and plasmids with a single copy of the intron inserted in the correct orientation were isolated. The structure was confirmed by DNA sequence analysis.

Transfection

3T6 cells were stably transfected with 80 µg of a TS minigene and 1 µg of a neomycin resistance gene using an electroporation procedure as described previously [Ash et al., 1993]. Cells that had integrated the transfected genes were selected in medium containing 400 µg/ml of G418 (GIBCO-BRL). Drug-resistant colonies were observed 10 to 12 days after transfection. At least 100 independent colonies were pooled and maintained as a mass culture. Transient transfections into COS cells were performed by the Lipofectamine (GIBCO-BRL) procedure following the manufacturer's instructions. Transient transfections into V79 cells were carried out by a calcium phosphate protocol as previously described [DeWille et al., 1988].

RNA Analysis

Total cytoplasmic RNA was isolated, purified by phenol-chloroform extraction, digested with RNase-free DNase (to remove any contaminating plasmid), and analyzed by S1 nuclease protection assays as described previously [Favaloro et al., 1980; Ke et al., 1996] and in the text.

RESULTS

Effect of PPE on Transiently Transfected TS Minigenes

Our initial goal was to determine the effect of the PPE element on the expression of intron-

Fig. 1. Construction and expression of PPE-containing TS minigenes. **A:** Structure of TS minigenes. The structures of the TS minigenes and the control gene ($SI_{56}S$) that were used in these studies are indicated. The drawings are not to scale. The PPE element (wild-type or altered) was inserted into exon 1 either in the 5' untranslated region or in the coding region, as described in the text. The probe that was used for the S1 nuclease protection assay and the sizes of the protected fragments are also shown. **B:** S1 protection analysis of RNA from transiently transfected cells. V79 cells were cotransfected with 12 pmol of the test TS minigene as well as 1.2 pmol of the control gene, $SI_{56}S$. The TS minigenes were tagged with a 57-nt deletion between two *Bam*HI (B) sites in exon 3, whereas the control gene retained the *Bam*HI fragment. This allowed us to distinguish between mRNA derived from the control and test minigenes. Forty micrograms of total cytoplasmic RNA were analyzed in each lane. The probe, which was derived from an intronless TS minigene that retained the internal *Bam*HI fragment, was 5' end-labeled at the *Bgl*II (G) site in exon 5 and extended into the plasmid. Radioactive fragments corresponding to mRNA from the control gene and test TS minigenes are shown. Size markers (in nucleotides) are shown on the right of the autoradiogram.

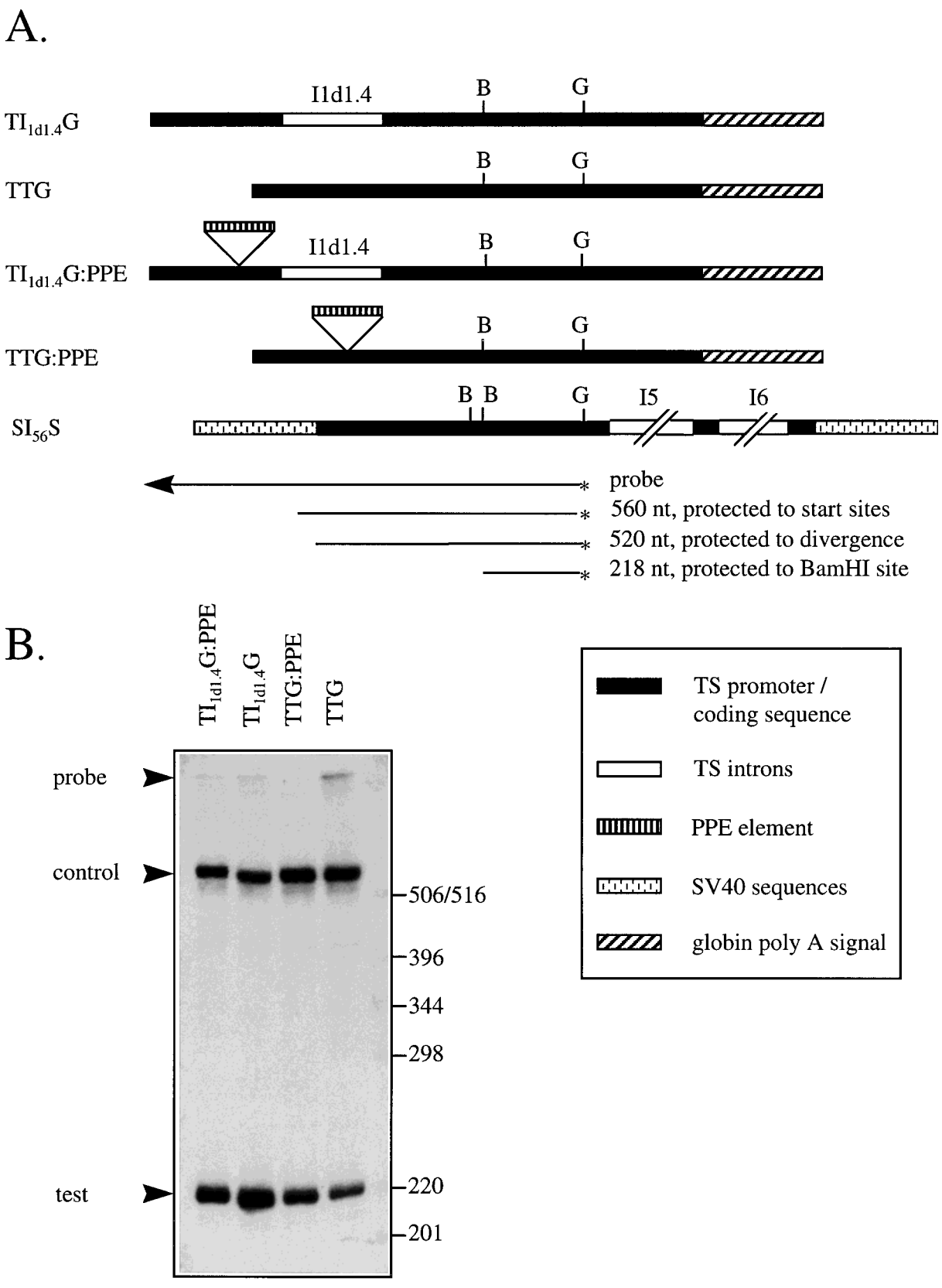
A.
I1d1.4
B
G
$TI_{1d1.4}G$
TTG
$TI_{1d1.4}G$:PPE
TTG:PPE
B B
I5
I6
$SI_{56}S$
probe
560 nt, protected to start sites
520 nt, protected to divergence
218 nt, protected to BamHI site
B.
$TI_{1d1.4}G$:PPE
$TI_{1d1.4}G$
TTG:PPE
TTG
probe
control
test
506/516
396
344
298
220
201
TS promoter / coding sequence
TS introns
PPE element
SV40 sequences
globin poly A signal

Figure 1.

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PPE element    +361
wt             GATGACTTACTGGCAGGTGCTGGGGGCTTCCGAGACAATCGCGAACATCTACACCACACA
utr              ..........................................................
utr-atg          ..........................................................
orf            ..........................................................

                                                                      +480
wt             ACACCGCCTCGACCAGGGTGAGATATCGGCCGGGGACGCGGCGGTGGTAATGACAAGCGC
utr            ...........................................................TAA
utr-atg        ...................................................C......
orf            ..........................................................
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Fig. 2. The nucleotide sequence of the PPE element. The sequence of the wild-type (wt) HSV-TK gene between +361 and +480 relative to the transcriptional start site is shown. The AUG codons are underlined. The regions indicated by dots or letters were incorporated into the TS minigenes. For the PPE element "utr," a translational stop codon (UAA) was engineered downstream of the element to prevent possible translational readthrough from the PPE element into the TS coding region (which is in a different reading frame). In the PPE element "utr-atg," all of the AUG codons in the PPE element were eliminated. In the PPE element "orf," several additional nucleotides were included to maintain the TS open reading frame. The sequences of the engineered restriction sites that were adjacent to the PPE element are not shown.

less and intron-containing TS minigenes. Expression was determined by measuring the steady state amount of mRNA derived from the minigenes that had been transiently transfected into cultured cells. The minigenes that were used in these analyses are summarized in Figure 1A. TTG is an intronless TS minigene that includes the wild-type TS promoter/5′ flanking region linked to the TS coding region. The efficient polyadenylation signal of the human β-globin gene was substituted for the relatively weak TS polyadenylation signal to increase the level of expression [Ash et al., 1993]. $TI_{1d1.4}G$ is the same as TTG except that TS intron 1 (with a 1.4-kb internal deletion) was included at its normal position in the coding region [Ke et al., 1996].

The sequence of the PPE element is shown in Figure 2. The element was initially inserted in the 5′ untranslated region at an *Nhe*I site that was engineered between 5 and 10 nt upstream of the AUG start codon. This site was selected since it is downstream of the 3' boundary of the transcriptional initiation window (approximately 14 nt upstream of the AUG codon) of the mouse TS promoter. Thus all of the transcripts should contain the PPE element. We showed previously that the creation of the *Nhe*I site at this position had no detectable effect on TS promoter activity. Furthermore, insertion of a variety of "random" DNA sequences at this position had little effect on promoter activity [Geng and Johnson, 1993]. The element was also cloned into the coding region of exon 1 at a *Bsu*36I site that is 81 nt downstream of the AUG codon. Insertion of the PPE element did not disrupt the TS open reading frame. The TS minigenes were tagged with a 57-nt deletion by eliminating the region between two adjacent *Bam*HI sites at the beginning of exon 3 [Perryman et al., 1986]. The control gene ($SI_{56}S$) that was used to correct for differences in transfection efficiency and RNA recovery consisted of the SV40 early promoter linked to the TS coding region (including TS introns 5 and 6) and the SV40 early polyadenylation signal. The 57-nt segment was not deleted from the control gene.

The levels of expression of the test and control minigenes were determined by transiently transfecting the minigenes into hamster V79 cells and quantitating the amounts of cytoplasmic mRNA derived from each minigene by S1 nuclease protection assays, as shown in Figure 1A and B. V79 cells were used in these assays since they transfect with high efficiency. The amount of mRNA corresponding to the test minigene was normalized to the amount of mRNA corresponding to the control minigene, and the results are presented in Table I.

Insertion of the internally deleted TS intron 1 into the intronless TS minigene led to a 3–4 fold increase in mRNA content (compare the value for TTG with that of TIG). This increase is in line with our previous observations [Ke, 1995]. To determine the effects of the PPE element on the expression of TS minigenes, we compared the amount of mRNA produced from TS minigenes that lacked or contained the PPE element. In our initial experiments, the PPE element was inserted into the 5′ untranslated region to form TTG:PPE(utr) and $TI_{1d1.4}G$:

TABLE I. Expression of PPE-Containing TS Minigenes*

Minigene	Expt.	Amount of mRNA					
		TIG	TTG	TIG/TTG	TIG:PPE	TTG:PPE	TIG:PPE/TTG:PPE
utr	1	1.00	0.32	3.1	0.40	0.38	1.05
	2	1.00	0.30	3.3	0.44	0.45	0.98
utr-atg	1	1.00	0.31	3.2	0.31	0.33	0.94
	2	1.00	0.24	4.2	0.23	0.22	1.05
orf	1	1.00	0.30	3.3	0.69	0.52	1.33
	2	1.00	0.28	3.6	0.55	0.39	1.41

*Transient transfection assays were performed with minigenes that lacked PPE (TIG, TTG) or that contained the wild-type PPE element integrated into the 5′ untranslated region (utr) or in the open reading frame of exon 1 (orf) of the indicated TS minigenes. In the PPE element designated utr-atg, the ATG codons have been altered by site-directed mutagenesis. The results of two independent experiments are shown. TIG is an abbreviation for $TI_{1d1.4}G$. RNA was quantitated by S1 nuclease protection assays as described in Figure 3. Radioactivity corresponding to mRNA of the test minigene was quantitated by phosphorimager and normalized to that of the control gene ($SI_{56}S$). The values were then normalized to the value observed for TIG.

PPE(utr). In contrast to the observations with the β-globin gene (see below), we found that insertion of the PPE element into the intron-containing minigene led to a 2.5-fold reduction in mRNA content (compare the value for TIG with that of TIG:PPE). This suggests that the PPE element exerted a modest inhibitory effect on expression of the TS gene, possibly due to a slight decrease in mRNA stability. However, the intronless TS minigene was expressed at approximately the same level in the presence or absence of the element (compare the value for TTG with that of TTG:PPE). This could be due to the fact that the inhibitory effect of the PPE element was balanced by a stimulatory effect of the element on the expression of the intronless minigene. Minigenes that contained the PPE element were expressed at approximately the same level in the presence or absence of the internally deleted TS intron 1, as shown by the fact that TTG:PPE/TIG:PPE is approximately equal to 1.0. This is consistent with the ability of the element to reduce the requirement for introns to achieve efficient expression of transfected genes.

It was possible that the inhibitory effect of the PPE element on the expression of $TI_{1d1.4}G$ might be due to the introduction of an alternative short open reading frame prior to the intron [Maquat, 1995]. However, when all of the AUG codons in the PPE element were eliminated to form PPE(utr-aug) (Fig. 2), the element functioned in the same manner as the wild-type PPE (Table I).

To determine if the PPE element might have a greater stimulatory effect at a different location, we inserted the PPE element into a *Bsu*36I site that is 81 nt downstream of the AUG start codon to create TTG:PPE(orf) and $TI_{1d1.4}G$:PPE(orf). The sequence of PPE(orf) is shown in Figure 2. Insertion of the PPE(orf) element did not disrupt the open reading frame. Table I shows that inclusion of the PPE element in the open reading frame had a smaller inhibitory effect on expression of the intron-containing TS minigene and led to a minor increase in expression of the intronless TS minigene. Again, the minigenes that contained the PPE(orf) element were expressed at approximately the same level in the presence or absence of the intron, indicating that the element was able to reduce the dependence on introns for efficient expression of the minigene.

PPE Element Is Active in Rodent Cells

The above studies suggest that the PPE element has a much smaller stimulatory effect on the expression of the intronless mouse TS minigene than on the expression of the intronless human β-globin gene. An alternative possibility is that the PPE element is much less active in rodent cells than in primate cells, which were used in the analyses of the β-globin gene [Liu and Mertz, 1995]. To test this possibility, we compared the effect of the PPE element on the production of mRNA from intronless and intron-containing β-globin genes that were transiently transfected into V79 (hamster lung) or COS (monkey kidney) cells. The globin genes used in this study were the same as those used by Liu and Mertz [1995] and were kindly provided by these investigators.

Figure 3 shows that when the globin genes lacked the PPE element, at least 100 times

more mRNA was produced in cells transfected with the intron-containing β-globin gene [pβ1(+)2(+)] than in cells transfected with the intronless version of the gene [pβ1(-)2(-)]. Insertion of the PPE element into the 5′ untranslated region had little effect on the expression of the intron-containing globin gene [compare pβ1(+)2(+) with pTK119β1(+)2(+)]. However, the PPE element stimulated the expression of the intronless globin gene at least 15-fold [compare pβ1(-)2(-) with pTK119β1(-)2(-)], so that it was expressed at 7.5 to 15% of the level of the intron-containing version of the gene. The results were similar for both cell lines and closely resembled those obtained by Liu and Mertz [1995]. Therefore the PPE element functions in the same manner in rodent and primate cell lines.

Growth-Dependent Regulation of PPE-Containing TS Minigenes

A second goal of our investigation was to use the PPE element as a tool to explore more deeply the role of introns in TS gene regulation in growth-stimulated cells. As mentioned above, S phase-specific expression of stably transfected TS minigenes depends on the presence of a spliceable intron within the minigene. It was possible that insertion of the PPE element into a TS minigene might perturb normal (intron-dependent) TS gene regulation in two different ways. Insertion of the PPE element into an intronless TS minigene might overcome the requirement for introns for S phase-specific expression. Alternatively, the presence of the PPE element in an intron-containing TS minigene might alter the normal pattern of S phase-specific expression.

To examine these possibilities, TTG:PPE(orf) and $TI_{1d1.4}G$:PPE(orf) were stably transfected into mouse 3T6 fibroblasts. Mass cultures derived from greater than 100 independent clones were growth-arrested in medium containing 0.5% serum for 7 days, then serum-stimulated to re-enter the cell cycle. The mRNA corresponding to the endogenous TS gene, the transfected TS minigene, and the constitutively expressed endogenous rpL32 gene were analyzed by S1 nuclease protection assays at various times following growth-stimulation. The amount of mRNA corresponding to the endogenous TS gene and the transfected TS minigenes were normalized to the amount of rpL32 mRNA at each time point to correct for differences in

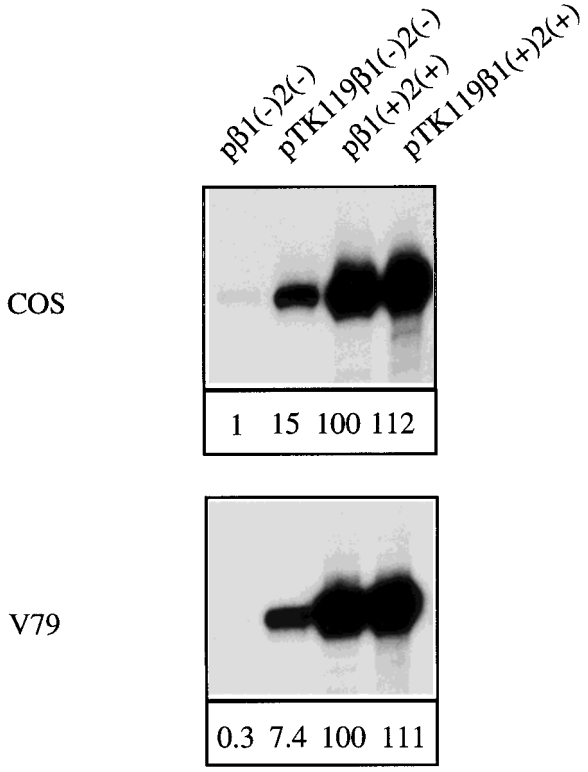

Fig. 3. The PPE element is active in hamster cells. The pβ1(-)2(-) and pβ1(+)2(+) genes are intronless and intron-containing versions of the human β-globin gene, respectively. The pTK119β1(-)2(-) and pTK119β1(+)2(+) genes contain the 119-nt PPE element in the 5′ untranslated region of the first exon. The plasmids were provided by Liu and Mertz [1995]. The same amounts of each construct were transiently transfected into V79 (hamster fibroblasts) using the calcium phosphate protocol and 12 pmol of minigene, or into COS (monkey kidney) cells using the Lipofectamine protocol and 1.2 pmol of minigene. Total cytoplasmic RNA (40 μg) was analyzed by an S1 nuclease protection assay using a human β-globin probe as described [Liu and Mertz, 1995]. The radioactive band corresponding to globin mRNA is shown in the autoradiogram. Radioactivity was quantitated in a phosphorimager and the values were normalized to that observed for pβ1(+)2(+), which was set at 100. The normalized values are shown below the autoradiogram.

RNA recovery. We have shown previously that the ratio of rpL32 mRNA/total cytoplasmic RNA remains approximately constant as cells progress from G0 through S phase of the cell cycle [Geyer et al., 1982]. As shown in Figure 4A, the amount of mRNA derived from the endogenous TS gene increased approximately 17-fold as cells progressed from G1 through S phase (which begins approximately 12 hr after serum stimulation). However, the amount of mRNA corresponding to the intronless TS minigene, TTG:PPE, remained constant following growth-stimulation, as observed previously for TTG [Ash et al., 1993].

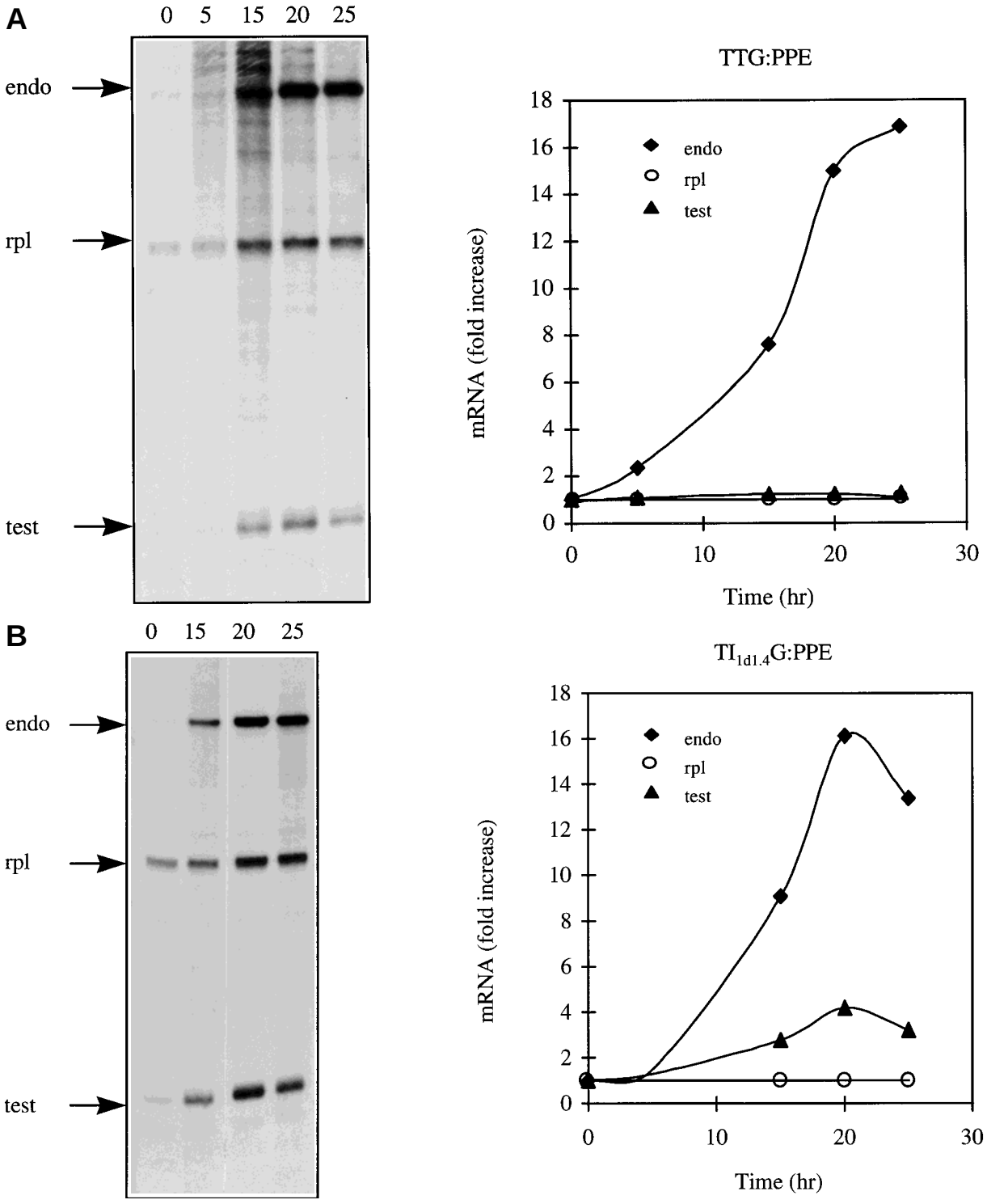

Fig. 4. Regulation of PPE-containing TS minigenes. 3T6 cells that were stably transfected with the TTG:PPE (**A**) or $TI_{1d1l4}G$:PPE (**B**) were allowed to rest in 0.5% calf serum for 7 days and then stimulated to reenter the cell cycle by feeding them at time = 0 with fresh medium containing 10% serum. At the indicated times, cells were harvested and total cytoplasmic RNA was isolated. Equal amounts of mRNA (approximately 20 µg) were analyzed by S1 nuclease protection assays. The probe for the TS mRNAs was derived from $SI_{56}S$ (Fig. 1). The probe for the rpL32 mRNA (a constitutively expressed control gene) was derived from an intronless version of the rpL32 gene, as described previously [Ke et al., 1996]. The fragments labeled endo, rpl, and test correspond to mRNA derived from the endogenous TS gene, the rpL32 gene, and the transfected TS minigene, respectively. The amount of radioactivity was quantitated by a phosphorimager. The values observed for TS mRNA were normalized to the values observed for rpL32 mRNA (to correct for differences in RNA recovery) and then normalized to the value observed at time 0. The final values are plotted in the graph. At least two independent transfections were performed for each minigene, and each transfected cell line was analyzed twice. Similar results were obtained in each analysis.

This analysis was also performed with the intron-containing version of the TS minigene, $TI_{1d1.4}G$:PPE. In this case, the minigene was expressed in an S phase-specific manner (Fig. 4B). The amount of mRNA derived from $TI_{1d1.4}G$:PPE increased approximately 4-fold as the cells progressed from G1 through S phase, which was the same as the increase that was observed previously when analyzing $TI_{1d1.4}G$ [Ke et al., 1996]. Thus insertion of the PPE element did not overcome the dependence on introns for S phase-specific expression of TS minigenes in growth-stimulated cells.

Regulation of TS-TK Chimeric Genes

To determine if the PPE element functions in a different manner in its natural context, we examined the expression of the HSV-TK gene that was driven by the mouse TS promoter. The TS promoter region (from -1,000 to -11 nt relative to the AUG codon) was linked to the TK coding region and polyadenylation signal to form TS-TK (Fig. 5A). An intron-containing version (TS-TK:$I_{1d1.0}$) was also created by inserting TS intron 1 (with a 1.0-kb internal deletion) at the junction site between the TS promoter and TK coding region. When this intron is included in an intronless TS minigene, expression increases approximately 4-fold [Ke, 1995]. In contrast, the addition of this intron to the intronless TS-TK gene had no detectable effect on the level of expression (Fig. 5B). This experiment was repeated 4 times with similar results. This is consistent with the idea that the PPE element in the HSV-TK gene leads to efficient expression of the gene in the absence of splicing.

We next examined the expression of the two TS-TK chimeric genes in growth-stimulated cells. Figure 6A shows that the intronless TS-TK gene was expressed at a low but constant level as cells progressed from G1 through S phase. However, Figure 6B shows that when the intron was included in the TS-TK gene, expression was regulated in an S phase-dependent manner. These experiments were repeated several times with similar observations in each experiment. These observations are consistent with those made with the PPE-containing TS minigenes and reinforce our previous observations that a spliceable intron is necessary for proper S phase regulation.

DISCUSSION

When the PPE element is included in an intronless human β-globin gene, the amount of cytoplasmic mRNA produced by the gene increases at least 15-fold. We found that the PPE element is able to stimulate the production of mRNA from an intronless TS minigene and partially overcome the dependence on introns for efficient expression. However, the stimulatory effect is far less than that observed with the highly intron-dependent human β-globin gene.

The reason why the PPE element has a much smaller effect on the expression of the TS gene than the β-globin gene may be related to the fact that removal of introns leads to a 100-fold decrease in expression of the β-globin gene but only a 10–20-fold decrease in expression of the TS gene. Addition of the PPE element may increase the level of expression of an intronless gene to a value that approaches (but is still less than) that of the intron-containing gene. For the β-globin gene, this represents a major increase in expression. However, for the TS gene, the increase is much less. The generality of this explanation could be tested by examining the effect of the PPE element on other genes whose expression levels are either strongly or weakly dependent on the presence of introns.

The reason why the expression of some genes is highly dependent on the presence of introns whereas the expression of other genes is affected to a lesser extent by the presence of introns is not known. Genes that are affected to only a minor extent by introns may contain positive-acting elements (similar to those identified in the HSV-TK or histone genes) that reduce the need for introns for efficient production of mRNA. Genes that are completely intron-independent may contain several such elements [Liu and Mertz, 1995; Huang and Carmichael, 1997]. It will be interesting to identify the elements that permit efficient intron-independent expression of different genes to determine if they function in an analogous manner and if they can function synergistically. It is also possible that intron-dependent genes contain negative-acting elements in their exons that inhibit the expression of intronless versions of the genes. A gene may contain both positive and negative elements. The extent to which the expression of a particular intronless gene is stimulated by the inclusion of an intron

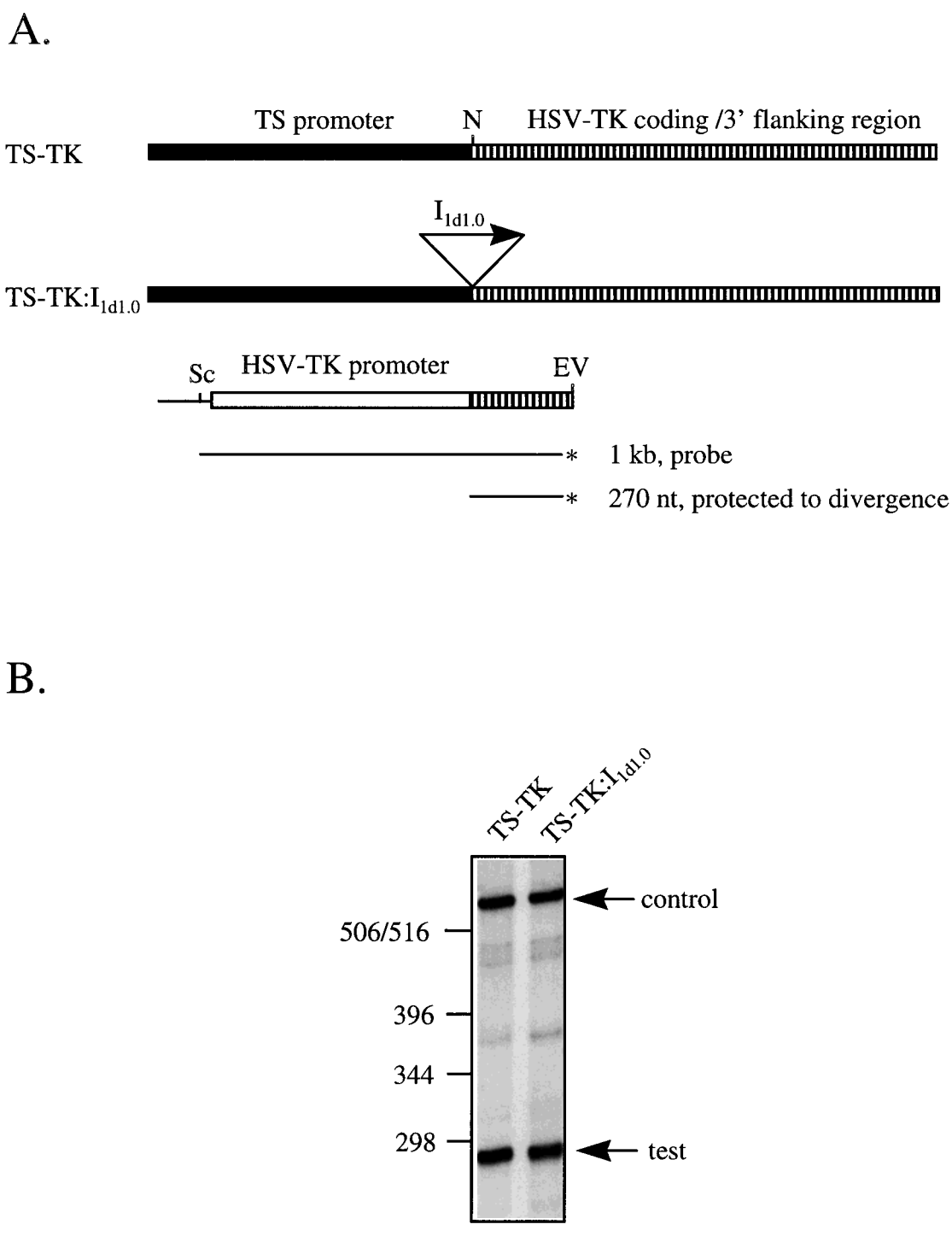

Fig. 5. Construction and expression of chimeric TS-TK genes. **A:** Structures of the TS-TK genes. TS-TK consists of the promoter of the TS gene (between -1000 and -11 nt relative to the AUG start codon of the TS gene) linked to the HSV-TK gene at an engineered *Nhe*I (N) site 41 nt upstream of the AUG start codon of the TK gene. TS-TK:$I_{1d1.0}$ is the same as TS-TK except that it contains TS intron 1 (with a 1-kb internal deletion) at the *Nhe*I site between the TS promoter and TK coding region. The probe that was used in the S1 nuclease protection assay to detect mRNA from the TS-TK genes was derived from the HSV-TK gene and is indicated at the bottom of A. The probe was end-labeled at an *Eco*RV (EV) site in the coding region and extended to a *Sac*I (Sc) site in the plasmid. **B:** Expression of the TS-TK genes. Hamster V79 cells were transiently co-transfected with a TS-TK gene and the control gene, $SI_{56}S$, as described in Figure 1. Total cytoplasmic RNA was isolated and analyzed by S1 protection assays using the probe described in A to detect mRNA from the TS-TK gene and the probe described in Figure 1 to detect RNA from the control gene. Size markers (in nucleotides) are shown to the left of the autoradiogram.

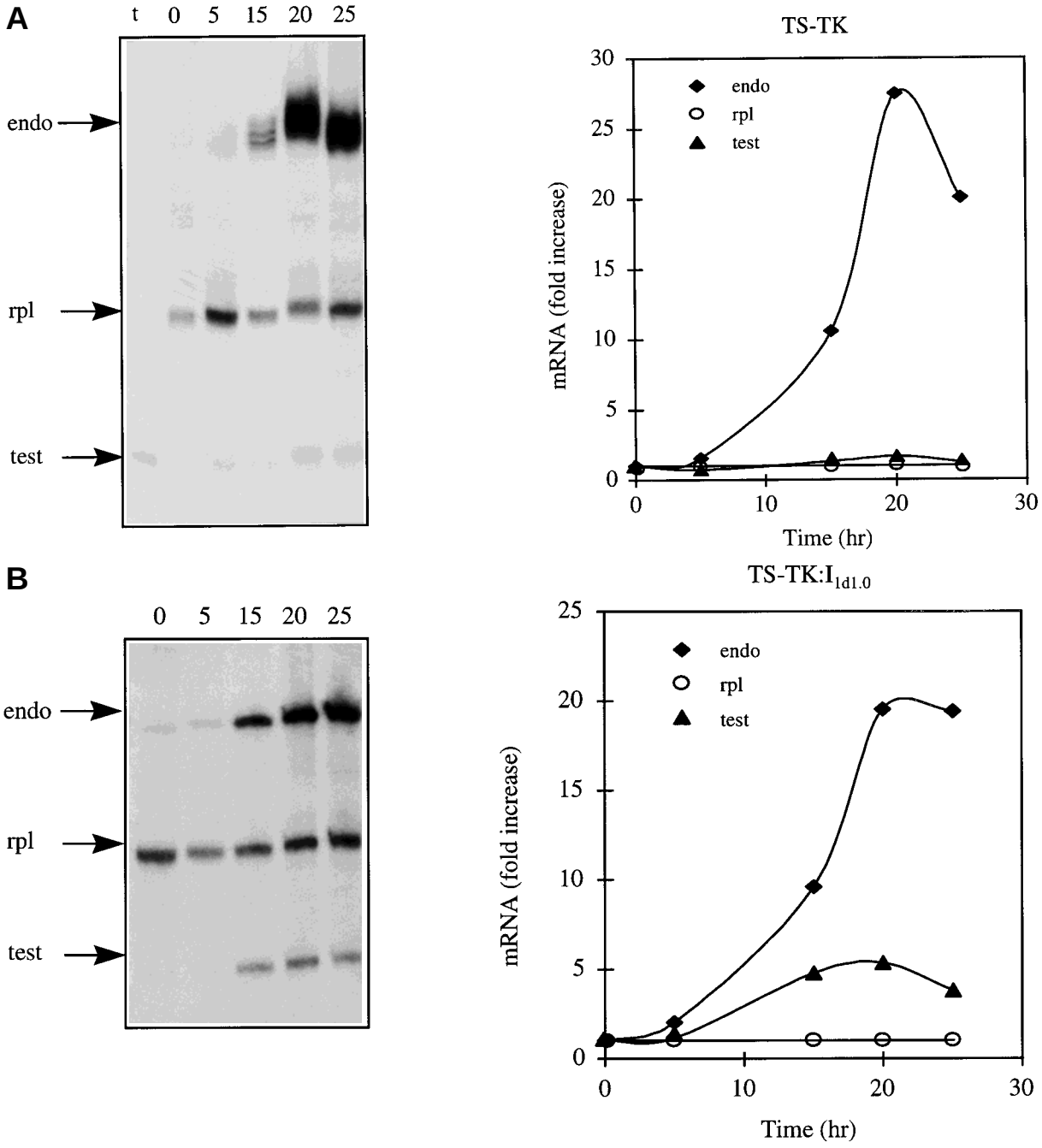

Fig. 6. Regulation of TS-TK genes in growth-stimulated cells. 3T6 cells were stably transfected with TS-TK (**A**) or TS-TK:$I_{1d1.0}$ (**B**). Growth stimulation studies were performed as described in Figure 4. RNA corresponding to the endogenous TS gene (endo), the transfected TS-TK gene (test), and the endogenous rpL32 (rpl) gene were determined by S1 nuclease protection assays using probes that were specific for each mRNA species. The data were normalized as described in Figure 4 and are plotted in the graph. **Lane t** contains RNA from V79 cells that were transiently transfected with the TS-TK gene (to facilitate identification of mRNA derived from the transfected gene).

may depend on the relative activities of the positive and negative elements within the gene as well as the efficiency of splicing of the intron [Korb et al., 1993].

In the present study, we also found that the inclusion of the PPE element did not overcome the dependence on introns for S phase-specific expression of TS minigenes or TS-TK chimeric genes in growth-stimulated cells. Intronless genes were expressed at a constant level in the presence or absence of the PPE element, whereas intron-containing genes were ex-

pressed in an S phase-dependent manner in growth-stimulated cells. These results are in line with our earlier observations with intronless and intron-containing TS minigenes [Ke et al., 1996; Ash et al., 1993] and further strengthen our conclusion that proper regulation of the TS gene requires the presence of both the TS promoter as well as a spliceable intron.

We have previously suggested that S phase regulation of the mouse TS gene depends on some form of communication between the TS promoter and the RNA processing machinery [Johnson, 1994; Ke et al., 1996]. Similar observations have recently been made for the PFK-2 gene, which is also expressed in an S phase-dependent manner [Darville and Rousseau, 1997]. The biochemical mechanism for this communication is not known. However, observations from a number of laboratories have shown that transcription and RNA processing are not independent events [Sisodia et al., 1987; Hernandez and Weiner, 1986; Xing et al., 1993; Beyer and Osheim, 1988]. Mechanisms for communication between transcription and RNA processing that involve the C-terminal domain of RNA polymerase have been proposed [Greenleaf, 1993; McCracken et al., 1997; Yuryev et al., 1996; Steinmetz, 1997]. The mouse TS gene may represent an example of a gene whose regulation depends directly on this communication process.

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