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Expression from herpesvirus promoters does not relieve the intron requirement for cytoplasmic accumulation of human β-globin mRNA

Xian-ming Yu[+], Gregory W.Gelembiuk, Chung-Yih Wang[§], Wang-Shick Ryu[†] and Janet E.Mertz*
McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI 53706, USA

ABSTRACT

Expression plasmids were constructed in which the human β-globin gene or a variant of it precisely lacking its two introns was transcribed from its own promoter, the herpes simplex virus type 1 thymidine kinase (HSV-tk) promoter, or the immediate early promoter of human cytomegalovirus (CMV-IE). Forty two hours after transfection of these plasmids into monkey kidney cells, nuclear and cytoplasmic RNA were isolated. Quantitative S1 nuclease mapping and primer extension analysis were used to determine the relative abundances, cellular locations, and leader sizes of the accumulated β-globin RNAs. Whereas transcripts of all of the intron-containing genes accumulated in the cytoplasm to high levels, transcripts of their cDNA variants were neither stably maintained in the nucleus nor accumulated in the cytoplasm, irrespective of the promoter from which transcription was driven. We conclude that the intron requirement for cytoplasmic accumulation of β-globin RNA can not be circumvented by synthesis from either the promoter of the intronless HSV-tk gene or the CMV-IE promoter.

INTRODUCTION

Most genes in higher eukaryotes contain introns. Whereas many of these genes require introns for efficient expression, some do not. For example, the naturally intronless herpes simplex virus type 1 thymidine kinase (HSV-tk) gene is expressed efficiently regardless of the presence of introns (1,2). Efficient expression of some genes is intron dependent because transcriptional enhancer elements are encoded within the introns. However, the intron dependence of at least some genes (e.g.'s, SV40 late, β-globin) is post-transcriptional in nature [see (2,3) and references cited therein].

Two reports have appeared recently indicating that expression from certain herpesvirus promoters (e.g.'s, cytomegalovirus immediate early (CMV-IE), HSV-tk) can overcome the intron requirement of at least some genes (e.g.'s, immunoglobulin, β-globin) for proper processing and nuclear export of their transcripts (1,4). However, CMV transcripts synthesized from the IE promoter in its natural context do not accumulate as unspliced mRNAs; rather, multiple alternatively spliced introns are excised from them. We show here that synthesis from the CMV-IE promoter does not obviate the intron dependence of β-globin. Furthermore, during the course of followup experiments designed to map the *cis*-acting element(s) within the HSV-tk promoter responsible for intron independence, we discovered that these elements mapped entirely upstream of the HSV-tk promoter sequences (5), leading us to propose an alternative explanation for the cytoplasmic accumulation of RNA synthesized from our intronless constructs. As we show here, what appeared in our case to be intron-independent accumulation of β-globin transcripts synthesized from the HSV-tk promoter (1) was, in actuality, efficient accumulation of transcripts initiated at upstream sequences within the plasmid.

MATERIALS AND METHODS

Plasmids

All plasmids were constructed by standard recombinant DNA techniques (6). The starting plasmid, pβ-βIVS(+), has been described in detail elsewhere (1,7). It contains the nt 812 to nt +2156 (relative to the site of transcription initiation) region of the human β-globin gene in an SV40 ori-containing, pBR322-based vector (Fig. 1). Plasmid pβ-βIVS(−) is identical in sequence to pβ-βIVS(+) except for the precise lack of the two β-globin introns. It was constructed by recombination of pβ-βIVS(+) with a plasmid containing a cDNA version of the human β-globin gene. The promoter region of pSVTK-βIVS(+) (Fig. 1) was obtained from plasmid pDG013 (8), a gift from D. Greenspan. It contains the enhancer region of SV40 inserted into a KpnI site located upstream of the HSV-tk promoter of HSV-1 strain CL101. The novel HSV-tk/β-globin joint was constructed by cutting the β-globin DNA with NcoI, filling in the NcoI site, adding BglII linker DNA (CAGATCTG), cleaving with BglII, and ligating to BglII-cut HSV-tk DNA. Plasmid pTK-βIVS(+) (Fig. 1) was constructed from pSVTK-βIVS(+) by excision of the SV40 enhancer region fragment with PvuII. The promoter region of pCMV-βIVS(+) was taken from the HCMV-IE1 gene (approximately nt −1200 to nt −17 relative to the start site of transcription). The distal (HindIII-to-NcoI) and

* To whom correspondence should be addressed

Present addresses: [+]Shanghai Institute of Biochemistry, Academia Sinica, Shanghai, China, [§]Howard Hughes Medical Institute, University of Michigan, Medical Center, Ann Arbor, MI and [†]Fox Chase Cancer Center, Institute for Cancer Research, Philadelphia, PA, USA

proximal (NcoI-to-AluI) fragments of this segment were obtained, respectively, from pHCMV-Vμ[In1] (4) and pCATwt760 (9), regenerating the intact pHCMV-IE1 promoter region. Nt −9 to +53 of the human β-globin gene was replaced by the homologous segment of the mouse major β-globin gene taken from pMHβSV-330 LS21/10, a linker scanning mutant in which a BglII linker is substituted for nt −21 to −10 [an additional 12 bp linker is present at nt +28] (10). The CMV/β-globin joint was constructed by cutting pMHβSV-330 LS-21/−10 with BglII, filling in the BglII end, and ligating to AluI-cut HCMV DNA. Plasmids pβ-βIVS(−), pCMV-βIVS(−), pTK-βIVS(−), and pSVTK-βIVS(−) are identical in sequence to plasmids pβ-βIVS(+), pCMV-βIVS(+), pTK-βIVS(+), and pSVTK-βIVS(+), respectively, except for their precise lack of the two β-globin introns. The origin of pRSV-Tori is described elsewhere (1,7).

Transfections and RNA analyses

In all experiments, the intron dependence of gene expression was tested by co-transfection of the African green monkey kidney cell line, CV-1PD, with 2 μg of test plasmid DNA and 1 μg of pRSV-Tori per 100 mm dish of cells by the DEAE-dextran followed by chlorquine method as described previously (3). Cells were harvested 42 h after transfection and fractionated into nuclear and cytoplasmic components by treatment with 0.5% Nonidet P40 as described previously (3). Nuclear and cytoplasmic RNAs were purified as described previously (3). Cellular β-actin RNA served as an internal control for recovery of the RNA samples and purity of the nuclear RNA. Southern blot analysis, performed as described elsewhere (1,7), of the relative amounts of DpnI-resistant, β-globin-encoding plasmid DNA present in each nucleic acid sample prior to treatment with DNase I was used to assay both (i) for nuclear contamination of cytoplasmic nucleic acid and (ii) for differences in transfection efficiencies. The latter were found to vary by at most two fold within an experiment (data not shown).

Quantitative S1 nuclease mapping analysis was performed by the trichloroacetic acid method of Murray (11). The probes used are shown in Figures 2B and 2C. Primer extension analysis was done as described by Good (12) with a 5′ end-labeled nt +90 to nt +114 (relative to the β-globin site of transcription initiation) antisense synthetic oligonucleotide serving as primer. In brief, RNA was mixed with 2×10^6 cpm of 5′ end-labeled primer and 20 μg carrier poly(A) (Pharmacia) in 10 μl of 0.3 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, denatured at 80° for 5 min, and annealed at 65° for 30 min. The primer was extended by the addition of 5 units of AMV reverse transcriptase (Life Sciences, Inc.), 20 units of RNasin (Promega), and 40 μl reverse transcriptase buffer [12.5 mM Tris-HCl (pH 8.4), 12.5 mM DTT, 1.25 mM each of dGTP, dCTP, dATP, and TTP, 62.5 μg/ml actinomycin D, and 15 mM $MgCl_2$] and incubation at 48° for 2 h. The reaction was terminated and RNA was hydrolyzed by addition of 6 μl 1M NaOH and 1 μl 0.5 M EDTA, incubation at 60° for 1 h, then neutralization with HCl. The resulting DNAs were precipitated with ethanol, resuspended in 80% formamide, 10 mM NaOH, 1 mM EDTA, and electrophoresed in an 8M urea, 6% polyacrylamide gel.

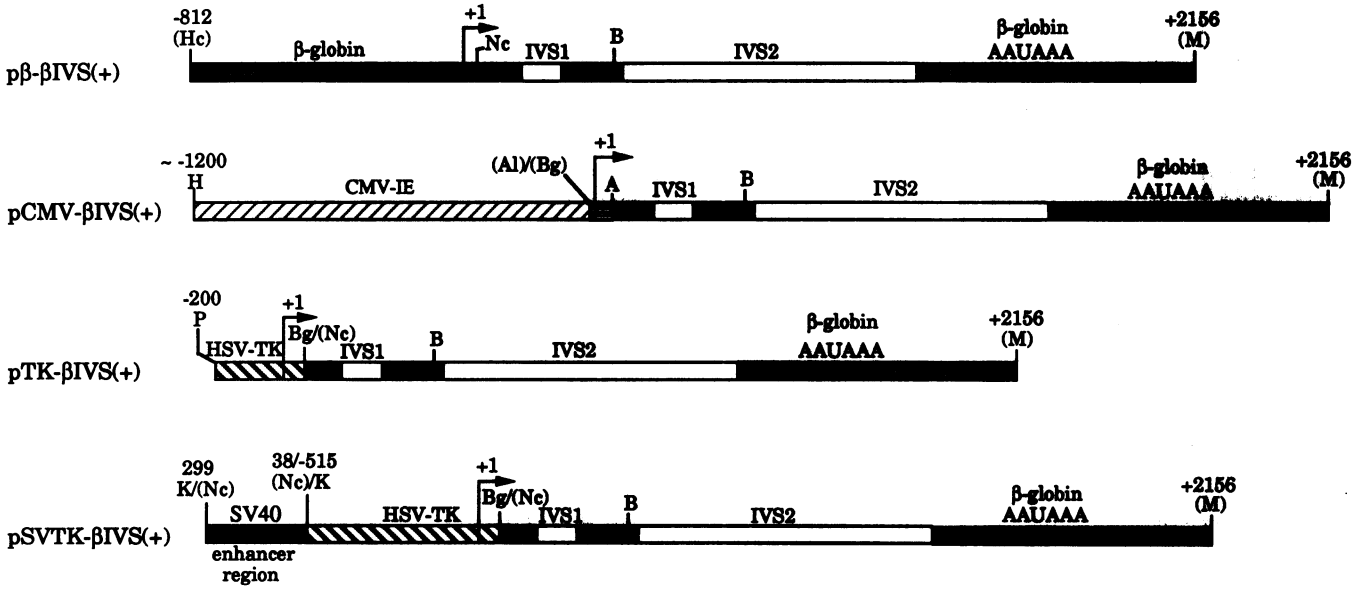

Figure 1. Structures of the plasmids used in this study. Only the regions of each plasmid involved in synthesis of β-globin RNA are shown. The numbers above each line are the nucleotide residues relative to the major transcription initiation site indicated by the rightward arrow. The polyadenylation signal is indicated by AAUAAA. The vectors are not shown. The construction of the pβ-βIVS(+) vector is described in detail elsewhere (1,7). It contains the following sequences: the enhancer region of SV40 (SV40 nt 38 through 299); a spacer consisting of HSV-1 DNA nt −510 to −722 relative to the transcription initiation site of the TK gene; a minimal origin of SV40 DNA replication (SV40 nt 5171 to 34 plus nt 198 to 272, inclusive); the BalI-to-EcoRI region of the poison-minus derivative of pBR322, pAT153 (14); and a spacer consisting of SV40 nt 2297 to 2533. The vectors in the other plasmids are the same as the one in pβ-βIVS(+) except for the deletion of SV40 nt 38 through 269 from pTK-βIVS(+) and pTK-βIVS(−), the precise deletion of the HSV spacer and SV40 enhancer regions in pCMV-βIVS(+) and pCMV-βIVS(−), and the inclusion of the SV40 enhancer region of the vector in the schematic of pSVTK-βIVS(+). The plasmids pβ-βIVS(−), pCMV-βIVS(−), pTK-βIVS(−), and pSVTK-βIVS(−) are identical in sequence to their corresponding IVS(+) counterparts except for the precise lack of IVS1 and IVS2. Crosshatched boxes, human β-globin promoter, exon, and 3′ region sequences; open boxes, human β-globin intron sequences; horizontally striped box, mouse β-globin sequence; hatched boxes, herpesvirus promoter region sequences; stippled box, SV40 promoter region sequence. Restriction sites inactivated during construction of the plasmids are in parentheses. Abbreviations: A, ApaLI; Al, AluI; B, BamHI; Bg, BglII; H, HindIII; Hc, HincII; IVS, intervening sequence; K, KpnI; M, MspI; Nc, NcoI; P, PvuII.

RESULTS

Synthesis from HSV-tk promoter does not relieve intron dependence of β-globin RNA accumulation

In the experiments presented here, CV-1PD cells, an African green monkey kidney cell line, were co-transfected with plasmids containing the genes of interest to be tested and pRSV-Tori. The latter plasmid, encoding the SV40 large T antigen, results in replication of the test SV40 ori$^+$ plasmids to high copy number (1,7). Consequently, transcription of the test genes occurs at high levels when synthesized from the human β-globin, HSV-tk, or CMV-IE promoter. Intron dependence was determined by comparing the amounts of β-globin RNA accumulated in the cytoplasm of cells transfected in parallel with plasmids containing genomic and their corresponding cDNA variant genes.

In confirmation of previous reports [see (1,2) and references cited therein], cytoplasmic accumulation of human β-globin mRNA was found to be highly intron dependent when synthesized from the human β-globin promoter—i.e., cells transfected with pβ-βIVS(−) accumulated β-globin RNA to at most 1/500th the level that cells transfected with pβ-βIVS(+) did (Fig. 2A, lane 6 vs. 4). As noted earlier (1,2), the nuclear accumulation of β-globin RNA was also intron dependent (Fig. 2A, lane 5 vs. 3). This latter finding is probably the consequence of unutilizable transcripts being degraded rapidly within the nucleus (1,7).

Prior findings from our laboratory indicated that synthesis from the HSV-tk promoter could at least partially alleviate this intron dependence of β-globin RNA accumulation (1; Fig. 2A, lane 18 vs. 16). However, followup studies involving the use of HSV-tk/human β-globin hybrid promoters indicated that the element(s) enabling intron-independent accumulation of β-globin RNA mapped more than 200 bps upstream of the β-globin transcription initiation site (5). Therefore, a plausible alternative hypothesis was that these β-globin RNAs accumulated, instead, as a consequence of readthrough transcription from promoters lying upstream of the HSV-tk promoter (e.g., SV40 promoter region sequences present within the vector; see Fig. 1).

To test this hypothesis, we determined by primer extension analysis the sizes of the leader regions of the β-globin RNAs accumulated in these transfected cells. As expected, most of β-globin RNAs accumulated in the pβ-βIVS(+)- and pSVTK-βIVS(+)-transfected cells had leader sizes consistent with their

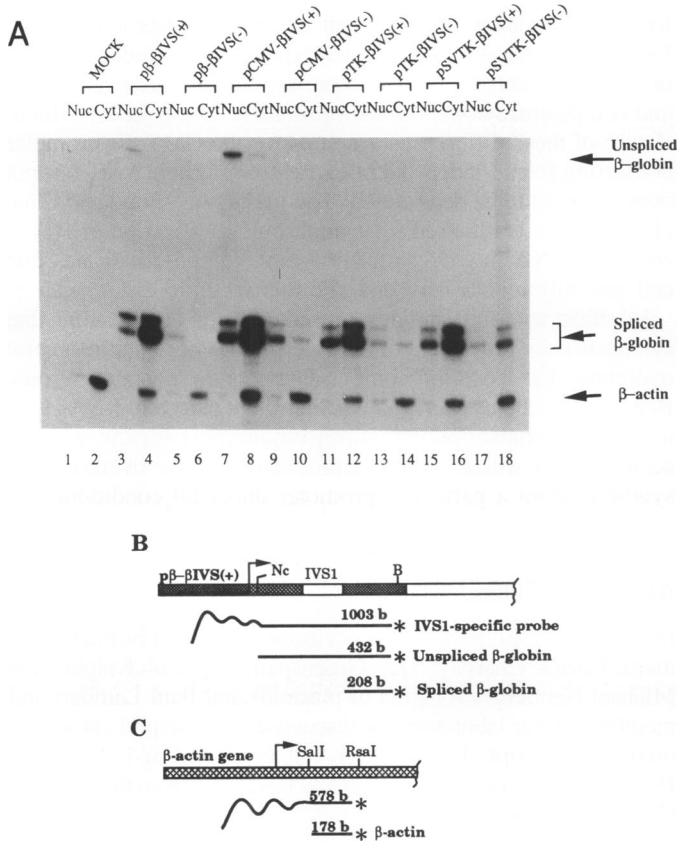

Figure 2. Excisable introns are required for both stabilization and nuclear export of human β-globin RNA. A. Autoradiogram of S1 nuclease mapping analysis of β-globin RNA accumulated in the nucleus (Nuc) and cytoplasm (Cyt) of cells transfected with the plasmids containing IVS(+) and IVS(−) counterparts of the various promoter/human β-globin hybrid genes shown in Fig. 1. Assays were performed as described in Materials and Methods with the probes shown in panels B and C. The labels at the right show the expected sizes of the probes protected by the RNA species indicated. The doublet band for IVS1-spliced globin RNA is an S1 mapping artifact due to the presence of a nearly perfect duplication of sequence at the exon 1-exon 2 joint. B. Schematic diagram of the β-globin probe used for the S1 nuclease mapping analysis in panel A and the resulting fragments protected by hybridization with the corresponding RNAs. The structure of the relevant portion of the β-globin gene is as shown in Fig. 1. The β-globin probe, the 1003-bp KpnI-to-BamHI fragment of pSVTK-βIVS(+), was 5′ end-labeled at the BamHI site; the wavy line indicates the HSV-tk sequences. C. Schematic diagram of the cellular β-actin probe used as a control in the S1 nuclease mapping experiment in panel A and the resulting fragment protected by hybridization with the corresponding RNA. The structure of the relevant portion of the cellular β-actin gene is shown. The actin probe, the 578-bp RsaI-to-XmnI fragment of pHFβA-1ΔSV (15), was 5′ end-labeled at the RsaI site. This probe has pBR322 sequence, indicated by the wavy line, adjacent to the SalI site of the β-actin gene.

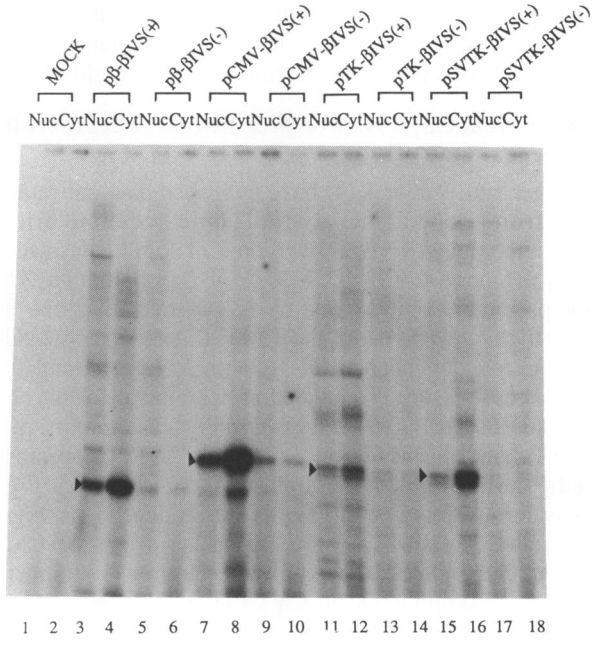

Figure 3. 5′ end analysis of the β-globin RNAs from the experiment described in Fig. 2. Shown here is an autoradiogram of a primer extension analysis performed as described in Materials and Methods. The RNA sample analyzed in each numbered lane is identical to the one analyzed in the like numbered lane in Fig. 2A. The arrows indicate the expected sizes of cDNAs synthesized from RNAs in which the 5′ ends were determined by the TATA boxes present within the promoters shown in Fig. 1.

having been synthesized from the β-globin and HSV-tk promoters, respectively (Fig. 3, lanes 4 and 16, respectively). On the other hand, the leaders of the β-globin RNAs accumulated in the pSVTK-βIVS(−)-transfected cells were quite heterogeneous in size (Fig. 3, lane 18). Most of the accumulated β-globin RNA had been synthesized from upstream promoters rather than from the HSV-tk promoter. Noteworthy is the fact that the transcripts initiated from the HSV-tk TATA box (indicated by the arrow adjacent to lane 15 of Fig. 3) failed to accumulate, while most of those initiated from other sites accumulated to similar levels whether or not the β-globin introns were present (Fig. 3, lane 16 vs. 18). Therefore, synthesis from the HSV-tk promoter does not relieve, even partially, intron dependence of β-globin RNA accumulation. Our finding that initiation from other, upstream promoters appears to do so may be a consequence of these β-globin transcripts possessing additional sequences at their 5′ ends that (i) enable cryptic splicing out of introns and/or (ii) contain elements specifying intron independence analogous to those present within the coding region of the HSV-tk gene (1,2,7).

In an attempt to eliminate readthrough transcription from upstream promoters, we deleted from pSVTK-βIVS(+) and pSVTK-βIVS(−) the non-essential SV40 enhancer region present within the vector. The accumulation of β-globin RNA synthesized from the resulting plasmids, pTK-βIVS(+) and pTK-βIVS(−), was analyzed as above. Cytoplasmic accumulation of β-globin RNA was largely intron dependent, with these RNAs having 5′ ends that mapped to the HSV-tk promoter (Fig. 2A, lane 12 vs. 14; Fig. 3, lane 12 vs. 14). Furthermore, insertion of this SV40 enhancer-containing restriction fragment into pβ-βIVS(−) at nt −266 (relative to the authentic transcription initiation site) resulted in high levels of readthrough transcription, partially restoring RNA accumulation in the absence of the two natural β-globin introns (5). These results clearly establish that synthesis from the HSV-tk promoter does not supplant the intron requirement for cytoplasmic accumulation of β-globin mRNA.

Synthesis from CMV-IE promoter also does not relieve intron dependence

Neuberger and Williams (4) have published data indicating that the intron dependent, cytoplasmic accumulation of immunoglobulin μ RNA is overcome in J558L plasmacytoma cells by synthesis from the CMV-IE or Drosophila *hps70* heat-shock promoter. If their finding is generalizable, one would predict that synthesis from the CMV-IE promoter would also relieve intron dependence of β-globin RNA accumulation in CV1-PD cells. To test this prediction, we constructed the plasmid pCMV-βIVS(+) (Fig. 1) and its intronless counterpart, pCMV-βIVS(−). Analysis as above of the β-globin RNA accumulated in cells transfected with these plasmids indicated that β-globin RNA accumulation was still highly intron dependent (Figs. 2A and 3, lanes 7−10). Therefore, synthesis from the CMV-IE promoter does not alleviate β-globin's intron requirement for cytoplasmic accumulation in CV1-PD cells.

DISCUSSION

In the study presented here, we have shown clearly that the very strong intron dependence for cytoplasmic accumulation of human β-globin RNA (Figs. 2A and 3, lanes 3−6) cannot be overcome by synthesis from the HSV-tk promoter (Figs. 2A and 3, lanes 11−18). Our previous data indicating that synthesis from the HSV-tk promoter might at least partially relieve intron dependence (1) was herein shown to be the result of readthrough transcription from upstream promoters (Fig. 3, lanes 15−18). These findings are consistent with those of Buchman and Berg (2) who reported that the presence of the rabbit β-globin second intron increased 20 fold cytoplasmic accumulation of a rabbit β-globin-like RNA synthesized from the HSV-tk promoter. Extending this conclusion, we have found that synthesis from the CMV-IE promoter also can not overcome this intron dependence for β-globin RNA accumulation (Figs. 2A and 3, lane 7−10).

On the other hand, Neuberger and Williams (4) have reported that the intron requirement for immunoglobulin μ expression can be obviated by synthesis from the CMV-IE promoter. The reason for the discrepancy between their and our findings is not clear. Differences between their and our experiments included their use of (i) a different test gene, (ii) expression from an integrated state, and (iii) plasmacytoma instead of monkey kidney cells. Which, if any, of these differences accounts for the CMV-IE promoter conferring intron independent expression in their case, but not ours, remains to be determined. The finding of Arrigo and Chen (13) that Rev is required for cytoplasmic localization of HIV-1 *env/vpu2* RNA in COS, but not lymphoid cells, indicates that cell line differences may underlie this seeming discrepancy.

In summary, the findings presented here demonstrate that synthesis from promoters of intronless genes does not in general overcome the need of some transcripts to contain introns. Remaining to be answered is whether there exist any RNAs with a clear post-transcriptional intron requirement for cytoplasmic accumulation whose intron requirement can be overcome by synthesis from a particular promoter under all conditions.

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REFERENCES

1. Ryu, W.-S. (1989) Ph.D. thesis, University of Wisconsin, Madison, WI.
2. Buchman, A. R. and Berg, P. (1988) Mol. Cell. Biol., 8, 4395−4405.
3. Ryu, W.-S. and Mertz, J. E. (1989) J. Virol., 63, 4386−4394.
4. Neuberger, M. S. and Williams, G. T. (1988) Nucleic Acids Res, 16, 6713−6724.
5. Wang, C.-Y. and Mertz, J. E. (unpublished).
6. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual*, Second edition, Cold Spring Harbor Laboratory.
7. Ryu, W.-S., Gelembiuk, G. W. and Mertz, J. E. (manuscript in preparation).
8. Greenspan, D. S. and Weissman, S. M. (1985) Mol. Cell. Biol., 5, 1894−1900.
9. Stinski, M. F. and Roehr, T. J. (1985) J. Virol., 55, 431−441.
10. Charnay, P., Mellon, P. and Maniatis, T. (1985) Mol. Cell. Biol., 5, 1498−1511.
11. Murray, M. G. (1986) Anal. Biochem., 158, 165−170.
12. Good, P. J. (1987) Ph.D. thesis, University of Wisconsin, Madison, WI.
13. Arrigo, S. J. and Chen, I. S. Y. (1991) Gene Dev., 5, 808−819.
14. Twigg, A. J. and Sherratt, D. (1980) Nature, 283, 216−218.
15. Sedman, S. A., Good, P. J. and Mertz, J. E. (1989) J. Virol., 63, 3884−3893.