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Intronless mRNA transport elements may affect multiple steps of pre-mRNA processing

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We have reported recently that a small element within the mouse histone H2a-coding region permits efficient cytoplasmic accumulation of intronless β-globin cDNA transcripts. This sequence lowers the levels of spliced products from intron-containing constructs and can functionally replace Rev and the Rev-responsive element (RRE) in the nuclear export of unspliced HIV-1-related mRNAs. In work reported here, we further investigate the molecular mechanisms by which this element might work. We demonstrate here through both *in vivo* and *in vitro* assays that, in addition to promoting mRNA nuclear export, this element acts as a polyadenylation enhancer and as a potent inhibitor of splicing. Surprisingly, two other described intronless mRNA transport elements (from the herpes simplex virus thymidine kinase gene and hepatitis B virus) appear to function in a similar manner. These findings prompt us to suggest that a general feature of intronless mRNA transport elements might be a collection of phenotypes, including the inhibition of splicing and the enhancement of both polyadenylation and mRNA export.

Keywords: intronless mRNA/mRNA transport/polyadenylation/splicing

Introduction

Most higher eukaryotic messages contain introns that are removed by splicing prior to mRNA transport to the cytoplasm. Since unspliced messages usually do not leave the nucleus, splicing is widely thought to be intimately associated with mRNA transport. One mechanism which might prevent the transport of unspliced messages would be that such transcripts are retained in the nucleus due to their association with splicing factors (Chang and Sharp, 1989; Legrain and Rosbash, 1989; Huang and Carmichael, 1996b). Many retroviruses, however, require unspliced and partially spliced viral messages to be transported to the cell cytoplasm during their life cycle. This bypass of the requirement for splicing is often accomplished by specific interactions between *cis*-acting mRNA transport elements and corresponding viral or cellular factors (Cullen, 1992; Bray *et al*., 1994; Ogert *et al*., 1996; Tang *et al*., 1997). For example, nuclear export of unspliced and partially spliced HIV-1 mRNAs is facilitated by the interaction between the viral encoded Rev protein and the Rev-responsive element (RRE) present within the target mRNAs (reviewed in Tang *et al*., 1997). In the case of simple retroviruses such as the Mason-Pfizer monkey virus, efficient cytoplasmic accumulation of unspliced mRNAs may depend on the interaction of a *cis*-acting RNA element with adenosine 5′-triphosphate-dependent RNA helicase A (Bray *et al*., 1994; Tang *et al*., 1997) or with other cellular factors (Pasquinelli *et al*., 1997; Gruter *et al*., 1998).

A number of naturally intronless messages are transported to the cell cytoplasm without being spliced (Kedes, 1979; Nagata *et al*., 1980; Hentschel and Birnstiel, 1981; Koilka *et al*., 1987; Hattori *et al*., 1988). This is in contrast to mRNAs transcribed from cDNAs of many intron-containing genes which fail to be exported to the cytoplasm in the absence of splicing (Hamer and Leder, 1979; Kedes, 1979; Nagata *et al*., 1980; Hentschel and Birnstiel, 1981; Koilka *et al*., 1987; Hattori *et al*., 1988; Neuberger and Williams, 1988; Ryu and Mertz, 1989; Jonsson *et al*., 1992; Nesic *et al*., 1993). Recently, a growing body of data has suggested that efficient nuclear export of intronless messages is facilitated by specific sequences present within these messages. Examples include the herpes simplex virus thymidine kinase (HSV-TK) message (Liu and Mertz, 1995), the hepatitis B virus (HBV) message (Huang and Liang, 1993; Huang and Yen, 1995) and the mouse histone H2a message (Huang and Carmichael, 1997). HnRNP L, which interacts specifically with the HSV-TK element, has been implicated in the nuclear export of HSV-TK messages (Liu and Mertz, 1995).

The naturally intronless higher eukaryotic histone genes can be divided into two classes: replication-dependent histones and replacement variants (Stein *et al*., 1984). Replication-dependent histone messages are expressed predominantly in the S phase of the cell cycle and their 3′ termini form a stem–loop structure processed by a mechanism different from polyadenylation (Marzluff, 1992). The replacement variant messages, on the other hand, are polyadenylated and expressed primarily in quiescent and differentiated cells (Stein *et al*., 1984).

Histone stem–loop 3′-end processing has been shown to play an important role in cell cycle regulation of histone expression (Sittman *et al*., 1983; Harris *et al*., 1991). Replacement of the histone 3′-end processing sequences with polyadenylation signals leads to cell cycle-independent histone expression (Levine *et al*., 1987; Cheng *et al*., 1989; Kirsch *et al*., 1989). In addition, some histone messages undergo an alternative pathway in their 3′-end processing, depending on cell type or growth state. For example, in growing cells the predominant *Xenopus* H2a mRNA ends at the stem–loop characteristic of replication-dependent histone mRNAs. In non-growing cells the predominant H2a mRNA is polyadenylated (Mannironi *et al*., 1989). Similarly, a mouse histone H1 gene forms stem–loop-processed histone 3′-ends in S phase cells but polyadenylated mRNAs

in quiescent cells (Cheng *et al*., 1989). For avian H2a, H2b and H3 genes, mRNAs are always polyadenylated in spermatocytes, but are stem–loop-processed in somatic cells (Challoner *et al*., 1989). The mouse histone H2a message described in this work also undergoes an alternative 3′-end processing pathway. In somatic cells, the H2a message ends in a stem–loop structure, while in spermatid cells it is polyadenylated (Moss *et al*., 1994). Importantly, both messages are transported to the cytoplasm where they are translated into proteins (Moss *et al*., 1994). Since mouse histone H2a message can be polyadenylated and exported to the cytoplasm under physiological conditions, we considered it an ideal model system to study export of intronless mRNAs.

We have shown previously that polyadenylated mouse histone H2a messages are efficiently transported to the cytoplasm of COS7 cells (Huang and Carmichael, 1997). We have gone on to identify a 101 bp sequence within the H2a-coding region that induces efficient nuclear export of human β-globin cDNA transcripts, which otherwise accumulate predominantly in the nucleus (Huang and Carmichael, 1997). In this study, we have further examined the mechanism of action of the histone element and have unexpectedly found that, in addition to promoting mRNA transport, this element exhibits strong activity not only in inhibition of splicing, but also in stimulation of polyadenylation. Surprisingly, the reported mRNA transport elements from HSV-TK (Liu and Mertz, 1995) and HBV (Huang and Liang, 1993; Huang and Yen, 1995) act in the same manner. Moreover, each of these three elements can promote unspliced mRNA export as well as lower the levels of spliced products in an HIV-1 based system. We therefore hypothesize that a general feature of transport elements from intronless messages might be to perform three functions: inhibit splicing, enhance mRNA 3′-end processing and promote nuclear export.

Results

The histone element increases the level of polyadenylated β-globin cDNA transcripts

We have previously shown that a 101-bp sequence from the mouse histone H2a gene (nt 201–301 relative to the transcriptional start site) significantly stimulates the cytoplasmic accumulation of human β-globin cDNA transcripts (Huang and Carmichael, 1997). To understand further how this sequence might work, plasmid construct 2B-βG, in which two tandem repeats of the 101-bp sequence were inserted into the 5′ untranslated region of a human β-globin cDNA construct, was created (Figure 1A). As always, inserts were created so as to avoid possible effects of nonsense codons on mRNA stability (Huang and Carmichael, 1997). This plasmid, together with an internal control plasmid H-XβG, was transiently transfected into COS7 monkey kidney cells. The nucleocytoplasmic distribution of RNAs was examined by an RNase protection assay. RNAs expressed from the globin cDNA construct pβ1(–)2(–) were examined in a parallel assay.

As shown in Figure 1B, the steady-state cytoplasmic level of intronless globin mRNA from the cDNA construct is low, indicated by the low c/n ratio as well as the overall low levels of globin mRNAs (lanes 1 and 2). Inclusion of the histone sequence in the mRNA, however, dramatically increases the c/n ratio and the apparent mRNA levels (Figure 1B, compare lanes 3 and 4 with 1 and 2). The increased RNA levels are not due to increased RNA stability or transcription rates; rather, they are largely due to the suppression of cryptic splicing events (see below). These results are consistent with our previous observations that a single 101-nt histone sequence was capable of significantly elevating the cytoplasmic accumulation of globin mRNA (Huang and Carmichael, 1997). The purpose of using two tandem elements instead of only one in this assay is that the former has a modestly stronger effect than the latter in promoting mRNA transport (data not shown).

To determine whether 3′-end processing of mRNAs might contribute to this phenomenon, a 3′-end probe was used instead of the internal probe (Figure 1A). This probe spans the polyadenylation site of the globin mRNA, hence it distinguishes between properly cleaved/polyadenylated RNAs and readthrough globin transcripts (Figure 1A and C). Oligo(dT)-affinity chromatography confirmed the presence of poly(A) in the pA^+ fractions (Figure 1D). Only a small fraction of the globin transcripts (32%) is found in the pA^+ fraction (Figure 1C and D, lanes 1 and 2), suggesting that polyadenylation of the globin transcripts is inefficient. As expected, the readthrough transcripts are localized exclusively to the nucleus (Figure 1C, lanes 1 and 2). In marked contrast, when the histone sequence is present, 95% of the transcripts are cleaved/polyadenylated (Figure 1C and D, lanes 3 and 4).

Elevated levels of polyadenylated mRNAs are due in part to improved polyadenylation

To rule out the possibility that augmented levels of polyadenylated mRNAs result from increased mRNA stability, an actinomycin D time-course analysis was performed (Figure 1E). The plasmids indicated were each transfected into COS7 cells. Forty-eight hours after transfection, actinomycin D was added to the cells. Nuclear and cytoplasmic RNAs were isolated at the time points indicated, followed by RNase protection assays using the 3′-end probe. The half-lives of the nuclear and cytoplasmic polyadenylated globin mRNAs are ~4.5 and >10 h, respectively, while the half-lives of the nuclear and cytoplasmic polyadenylated, element-containing globin mRNAs are ~3.5 and 4 h, respectively (Figure 1E). Hence, the globin mRNA itself is no less stable than the globin messages containing any of the elements. In other control experiments, the half-lives of non-polyadenylated species were measured and were found to be comparable to those of the polyadenylated species experiments (data not shown). For reasons that are not yet clear, the presence of the histone element appears to render globin transcripts slightly less stable than transcripts lacking the element. Nevertheless, these results argue that enhanced polyadenylation efficiency contributes to the elevated level of polyadenylated mRNAs.

The histone element stimulates cleavage/polyadenylation in vitro

To further investigate the role of the histone element in mRNA 3′-end processing, *in vitro* cleavage/polyadenylation analyses were performed. Two plasmid constructs were used. As a reporter construct, B-SV was made by inserting the 101-nt histone element into the well-characterized plasmid pSVL, which contains the SV40 late polyadenylation site (Figure 2A). The histone sequence was

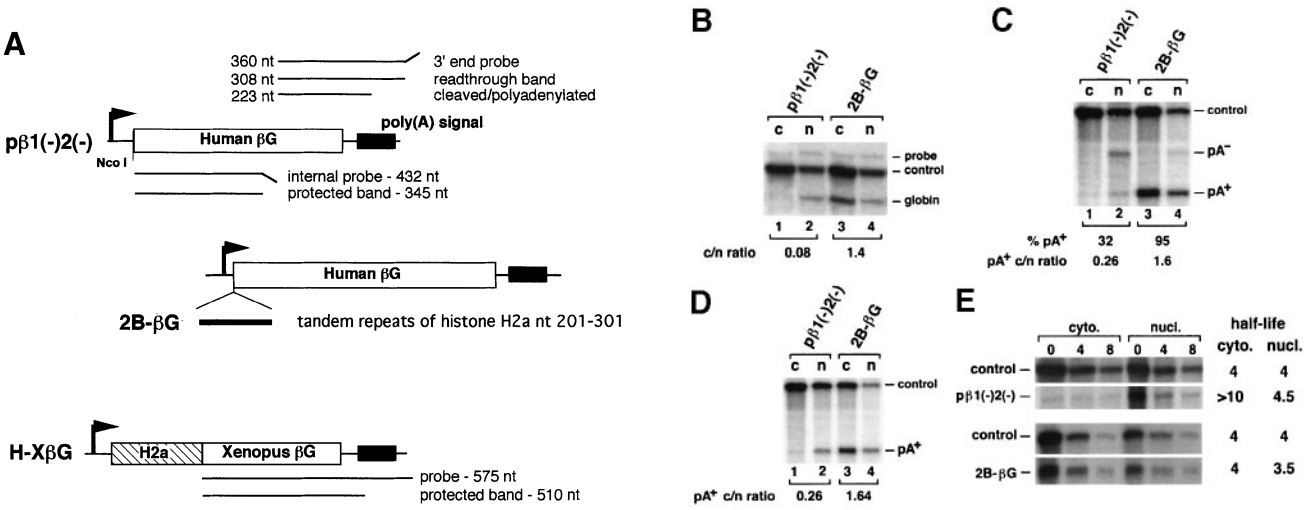

Fig. 1. The histone element stimulates polyadenylation and nuclear export of globin mRNA. (**A**) Plasmid constructs. The open boxes indicate either human or *Xenopus* β-globin cDNA sequences. The solid rectangle depicts the polyadenylation signal of the human β-globin gene. The hatched box denotes the mouse histone H2a sequence (nt –2 to 494 relative to the H2a transcription start site). The arrows indicate directions of transcription. The thick bar below the human globin gene construct represent tandem repeats of the histone element inserted in the sense orientation. The riboprobes used in RNase protection assays and the protected bands are indicated. The tilted portions of the probes depict non-homologous sequences derived from plasmid vectors. In pβ1(–)2(–), the unique *Nco*I site is marked. The control plasmid H-XβG is described in Materials and methods. Sizes are not to scale. (B, C, D and E) Autoradiograms of RNase protection assays of RNAs expressed in cells transfected with the indicated plasmids. The 432-nt internal probe was used in (**B**), and the 360-nt 3′-end probe was used in (**C**), (D) and (E). (**D**) The nuclear RNAs were oligo(dT)-selected before analysis. (**E**) Actinomycin D time course analysis of nuclear and cytoplasmic polyadenylated mRNAs. Plasmids pβ1(–)2(–) and 2B-βG were each transfected into COS7 cells, along with the control plasmid H-XβG. For the purpose of better quantitation, the amount of DNA used in transfection was doubled in the case of pβ1(–)2(–) DNA. At 48 h after transfection, actinomycin D was added to the media to a final concentration of 5 μg/ml. Nuclear and cytoplasmic RNAs were isolated at 0, 4 and 8 h after drug addition and subjected to RNase protection assays using the 3′-end probe. Only cleaved/polyadenylated mRNAs are shown. Numbers above the gels are the time point when RNAs were isolated. The approximate half-lives of the indicated mRNAs are shown in the columns on the right. c or cyto., cytoplasmic mRNAs; n or nucl., nuclear mRNAs; globin, reporter mRNAs from the indicated plasmids; control, mRNA from the co-transfected control plasmid H-XβG; probe, undigested 575-nt probe for the internal control mRNA (probes for other indicated mRNAs are not visible in the gels); c/n ratio, cytoplasmic and nuclear reporter mRNA distribution ratio after being normalized using the internal control mRNA; pA^-, uncleaved/unpolyadenylated transcripts from the indicated plasmids; pA^+, cleaved/polyadenylated transcripts from the indicated plasmids; % pA^+, percentage of cleaved/polyadenylated mRNAs in total mRNA fractions; pA^+ c/n ratio, cytoplasmic and nuclear distribution ratio of cleaved/polyadenylated mRNAs.

placed 135 nt upstream of the AAUAAA element of the SV40 poly(A) signal. For control purposes, the same histone sequence was inserted into pSVL, but in the opposite direction, to make Ba-SV. Our previous studies showed that this element exhibited no activity *in vivo* when placed in the opposite direction in all constructs tested (Huang and Carmichael, 1997). To address whether the histone element stimulates cleavage/polyadenylation, *in vitro* assays were carried out as described in the Materials and methods. As expected, the histone element significantly enhances cleavage/polyadenylation of the SV40 poly(A) signal when placed in the sense orientation (Figure 2B). The stimulation activity was observed as early as 15 min after the start of incubation and continued throughout the incubation period (Figure 2B and C). In multiple repeated experiments, similar results were obtained. The parental construct lacking the histone insert exhibits cleavage activity comparable to construct Ba-SV (data not shown). In addition, *in vitro* polyadenylation assays showed similar results, but were more difficult to quantitate owing to the heterogeneity of poly(A) addition (data not shown). Taken together, these results strongly suggest that the histone element acts to enhance polyadenylation/cleavage of nascent RNAs.

The histone element facilitates nuclear export of polyadenylated mRNAs

Although β-globin cDNA expression exhibits an apparent 3′-end processing defect, enhanced polyadenylation can only partially account for the transport defects measured with the internal probe (Figure 1C and D). Intronless globin transcripts are defective in nucleocytoplasmic transport regardless of whether they are polyadenylated (Figure 1C and D). Taken together with the experiments discussed above, these data suggest that the low level of cytoplasmic accumulation of globin mRNA as measured by the internal probe is due to at least two defects: poor 3′-end processing and inefficient nucleocytoplasmic transport. The histone sequence corrects both defects.

The histone element suppresses cryptic splicing

Early in our studies we noticed that the overall level of globin cDNA transcripts appears to be extremely low in comparison with that of the histone element-containing transcripts (Figure 1B, C and D). This raised the concern that the histone element might also influence transcription rates, but closer examination revealed that this is unlikely to be the case. The globin cDNA construct in fact produces high levels of RNA, most of which are cryptically spliced and polyadenylated, yielding products that were missed in previous assays. As shown in Figure 3, lanes 3 and 4, a 50-nt band is detected in RNA samples from cells transfected with the globin cDNA construct and not in the RNA of cells transfected with the element-containing globin construct (lanes 1 and 2). Insertion of the histone element in the antisense orientation does not inhibit cryptic splicing (Figure 3, lanes 5 and 6). In addition, the same 50-nt band is always seen in RNase protection assays of globin mRNAs into which sequences from regions of the histone H2a gene

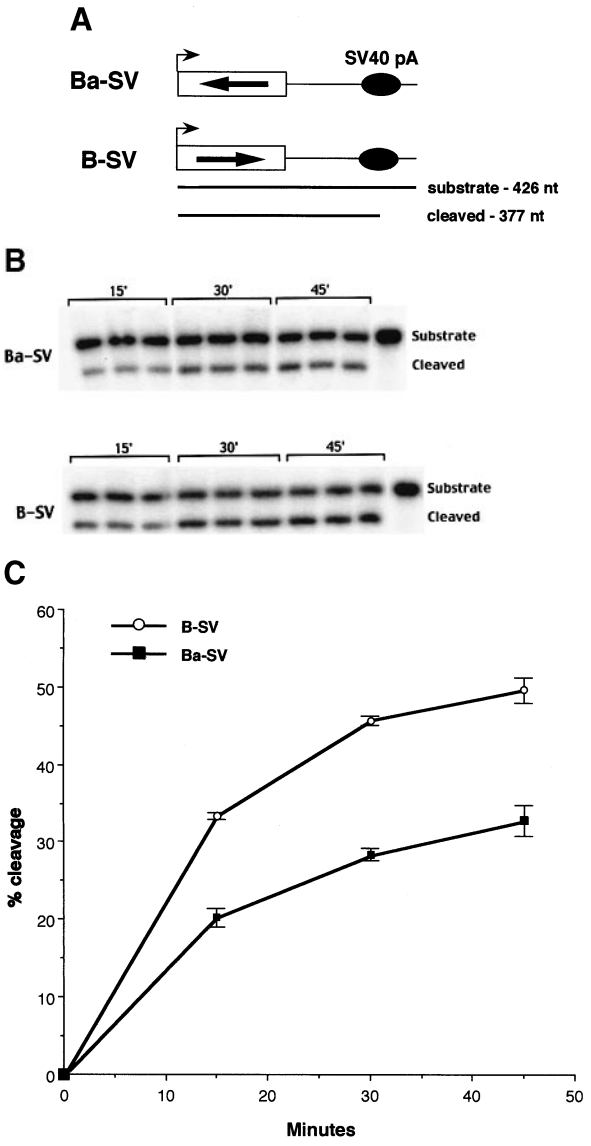

Fig. 2. The histone element stimulates cleavage/polyadenylation of the SV40 late poly(A) site. (**A**) Constructs. The open boxes represent the 101-nt histone element sequence. Arrows inside the boxes indicate the orientation of the element relative to the direction of the transcription which is marked by the angled arrows. The solid ovals depict the SV40 poly(A) site and the thin lines indicate bacterial vector sequences. The sizes of the substrate RNAs and the cleaved products are also shown beneath the constructs. Figures are not drawn to scale. (**B**) Time course (in minutes) of the reaction. The substrates were incubated under conditions as described in Materials and methods for the indicated time points and the experiments were performed in triplicate. (**C**) Quantification of the time course reaction. The cleavage efficiency is presented as the percentage of the cleaved products.

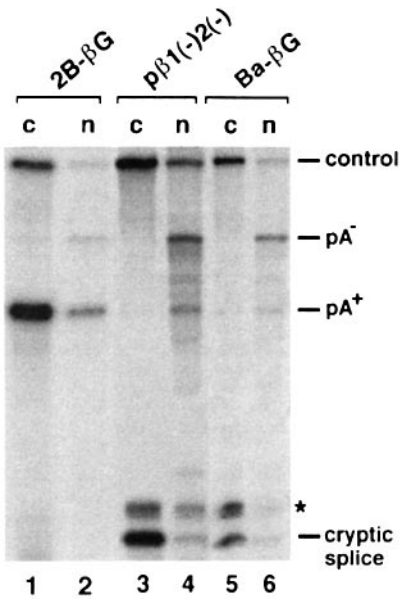

Fig. 3. The histone element appears to suppress cryptic splicing. The experiment was performed in the same way as that shown in Figure 1C, except that the bottom of the gel is also shown to reveal the cryptically spliced products. Lanes 1 and 2, cytoplasmic and nuclear RNAs from cells transfected with construct 2B-βG. Lanes 3 and 4, cytoplasmic and nuclear RNAs from cells transfected with construct pβ1(–)2(–). Lanes 5 and 6, cytoplasmic and nuclear RNAs from cells transfected with construct Ba-βG, which contains the 101 nt histone element in the antisense (nonfunctional) orientation. Cryptic splice, the 50-nt fragment representing a cryptically spliced mRNA species from the globin cDNA construct; *, non-specific background bands which appear variably in all lanes. Other labels are the same as described for Figure 1C.

which do not have effects on globin mRNA transport have been inserted (data not shown). Interestingly, this 50-nt product is detected only with the 575-nt internal control probe, which harbors part of the plasmid vector sequences and which has the potential to form base-paired interactions with globin cDNA transcripts that may have extended into the bacterial plasmid vector sequences downstream of the globin polyadenylation site. Since cleavage/polyadenylation of the cDNA transcripts at the normal site occurs at low efficiency (Figure 1), it was therefore highly likely that the bulk of the globin cDNA transcripts are cryptically polyadenylated downstream of the normal poly(A) site, and that cryptic splicing occurs, removing the globin coding region as an intron, hence resulting in the lower levels of the properly polyadenylated globin mRNA. Moreover, when corrected for the fact that the probes used in the RNase protection assays are internally labeled, the abundance of the 50-nt protected fragment suggests that the globin cDNA construct expresses the same, or even slightly higher levels of transcripts compared with the cDNAs into which the histone element has been inserted. A simple interpretation for these observations is that the presence of the histone element suppresses cryptic splicing.

In order to confirm cryptic splicing, and to map the site of the putative cryptic event within globin transcripts, we used a series of probes spanning the entire β-globin transcribed region in RNase protection assays. Results presented in Figure 4 revealed that, indeed, a cryptic 5′ splice site maps to a position 51 nt downstream of the *Nco*I site that was used for the insertion of all transport elements. The globin cDNA construct (Figure 4B, lane 2) expresses high levels of RNAs that are cryptically spliced from this site. When corrected for the small size of this band and the U-content of the protected radiolabeled probes, this spliced species is actually slightly more abundant than the full-length species, which includes both polyadenylated and non-polyadenylated RNAs. In numerous other assays, constructs that showed defects in the cytoplasmic accumulation of globin mRNAs also displayed the cryptic band (data not shown), and constructs which contain functional transport elements always lack this band (Figure 4B, lanes 3–5; data not shown). Other RNA probes spanning the globin gene failed to reveal the presence of any other major site of

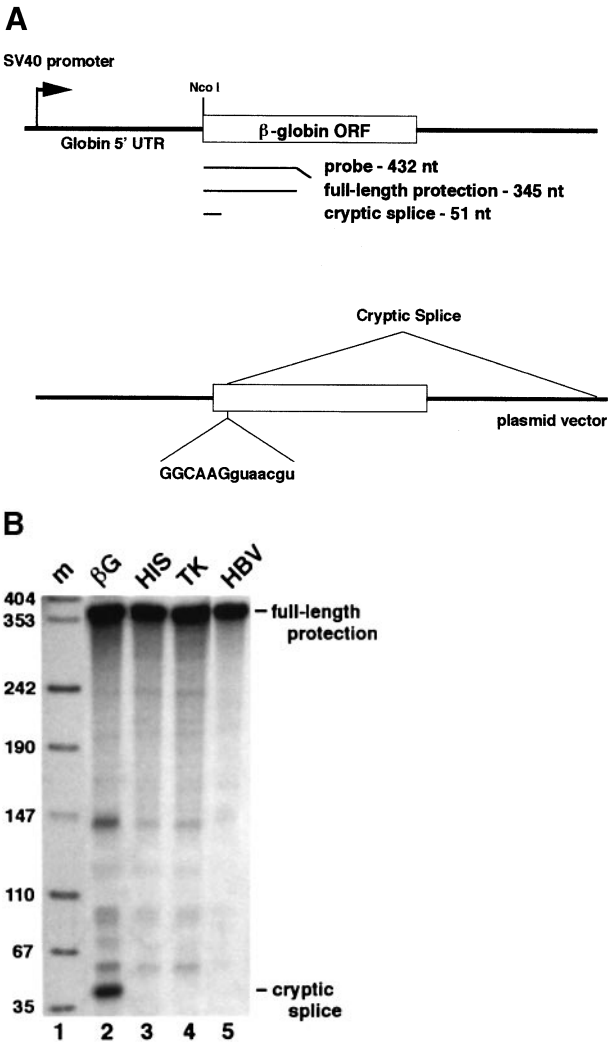

Fig. 4. Mapping the site of a cryptic 5′ splice. (**A**) Top, schematic diagram of the β-globin gene region in construct pβ1(–)2(–), and the probe used to map the cryptic splicing site. This is the same probe used in the experiments of Figures 1A and 6A. Bottom, the sequence of the cryptic 5′ splice site is shown below the diagram of the β-globin construct. (**B**) RNase protection assay of total cellular RNAs isolated from cells transfected with pβ 1(–)2(–), lane 2; 2B-βG, lane 3; p2×TK119 β 1(–)2(–), lane 4; and HBV- β G, lane 5. Lane 1 is molecular weight markers. In lane 2, 4-fold more total RNA was used than in lanes 3–5 in the protection assays. Other unmarked bands evident in this panel were variable, non-specific protection results commonly seen in our RNase protection assays.

cryptic splicing (data not shown). Note that in Figure 4B, lane 2, 4-fold more total RNA was used than in lanes 3–5 in the protection assays.

The histone element inhibits splicing in vitro

To further examine the role of the histone element in splicing, *in vitro* splicing experiments were performed (Figure 5). The constructs B-BSAd and Ba-BSAd were derived from plasmid pBSAd1, which was a generous gift from E.Izzauralde. The reporter construct B-BSAd has the 101-nt histone element inserted in the sense orientation into the pBSAd1 intron region 91 nt downstream of the 5′ splice site. RNAs transcribed from this construct consist of a 149-nt intron which is flanked by a 92-nt exon at the 5′ end and a 101-nt exon at the 3′ end. Plasmid, Ba-BSAd, differs from B-BSAd in that the histone element was inserted in the antisense orientation. ^{32}P-labeled, *in vitro*-transcribed substrate RNAs were incubated with HeLa cell nuclear extracts under splicing conditions for the indicated time periods. The splicing activity of the reporter RNAs was compared with that of the control RNAs. Spliced products and splicing intermediates accumulate as the incubation time increases for the control substrates BSAd1 RNA (Figure 5, lanes 1–4), and Ba-BSAd (lanes 5–8), which contains the histone element in the antisense orientation. In striking contrast, however, B-BSAd RNA, which contains the histone element in the sense orientation, failed to produce detectable amounts of splicing products (Figure 5, lanes 9–12). The same results have been seen when the histone element was inserted in other *in vitro* splicing cassettes (data not shown). These results, taken together with those described above, strongly suggest that the histone element acts to inhibit splicing in an orientation-dependent manner.

Two viral mRNA transport elements share functional similarity with the histone element

Two viral transport elements, a 119-nt sequence from the HSV-TK gene (Liu and Mertz, 1995) and a 534-nt sequence from HBV (Huang and Liang, 1993; Huang and Yen, 1995; Donello *et al*., 1996) have been shown to stimulate cytoplasmic accumulation of human β-globin cDNA transcripts. Since both elements derive from naturally intronless genes, we wondered whether they act in a similar way to the cellular histone H2a element. To test this hypothesis, two plasmid constructs containing either the TK or the HBV element in the β-globin cDNA vector pβ1(–)2(–) were used. Plasmid p2XTK119β1(–)2(–) contains two tandem repeats of the 119-nt TK sequence inserted into pβ1(–)2(–) (Figure 5A; Liu and Mertz, 1995). In plasmid HBV-βG, the 534-nt HBV sequence was inserted at the same position (Figure 6A). Each of the two reporter constructs was transiently transfected into COS7 cells and the nucleocytoplasmic distribution of RNAs was examined by RNase protection assays (Figure 6B and C).

As in the case of the histone element, the presence of the TK or the HBV sequences leads to an increase in cytoplasmic levels of globin mRNAs as revealed using the internal probe (Figure 6B, compare lanes 3, 5 and 7 with 1). This is consistent with results reported by others (Huang and Liang, 1993; Huang and Yen, 1995; Liu and Mertz, 1995; Donello *et al*., 1996). Strikingly, however, when the 3′-end probe was used, the two viral elements not only appear to significantly enhance the level of polyadenylated globin mRNA, but also to increase the c/n ratio of the mRNA, as does the histone element (Figure 6C, compare lanes 6–11 with 4 and 5). The presence of poly(A) on cleaved messages was confirmed by oligo(dT)-affinity chromatography (data not shown). Intriguingly, the presence of either of the viral elements also leads to disappearance of cryptic splicing products (Figure 6C, lanes 6–11; also see Figure 4B, lanes 3–5). mRNA stability analysis indicated that the element-containing, polyadenylated mRNAs are not more stable than the globin mRNA itself in either compartment (Figure 6D).

The three intronless message transport elements mimic the function of HIV-1 Rev/RRE

We have shown previously that the histone element, perhaps by interacting with an as yet unknown cellular factor(s),

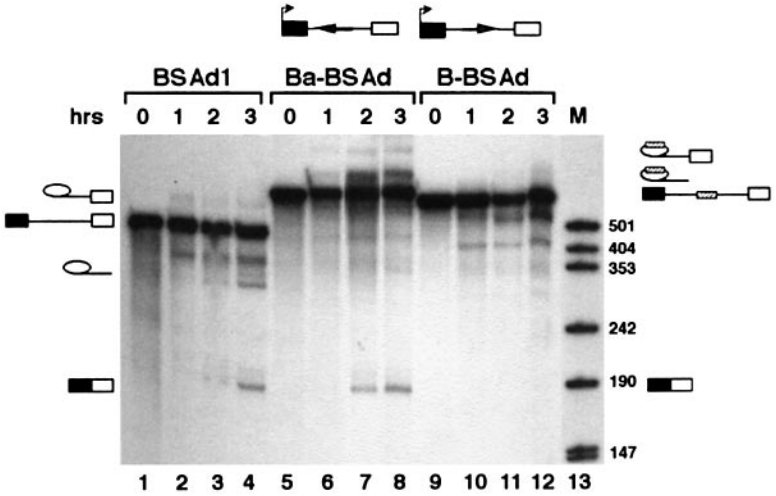

Fig. 5. The histone element inhibits splicing. Top: time course (in hours) of the *in vitro* splicing reaction. Shown above this are the structures of the constructs for making the RNA substrates. The solid and the empty boxes represent the pBSAd1 exons. The arrows indicate the 101-nt histone element inserted into the intron region with its orientation marked. The substrate RNAs were incubated for the indicated time points and the resulting products were resolved on 8% denaturing polyacrylamide gels. The identities of the substrate RNAs and the splicing products are marked on the sides of the gel. M, molecular markers in nucleotides.

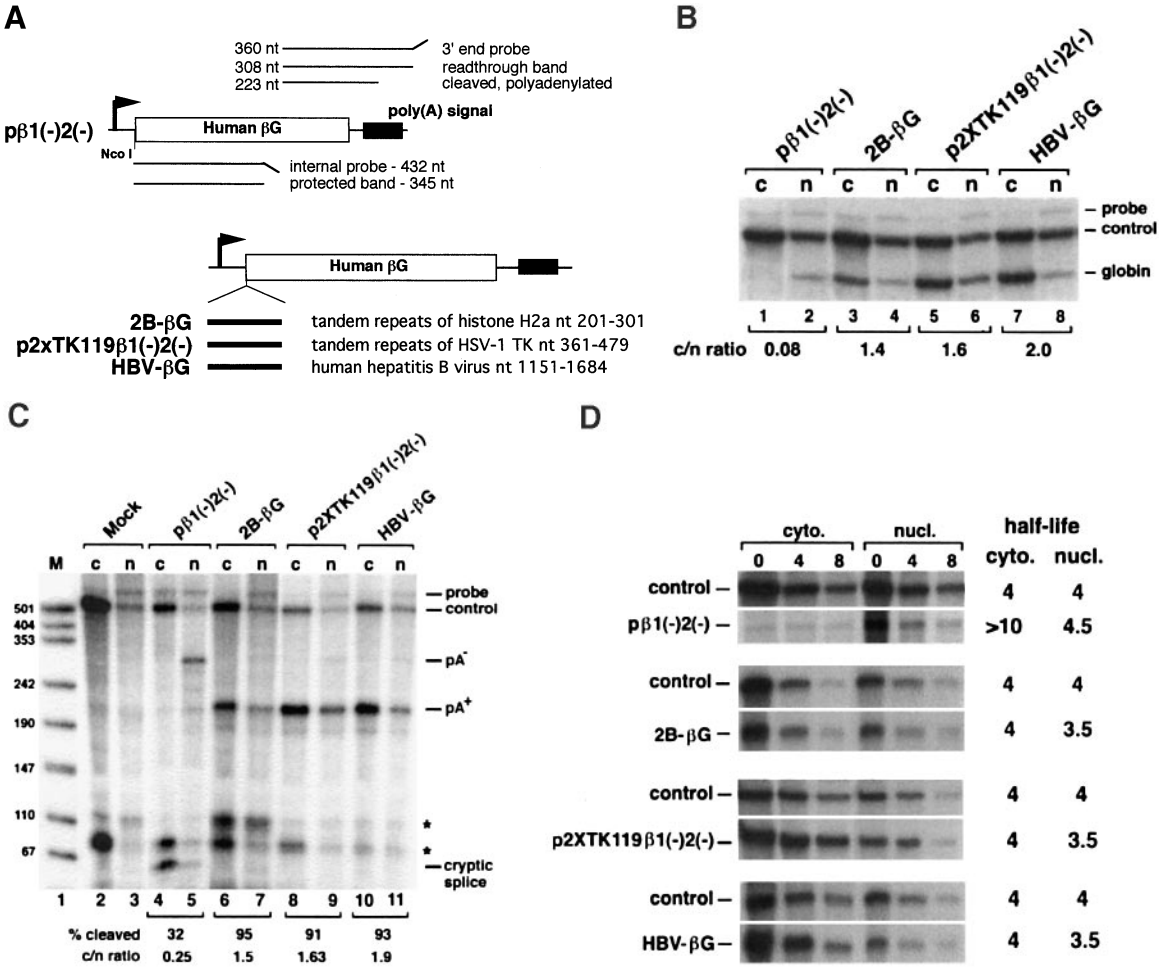

Fig. 6. The TK and the HBV elements mimic the function of the histone H2a element. (**A**) The structure of the plasmid constructs. (**B**, **C** and **D**) Autoradiograms of RNase protection assays of RNAs expressed in cells transfected with the indicated plasmids. The 432 nt internal probe was used in (B), and the 360 nt 3′-end probe was used in (C) and (D). In all the cases, the 575-nt internal control probe was also used. (D) Results of the actinomycin D time course analyses of nuclear and cytoplasmic polyadenylated mRNAs. See Figure 1B, C and E for other labels and descriptions.

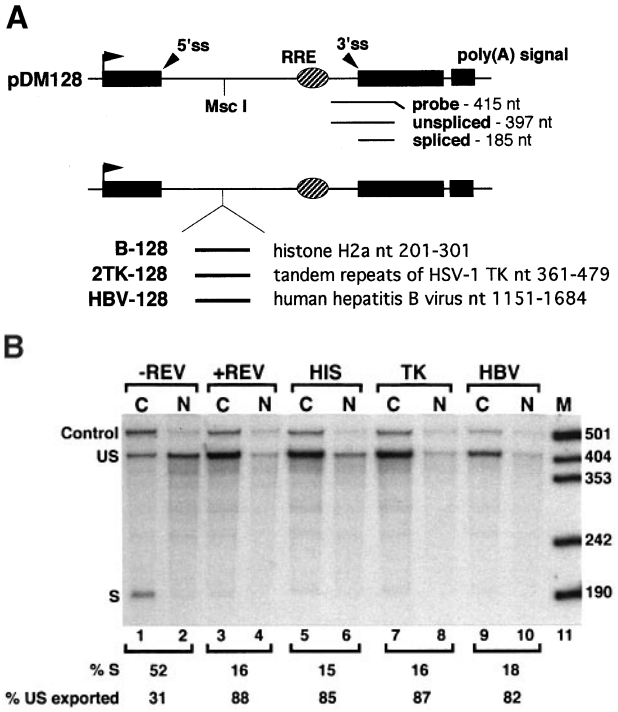

Fig. 7. The three intronless mRNA transport elements share functional similarity with Rev/RRE. (**A**) Structures of the plasmid constructs. The solid boxes and the thin lines in between denote the exon and the intron sequences from the HIV-1 gene, respectively. The RRE sequence, the HIV-1 polyadenylation sequence and the unique *Msc*I site are also marked. The thick bars underneath the second construct indicate the inserted elements. The probe and the predicted protected bands are indicated. 5′ss, 5′ splice site; 3′ss, 3′ splice site. (**B**) RNase protection assays of RNAs prepared from COS7 cells transfected with the indicated plasmids. Lanes 1–4 are mRNAs expressed from the parental pDM128 construct. +Rev, with co-transfection of the Rev-expressing vector; –Rev, without co-transfection of the Rev-expression vector. Lanes 5–10, mRNAs expressed from the indicated plasmids in the absence of Rev expression. M, molecular markers in nucleotides; HIS, mRNAs from B-128; TK, mRNAs from 2TK-128; HBV, mRNAs from HBV-128; US, unspliced mRNA; S, spliced mRNA. The quantification of the gel is shown at the bottom.

plays a similar role to the HIV-1 Rev/RRE in inducing efficient nuclear export of unspliced mRNAs (Huang and Carmichael, 1997). It has also been reported that the HBV element mimics the function of Rev and RRE (Huang and Liang, 1993; Huang and Yen, 1995; Donello *et al*., 1996). We therefore wanted to know whether the three intronless message transport elements (histone, TK and HBV) act in a similar manner to HIV-1 Rev/RRE in our experimental system. To this end, each of the three elements was inserted into the HIV-1-based construct, pDM128, in the sense orientation (Figure 7A). Following transfections, intracellular RNA distributions were measured by RNase protection assays, either in the presence or absence of Rev.

Rev, through its specific interaction with the RRE, enhances the nuclear export of unspliced mRNAs expressed from subgenomic HIV-1 constructs, with a concomitant decrease in the level of spliced mRNAs in COS7 cells (Malim and Cullen, 1993). Consistent with this, the cytoplasmic level of the unspliced mRNA from the reporter construct is increased ~3-fold in the presence of Rev (Figure 7B, compare lanes 1 and 3). Importantly, the increased level of the unspliced mRNA is accompanied by a decreased level of spliced mRNA (Figure 7B, compare lanes 3 and 4 with lanes 1 and 2).

Intriguingly, each of the three elements appears sufficient to replace the function of Rev and RRE in construct pDM128. In the absence of Rev, inclusion of any of these elements leads to elevated cytoplasmic levels of unspliced reporter mRNAs. At the same time, the levels of the spliced mRNAs are decreased (Figure 7B, compare lanes 5–10 with 1 and 2). Like RRE, each of the elements is functional only in the sense orientation (Huang and Carmichael, 1997; data not shown). Moreover, addition of Rev does not enhance the effects of these elements (data not shown).

Discussion

Until recently it has been unclear why naturally intronless mRNAs, such as histone mRNAs, are capable of accumulating abundantly in the cell cytoplasm, while messages from cDNAs of many intron-dependent genes are not (Gruss *et al*., 1979; Hamer and Leder, 1979; Gasser *et al*., 1982; Callis *et al*., 1987; Buchman and Berg, 1988; Neuberger and Williams, 1988; Chung and Perry, 1989; Deng *et al*., 1989; Ryu and Mertz, 1989; Jonsson *et al*., 1992; Nesic *et al*., 1993). We have reported previously that a 101-nt sequence from the mouse H2a gene enables efficient cytoplasmic accumulation of human β-globin cDNA transcripts, and that this element mimics the function of the HIV-1 Rev/RRE in promoting nuclear export of unspliced mRNAs (Huang and Carmichael, 1997). In work to be presented elsewhere (J.Podoloff, Y.Huang and G.Carmichael, in preparation), we have identified a similar element within the mouse histone H3.2 gene.

In this study, we have further elucidated the mechanism by which the H2a element works. Using internal and 3′-end probes in RNase protection assays, combined with *in vitro* studies, we have demonstrated that: (i) the majority of globin cDNA transcripts are not polyadenylated at the natural site; (ii) properly polyadenylated globin mRNAs are defective in nucleocytoplasmic transport despite being polyadenylated; (iii) cryptic splicing and polyadenylation downstream of the globin poly(A) site appear to partly account for the low level of correctly processed globin mRNAs; (iv) the histone H2a element, when present in the globin construct, corrects all the defects; (v) *in vitro* experiments further support the conclusion that the same element acts not only to stimulate polyadenylation, but also to inhibit splicing; and (vi) two intronless viral mRNA transport elements, the HSV-TK element and the HBV element, may share functional similarity with the histone element.

That the histone element, as well as the two viral elements, functions to directly facilitate mRNA nuclear export is supported by several lines of evidence. First, the presence of each of the elements *in cis* is able to rescue the nuclear export defect of polyadenylated globin mRNA, and enhancement of polyadenylation alone does not fully account for the elevated cytoplasmic level of globin mRNA measured by the internal probe (Figure 6B and C). Secondly, each of the elements exhibits functional similarity to the HIV-1 Rev/RRE in stimulating nuclear export of unspliced mRNAs (Figure 7B). It has been shown that the Rev protein harbors a nuclear export signal (NES) capable of directly promoting nuclear export of RRE-containing, unspliced

mRNAs (Kalland *et al*., 1994; Meyer and Malim, 1994; Bogerd *et al*., 1995; Fischer *et al*., 1995; Fritz *et al*., 1995; Stutz *et al*., 1995). It will be intriguing to discover whether each of the intronless mRNA transport elements interacts with NES-containing proteins. It should be noted that the HSV-TK element has been shown to interact functionally with hnRNP L (Liu and Mertz, 1995). Whether the H2a and HBV elements also interact with this protein remains to be determined.

It has been demonstrated that the specific interaction between Rev and the RRE is able to directly promote the nuclear export of unspliced mRNAs (Malim and Cullen, 1993; Kalland *et al*., 1994; Meyer and Malim, 1994; Bogerd *et al*., 1995; Fischer *et al*., 1995; Fritz *et al*., 1995; Stutz *et al*., 1995). Recently, the cellular export receptor for Rev, CRM1, has been identified (Nishi *et al*., 1994; Wolff *et al*., 1997). CRM1 belongs to the importin-β family and its gene product is the target for the cytotoxin leptomycin B (Fornerod *et al*., 1997a,b; Görlich *et al*., 1997). Experiments of microinjection into *Xenopus* oocytes have shown that export of both Rev and U snRNA are blocked by leptomycin B, indicating that Rev and U snRNA access the same export pathway (Fornerod *et al*., 1997a). Since the three elements discussed here all functionally mimic Rev/RRE in promoting nuclear export of unspliced mRNAs, it is possible that they might act by similar mechanisms. It would be interesting to find out whether export of these RNAs are also sensitive to the inhibititory effect of leptomycin B. As Rev/RRE has been reported to directly suppress splicing (Chang and Sharp, 1989; Felber *et al*., 1989; Lu *et al*., 1990; Schwartz *et al*., 1992; Stutz and Rosbash, 1994), which might contribute to the lowered levels of unspliced mRNAs (Figure 7B, compare lanes 3 and 4 with 1 and 2), we suggest that the three elements might each act to inhibit the assembly or resolution of spliceosomes. This is further supported by the notion that the histone element suppresses splicing both *in vitro* and *in vivo*. Although less consistent with our results for the intronless mRNA elements, it has been suggested that Rev/RRE may induce the export of unspliced mRNAs via a distinct pathway which precludes splicing (Malim and Cullen, 1993; Kalland *et al*., 1994; Meyer and Malim, 1994; Bogerd *et al*., 1995; Fischer *et al*., 1995; Fritz *et al*., 1995; Stutz *et al*., 1995). If this were true for the elements studied here, then the lower levels of spliced products in Figure 7B would be an indirect result of a transport phenomenon. However, the *in vitro* splicing results (Figure 5) would then remain to be explained.

Polyadenylation has been shown to play an important role in nucleocytoplasmic mRNA transport (Eckner *et al*., 1991; Huang and Carmichael, 1996a). The polyadenylation machinery involves a multi-protein complex that specifically interacts with *cis*-acting elements present within the target transcripts (reviewed by Collis *et al*., 1990). Our transfection and RNase protection experiments have revealed that the histone element stimulates nuclear export of globin cDNA transcripts partly by enhancing polyadenylation. This is further confirmed by our *in vitro* cleavage/polyadenylation studies. More intriguingly, two viral intronless mRNA transport elements, the HSV-TK element and the HBV element, appear to act in the same manner (Figure 6B and C). The stimulation of polyadenylation by the histone element *in vitro* is only ~2-fold, while it appears to be much greater than this *in vivo*. In the *in vitro* assays the control constructs already exhibit significant activity and therefore cannot be stimulated more than several fold by the histone element. In an independent study, Liu and Mertz reported that the HSV-TK element increases the accumulation of polyadenylated globin mRNA (unpublished data cited in Liu and Mertz, 1995). These results taken together point to the notion that enhancement of polyadenylation might be an intrinsic and perhaps general property of intronless mRNA transport elements.

For intron-containing messages, 3′-terminal introns have been reported to enhance polyadenylation (Collis *et al*., 1990; Huang and Gorman, 1990; Niwa *et al*., 1990, 1992; Pandey *et al*., 1990; Liu and Mertz, 1993, 1996; Nesic *et al*., 1993). Evidence exists that this enhancement is achieved through direct interactions between splicing factors that bind to the terminal 3′-splice site and the polyadenylation machinery (Wassarman and Steitz, 1993; Lutz and Alwine, 1994). Efficient nucleocytoplasmic transport of human β-globin messages can be obtained by inclusion of introns (Buchman and Berg, 1988; Collis *et al*., 1990) or by introducing intronless transport elements (Huang and Yen, 1995; Liu and Mertz, 1995; Huang and Carmichael, 1997; Figure 6B and C). It is thus tempting to postulate that intronless mRNA transport elements might function analogously to the terminal intron for the recruitment or stability of the polyadenylation machinery. However, the histone element along with the other two elements is unlikely to actually contain cryptic 3′ splice sites because (i) there appears to be no good 3′ splice site consensus sequences within these elements, and (ii) we have been unable to detect any use of a cryptic splice site when these elements were cloned into the intron of the pDM128 vector (data not shown).

In addition to stimulating polyadenylation and mRNA export, our studies have revealed that the three elements exert negative effects on splicing. This is supported by the following observations. First, each of the elements when present in the globin messages leads to the disappearance of a 51-nt band which most likely represents a cryptically spliced product (Figure 4B). Secondly, the level of the spliced product is significantly reduced from the HIV-1-related RNAs when each of the elements is present *in cis* (Figure 7B). Finally, *in vitro* splicing experiments indicated that, when inserted into an intron, the histone element abolishes splicing from the substrate RNAs (Figure 5). It would thus not be surprising if the two viral elements would also turn out to inhibit splicing in the same *in vitro* assays. Considering that naturally intronless messages may harbor cryptic splice sites, the presence of these elements may help to prevent unwanted cryptic splicing events to occur *in vivo*. In so doing, these elements might also facilitate mRNA nuclear export, since splice sites and/or introns have been shown to cause nuclear mRNA retention (Chang and Sharp, 1989; Legrain and Rosbash, 1989; Huang and Carmichael, 1996b).

It has been reported that inhibitory sequences (INS) present within the HIV-1 messages prevent the messages from being exported to the cytoplasm, and that interaction between Rev and RRE is able to overcome this inhibition (Rosen *et al*., 1988; Hadzopoulou-Cladaras *et al*., 1989; Cochrane *et al*., 1991; Schwartz *et al*., 1992; Nasioulas *et al*., 1994; Mikaélian *et al*., 1996). It is possible that naturally intronless messages may contain INS-like

sequences as well and that the three elements studied here overcome the inhibitory effects. This would help them to mimic the function of Rev/RRE (Figure 6B).

In summary, we have shown that the histone element performs three functions: (i) it enhances cleavage/polyadenylation of the intronless globin mRNAs; (ii) it exhibits splicing inhibition activity both *in vivo* and *in vitro*; and (iii) it directly promotes nuclear export of both intronless and unspliced mRNAs. Curiously, the HSV-TK and the HBV elements act the same way in our assays. It is not known yet whether these three functions are fulfilled by a single protein factor or multiple factors that bind to these elements. In our favored model, a single protein factor might bind to each element and might then oligomerize along the transcript in a manner reminiscent of the HIV-1 Rev protein on RRE-containing transcripts (Zapp *et al*., 1991). Two important by-products of this interaction could be the recruitment or stabilization of polyadenylation machinery to the correct poly(A) site, and the interference with the association of spliceosomes to nearby splice sites. If this putative factor contained a NES, then the export of unspliced mRNAs to the cytoplasm would be further facilitated. As the HSV-TK element has been shown to functionally interact with hnRNP L (Liu and Mertz, 1995), we further speculate that some but not all hnRNP proteins may possess these properties. An alternative model would be that a single protein may bind to the elements and initiate the binding of other factors that perform the functions of splicing inhibition and enhancement of polyadenylation.

It will be of great interest and importance to identify the factor(s) that specifically interacts with the histone element. Studies toward this goal are already underway and results will be presented elsewhere. We have observed that the three intronless message elements studied here share no obvious sequence similarities, and we therefore suspect that they might be recognized by different factors. However, it is also equally plausible to hypothesize that they might interact with a common factor(s) that recognizes the elements based on their RNA secondary or tertiary structures. Finally, experiments are under way to determine whether the histone element plays a role in normal H2a 3′-end processing, considering that in somatic cells, the mouse histone H2a messages processed by a mechanism other than polyadenylation.

Materials and methods

Plasmid constructs

Plasmids pβ1(–)2(–), p2×TK119β1(–)2(–) (Liu and Mertz, 1995), pDM128, pRSV-Rev (Peterlin *et al*., 1986; Hope *et al*., 1990), B-128 and H-XβG (Huang and Carmichael, 1997) have been described previously. 2B-βG was built by inserting two sense orientation tandem repeats of the 101-bp mouse histone H2a sequence (nt 201–301 relative to the H2a transcription start site) into the *Nco*I site of pβ1(–)2(–) (Figure 1A). HBV-βG was made by cloning the 534-bp human HBV PCR fragment (nt 1151–1684) into the *Nco*I site of pβ1(–)2(–) in the sense orientation (Figure 6A). 2TK-128 and HBV-128 were made by inserting two tandem repeats of HSV-1 TK sequence (nt 361–479 relative to the TK transcription start site) and the 534-bp HBV PCR fragment into pDM128 opened with *Msc*I, respectively (Figure 7A). H-XβG (Figure 1A) was created by fusing a *Xenopus laevis* β-globin cDNA sequence immediately 3′ of the mouse histone H2a sequence (nt –2 to 494 relative to the H2a transcription start site without including the stem–loop processing sequence) (Huang and Carmichael, 1997). mRNAs expressed from this construct are polyadenylated and partition between the nucleus and the cytoplasm in a reproducible manner. This construct was therefore used in all experiments as an internal control both for transfection efficiency and for subcellular fractionation.

Plasmids for *in vitro* cleavage/polyadenylation assays originated from pSVL, which contains the SV40 late polyadenylation sequence and was kindly provided by J.Wilusz (Wilusz and Shenk, 1988). B-SV was created by cloning the 101-bp histone element into pSVL (Wilusz and Shenk, 1988) at the *Eco*RI site, 135 nt upstream of the AAUAAA sequence of the SV40 poly(A) site (Figure 2A). In construct Ba-SV, the 101-nt element was inserted in the opposite direction.

Constructs B-BSAd and Ba-BSAd for *in vitro* splicing experiments were derived from the adenovirus major late transcription unit (Figure 5). The parent plasmid for the constructions is pBSAd1, a generous gift from Dr E.Izzauralde. T3 RNA polymerase transcripts from this plasmid contain a 149-nt intron flanked by two short exons. To make B-BSAd, the 101-bp histone H2a sequence was inserted pBSAd1 at the *Sac*II site, 92 nt downstream of the 5′ splice site. Ba-BSAd differs from B-BSAd in that the 101-bp element was inserted into pBSAd1 in the opposite direction.

Cells and transfections

COS7 cells were maintained and propagated as described previously (Huang and Carmichael, 1997). Transfections were carried out with a modified $CaPO_4$ DNA coprecipitation method (Cahill and Carmichael, 1989). Approximately 16 h prior to transfection, cells were diluted 2-fold and replated in 150 mm plates. Five micrograms of reporter DNA [pβ1(–)2(–), 2B-βG, p2×TK119β1(–)2(–), HBV-βG, pDM128, B-128, 2TK-128 or HBV-128], 2 μg of the control DNA H-XβG and 27 μg of pBluescript DNA or of Rev-expressing plasmid pRSV-Rev were used per plate for transfection.

Total, nuclear and cytoplasmic RNA preparation. RNAs were isolated 48 h after transfection. For the preparation of cytoplasmic RNA, cells were rinsed free of media with ice-cold phosphate-buffered saline and were then disrupted with an NP-40 lysis buffer (10 mM HEPES pH 7.6, 10 mM NaCl, 3 mM $MgCl_2$ and 0.5% NP-40) on ice for 30 s. Cytoplasmic lysates were collected in new tubes and appropriate amounts of guanidinium thiocyanate crystals were added to give a final concentration of 4 M. Cytoplasmic RNA was then purified by centrifugation at 30 000 r.p.m. through 5.7 M CsCl step gradient in an SW41 rotor. For nuclear RNA, the above intact cell nuclei which were still attached to the plates were rinsed with ice-cold NP-40 buffer followed by lysis in 4 M guanidinium isothiocyanate, 20 mM sodium acetate pH 5.2, 0.1 mM dithiothreitol (DDT), and 0.5% *N*-lauryl sarcosine. For total RNA, cells were washed, then lysed directly with 4 M guanidinium isothiocyanate, 20 mM sodium acetate pH 5.2, 0.1 mM DDT, and 0.5% *N*-lauryl sarcosine. As described above, RNA was then pelleted through cesium chloride.

In the actinomycin D time course analyses, nuclear and cytoplasmic RNAs were extracted 0, 4 and 8 h after treatment with a 5 μg/ml final concentration of actinomycin D and were analyzed by the quantitative RNase protection assays. Due to low levels of the mRNAs expressed from pβ1(–)2(–), 10 rather than 5 μg of pβ1(–)2(–) DNA was used in the transfections to facilitate quantitation.

RNase protection assays

Internally labeled RNA probes were made by *in vitro* transcription by T3 or T7 RNA polymerase in the presence of [α-^{32}P]UTP. DNA templates were removed by RQ1 DNase digestion followed by phenol/chloroform extraction. Internally labeled riboprobes were hybridized to target RNAs at 60°C overnight, as described previously (Huang and Carmichael, 1997). The hybridization products were digested with a T1/T2 mixture (Huang and Carmichael, 1997) at 37°C for 1.5 h and the resulting samples were resolved on 6% denaturing polyacrylamide gels. Routinely, 50% volume of total nuclear and cytoplasmic RNA samples were used for each RNase protection assay.

Quantitation of RNase protection data

Protected bands were quantitated using a Packard Instant Imager. Background was subtracted using regions of identical size located immediately below each of the experimental bands. When bands within the same lane were compared, values were adjusted for length and uridine content, because the radioactive probes used were internally labeled with [^{32}P]UTP.

In order to accurately determine the subcellular distribution of various RNAs, the c/n ratio of RNA expressed from the internal control plasmid H-XβG was used to normalize all experimental results. In different experiments, this ratio varied slightly. We arbitrarily set 0.50 as a standard ratio for normalization purposes, and values for other RNAs were adjusted by the same amount.

In vitro experiments

Nuclear extracts were prepared from HeLa cells by the procedure of Dignam *et al*. (1983) with the modifications described by Wilusz and Shenk (1988). Substrate RNAs were transcribed by Sp6 RNA polymerase (Gibco-BRL) for *in vitro* cleavage reactions, or by T7 RNA polymerase for *in vitro* splicing reactions in the presence of 50 μCi of [^{32}P]CTP and m7GpppG. RNAs were gel purified, eluted and ethanol precipitated. Briefly, cleavage reactions were carried out in a volume of 12 μl containing 58% HeLa cell nuclear extract, 2.5% polyvinyl alcohol, 1 mM 3′-dATP, 1 mM EDTA and 2×10^4 c.p.m. of ^{32}P-labeled RNA substrates. 3′-dATP was included to terminate the poly(A) addition in order to facilitate quantitation of products that had undergone cleavage followed by addition of the first adenosine residue. The reactions were incubated for the indicated time period at 30°C and the resulting products were phenol/chloroform extracted and ethanol precipitated. The RNAs were resolved on 6% denaturing polyacrylamide gels. Splicing reactions were performed in a volume of 20 μl containing 1.5% PEG, 0.1 mM ATP, 20 mM phosphocreatine, 1.5 mM HEPES pH 7.9, 40% HeLa nuclear extract, 2.5 mM $MgCl_2$ and 2×10^4 c.p.m. of substrate RNAs. The reactions were incubated at 30°C for the indicated times and resulting products were phenol/chloroform extracted and ethanol precipitated. The RNAs were analyzed on 8% denaturing polyacrylamide gels.

Quantitation of in vitro reactions

RNA products from processing reactions on polyacrylamide gels were quantitated using a Packard Instant Imager. For *in vitro* cleavage assays, the processing efficiency was determined from the percentage of cleaved products. Substrates were prepared to the same specific activity to ensure proper comparison. Experiments were repeated at least three times and representative results are presented.

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