

# Pre-mRNA processing enhancer (PPE) elements from intronless genes play additional roles in mRNA biogenesis than do ones from intron-containing genes

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## ABSTRACT

**Most mRNA-encoding genes require introns for efficient expression in high eukaryotes. However, mRNAs can efficiently accumulate in the cytoplasm without intron excision if they contain *cis*-acting elements such as the post-transcriptional regulatory element (PRE) of hepatitis B virus (HBV), the constitutive transport element (CTE) of Mason–Pfizer monkey virus (MPMV), or the pre-mRNA processing enhancer (PPE) of herpes simplex virus' thymidine kinase (HSV-TK) gene. We compared the activities of these viral elements, the Rev-responsive element (RRE) of the human immunodeficiency virus (HIV), and the human *c-Jun* gene's enhancer (CJE), an element newly identified here, to enable expression of an intronless variant of the human  $\beta$ -globin gene. The PRE, PPE and CJE from naturally intronless genes, but not the CTE or RRE from intron-containing genes, significantly enhanced stability, 3' end processing and cytoplasmic accumulation. When the transcripts included the  $\beta$ -globin gene's first intron, the PRE, PPE and CJE still enhanced mRNA biogenesis, in some cases without intron excision. Thus, elements enabling stability, 3' end formation and nucleocytoplasmic export, not the presence of introns or their excision *per se*, are necessary for mRNA biogenesis. While the CTE and RRE primarily enhance nucleocytoplasmic export, PPE-like elements from naturally intronless genes facilitate polyadenylation as well.**

## INTRODUCTION

The biogenesis of mRNAs in higher eukaryotes requires numerous steps, including 5' end capping, stabilization of the

transcript, 3' end cleavage and polyadenylation, excision of introns and nucleocytoplasmic transport. All of these steps are coupled, from initiation of synthesis of the primary transcript through translation of the resulting mRNA for synthesis of the encoded protein (1–3). Only properly processed mRNAs are translated, with incorrectly synthesized ones being degraded by quality check surveillance or nonsense-mediated decay (4).

Most protein-encoding genes in higher eukaryotes contain introns. Their presence can significantly influence the metabolism of the primary transcripts [(5–11) and references cited therein]. Intron excision leads to further deposition 20–24 nt upstream of the exon–exon junctions of complexes (EJCs) containing the proteins eIF4AIII, SRm160, DEK, UAP56, RNPS1, Y14, Magoh and Aly/REF (12–18). These factors play roles in 3' end processing, nucleocytoplasmic transport and nonsense-mediated decay (1–4,19,20).

Complex retroviruses such as human immunodeficiency virus type 1 (HIV-1) need to express unspliced and partially spliced as well as fully spliced mRNAs during their life cycles [reviewed by (21,22)]. They bypass the requirement for excision of all introns prior to nuclear export by encoding a *trans*-acting factor, such as Rev, that sequence-specifically interacts with a viral *cis*-acting element, called the Rev-responsive element (RRE) (23). Simple retroviruses, such as Mason–Pfizer monkey virus (MPMV), contain a *cis*-acting RNA element, called a constitutive transport element (CTE), that directly binds the cellular protein TAP, thereby enabling export of intron-containing viral mRNAs without splicing (21,24,25). Rous sarcoma virus, another simple retrovirus, also exports its unspliced mRNA to the cytoplasm utilizing *cis*-acting elements, in this case via an as-yet-unidentified pathway (26).

Some genes are naturally intronless yet expressed in higher eukaryotes, presumably by the inclusion of specific sequences that facilitate the processing and export of intronless transcripts. Viral genes of this type include hepatitis B virus (HBV) (27–29) and most of the genes in herpes simplex virus type 1 (HSV-1) [reviewed in (22)]. Intronless cellular

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genes include *c-Jun* (30), some histones (31–34) and interferon  $\alpha$  (35). HSV's thymidine kinase (TK) gene contains a pre-mRNA processing enhancer (PPE) that enables efficient processing and cytoplasmic accumulation of transcripts synthesized from a cDNA version of the human  $\beta$ -globin gene by binding the cellular protein heterogeneous nuclear ribonucleoprotein L (hnRNP L) (36). The mouse histone H2a gene contains an element that enables cytoplasmic accumulation of globin-like mRNA in the absence of introns by binding the cellular SR proteins SRp20 and 9G8 (33,34). HBV's post-transcriptional regulatory element (PRE) presumably also functions by binding cellular factor(s) (37,38). Unfortunately, the precise mechanism by which these latter elements enable intron-independent gene expression is only beginning to be understood (34).

MPMV's CTE, HBV's PRE, HSV-TK's PPE and HIV-1's RRE in the presence of Rev all enhance efficient nuclear export of mRNA without splicing in an intron-containing reporter derived from the HIV-1 genome (32,39). Recently, Lu and Cullen (40) showed that the PRE may act, at least in part, by a mechanism distinct from that of the CTE. Given that the CTE and RRE are naturally located in intron-containing genes while the PRE and PPE are naturally located in intronless genes, we hypothesized that CTE-like elements from intron-containing genes may not provide all of the same functions as do PPE-like elements from intronless ones.

To test the validity of this hypothesis, we examined here the effects of these various elements on stabilization, 3' end processing and cytoplasmic accumulation of transcripts synthesized from variants of the human  $\beta$ -globin gene containing either no intron or only the globin gene's first intron. Insertion into an intronless variant of the  $\beta$ -globin gene of HSV-TK's PPE, HBV's PRE or CJE, an enhancer element(s) in the human *c-Jun* gene newly identified here, but not MPMV's CTE or HIV's RRE in the presence of Rev, significantly enhanced stabilization, 3' end processing and cytoplasmic accumulation of  $\beta$ -globin-like mRNA. When the first intron of  $\beta$ -globin was included in the gene, the PRE, PPE and CJE still enhanced stabilization, 3' end processing and cytoplasmic accumulation of  $\beta$ -globin-like mRNA, in some cases without excision of the intron. We conclude that while CTE-like elements enhance nucleocytoplasmic export with the introns in these genes facilitating proper 3' end formation, PPE-like elements from naturally intronless genes facilitate both these crucial steps in mRNA biogenesis regardless of the presence of introns.

## MATERIALS AND METHODS

### Cells and transfections

The African green monkey kidney cell line CV-1PD was grown in DMEM supplemented with 5% fetal bovine serum as described previously (41). Co-transfections were performed by the DEAE-dextran/chloroquine procedure as described previously (36,42). COS-M6 cells were grown in 35-mm dishes in DMEM supplemented with 10% fetal bovine serum. They were transfected by addition to the media in the presence of serum of TransIT LT-1 reagent (Mirus Corp.) following the manufacturer's protocols and incubated for 48 h.

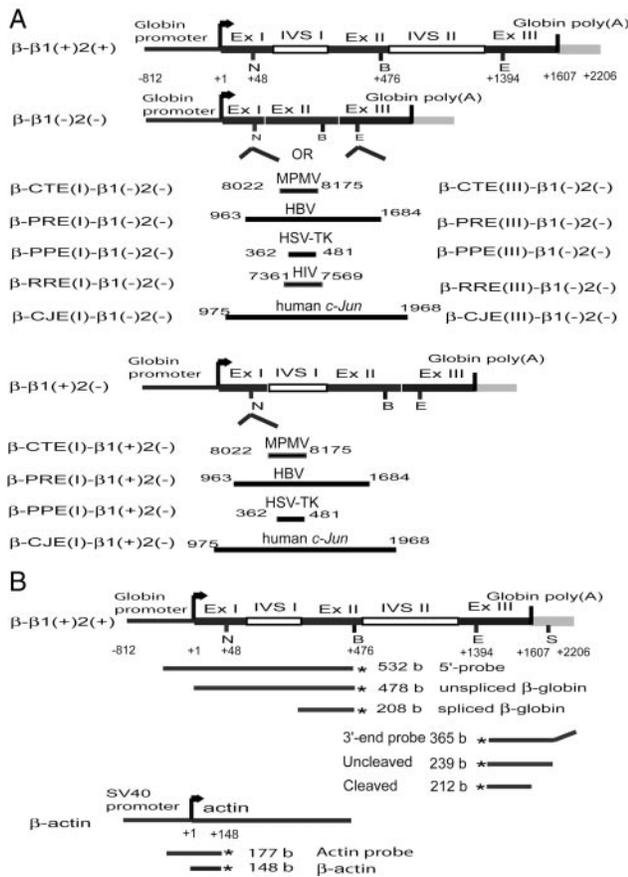
### Recombinant plasmids

Plasmids p $\beta$ - $\beta$ 1(+) $\beta$ 2(+), p $\beta$ - $\beta$ 1(-) $\beta$ 2(-), p $\beta$ - $\beta$ 1(+) $\beta$ 2(-) and p $\beta$ -PPE(I)- $\beta$ 1(-) $\beta$ 2(-) have been described previously (36,43,44). They contain the nucleotides -812 to +2206 region of the human  $\beta$ -globin gene relative to the transcription initiation site with (+) or without (-) the first (1) or second (2) intron in a pBR322-based cloning vector containing an SV40 origin of DNA replication. HSV-TK's 119-nt PPE includes nucleotides 361–479 of the thymidine kinase gene relative to its transcription initiation site (36). HBV's PRE was PCR amplified from pDM138/PRE (28) to include nucleotides 963–1694 of HBV. MPMV's CTE was PCR amplified from pGEM/CTE566 (45) to include MPMV nucleotides 8022–8175 relative to the transcription initiation site. HIV-1's RRE was PCR amplified from pDM128 to include nucleotides 7361–7569 (46). Plasmid pcRev (47), encoding HIV-1's Rev protein, was obtained from Dr B. Cullen's laboratory along with its parental empty vector pBcCMV. The nucleotides 975–1968 region of the human *c-Jun* gene relative to the transcription initiation site was obtained by PCR amplification. All PCR primers used here are listed in Table 1. Each of the PCR products was cleaved with NcoI or EcoRI and inserted into exon I at the NcoI site of p $\beta$ - $\beta$ 1(-) $\beta$ 2(-) or p $\beta$ - $\beta$ 1(+) $\beta$ 2(-) or exon III at the EcoRI site of p $\beta$ - $\beta$ 1(-) $\beta$ 2(-), respectively, in the sense orientation (Figure 1A). Plasmid pRSV-Tori, encoding SV40 large T antigen transcribed from the RSV promoter, has been described previously (36).

To construct the reporter plasmid pGLiresLucGA, plasmid pGL3control/Luc (Promega) was first digested with HindIII, and the ends were filled in with T4 DNA polymerase. Plasmid pIRES2/EGFP (BD Science, Clontech) was digested with NheI and BstXI, and the ends were filled in with T4 DNA polymerase. The MCS-IRES2 sequence was cloned into pGL3control/Luc by blunt-end ligation to form pGL3control/IRES2/Luc. The  $\beta$ -globin polyadenylation signal (nucleotides -100 to +416 relative to the cleavage site for polyadenylation) was PCR amplified from p $\beta$ - $\beta$ 1(+) $\beta$ 2(+) with primers 5'-TGTTCTAGAGGTTCCCTTTGTTCCC-3' and 5'-TGTTCTAGAGAAACC-ATCTCGCCG-3', cleaved with XbaI, and inserted into the XbaI site of pGL3control/IRES2/Luc to create pGL3control/IRES2/Luc/ $\beta$  (A), abbreviated pGLiresLucGA (Figure 3). RRE, PRE and CTE were PCR amplified and inserted into the EcoRI site of pGLiresLucGA to construct pRREiresLucGA, pPREiresLucGA and pCTEiresLucGA, respectively. HSV-TK's 2xTK49 PPE, a duplicated subelement of TK119

**Table 1.** Sequences of primers used for cloning RRE, CTE, PRE, PPE and CJE

Primer name	Sequence (5' → 3')
PRE forward	GGGGAATTCACATGTAAACAGGCCTATTGATTG
PRE reverse	GGGGAATTCACATGTTCCCTTATGCAAGACCT
TK119 PPE forward	GGGGAATTCATGGATGACTTACTGGCAGGTG
TK119 PPE reverse	GGGGAATTCATGGGGCGCTTGTCAATTACCAC
RRE forward	GGGCCATGGAATTCAGTAGGAGCTATGTTCC
RRE reverse	TTTCCATGGAATTCCTAGGAGCTGTTGATCCC
CTE forward	TTTCCATGGAATTCAGACTGGACAGCCAATGA
CTE reverse	TTTCCATGGAATTCACACATCCCCTCGGAGGCT
CJE forward	GGGGAATTCATGGATGACTGCAAAAGATG
CJE reverse	GGGGAATTCATGGAAATGTTTGAACCTG



**Figure 1.** Schematic diagrams showing structures of plasmids and probes used in this study. (A) Structures of plasmids containing insertions of MPMV's CTE, HBV'S PRE, HSV-TK's PPE, HIV-1's RRE or the human cellular *c-Jun* gene's enhancer (CJE) into either the NcoI site in exon I or the EcoRI site in exon III of a cDNA variant of the human  $\beta$ -globin gene. All numbers are given relative to the site of transcription initiation from each gene, with those of the inserted elements denoting the inserted sequences. Ex, exon; N, NcoI; B, BamHI; E, EcoRI; S, SspI. (B) Structure of the human  $\beta$ -globin and human  $\beta$ -actin probes used for quantitative S1 nuclease mapping analysis. Probes were amplified by PCR and end-labeled with  $^{32}\text{P}$ , indicated by the asterisk, as described in Methods. The sizes of the DNA fragments resulting from protection by hybridization with the corresponding RNAs are indicated.

PPE (48), and its mutation variant 2xTK49LS0 were PCR amplified from pSP72/2xTK49 and pSP72/2xTK49LS0 (48), respectively, and inserted into the EcoRI site of pGLiresLucGA to construct p2xTK49wtPPEiresLucGA and p2xTK49LS0PPEiresLucGA, respectively.

### RNA purification and S1 nuclease mapping analysis

Cells were harvested 48 h post-transfection. Nuclei and cytoplasm were separated by treatment with 0.5% NP-40 as described previously (36,42). RNA from these fractions was purified using TRIzol reagent following the manufacturer's directions (Gibco). The relative amounts of globin-like RNA accumulated in the nuclear and cytoplasmic fractions were determined by quantitative S1 nuclease mapping as described previously (36) and quantified with a PhosphorImager. Cellular  $\beta$ -actin RNA served as an internal control for recovery of the RNA samples (36,44). Southern blot analysis of the relative amounts of DpnI-resistant,  $\beta$ -globin-encoding plasmid

DNA present in the nuclear and cytoplasmic fractions prior to treatment with DNase I was performed as described previously (6,42) to assay both relative transfection efficiencies and contamination of cytoplasmic RNA with nuclear nucleic acid. Contamination of nuclear RNA by cytoplasmic RNA was determined by northern blot analysis of 18S ribosomal RNA. The occasional sample with >20% cross-contamination of nuclear RNA with cytoplasmic RNA or *vice versa* was discarded.

The probes used in the S1 nuclease mapping assays shown in Figure 1B were prepared by PCR amplification followed by 5' end-labeling with T4 DNA kinase or 3' end-labeling with Klenow polymerase after cleavage with EcoRI endonuclease. The templates used for synthesis of the 5' end-labeled  $\beta$ -globin and  $\beta$ -actin probes are indicated in Figure 1B. The template used for synthesis of the 3' end-labeled  $\beta$ -globin probe was prepared by subcloning into the EcoRV site of pGEM5z(+) (Promega) an SspI fragment (460 bp) of p $\beta$ - $\beta$ 1(+)(+)(+) which contains part of intron II, exon III and an additional 27 bp downstream of the site of cleavage for polyadenylation. S1 nuclease mapping assays were performed as described previously (36,44).

### RT-PCR analysis

Prior to RT-PCR analysis, each RNA sample was treated with RNase-free DNase I (Ambion) to degrade contaminating DNA. Reverse transcription (RT) reactions contained 300 ng RNA, 20 U AMV reverse transcriptase (Roche), and 15 pmol  $\beta$ -globin reverse primer (5'-CCAGATGCTCAAGGCC-3'), corresponding to the 3' end of the  $\beta$ -globin gene, in a total volume of 20  $\mu\text{l}$ . After incubation at 42°C for 1 h, the reaction mixture was incubated at 99°C for 5 min. The PCR reaction was performed as described previously (34) with Taq DNA polymerase (Promega) and the  $\beta$ -globin forward primer (5'-ACATTTGCTTCTGACACAACCTG-3'), which corresponds to the 5' end of  $\beta$ -globin mRNA, using a thermal cycler (Techne), with denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 2 min for 40 cycles (36).

### Luciferase assays

Forty-eight hours after transfection of COS-M6 cells, luciferase activities were determined with a Dual-Glu Luciferase Assay system (Promega) according to the manufacturer's protocol. Each plasmid was assayed in triplicate on at least three different occasions. Data were normalized both to the co-transfected plasmid phRL-TK (Promega) and to pGLiresLucGA transfected in parallel.

## RESULTS

### PRE and PPE, but not CTE or RRE, enhance cytoplasmic accumulation of intronless $\beta$ -globin-like transcripts

To test whether MPMV's CTE or HIV's RRE can provide the same functions as HBV's PRE and HSV's PPE, we constructed plasmids containing the CTE, RRE, PRE or PPE inserted into exon I of p $\beta$ - $\beta$ 1(-)(-)(-), a plasmid containing a cDNA version of the human  $\beta$ -globin gene including the  $\beta$ -globin promoter and polyadenylation signals (Figure 1A). Each

plasmid was transiently co-transfected in parallel into CV-1PD cells together with pRSV-Tori, a plasmid encoding the SV40 large T antigen. The presence of the latter plasmid results in replication of the test plasmid to high copy number in the nucleus. Thus, transcription of the  $\beta$ -globin gene occurs at high levels, enabling the resulting  $\beta$ -globin-like RNAs to be readily analyzed by quantitative S1 nuclease mapping. Transfection efficiencies and cross-contamination of the nuclear and cytoplasmic fractions were determined for all samples by Southern blot analysis of replicated, nuclear DpnI-resistant globin plasmid DNA and northern blot analysis of cellular 18S rRNA (data not shown). Globin RNA levels were normalized to globin DNA levels. The occasional sample with >20% cross-contamination of nuclear RNA with cytoplasmic RNA or vice versa was discarded.

As expected (5,36,43,49,50), the absence of introns in the  $\beta$ -globin primary transcripts resulted in cytoplasmic accumulation of  $\beta$ -globin mRNA to approximately one-sixtieth the level observed with the wild-type, intron-containing transcripts (Figure 2A, lane 4 versus lane 2) even though precise deletion of the  $\beta$ -globin gene's introns does not significantly change the rate of transcription of this gene as determined by nuclear run-on assays (49,50). As expected (5,36), the transcripts synthesized from the cDNA version of the  $\beta$ -globin gene were also highly defective in stabilization within the nucleus and cleavage for polyadenylation compared to the ones synthesized from the wild-type parental gene (Figure 2A and B, lanes 3 and 4 versus lanes 1 and 2). Insertion of the 154-base CTE of MPMV into exon I led to a 0.6-fold increase in cytoplasmic accumulation of RNA synthesized from the cDNA version of the  $\beta$ -globin gene (Figure 2A, lane 6 versus lane 4). On the other hand, insertion of a 722-base version of HBV's PRE or one copy of the 120-base version of HSV-TK's PPE into exon I resulted in 7.6-fold and 4.2-fold more  $\beta$ -globin-like mRNA, respectively, accumulating in the cytoplasm from intronless primary transcripts (Figure 2A, lane 10 and lane 14 versus lane 4, respectively). Thus, contrary to the results obtained with the PRE, PPE and histone H2a elements (32), the CTE barely functions, if at all, to enhance intron-independent gene expression, at least when located near the 5' end of the primary transcript. This small, statistically insignificant increase observed with the CTE could easily be due to experimental variability or small changes in the half-life of the globin-like RNA as a consequence of the alteration in its primary sequence. Likewise, insertion of HIV-1's RRE into exon I of p $\beta$ - $\beta$ 1(-)2(-) together with co-expression of HIV's Rev protein also led to little, if any change in cytoplasmic accumulation of the intronless RNA (Figure 2A, lane 18 versus 4). Similar results were observed when these plasmids were transfected into COS-M6 cells (data not shown). Previous studies of others have shown that the RRE, CTE, PRE and PPE can all enhance nucleocytoplasmic export in CV-1 and COS cells of an intron-containing mRNA derived from the HIV genome without excision of the intron (28,32,51,52). Thus, the RRE and CTE can function in these cell lines in the context of some, but not all genes.

To test the possibility that the PRE or PPE enhanced cytoplasmic accumulation of the  $\beta$ -globin-like RNA because of the presence of cryptic splice sites within these elements, we performed RT-PCR assays with primers corresponding to sequences immediately adjacent to the 5' and 3' ends of

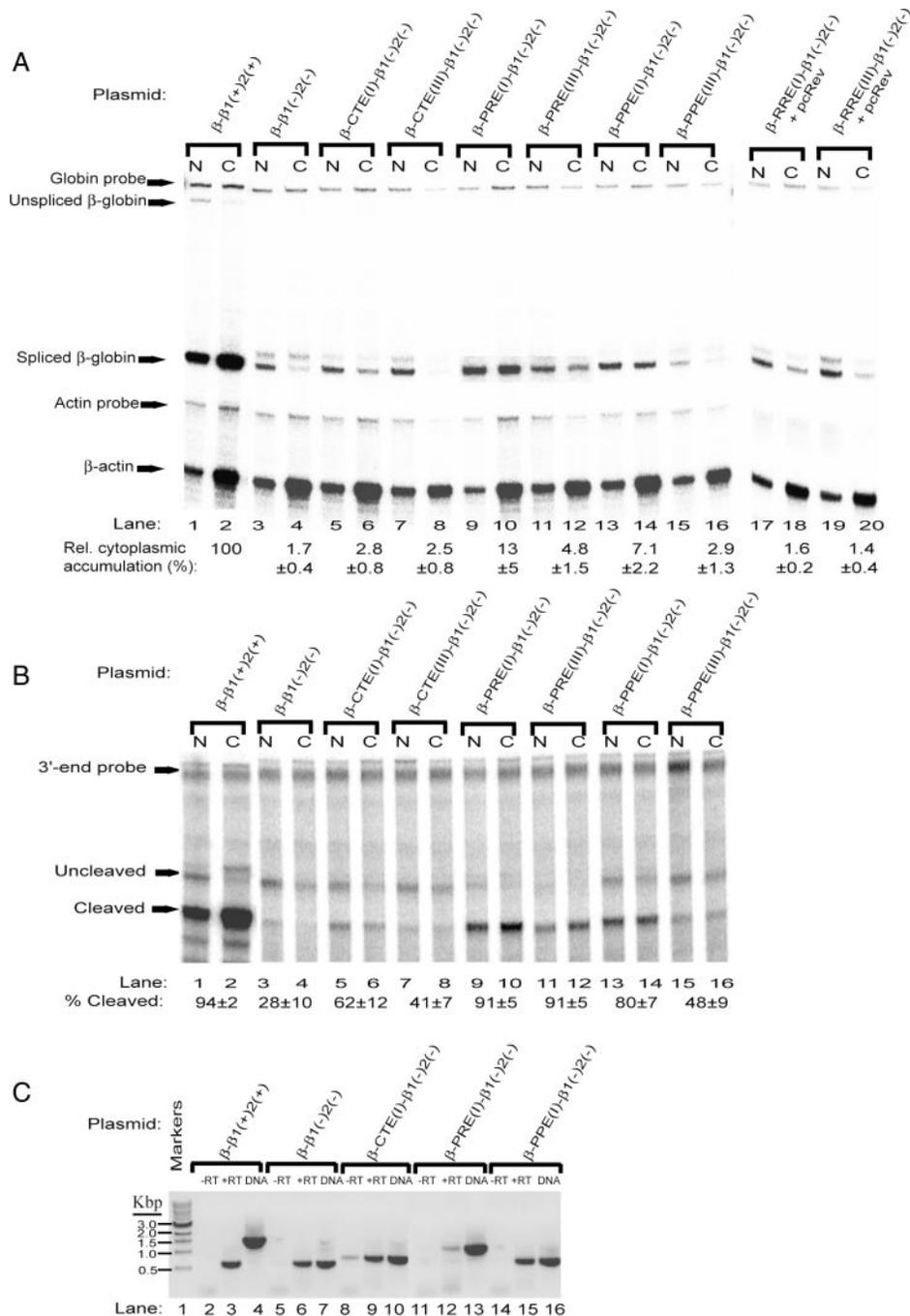
these RNAs. The globin-like RNAs that accumulated in the cytoplasm of cells transfected with p $\beta$ -CTE(I)- $\beta$ 1(-)2(-), p $\beta$ -PRE(I)- $\beta$ 1(-)2(-) and p $\beta$ -PPE(I)- $\beta$ 1(-)2(-) were similar in size to their corresponding template DNAs (Figure 2C). No smaller bands corresponding to products of cryptic splicing within the gene were observed. Primer extension analysis did not indicate any utilization of alternative, upstream 5' ends (data not shown). Cryptic polyadenylation followed by splicing to sequences situated downstream of the known cleavage site for polyadenylation also could not have accounted for a significant portion of the accumulated RNA since the relative amounts of globin-like RNA present in the cytoplasm as quantified with this 5' end probe were similar to the relative amounts as quantified with a 3' end probe (Figure 2B below). Therefore, we conclude that most, if not all of the globin-like RNA accumulated in the cytoplasm was processed and transported without being spliced.

The failure of the CTE to significantly enhance cytoplasmic accumulation of the globin-like mRNA in the above experiment might be a consequence of insertion of the CTE into an unfavorable context near the 5' end of the primary transcript, rather than its needing an intron to function. To distinguish between these two possibilities, the above experiment was repeated with the CTE, PRE and PPE inserted within exon III, rather than exon I, of the cDNA variant of the  $\beta$ -globin gene (Figure 1A). The PRE still functioned, albeit less well than it did in the 5' position (Figure 2A, lane 12 versus lane 10). The PPE no longer significantly enhanced globin RNA accumulation (Figure 2A, lane 16), confirming prior findings (36). The CTE and RRE also failed to significantly enhance cytoplasmic accumulation of the intronless transcripts (Figure 2A, lane 8 and lane 20, respectively). Thus, we conclude that the location of these elements does, indeed, affect their ability to enhance mRNA biogenesis in the absence of introns; however, whereas the PRE and PPE can significantly enhance cytoplasmic accumulation of intronless RNA when located near the 5' end of the primary transcript, the CTE and RRE fail to do so from either a 5' or 3' location.

### Effects of PRE, PPE and CTE on 3' end processing of intronless transcripts

One hypothesis to explain the difference in the functional activities of these elements is that the CTE and RRE primarily function to enhance nucleocytoplasmic export of intron-containing RNAs in the absence of intron excision, with proper 3' end formation being determined in large part by the 3'-terminal intron normally present in these retroviral transcripts defining the 3'-terminal exon and site of cleavage for polyadenylation. On the other hand, the PRE and PPE, coming from naturally intronless genes, substitute for introns by enhancing proper 3' end formation as well as nucleocytoplasmic export.

To begin to test this hypothesis, the RNA samples from the above experiment were also analyzed by quantitative S1 nuclease mapping with a <sup>32</sup>P-labeled, 3' end probe (Figure 1B). Most of the  $\beta$ -globin RNA accumulated in cells transfected with p $\beta$ - $\beta$ 1(+)-2(+), the intron-containing genomic version of the  $\beta$ -globin gene, was processed at the  $\beta$ -globin polyadenylation site (Figure 2B, lanes 1 and 2). Some of the polyA<sup>+</sup> RNA observed in the nuclear fraction (Figure 2B, lane 1) was, likely,



**Figure 2.** Effects of the presence of a PRE, PPE, CTE or RRE in obviating the intron requirement for efficient 3' end processing and cytoplasmic accumulation of human  $\beta$ -globin-like RNA. (A) Autoradiogram of quantitative S1 nuclease mapping analysis of the human  $\beta$ -globin-like RNAs accumulated in the nucleus and cytoplasm of CV-1PD cells transfected with the plasmids shown in Figure 1A. CV-1PD cells were co-transfected with 2  $\mu$ g of the indicated plasmid along with 1  $\mu$ g of the SV40 T antigen-encoding plasmid pRSV-Tori. Nuclear (N) and cytoplasmic (C) RNAs were harvested 48 h later and analyzed by concurrent quantitative S1 nuclease mapping with the 5' end-labeled  $\beta$ -globin and  $\beta$ -actin probes shown in Figure 1B. Transfection and RNA fractionation efficiencies were analyzed as described in Methods. The amount of  $\beta$ -globin-like RNA accumulated in the cytoplasm was internally normalized to the amount of cellular  $\beta$ -actin RNA present in the same sample. The numbers shown below the lanes are the percentages relative to the amount of  $\beta$ -globin-like RNA accumulated in the cytoplasm of cells transfected in parallel with  $\beta$ - $\beta 1(+)$ 2(+) RNA, with normalization as well to the relative amounts of replicated  $\beta$ -globin plasmid DNA present in the nuclear fractions of those samples. These numbers are mean  $\pm$  SEM values of data obtained from five independent experiments similar to the one shown here. (B) Autoradiogram of quantitative S1 nuclease mapping analysis of the 3' ends of the  $\beta$ -globin-like RNAs accumulated in the nucleus and cytoplasm of CV-1PD cells. The RNA samples from the experiment in panel A were analyzed with the 3' end-labeled probe shown in Figure 1B. The numbers below the pairs of lanes are mean  $\pm$  SEM values of the cleaved plus uncleaved RNA (N + C) times 100% of data obtained from three independent experiments similar to the one shown here. (C) The  $\beta$ -globin-like RNAs accumulated in the cytoplasm are unspliced. Portions of the cytoplasmic RNA samples from the experiment shown in (A) were reverse-transcribed and then amplified by PCR with the primers described in Methods (+RT, lanes 3, 6, 9, 12 and 15). As controls, PCR amplification reactions were performed on the RNA samples without prior reverse transcription (-RT, lanes 2, 5, 8, 11 and 14) and on the plasmid DNAs used in the transfections (DNA, lanes 4, 7, 10, 13 and 16). Shown here is a photograph of an ethidium bromide-stained 1% agarose gel in which the PCR products were electrophoresed. Lane 1 contained 1 kilobase pair ladder DNA as size markers.

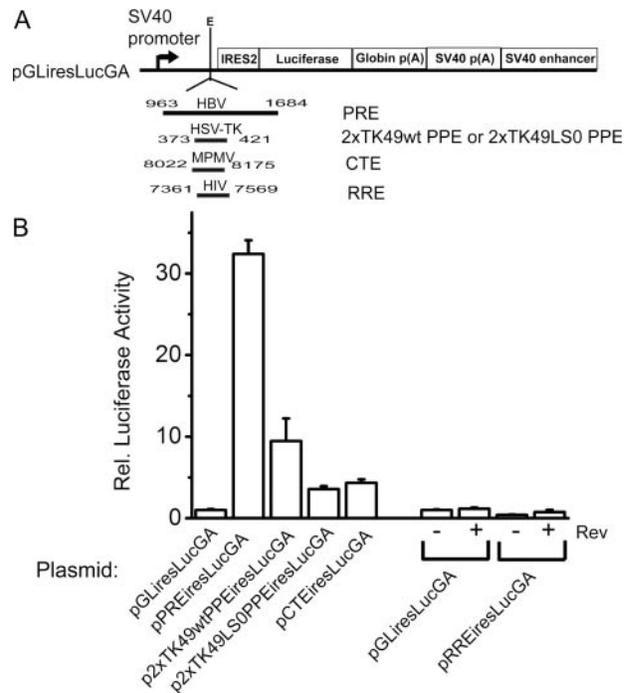
actually from the cytoplasm since nuclear fractions are usually contaminated with perinuclear material (53). As expected, cells transfected with p $\beta$ - $\beta$ 1(-)2(-), the cDNA variant, contained as much uncleaved primary transcript as the genomic-transfected cells did, but little RNA that had been cleaved at the site of polyadenylation (Figure 2B, lanes 3 and 4 versus lanes 1 and 2). Some of the uncleaved RNA observed in this and many of the other cytoplasmic samples (Figure 2B) was, probably, due to contamination with some nuclear material. However, since nuclear contamination was at most 20% as assayed by the presence of DpnI-resistant  $\beta$ -globin plasmid DNA (data not shown), at least some of this uncleaved RNA must have been exported to the cytoplasm despite not having been polyadenylated [see also (40)].

When either the PRE or PPE was present in exon I of p $\beta$ - $\beta$ 1(-)2(-), most of the accumulated  $\beta$ -globin-like mRNA was properly cleaved at the  $\beta$ -globin polyadenylation site (Figure 2B, lanes 9, 10, 13 and 14). On the other hand, the presence of the CTE at this same site in exon I led to a much smaller increase in the amount of globin-like RNA properly cleaved at the  $\beta$ -globin polyadenylation site (Figure 2B, lanes 5 and 6). Likewise, whereas 3' end processing also occurred very efficiently when the PRE was present in exon III (Figure 2B, lanes 11 and 12), it occurred quite inefficiently when either the PPE or CTE was present in this same site in exon III (Figure 2B, lanes 15 and 16 and lanes 7 and 8, respectively). These findings correlated well with the results obtained above for enhancement of cytoplasmic accumulation (Figure 2A). Furthermore, the relative amounts of globin-like RNA present in the cytoplasm as quantified with this 3' end probe were similar to the relative amounts as quantified with the 5' end probe. Thus, it was unlikely that cryptic polyadenylation or splicing downstream of the known cleavage site for polyadenylation accounted for a significant portion of the accumulated RNA. Similar results were observed when these plasmids were transfected into COS-M6 cells (data not shown). Thus, we conclude that the PRE and PPE, but not the RRE and CTE, can play significant roles in facilitating 3' end formation.

### Effects on expression of other intronless genes

Another plausible explanation for the observation that the PRE and PPE enhanced intronless  $\beta$ -globin gene expression while the CTE and RRE did not is that these findings are specific to the precise sequence context or gene. However, Lu and Cullen (40) have recently reported that HBV's PRE is much more efficient than either the CTE or RRE in the presence of Rev in rescuing expression of an intronless gene, in their case a chimeric  $\beta$ -globin/preproinsulin II one in which the element was inserted into the 3'-untranslated region (UTR) of the preproinsulin II sequences. To further test the generality of the differences observed in the functional activities of these elements in intronless versus intron-containing genes, we also inserted them into the 5'-UTR of pGLiresLucGA (Figure 3A). Plasmid pGLiresLucGA contains an intronless gene with an internal ribosome entry site (IRES) situated between its 5'-UTR and luciferase-coding sequence. Thus, alterations in the sequence of its 5'-UTR should have minimal effects on translation of its coding region.

As expected, insertion of the PRE enhanced expression of this intronless gene, in this case a very dramatic 32-fold



**Figure 3.** Effects of PRE, PPE, CTE and RRE in the presence of Rev on expression of an intronless luciferase reporter gene. (A) Structures of pGLiresLucGA and variants of it containing the indicated sequences inserted into the EcoRI site within the 5'-untranslated region. E, EcoRI. (B) Relative luciferase activities of COS-M6 cells transiently transfected for 48 h with the indicated plasmids. The four rightmost samples were co-transfected as well with the Rev-encoding plasmid pCRev (+) or its parental vector pBcCMV (-). Data are mean + SEM values from three independent transfections, each performed in triplicate.

(Figure 3B). Insertion of HSV-TK's 2xTK49 PPE (48) enhanced expression 9-fold, while its cluster base substitution mutant variant, 2xTK49LS0, enhanced expression only 3-fold. Again, the RRE in the presence of Rev completely failed to enhance expression of yet another intronless gene. The presence of the CTE enhanced luciferase synthesis 4-fold; however, much, if not all of this effect could have been at the level of translation (52) rather than mRNA biogenesis.

Thus, the failure of the CTE and RRE to function well in enhancing expression of intronless genes is independent of position, sequence context and gene. We conclude that the PRE and PPE probably enhance cytoplasmic accumulation of intronless transcripts by facilitating 3' end processing as well as export. On the other hand, the CTE and RRE enhance cytoplasmic accumulation of intron-containing transcripts by facilitating nucleocytoplasmic export, but fail to enhance cytoplasmic accumulation of intronless transcripts because they cannot compensate well for the defect in 3' end processing.

### Elements within the naturally intronless *c-Jun* gene also enhance 3' end processing and cytoplasmic accumulation of intronless transcripts

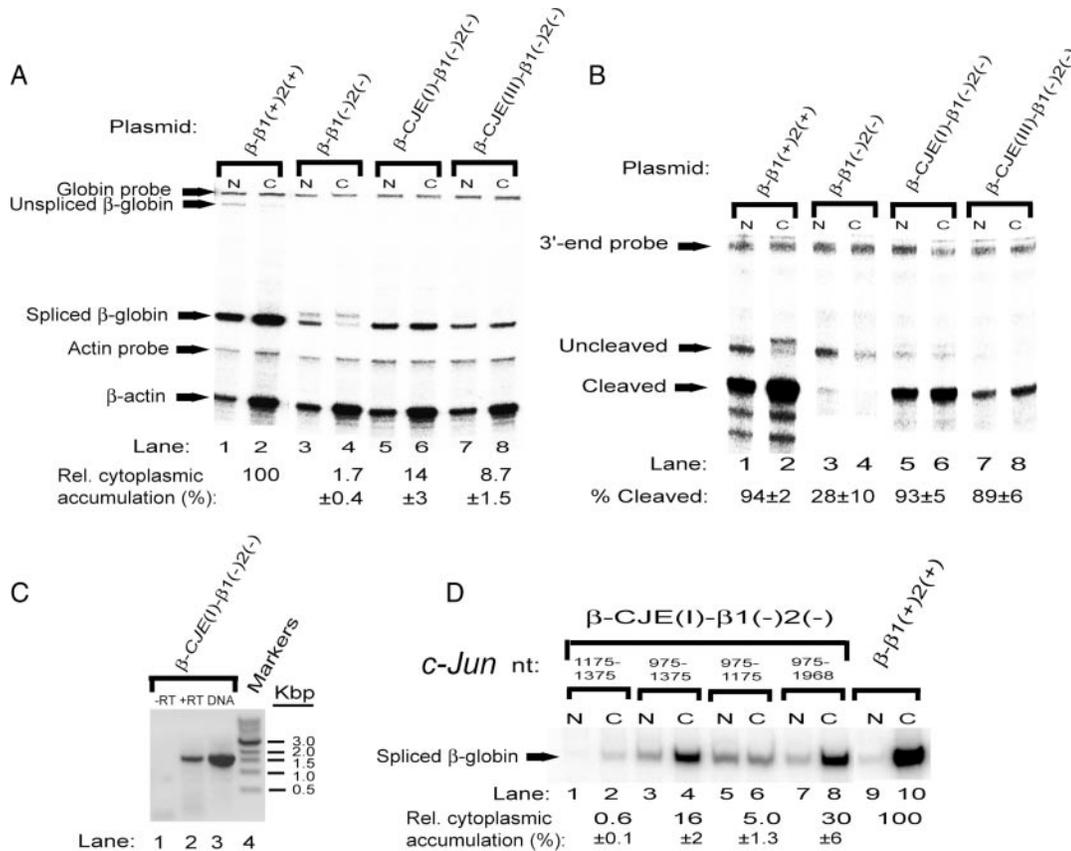
To test whether naturally intronless cellular genes also contain elements that enhance 3' end processing and cytoplasmic accumulation of transcripts lacking introns, we inserted part of the protein-coding region of the human *c-Jun* gene into

either exon I or exon III of  $\beta\text{-}\beta 1(-)2(-)$  (Figure 1A). These plasmids were transfected into CV-1PD cells together with pRSV-Tori, and the accumulated RNAs were analyzed as described above. The presence of the *c-Jun* sequences led to 8.4-fold more  $\beta$ -globin-like mRNA accumulating in the cytoplasm (Figure 4A, lane 6 versus 4), along with highly efficient, proper 3' end processing of the intronless transcripts (Figure 4B, lanes 5 and 6 versus lanes 3 and 4). Once again, this enhanced cytoplasmic accumulation of the RNA was less dramatic when this element(s) was inserted into exon III (Figure 4A, lane 8 versus 6). This enhancement occurred with neither cryptic splicing (Figure 4C) nor cryptic polyadenylation, the latter concluded, in part, from the relative amounts of RNA quantified with the two probes being similar. A single copy of the 201-base nucleotides 975–1175 region of *c-Jun* was sufficient to provide several-fold enhancement in intronless gene expression (Figure 4D, lane 6 versus lane 2). Furthermore, as with the PRE (28) and PPE (36), even greater enhancement occurred when a larger region of *c-Jun* was present (Figure 4D, lane 6 versus lane 4 versus lane 8). Similar

results were observed when these plasmids were transfected into COS-M6 cells (data not shown). Thus, we conclude that the naturally intronless human *c-Jun* gene contains two or more elements comparable in function to the previously identified viral ones that enhance stabilization, 3' end cleavage and polyadenylation, and nucleocytoplasmic export of intronless transcripts. We name these elements CJEs, for *c-Jun* processing enhancers.

**Effects of presence of intron on functioning of PPE-like elements**

The CTE, PRE and PPE enhance nucleocytoplasmic export of an intron-containing reporter derived from the HIV-1 genome in the absence of intron excision when located within or 3' of the intron (32,39). To examine whether these elements also enhance 3' end processing and export without intron excision when placed 5' of an intron, we inserted them into  $\beta\text{-}\beta 1(+ )2(-)$ , a variant of the human  $\beta$ -globin gene retaining its first intron but lacking its second intron (Figure 1A).



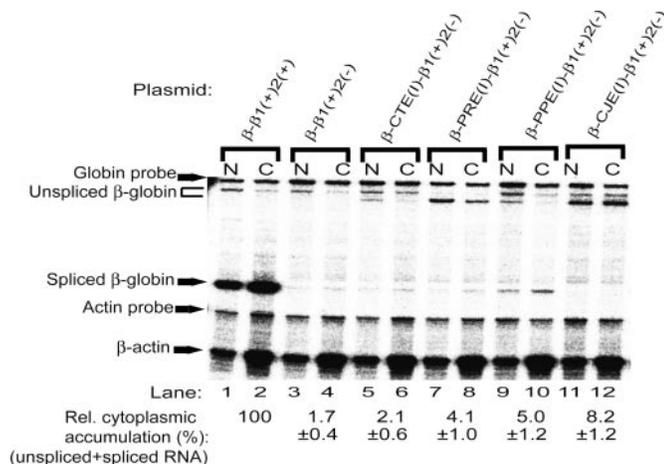
**Figure 4.** The human *c-Jun* gene contains elements that enhance stabilization, 3' end processing and cytoplasmic accumulation of intronless transcripts. (A) Autoradiogram of quantitative S1 nuclease mapping analysis of  $\beta$ -globin-like RNAs accumulated in the nucleus (N) and cytoplasm (C) 48 h after co-transfection of CV-1PD cells with the indicated plasmids and pRSV-Tori. The S1 nuclease mapping probes were the 5' end-labeled ones shown in Figure 1B. The samples were analyzed as described in the legend to Figure 2A. The numbers below the lanes are mean  $\pm$  SEM values of data obtained from three independent experiments similar to the one shown here. (B) Autoradiogram of quantitative S1 nuclease mapping analysis of the 3' ends of the  $\beta$ -globin-like RNAs accumulated in the nucleus and cytoplasm of CV-1PD cells. Portions of the RNA samples from the experiment shown in (A) were mapped with the 3' end-labeled probe shown in Figure 1B. The numbers below the lanes are mean  $\pm$  SEM values of data obtained from three independent experiments similar to the one shown here; they were calculated as described in the legend to Figure 2B. (C) The  $\beta$ -globin-like RNAs accumulated in the cytoplasm are unspliced. A portion of the RNA sample from the experiment shown in (A), lane 6, was reverse-transcribed and amplified by PCR as described in Figure 2C. Shown here is a photograph of an ethidium bromide-stained 1% agarose gel in which the PCR products were electrophoresed. The rightmost lane contains a 1 kilobase pair DNA ladder. (D) Autoradiogram of quantitative S1 nuclease mapping analysis showing that a 201 nt region of *c-Jun* is sufficient to enhance cytoplasmic accumulation of intronless  $\beta$ -globin-like transcripts. COS-M6 cells were transfected with the indicated plasmids and processed as described in (A).

These plasmids were co-transfected into CV-1PD cells together with pRSV-Tori. The  $\beta$ -globin-like RNAs accumulated in the cells by 48 h post-transfection were analyzed as described above.

As noted previously (44), the absence of the second intron led to defects in stabilization of the primary transcript, intron excision and cytoplasmic accumulation of the mRNA (Figure 5, lanes 3 and 4 versus lanes 1 and 2) even though the first intron was present. Interestingly, the CTE, PRE, PPE and CJE behaved differently in their abilities to rescue these defects. Once again, the CTE failed to significantly enhance cytoplasmic accumulation (Figure 5, lane 6 versus lane 4). On the other hand, the PRE enhanced cytoplasmic accumulation, doing so largely in the absence of intron excision (Figure 5, lane 8). The PPE also significantly enhanced cytoplasmic accumulation, but with efficient removal of the intron (Figure 5, lane 10). Strikingly, the CJE was the most efficient of these elements at enhancing cytoplasmic accumulation, doing so in the complete absence of intron excision (Figure 5, lane 12). The appearance of the smaller of the two unspliced bands is an artifact of S1 nuclease sometimes cleaving the 5' end-labeled  $\beta$ -globin probe opposite the sequences inserted at the NcoI site as well as at the 5' end of the transcripts. Therefore, we conclude that these elements probably function in mRNA biogenesis at least in part via different mechanisms, pathways or kinetics.

#### Cell type-specific effects on functioning of PPE-like elements

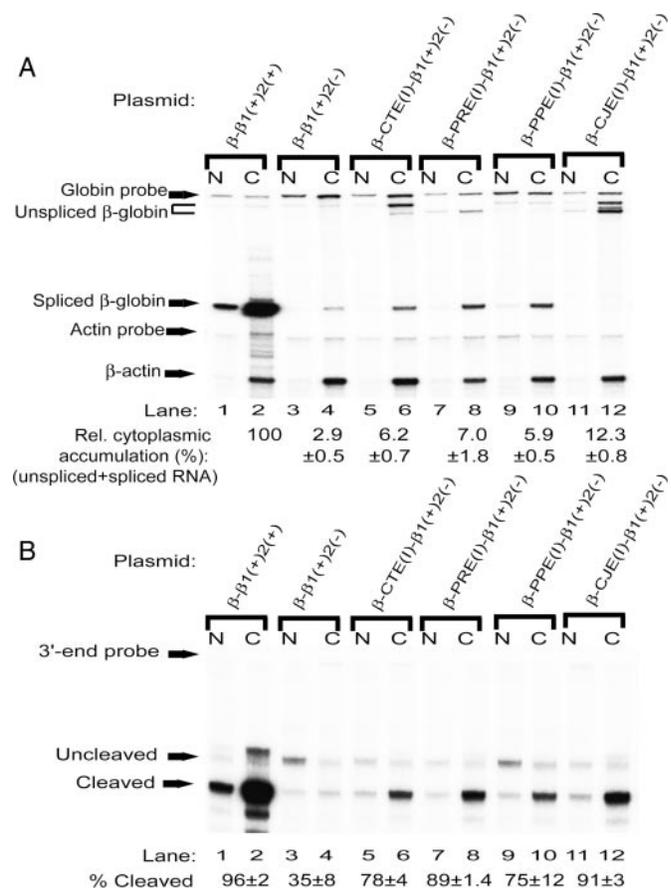
To further examine the relationships among proper 3' end formation, intron excision and cytoplasmic accumulation of the  $\beta$ -globin-like mRNAs, we also transfected these



**Figure 5.** Effects of presence of the first intron of the human  $\beta$ -globin gene on functioning of CTE-like elements in CV-1PD cells. Shown here is an autoradiogram of quantitative S1 nuclease mapping analysis of the  $\beta$ -globin-like RNAs accumulated in the nucleus and cytoplasm of CV-1PD cells co-transfected with the indicated plasmids together with pRSV-Tori. The nuclear (N) and cytoplasmic (C) RNAs were harvested 48 h after co-transfection and analyzed as described in Figure 2 by concurrent S1 nuclease mapping with  $\beta$ -actin and  $\beta$ -globin 5' end-labeled probes. The numbers shown below the lanes are mean  $\pm$  SEM values of data obtained from three independent experiments similar to the one shown here. Accumulation was measured as the sum of unspliced plus spliced  $\beta$ -globin RNA present in the cytoplasm, with normalizations to  $\beta$ -actin, replicated  $\beta$ -globin DNA and  $\beta$ -globin RNA made from genomic plasmid calculated as described in Figure 2.

$\beta$ - $\beta 1(+)$ 2(-)-based plasmids into COS-M6 cells. COS-M6 cells are an SV40-transformed derivative of CV-1 cells from which CV-1PD cells were derived as well. These cells constitutively express SV40 large T antigen, but at a significantly lower level than is achieved when CV-1PD cells are co-transfected with pRSV-Tori. They also exhibit a highly transformed phenotype whereas CV-1PD cells exhibit contact inhibition when cultured in dishes.

Transcripts synthesized from  $\beta$ - $\beta 1(+)$ 2(-) still accumulated very inefficiently in the cytoplasm in COS-M6 cells, albeit spliced (Figure 6A, lane 4), with the majority of them not properly cleaved for polyadenylation (Figure 6B, lanes 3 and 4). Insertion of the CTE led to a doubling in the cytoplasmic accumulation of the  $\beta$ -globin-like RNA (Figure 6A, lane 6) and a large enhancement in proper 3' end cleavage (Figure 6B, lanes 5 and 6), with the majority of these RNAs retaining the first intron (Figure 6A, lane 6). Insertion of either the PRE or PPE also led to significant enhancement in both cytoplasmic accumulation of  $\beta$ -globin-like RNA (Figure 6A, lane 8 and lane 10, respectively) and proper 3' end cleavage (Figure 6B, lanes 7 through 10). In these latter cases, the intron was efficiently excised as well (Figure 6A). Insertion of the CJE led to



**Figure 6.** Cell type-specific effects of CTE-like elements on (A) splicing and cytoplasmic accumulation, and (B) proper 3' end cleavage of  $\beta$ -globin-like RNAs. Shown here are autoradiograms of quantitative S1 nuclease mapping analyses of the human  $\beta$ -globin-like RNAs accumulated in the nucleus (N) and cytoplasm (C) of COS-M6 cells co-transfected with the indicated plasmids. The RNA samples were processed and analyzed as described in Figure 2. The numbers shown below the lanes are mean  $\pm$  SEM values of data obtained from three independent experiments similar to the one shown here.

the greatest enhancement in cytoplasmic accumulation of  $\beta$ -globin-like RNA (Figure 6A, lane 12), with highly efficient 3' end cleavage (Figure 6B, lane 12). However, as opposed to the PRE and PPE, it enabled these steps in mRNA biogenesis to occur quite efficiently in the total absence of excision of the first intron (Figure 6A, lane 12). Thus, we conclude that these elements modulate pre-mRNA processing and nucleocytoplasmic export in a cell-type-dependent manner.

**DISCUSSION**

In this report, we systematically analyzed the effects of RNA elements from several viruses, MPMV's CTE, HIV's RRE, HSV-TK's PPE, HBV's PRE, and one cellular gene, *c-Jun*'s CJE, on processing and cytoplasmic accumulation of transcripts synthesized from variants of the highly intron-dependent human  $\beta$ -globin gene. We showed that the PRE, PPE and CJE, but not the RRE and CTE, can significantly rescue defects in mRNA biogenesis from  $\beta$ -globin gene variants containing either no introns (Figures 2 and 4) or only the first intron, sometimes doing so without intron excision (Figures 5 and 6). They enhance steady-state mRNA levels, in part, by facilitating 3' end processing (Figures 2C, 4B and 6B; summarized in Figure 7). The different effects on intronless gene expression are independent of position (Figure 2) and gene (Figure 3). Thus, we conclude that the need for mechanisms to facilitate proper 3' end formation and nucleocytoplasmic export, not the presence of introns or their excision *per se*, is required for mRNA biogenesis in high eukaryotes. While PPE-like elements from intronless genes facilitate both these crucial steps in mRNA biogenesis regardless of the presence of introns, CTE-like elements from intron-containing genes are

deficient in facilitating proper 3' end formation given the gene's 3'-terminal intron is normally available to provide this function.

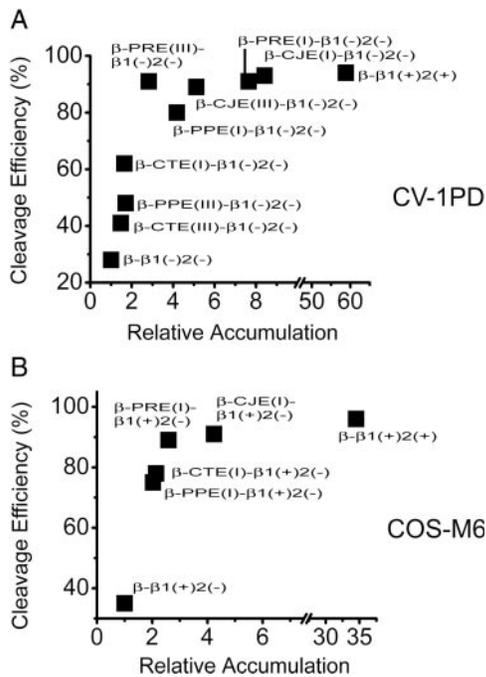
**$\beta$ -globin mRNA biogenesis requires a mechanism for proper 3' end formation, not the presence of introns or their excision**

$\beta$ -globin primary transcripts lacking both the  $\beta$ -globin's introns or only the 3'-terminal second intron are defective in RNA stability, proper 3' end formation and nucleocytoplasmic export [(5,36,44,49,50); Figures 2 and 5]. Interestingly, intron 1 was excised from human  $\beta$ -globin transcripts lacking intron 2 (Figure 6A, lane 4), yet accumulation (Figure 6, lane 4) and 3' end cleavage for polyadenylation (Figure 6B, lane 4) remained defective in COS-M6 cells. Insertion of the CJE dramatically enhanced both cytoplasmic accumulation and 3' end processing, doing so in the complete absence of intron excision (Figures 5 and 6, lanes 12). Polyadenylation usually precedes splicing in higher eukaryotes (7,42), with the presence of the 3'-terminal intron's branchpoint, polypyrimidine tract and 3' splice site influencing the efficiency of polyadenylation [(7,54), and references cited therein]. CTE-like elements then enable nucleocytoplasmic export to occur prior to intron excision. Taken together, these findings indicate that the existence of a mechanism for proper 3' end processing, not the presence or excision of introns *per se*, is a major requirement for biogenesis of human  $\beta$ -globin mRNA. This conclusion is also consistent with findings of others indicating coupling of 3' end processing with surveillance (55,56).

The effect of splicing on nucleocytoplasmic transport of mRNAs remains controversial. While splicing enhances mRNA export in *Xenopus* oocytes (3,57), only marginal effects of splicing on nucleocytoplasmic distribution of mRNAs were found in transient transfection assays in mammalian cells (11,40). Intronless  $\beta$ -globin transcripts are defective in mRNA export [(5,32,36,43,49,50); Figure 2A]. Probably, differences in cell types, transcriptional promoters, specific sequences within the mRNAs and polyadenylation signals all affect the degree to which mRNA export is dependent upon splicing.

**PRE, PPE and CJE functions are distinguishable from CTE and RRE ones**

Since mRNA biogenesis involves multiple coupled post-transcriptional steps, it was not surprising to find that the elements studied here were functionally distinguishable. After completion of this study, Lu and Cullen (40) published that HBV's PRE is better than either MPMV's CTE or HIV-1's RRE in rescuing expression of an intronless  $\beta$ -globin-like gene. The quantitative differences between their and our findings are probably due to differences in the cell lineages, promoters and polyadenylation signals used in the two sets of experiments. Lu and Cullen (40) also reported the converse that HBV's PRE fails to rescue expression of Gag encoded by a Rev-negative HIV-1 provirus that can be rescued by MPMV's CTE or HIV-1's RRE in the presence of Rev. HSV-TK's PPE, likewise, fails in this latter assay (K. Boris-Lawrie, X. Liu, and J.E. Mertz, unpublished data). Taken together, we conclude that PPE-like elements from intronless genes provide partially different functions in



**Figure 7.** Summary of the relationship observed here between efficiency of cleavage for proper 3' end formation and cytoplasmic accumulation of  $\beta$ -globin-like RNAs. Data were compiled from (A) Figures 2 and 4 and (B) Figure 6.

mRNA biogenesis than do CTE-like elements from intron-containing genes. Unfortunately, one cannot precisely determine solely by the accumulation assays used here and elsewhere by others which steps are being facilitated by each of these elements. Given that coupling exists among all of the steps in mRNA biogenesis (1–3), defects in any one step will be observed at multiple levels *in situ*. Thus, definitive conclusions regarding the functionality of these elements require studies employing cell-free systems in which these steps have been uncoupled.

### **PPE-like elements can substitute for introns to enhance polyadenylation efficiency**

Polyadenylation is essential in both *S.cerevisiae* and metazoans for efficient mRNA biogenesis, with improperly processed transcripts being actively retained by the nuclear exosome (56,58–60). Huang *et al.* (32) have shown that the histone H2a element can enhance 3' end cleavage in a cell-free system. We have found that HSV-TK's PPE can also enhance the kinetics of polyadenylation in a cell-free system (48). Huang *et al.* (32) have also reported similar half-lives for the full-length unpolyadenylated and polyadenylated RNAs synthesized from our intronless  $\beta$ -globin gene variant, with insertion of the histone H2a, PPE or PRE element not significantly changing the half-life. Taken together, these findings indicate that insertion of HBV's PRE, HSV-TK's PPE or *c-Jun*'s CJE enhances stabilization of the primary transcript during its initial synthesis, proper 3' end formation and cytoplasmic accumulation of  $\beta$ -globin-like RNAs (Figures 2 and 4), but not mRNA half-life. Consistent with this conclusion was the finding that a strong positive correlation exists between the abilities of these elements to enhance 3' end cleavage and cytoplasmic accumulation of the mRNA [(11); Figure 7].

Interestingly, these elements did not fully rescue back up to genomic level the defects caused by lack of the introns in the  $\beta$ -globin gene (e.g. Figures 2 and 4). This latter finding implies that these elements either efficiently provide only a subset of the multiple functions performed by introns or provide them all, but at lower efficiency. The fact that these elements function better and better as their copy number is increased [(28,33,36,48); Figure 4] is consistent with the latter hypothesis.

The PRE, PPE and CJE more efficiently enhanced cytoplasmic accumulation and 3' end processing of intronless transcripts when inserted into exon I than when inserted into exon III of the cDNA variant of the  $\beta$ -globin gene (Figures 2 and 4). Likewise, Nott *et al.* (11) noted that a strong correlation exists between polyadenylation efficiency and intron position, with a 5'-proximal intron enhancing polyadenylation and RNA accumulation more efficiently than a 3'-proximal intron. The PPE is naturally situated in the 5'-half of the protein-coding region of HSV-1's TK gene [(36); Figure 1A]. However, the PRE is naturally situated near the 3' end of HBV's mRNAs (27,28). The precise reason for the location dependence of introns and PPE-like elements on mRNA biogenesis remains unknown. Possibly, a 5'-proximal location helps bring hnRNPs to the transcript while it is being synthesized, thereby increasing its stability as well as enhancing 3' end formation.

### **PPE-like elements enhance the kinetics of several steps in mRNA biogenesis**

mRNA biogenesis requires numerous multi-component cellular complexes which are tethered together in gene expression factories to maximize the efficiency of production of correctly processed mRNAs (3). PPE-like elements from intronless genes and CTE-like elements from intron-containing genes both probably enhance the efficiency of specific post-transcriptional steps in mRNA biogenesis by increasing the kinetics of these steps via facilitating the recruitment of some of these complexes to the pre-mRNA. CTE-like elements recruit complexes involved in nucleocytoplasmic export (21). PPE-like elements recruit complexes involved in export (34,48) and, probably, proper 3' end processing (48). 3' end cleavage for polyadenylation usually takes place prior to intron excision for small transcripts containing 3'-terminal introns (7,42). Thus, CTE-like elements could inhibit splicing indirectly by increasing the kinetics of nucleocytoplasmic export such that intron-containing RNAs are usually exported to the cytoplasm prior to excision of all of their introns. However, CTE-like elements may fail to enhance cytoplasmic accumulation of intronless transcripts since these RNAs would still lack a mechanism for proper 3' end cleavage and polyadenylation and, thus, be degraded, instead. On the other hand, PRE, PPE and CJE probably (i) stabilize pre-mRNAs in the nucleus by recruiting hnRNPs and (ii) enhance the efficiency of proper 3' end processing by recruiting the 3' end cleavage/polyadenylation machinery. Afterward, they also recruit export factors, leading to the polyadenylated mRNAs being exported to the cytoplasm with or without intron excision depending upon the relative kinetics of these multiple steps in processing and transport. Unfortunately, in the absence of detailed kinetic data, one cannot definitively state to what extent these elements contribute to each of these coupled steps in mRNA biogenesis.

Also noteworthy is our finding that these elements modulate pre-mRNA processing and nucleocytoplasmic export in a cell-type-dependent manner (Figures 5 and 6). This conclusion is consistent with prior reports showing species specificities in the functionality of the CTEs of MPMV (51) and Rous sarcoma virus (26). Whether this cell-type dependence exists because of natural differences in the abundances or sequences of mRNA processing factors or alterations that arose during passage in culture (51,61) remains unclear.

### **Intronless cellular genes also contain PPE-like elements**

Huang and Carmichael (31) were the first to identify a PPE-like element in a cellular gene, mouse histone H2a. However, histone genes employ their own special machinery for 3' end formation of their transcripts (62). We report here the first identification of a PPE-like element, CJE, in a cellular protein-encoding gene whose 3' end is formed employing the cell's standard cleavage/polyadenylation machinery (30). We showed that the CJE can significantly enhance stabilization, proper 3' end formation and cytoplasmic accumulation of intronless transcripts (Figure 4). As yet unknown is the *trans*-acting factor(s) with which the CJE interacts and the precise mechanism(s) by which it functions. Presumably, other naturally intronless cellular genes also contain such elements that act via binding *trans*-acting factors involved

in mRNA biogenesis. One interesting question is whether expression of these genes is coordinately regulated, possibly to function at times when the machineries for intron-containing genes are inactive such as following infection with certain viruses.

### Intron-dependent versus intron-independent genes

Despite the functional existence of intronless genes such as *c-Jun*, most genes in higher eukaryotes actually contain introns. Introns are absolutely essential for some of them, partially dispensable for others, and completely unnecessary for yet others [(10,11) and references cited therein]. We hypothesize that all intronless and highly intron-independent genes contain PPE-like elements that facilitate multiple steps in mRNA biogenesis. These PPE-like elements are essential for the proper processing and export of intronless transcripts, but dispensable in most intron-containing transcripts since most introns can facilitate these same essential steps in mRNA biogenesis. Thus, during the course of evolution, most genes in higher eukaryotes acquired introns, with many of them subsequently then gradually losing their no-longer-essential PPE-like elements to become more and more dependent upon the presence of introns for proper processing and export of their transcripts. The end result is that genomes in higher eukaryotes now consist of a mixture of intronless, intron-independent and variably intron-dependent genes.

### Utility of PPE-like elements

One problem frequently encountered in working with exogenously added genes in mammalian cells is that their cDNA versions are often poorly expressed due to their intron dependence. Inclusion of PPE-like elements in expression vectors may enable one to achieve efficient expression of intron-dependent genes in the absence of splicing for use in gene therapy, the manufacture of proteins and research [(63–65); Figure 3].

In summary, we conclude that CTE- and PPE-like elements enhance several steps in mRNA biogenesis; ones from intron-containing genes primarily act in nucleocytoplasmic transport, while ones from naturally intronless genes probably facilitate stabilization and proper 3' end formation as well as export.

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*Conflict of interest statement.* None declared.

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