Activation of HIV-1 pre-mRNA 3' processing *in vitro* requires both an upstream element and TAR

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The architecture of the human immunodeficiency virus type 1 (HIV-1) genome presents an intriguing dilemma for the 3' processing of viral transcripts—to disregard a canonical 'core' poly(A) site processing signal present at the 5' end of the transcript and yet to utilize efficiently an identical signal that resides at the 3' end of the message. The choice of processing sites in HIV-1 appears to be influenced by two factors: (i) proximity to the cap site, and (ii) sequences upstream of the core poly(A) site. We now demonstrate that an *in vivo*-defined upstream element that resides within the U3 region, 76 nucleotides upstream of the AAUAAA hexamer, acts specifically to enhance 3' processing at the HIV-1 core poly(A) site *in vitro*. We furthermore show that efficient *in vitro* 3' processing requires the RNA stem-loop structure of TAR, which serves to juxtapose spatially the upstream element and the core poly(A) site. An analysis of the stability of 3' processing complexes formed at the HIV-1 poly(A) site *in vitro* suggests that the upstream element may function by increasing processing complex stability at the core poly(A) site.

Key words: HIV-1/polyadenylation/RNA processing/TAR

Introduction

The processing of a primary transcript to generate a mature mRNA 3' end is an obligatory step in the synthesis of a functional eukaryotic message. The 3' processing of polyadenylated mRNAs entails the endonucleolytic cleavage of the pre-mRNA and the subsequent addition of ~200 adenylate residues. In higher eukaryotes, a poly(A) site is defined by two *cis*-acting sequences—a highly conserved AAUAAA hexamer 10–30 nucleotides upstream of the cleavage site and an amorphous downstream U- or GU-rich element that possesses neither an identifiable consensus sequence nor a conserved secondary structure (for reviews see Birnstiel *et al.*, 1985; Manley, 1988; Wickens, 1990). A 'core' poly(A) site of 48 nucleotides, comprising only these two elements, has been shown to be sufficient to direct efficient 3' processing *in vivo* (Levitt *et al.*, 1989).

Recently, a second class of processing signals, distinct from the elements of the core poly(A) site, has been shown to play a role in the modulation of poly(A) site efficiency (reviewed by Coffin and Moore, 1990). Sequences that enhance 3' processing have been identified in SV40 (Carswell and Alwine, 1989), adenovirus (DeZazzo and Imperiale, 1989; DeZazzo *et al.*, 1991b; Prescott and Falck-

Pedersen, 1992), hepatitis B virus (Russnak and Ganem, 1990; Russnak, 1991), as well as human immunodeficiency virus type 1 (HIV-1) (Brown *et al.*, 1991a; DeZazzo *et al.*, 1991a; Valsamakis *et al.*, 1991; Cherrington and Ganem, 1992). In each case, sequences that modulate 3' processing have been found to reside upstream of the core poly(A) site. The upstream sequences function in an orientation-dependent manner, suggesting that recognition may occur at the level of RNA (Russnak and Ganem, 1990; Russnak, 1991; Cherrington and Ganem, 1992). Substitution experiments have demonstrated that several of these upstream elements are functionally analogous. The HIV-1 upstream sequences may functionally replace both the SV40 late (Valsamakis *et al.*, 1991) and the hepatitis B virus sequences (Russnak and Ganem, 1990). Despite their functional similarity, however, these sequences appear to possess little primary or secondary structure similarity. The lack of an obvious consensus sequence as well as the general U-richness of the upstream elements has led to the suggestion that these sequences may function in a manner similar to that of the poly(A) site downstream element, possibly interacting with the same factor (Valsamakis *et al.*, 1991).

The upstream sequences of the HIV-1 poly(A) site are of particular interest due to their possible role in poly(A) site selection. The body of the retroviral primary transcript is flanked by a direct repeat of the R/U5 sequences, within each of which is encoded a core poly(A) site. A mechanism must therefore exist that selects only the core poly(A) site within the 3' R/U5 repeat for processing. At least two factors appear to influence HIV-1 poly(A) site selection: (i) the proximity of the processing site to the cap site, and (ii) the presence of specific sequences upstream of the 3' R/U5 repeat. Processing at the core poly(A) site of HIV-1, as well as that of a second retrovirus, spleen necrosis virus (SNV), is greatly reduced when the processing site resides within ~500 nucleotides of the cap site (Iwasaki and Temin, 1990; Weichs an der Glon *et al.*, 1991; Cherrington and Ganem, 1992). The occlusion of processing by cap site proximity does not appear to be a general attribute of poly(A) sites, but rather appears to be specific to the retroviral processing sites. The mechanism by which proximity to the cap site occludes retroviral 3' processing, however, remains a matter of speculation. In contrast to the inhibition of processing at the 5' core poly(A) site, HIV-1 poly(A) site selection may be augmented by the activation of processing at the 3' core poly(A) site. Sequences within the U3 region, upstream of the 3' R/U5 repeat, have been shown to be required for efficient processing *in vivo* (Brown *et al.*, 1991a; DeZazzo *et al.*, 1991a; Valsamakis *et al.*, 1991; Cherrington and Ganem, 1992). The dual nature of the HIV-1 core poly(A) site suggests that an upstream element may be required as a consequence of the extraordinary susceptibility of the core poly(A) site to occlusion by cap site proximity.

The HIV-1 U3 region appears to contain a major 3' processing element (located ~76 nucleotides upstream of

the AAUAAA) as well as a minor element (located ~130 nucleotides upstream of the AAUAAA). Two adjacent linker scanning mutations within the major element reduced processing *in vivo* to 30 and 50% of wild-type levels, respectively, whereas a mutation within the minor element reduced processing to 80% of the wild-type level. Upon deletion of both of these regions, however, processing efficiency was found to be only 9% of wild-type (Valsamakis *et al.*, 1991). The enhancement of processing by U3 sequences is apparently mediated primarily, if not entirely, by cellular factors. The *in vivo* experiments described above demonstrated that the activation of the HIV-1 core poly(A) site by upstream sequences did not require the presence of virus-encoded proteins (Brown *et al.*, 1991a; DeZazzo *et al.*, 1991a; Valsamakis *et al.*, 1991; Cherrington and Ganem, 1992). This is of particular significance since the TAR element resides between the U3 sequences and the core poly(A) site, 14 nucleotides upstream of the AAUAAA. The TAR element encompasses a stable 59 nucleotide stem-loop structure that serves as the target for the Tat protein, the virus-encoded *trans*-activator of HIV-1 transcription (for review see Cullen, 1990).

In order to address the mechanism by which upstream sequences and cap site proximity influence processing at the HIV-1 core poly(A) site we have investigated the requirements for processing at this site *in vitro*. We have now demonstrated that an upstream element, identified by Valsamakis *et al.* (1991) *in vivo*, acts specifically to enhance 3' processing at the HIV-1 poly(A) site *in vitro*. In addition, the activation of the core poly(A) site by the upstream element *in vitro* was found to require the RNA stem-loop structure of TAR for the spatial juxtaposition of the upstream element and the core poly(A) site. Our results furthermore suggest that the upstream element may increase the stability of the 3' processing complex at the HIV-1 core poly(A) site.

Results

U3 sequences are required for efficient cleavage at the HIV-1 poly(A) site *in vitro*

By standard criteria, the HIV-1 core poly(A) site (encoded within the R/U5 region and therefore present at both ends of the pre-mRNA) appears to possess all the elements of an authentic mRNA 3' processing signal. It contains an AAUAAA hexamer 19 nucleotides upstream of the cleavage site as well as a GU-rich element immediately downstream. Despite appearances, however, only the core poly(A) site within the 3' R/U5 repeat is processed *in vivo* (Cherrington and Ganem, 1992). *In vivo* analysis has demonstrated that efficient processing at the HIV-1 3' core poly(A) site requires sequences that reside upstream of the AAUAAA hexamer within the U3 region (Brown *et al.*, 1991a; DeZazzo *et al.*, 1991a; Valsamakis *et al.*, 1991; Cherrington and Ganem, 1992). Our initial objective was to determine whether the HIV-1 core poly(A) site is recognized *in vitro* and, if so, is processing enhanced by upstream sequences.

The HIV-1 5' and 3' core poly(A) sites are schematically diagrammed in Figure 1. The AAUAAA of the core poly(A) site resides 14 nucleotides 3' to TAR. The TAR element encompasses a stable 59 nucleotide stem-loop structure that forms the target for the Tat protein, the virus-encoded *trans*-activator of HIV-1 transcription (Cullen, 1990). The downstream element, as defined *in vivo* by Böhnlein *et al.*

(1989), resides within 26 nucleotides downstream of the cleavage site. An upstream element (use), defined operationally *in vivo* by a linker scanning mutation [NXS 9571–9588, Valsamakis *et al.* (1991)], resides 76 nucleotides 5' to the AAUAAA hexamer of the 3' core poly(A) site. While the mutagenesis and deletion results of several groups have indicated that this is not the only upstream sequence that influences processing efficiency (Brown *et al.*, 1991a; DeZazzo *et al.*, 1991a; Valsamakis *et al.*, 1991), we have confined our initial work to the analysis of this element.

The CPS (Core Poly(A) Site) pre-mRNA (Figure 2A) was designed such that its 5' end is colinear with the 5' end of authentic HIV-1 mRNA. Each of the HIV-1 pre-mRNAs contains 37 nucleotides of sequence downstream of the cleavage site. The upstream sequence used in these experiments encompasses 54 nucleotides of U3 sequence, immediately 5' to the R region, from position 9538 to 9591 (numbering according to Valsamakis *et al.*, 1991).

A set of capped pre-mRNAs, depicted schematically in Figure 2A, were transcribed by SP6 or T7 RNA polymerase in the presence of [α - 32 P]GTP and subsequently gel purified. Pre-mRNAs containing only the core poly(A) site (CPS), the core site plus U3 sequences (*use*/CPS), or the core site plus the upstream element linker scanning mutation NXS 9571–9588 of Valsamakis *et al.* (1991) (*duse*/CPS) were incubated in a HeLa cell nuclear extract in the presence of 3'dATP (Figure 2B, lanes 1–3). Each pre-mRNA was found to yield the products predicted of accurate endonucleolytic cleavage at the HIV-1 poly(A) site. The generation of two 5' products *in vitro* is consistent with the results of RNase protection analysis of *in vivo* synthesized HIV-1 mRNA performed by Cherrington and Ganem (1992). The *in vitro* 3' products comprise a set of RNAs that are partially degraded from their uncapped 5' end, as initially observed for the 3' products of the adenovirus L3 poly(A) site by Moore *et al.* (1986). The identity of the 3' cleavage products was confirmed by the analysis of 3'-end labeled pre-mRNAs (Figure 2B, lanes 6–8).

Processing at the HIV-1 poly(A) site *in vitro* was found to be strictly dependent upon the integrity of the AAUAAA and downstream element of the core poly(A) site. As illustrated in Figure 2C, introduction of a U to C mutation in the AAUAAA hexamer (CPS/AACAAA and *use*/CPS/AACAAA, lanes 4 and 7), or replacement of the downstream element (dse) by vector sequences (CPS/ Δ dse and *use*/CPS/ Δ dse, lanes 5 and 8) eliminated poly(A) site processing. Therefore, the two elements that are required for processing *in vivo*, the AAUAAA hexamer (Wickens and Stephenson, 1984) and the downstream element (Böhnlein *et al.*, 1989), are also required for processing at the HIV-1 poly(A) site *in vitro*.

The 3' products of these reactions are illustrated, since, in contrast to the 5' cleavage products, their sequences (and therefore their specific activities) are identical among all the pre-mRNAs (with the exception of the two RNAs that contain the downstream element mutation). It should be noted that the RNA present in lanes 3–5 of Figure 2C that comigrated with the 36 nucleotide RNase T1 product is not a 3' cleavage product, since it is not observed upon processing of a 32 P-3'-end labeled CPS pre-mRNA (Figure 2B, compare lanes 1 and 6).

As illustrated in both Figure 2B and C, processing at the

Fig. 1. A schematic representation of the 5' and 3' core poly(A) sites of the HIV-1 pre-mRNA. (A) Structure of the 5' core poly(A) site. The sequence that encompasses the AAUAAA hexamer and downstream element (dse) constitutes the core poly(A) site. The stem-loop structure of the *trans*-activation response element (TAR) extends from +1 to +59. The illustrated sequence is encoded by the R and U5 regions of the HIV-1 long terminal repeat (LTR). (B) Structure of the 3' core poly(A) site. An upstream element (use), defined *in vivo* by a linker scanning mutation (NXS 9571–9588, Valsamakis *et al.*, 1991) resides 76 nucleotides 5' to the AAUAAA hexamer. The illustrated sequence is encoded by the U3, R and U5 regions of the HIV-1 LTR.

HIV-1 core poly(A) site is stimulated ~5-fold *in vitro* in the presence of the upstream element, as quantitated by radioanalytic imaging (Betagen). The specificity of the enhancement of processing efficiency was verified by the introduction into the upstream element of the mutation that was previously shown to reduce processing efficiency to 30% of wild-type levels *in vivo* (Valsamakis *et al.*, 1991) (Δ use/CPS). Figure 2C, lane 9, demonstrates that the introduction of this mutation reduced processing at the HIV-1 poly(A) site to a level comparable with that observed for the CPS pre-mRNA (lane 3) which lacks upstream sequences.

Sequences 5' to the cleavage site contribute to the inefficient processing of the HIV-1 core poly(A) site *in vitro*

In the absence of the upstream element, processing at the HIV-1 core poly(A) site *in vitro* is quite inefficient (Figure 2B, lane 1), relative to the well characterized adenovirus L3 poly(A) site for which 50–75% of the precursor is correctly processed (Moore and Sharp, 1985; Gilmartin *et al.*, 1988). To address the basis for the low efficiency of the core site, chimeric pre-mRNAs were synthesized that contained sequences from the HIV-1 core poly(A) site fused to sequences of the adenovirus L3 poly(A) site. A pre-mRNA containing L3 sequences 5' to the poly(A) cleavage site in combination with the HIV-1 downstream element (dse) (L3/HIV) was processed at a level 6-fold greater than that of the HIV-1 core poly(A) site alone (Figure 3B, compare lanes 1 and 5). In the converse arrangement, however, a chimeric pre-mRNA containing HIV-1 core poly(A) site sequences 5' of the cleavage site in combination with the L3 poly(A) site downstream element (HIV/L3) exhibited a 4-fold reduction in processing

efficiency as compared with the wild-type L3 poly(A) site (Figure 3C, compare lanes 1 and 4).

These results suggested that the region immediately upstream of the cleavage site may be responsible for the inefficient processing of the HIV-1 core poly(A) site *in vitro*. Several additional RNAs were examined to test whether the TAR element, which resides 14 nucleotides upstream of the AAUAAA hexamer, has a negative effect on 3' processing. Disruption of TAR by the replacement of the 5' half of the stem with vector sequences (Δ Bgl/CPS) had little effect on processing at the core poly(A) site (Figure 3B, compare lanes 1 and 2). In addition, insertion of the TAR element 34 nucleotides upstream of the AAUAAA hexamer of the L3 poly(A) site (TAR/ Δ L3) had no discernible effect on processing efficiency at the L3 poly(A) site (Figure 3C, compare lanes 1 and 3). As a control, analysis of the Δ L3 pre-mRNA demonstrated that the deletion of the L3 sequences replaced by the TAR element in TAR/ Δ L3 did not alter processing efficiency (Figure 3C, compare lanes 1 and 2). The RNA stem-loop structure of TAR therefore does not appear to adversely affect mRNA 3' processing *in vitro*. Thus, these results suggest that the low efficiency of the HIV-1 core poly(A) site may be a consequence of the sequences that flank the AAUAAA hexamer.

The upstream element enhances the stability of processing complexes at the HIV-1 core poly(A) site *in vitro*

RNA-protein complexes formed by the specific interaction of processing factors with the pre-mRNA can be detected *in vitro* by gel mobility shift analysis (Konarska, 1989). 32 P-labeled pre-mRNAs were incubated for 10 min at 30°C in a HeLa cell nuclear extract in the absence of ATP, which allowed for the assembly of specific complexes but blocked subsequent cleavage and polyadenylation. The reactions were

Fig. 2. An upstream element enhances cleavage at the HIV-1 poly(A) site *in vitro*. (A) Schematic depiction of the HIV-1 pre-mRNA 3' processing substrates. The dark box represents vector sequences (derived from the pGEM-2 polylinker) that replace the poly(A) site downstream element in the CPS/Δdse and use/CPS/Δdse RNAs. The lightly shaded box represents the disruption of the upstream element by the linker scanning mutation NXS 9571–9588 of Valsamakis *et al.* (1991). (B) The HIV-1 core poly(A) site directs 3' processing *in vitro*. Uniformly 32 P-labeled (lanes 1–3) and 32 P-3'-end labeled pre-mRNAs (lanes 6–8) were incubated in a HeLa cell nuclear extract in the presence of 3'dATP and the products were resolved on a denaturing 10% polyacrylamide gel. Partial RNase T1 and alkaline hydrolysis ladders of 32 P-3'-end labeled use/CPS RNA (lanes 4 and 5) serve as size markers. (C) Both elements of the HIV-1 core poly(A) site are required for processing *in vitro*. Uniformly 32 P-labeled pre-mRNAs were incubated in a HeLa cell nuclear extract in the presence of 3'dATP and the 3' products were analyzed on a denaturing 10% polyacrylamide gel (lanes 3–9). Partial RNase T1 and alkaline hydrolysis ladders of 32 P-3'-end labeled use/CPS RNA (lanes 1 and 2) serve as size markers.

then analyzed on a non-denaturing 3% polyacrylamide gel. Figure 4A illustrates the sequence requirements for the formation of specific complexes at the HIV-1 poly(A) site. Specific complex formation was found to be dependent upon the integrity of both elements of the core poly(A) site. As previously demonstrated for the adenovirus L3 poly(A) site (Gilmartin and Nevins, 1989), pre-mRNAs that contained

both elements of the core poly(A) site, the AAUAAA and downstream element (dse), formed the slowest migrating complex, denoted complex 'B' (Figure 4A, lanes 1, 4 and 5). Pre-mRNAs in which the downstream element was replaced by vector sequences formed complexes of faster mobility, denoted complex 'A' (lanes 3 and 7). RNAs containing a U to C point mutation in the AAUAAA hexamer

Fig. 3. Processing of chimeric HIV-1/adenovirus L3 pre-mRNAs *in vitro*. (A) Schematic depiction of pre-mRNA 3' processing substrates. The thin line represents HIV-1 sequences; the hatched line represents vector sequences (derived from the pGEM-1 polylinker); the heavy line represents adenovirus L3 poly(A) site sequences. The numbering refers to the HIV-1 sequence position at the junctions of the chimeric RNAs. (B) *In vitro* 3' cleavage products of pre-mRNAs containing the HIV-1 poly(A) site downstream element. Uniformly 32 P-labeled pre-mRNAs were incubated in a HeLa cell nuclear extract in the presence of 3'dATP and the products were resolved on a denaturing 10% polyacrylamide gel. (C) *In vitro* 3' cleavage products of pre-mRNAs containing the adenovirus L3 poly(A) site downstream element. Uniformly 32 P-labeled pre-mRNAs were incubated in a HeLa cell nuclear extract in the presence of 3'dATP and the products were resolved on a denaturing 10% polyacrylamide gel.

formed only non-specific complexes (lanes 2 and 6). Therefore, specific complex formation at the HIV-1 poly(A) site *in vitro* required the elements of the core poly(A) site that were shown to be required for processing both *in vivo* (Wickens and Stephenson, 1984; Böhnlein *et al.*, 1989) and *in vitro* (this work).

The stability of the processing complexes formed *in vitro* has been shown to be directly reflected in the efficiency of poly(A) site processing both *in vitro* and *in vivo* (Ahmed *et al.*, 1991; Weiss *et al.*, 1991). We have therefore examined the impact of the upstream element on the stability of the complex formed at the HIV-1 poly(A) site. Complexes

were formed by the incubation of the 32 P-labeled pre-mRNA substrates in the nuclear extract for 10 min at 30°C, in the absence of ATP, followed by the addition of a ~100-fold molar excess of unlabeled *use*/CPS pre-mRNA. Complex stability was assessed by the analysis of the amount of the original [32 P]RNA-protein complex that remained after incubation for 10 and 20 min at 30°C in the presence of the RNA competitor. During the second incubation, the excess unlabeled pre-mRNA served to sequester any factors that dissociated from the initial complexes formed with the 32 P-labeled pre-mRNA. The stability of the specific complexes formed with the HIV-1 pre-mRNAs is illustrated in Figure 4B. The quantitation of the amount of each pre-mRNA present in the B complex in this experiment by Betagen radioanalytic imaging is presented graphically in Figure 4C. Of the *use*/CPS pre-mRNA present in the B complex at time 0, 65% was found to remain in the specific complex after 20 min in the presence of the unlabeled competitor RNA. Though a comparable amount of the CPS pre-mRNA was present in the B complex at time 0, only 15% remained associated with the specific complex after 20 min with the competitor. The amount of the mutant Δ *use*/CPS pre-mRNA in the B complex at time 0, however, was only 19% that of the wild-type *use*/CPS pre-mRNA, and by 10 min in the presence of competitor RNA the amount of complex remaining was almost undetectable above the background.

Taken together, these results demonstrate that the efficiency of processing at the HIV-1 poly(A) site is reflected in the stability of the processing complex formed *in vitro*. These results furthermore suggest that the role of the HIV-1 upstream element may be to increase the stability of the processing complex at the core poly(A) site.

The RNA stem-loop structure of TAR is required for efficient processing at the HIV-1 poly(A) site *in vitro*

Situated between the upstream element and the HIV-1 core poly(A) site is the 59 nucleotide RNA stem-loop structure that constitutes the TAR element. The presence of such an RNA structure within a 3' processing site is reminiscent of the Rex response element (RexRE) which bisects the HTLV-1 poly(A) site. The secondary structure of the RexRE, which is required for HTLV-1 poly(A) site function, serves to juxtapose spatially the widely separated AAUAAA and downstream elements (Ahmed *et al.*, 1991). Mutagenesis of the HIV-1 upstream region by Valsamakis *et al.* (1991) had revealed that disruption of the base of the TAR stem-loop by a linker scanning mutation (NXS 9589–9606) reduced processing efficiency to 50% of the wild-type level. We have therefore addressed the impact of the RNA stem-loop structure of TAR on HIV-1 poly(A) site function *in vitro*. Specifically, the requirement for the spatial juxtaposition of the upstream element and the core poly(A) site mediated by the TAR secondary structure was investigated.

RNA secondary structure disruption mutants were constructed by the introduction of base substitutions into both the 5' and 3' halves of the TAR stem (*use*/stem1 and *use*/stem2, Figure 5A). A compensatory double mutant was also constructed (*use*/stem1/2) that contains the nucleotide substitutions present in both the *use*/stem1 and *use*/stem2 mutants. The double mutation resulted in the restoration of the RNA stem-loop structure with a base-pairing potential

Fig. 4. An upstream element enhances the stability of 3' processing complexes at the HIV-1 poly(A) site *in vitro*. (A) Formation of specific processing complexes requires the elements of the HIV-1 core poly(A) site. Uniformly ³²P-labeled pre-mRNAs were incubated in a HeLa cell nuclear extract, in the absence of ATP, for 10 min at 30°C. Following treatment with heparin at 0°C, the RNA-protein complexes were resolved on a non-denaturing 3% polyacrylamide gel. (B) Analysis of processing complex stability. Uniformly ³²P-labeled pre-mRNAs were incubated in a HeLa cell nuclear extract, in the absence of ATP, for 10 min at 30°C. An aliquot was removed, denoted time 0, and treated with heparin at 0°C. A ~100-fold molar excess of unlabeled *use/CPS* pre-mRNA was added to the remaining reaction and aliquots were removed after an additional incubation for 10 and 20 min at 30°C. Each aliquot was treated with heparin at 0°C. The RNA-protein complexes were resolved on a non-denaturing 3% polyacrylamide gel. (C) Quantitation of B complex stability. The B complexes were quantitated by Betagen radioanalytic imaging.

identical to the wild-type TAR, albeit with a reversed sequence (see Materials and methods). Cleavage analysis revealed that the disruption of the TAR stem reduced processing at the HIV-1 poly(A) site, in the presence of the upstream element, to a level comparable with that of the core site alone (Figure 5B, compare lanes 2, 4 and 6). In contrast, the *use/stem1/2* pre-mRNA, in which the potential RNA secondary structure was restored, was processed at a level nearly equivalent to that of the wild-type *use/CPS* pre-mRNA (compare lanes 2 and 8). In each case, alterations within the TAR stem had little impact on processing of pre-mRNAs containing the upstream element mutation (compare lanes 3, 5, 7 and 9). The conclusion that the TAR element is required for the spatial juxtaposition of the upstream element and the core poly(A) site is further supported by the analysis of two pre-mRNAs in which the TAR stem-loop was completely deleted (*use/ΔTAR* and *Δuse/ΔTAR*). Although the complete elimination of TAR resulted in a 2-fold reduction in processing, the upstream element still functioned to enhance processing efficiency at the core poly(A) site (Figure 5B, compare lanes 10 and 11). These results demonstrate that efficient processing at the HIV-1 poly(A) site *in vitro* requires both an upstream element and the RNA stem-loop structure of TAR.

The RNA stem-loop structure of TAR enhances the stability of processing complexes at the HIV-1 poly(A) site *in vitro*

The analysis of RNA-protein complexes formed at the HIV-1 poly(A) site discussed above demonstrated that the upstream element enhances processing complex stability *in vitro*. Since the RNA stem-loop structure of TAR is

required for efficient *in vitro* processing at the HIV-1 poly(A) site, we have addressed the contribution of TAR to the stability of 3' processing complexes. Specific complexes were formed with each of the TAR stem mutant pre-mRNAs, followed by the addition of a ~100-fold molar excess of unlabeled *use/CPS* pre-mRNA. Analysis of the amount of complex remaining at 10 and 20 min after the addition of the unlabeled pre-mRNA revealed that the disruption of the RNA stem-loop reduced the stability of processing complexes formed at the HIV-1 poly(A) site (Figure 6, lanes 4–9). The pre-mRNA *use/stem1/2*, containing the compensatory double mutation, however, formed a significantly more stable complex than either the *use/stem1* or *use/stem2* pre-mRNAs (Figure 6, lanes 10–12). Thus, consistent with our previous results, illustrated in Figure 4B, processing complex stability reflected the efficiency of processing at the HIV-1 poly(A) site *in vitro*.

Discussion

As a central step in mRNA biosynthesis, 3' processing may contribute to the regulation of gene expression through the modulation of both the level and coding capacity of the mature message (e.g. Higgs *et al.*, 1983; Falck-Pedersen and Logan, 1989; Guise *et al.*, 1989; Thekkumkara *et al.*, 1992; Wang and Rubenstein, 1992). Sequences that reside outside the 'core' poly(A) site, composed of the AAUAAA hexamer and downstream element, have recently been shown to modulate processing site efficiency. Such accessory sequences function in the regulation of adenovirus poly(A) site selection (DeZazzo and Imperiale, 1989; DeZazzo *et al.*, 1991b; Prescott and Falck-Pedersen, 1992) and in the

Fig. 5. The RNA stem-loop structure of TAR is required for efficient processing at the HIV-1 poly(A) site *in vitro*. (A) Schematic depiction of the pre-mRNAs containing alterations within the TAR element. The TAR RNA stem-loop structure was disrupted by substitutions in the 5' half of the stem (*use/stem1*) or the 3' half of the stem (*use/stem2*). The *use/stem1/2* pre-mRNA contains the nucleotide substitutions present in both the *use/stem1* and *use/stem2* mutants. The double mutation restores the base-pairing potential of the TAR stem, albeit with a reversed sequence. The TAR stem-loop structure is completely eliminated in *use/ΔTAR* and $\Delta use/\Delta TAR$. The lightly shaded box represents the disruption of the upstream element by the linker scanning mutation NXS 9571–9588 of Valsamakis *et al.* (1991). (B) *In vitro* 3' cleavage products of pre-mRNAs containing alterations within the TAR element. Uniformly ^{32}P -labeled pre-mRNAs were incubated in a HeLa cell nuclear extract in the presence of 3'dATP and the products were resolved on a denaturing 10% polyacrylamide gel.

3' processing of SV40 (Carswell and Alwine, 1989) and hepatitis B virus (Russnak and Ganem, 1990) mRNA. In HIV-1, sequences upstream of the core poly(A) site, within the U3 region of the LTR, serve to enhance processing efficiency (Brown *et al.*, 1991a; DeZazzo *et al.*, 1991a; Valsamakis *et al.*, 1991; Cherrington and Ganem, 1992) and have been suggested to play a role in poly(A) site selection. We have attempted to address the mechanism by which the HIV-1 upstream sequences function through the analysis of poly(A) site processing *in vitro*.

Our analysis revealed that the elements of the HIV-1 core poly(A) site, consisting of the AAUAAA and the downstream element, were both necessary and sufficient to direct accurate 3' processing *in vitro*, albeit at a relatively low level. Processing was enhanced significantly, however, by the addition of sequences upstream of the core poly(A) site, within the U3 region. The upstream sequences functioned specifically to enhance processing 5-fold at the HIV-1 poly(A) site *in vitro*. A mutation within the upstream sequence, previously demonstrated by Valsamakis *et al.* (1991) to reduce processing to 30% of wild-type levels *in vivo*, eliminated the stimulation of HIV-1 poly(A) site processing by the upstream element *in vitro*. These results therefore demonstrate that poly(A) site processing *in vitro* is responsive to *cis*-acting elements that reside outside of the core poly(A) site. The ability to reproduce faithfully the activation of the HIV-1 poly(A) site *in vitro*, dependent upon the *in vivo* defined upstream and core sequence motifs, will thus facilitate the biochemical characterization of the mechanism of HIV-1 poly(A) site recognition and processing.

Previous work has demonstrated that the stability of complexes formed between processing factors and the pre-

mRNA *in vitro* is reflected in the efficiency of poly(A) site processing both *in vivo* and *in vitro* (Ahmed *et al.*, 1991; Weiss *et al.*, 1991). Indeed, the upstream sequences required for efficient processing at the HIV-1 poly(A) site enhanced the stability of the 3' processing complex formed upon incubation of the pre-mRNA in a HeLa cell nuclear extract. This result suggests that the mechanism by which the upstream element facilitates processing may be through the stabilization of the processing complex at the core poly(A) site. Such a function may be mediated through the binding of a specific factor to the upstream element or through the direct interaction of the upstream sequences with the core poly(A) site processing factors (Takagaki *et al.*, 1990; Keller *et al.*, 1991; Gilmartin and Nevins, 1991; and references therein). Recent work that has established a herpes simplex virus 1-encoded protein, ICP27, as an activator of poly(A) site processing, provides a precedent for the existence of *trans*-acting factors that serve to modulate mRNA 3' processing (Sandri-Goldin and Mendoza, 1992). The similarity of upstream elements and core poly(A) site downstream elements, however, has led to the suggestion that these sequences may play similar roles, possibly interacting with the same processing factor (Valsamakis *et al.*, 1991). Both upstream and downstream elements are generally U-rich and lack an obvious consensus sequence. Recognition of the downstream element is mediated by the core processing factor CF1 (Gilmartin and Nevins, 1989, 1991). The cooperative interaction of CF1 with PF2, which recognizes the AAUAAA hexamer, results in the formation of a stable complex at the core poly(A) site. The interaction of poly(A) polymerase and factor CF2 with the PF2–CF1–RNA complex is required for the subsequent cleavage and polyadenylation of the pre-mRNA (Gilmartin

Fig. 6. The RNA stem-loop structure of TAR enhances the stability of processing complexes at the HIV-1 poly(A) site *in vitro*. Analysis of the stability of 3' processing complexes formed on pre-mRNAs containing alterations within TAR. Processing complex stability was assessed as described in Figure 4B. Uniformly ^{32}P -labeled pre-mRNAs were incubated in a HeLa cell nuclear extract, in the absence of ATP, for 10 min at 30°C. An aliquot was removed, denoted time 0, and treated with heparin at 0°C. A ~100-fold molar excess of unlabeled *use/CPS* pre-mRNA was added to the remaining reaction and aliquots were removed after an additional incubation for 10 and 20 min at 30°C. Each aliquot was treated with heparin at 0°C. The RNA-protein complexes were resolved on a non-denaturing 3% polyacrylamide gel.

and Nevins, 1989). Alterations within the downstream element that reduced processing efficiency both *in vivo* and *in vitro* were also found to reduce the stability of the PF2-CF1-RNA complex *in vitro* (Weiss *et al.*, 1991). Therefore, the possible interaction of CF1 with both the upstream and downstream elements of the HIV-1 poly(A) site might serve to increase processing complex stability and, consequently, processing efficiency. Preliminary experiments in which partially purified PF2 and CF1 were incubated with the HIV-1 pre-mRNA indicated that the disruption of the upstream element significantly reduced the stability of the PF2-CF1-RNA complex (unpublished data). These results therefore suggest that the upstream element may interact directly with factors bound at the core poly(A) site.

A striking feature of the HIV-1 poly(A) site is the presence of the RNA stem-loop structure of TAR that resides between the upstream and core elements. The presence of such an RNA structure within a 3' processing site is reminiscent of the Rex response element (RexRE) which bisects the HTLV-1 poly(A) site. The RexRE comprises a 255 nucleotide RNA stem-loop structure that, in addition to its role in the transport of unspliced and singly spliced mRNAs from the nucleus to the cytoplasm, is required for HTLV-1 poly(A) site processing. The RexRE stem-loop structure serves to facilitate 3' processing of HTLV-1 transcripts through the spatial juxtaposition of the AAUAAA hexamer and the downstream element, which are separated by 274 nucleotides (Ahmed *et al.*, 1991). The physical approximation of the two RNA elements is required for the establishment of a stable PF2-CF1 complex with the pre-mRNA. The work of Brown *et al.* (1991b) has shown that the TAR RNA stem-loop structure is capable of serving

an analogous role when inserted between the AAUAAA hexamer and the downstream element of a synthetic poly(A) site. In such a model system, 3' processing was shown to be dependent upon the approximation of the widely separated AAUAAA and downstream elements by the stem-loop structure of TAR. We have now shown that TAR RNA stem-loop facilitates HIV-1 3' processing *in vitro* through the spatial juxtaposition of an upstream element and the core poly(A) site. The enhancement of both processing efficiency and complex stability by the HIV-1 upstream element *in vitro* required its approximation to the core poly(A) site by either the formation of the RNA stem-loop or the complete deletion of 57 nucleotides of TAR. The observation that a linker scanning mutation that disrupted the base of the TAR stem (NXS 9589-9606) reduced processing efficiency to 50% of wild-type levels *in vivo* (Valsamakis *et al.*, 1991) provides further support for the role of the TAR stem-loop structure in 3' processing. The impact of the viral TAR binding protein, Tat, on poly(A) site processing remains unclear. Certainly the upstream sequences are capable of functioning *in vivo* in the absence of virally encoded proteins (Brown *et al.*, 1991a; DeZazzo *et al.*, 1991a; Valsamakis *et al.*, 1991; Cherrington and Ganem, 1992). The addition of purified Tat protein (a gift of M.Laspias, Cold Spring Harbor) had no detectable effect on HIV-1 pre-mRNA 3' processing *in vitro* (unpublished data). Furthermore, RNase A footprinting of processing complexes formed in nuclear extracts in the presence of Tat protein indicated that the binding of Tat and 3' processing factors were not mutually exclusive *in vitro* (unpublished data).

While the evidence from several laboratories suggests that upstream sequences play a key role in the activation of the HIV-1 3' core poly(A) site (Brown *et al.*, 1991a; DeZazzo *et al.*, 1991a; Valsamakis *et al.*, 1991), the block to processing at the HIV-1 mRNA 5' core poly(A) site, as well as that of a second retrovirus, spleen necrosis virus (SNV), may be attributable primarily to cap site proximity (Iwasaki and Temin, 1990; Weichs an der Glon *et al.*, 1991; Cherrington and Ganem, 1992). We have observed that the core poly(A) site of the pre-mRNA CPS, which occupies the same position relative to the cap site as the authentic HIV-1 mRNA 5' core poly(A) site, is accurately processed *in vitro* with low efficiency. This low level of *in vitro* processing suggests that the occlusion of the 5' core poly(A) site *in vivo* may be directly coupled to active transcription. The 5' end of the nascent transcript may be sequestered in a manner that is apparently not reproducible upon incubation of an exogenous pre-mRNA in a nuclear extract. The mechanism by which processing at the HIV-1 and SNV poly(A) sites is occluded when present within the first 500 nucleotides of the nascent transcript may be due to a compartmentalization of the 5' end of the RNA prior to recognition by processing factors. Poly(A) sites which are not occluded by cap site proximity, such as the SV40 late, thymidine kinase, or α -globin poly(A) sites (Iwasaki and Temin, 1990; Weichs an der Glon *et al.*, 1991) may be capable of establishing a complex that commits the site to processing before the transcript is sequestered.

The demonstration that processing at the HIV-1 poly(A) site is responsive to the presence of an upstream element *in vitro* will permit the biochemical analysis of the mechanism of upstream element function. Furthermore, the coupling HIV-1 transcription and processing in nuclear

extracts may permit the investigation of the mechanism of retroviral poly(A) site occlusion.

Materials and methods

Cells and extracts

HeLa cells were grown in suspension in Joklik's modified minimal essential medium containing 5% calf serum. Nuclear extracts were prepared by the method of Dignam *et al.* (1983) except that the nuclei were extracted with 350 mM KCl and all buffers contained 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The extracts were dialyzed to 75 mM KCl, 20 mM HEPES (pH 7.9), 10% glycerol, 0.2 mM EDTA, 0.5 mM DTT and 0.1 mM PMSF.

Preparation of RNA substrates

Pre-mRNA substrates were prepared by *in vitro* transcription with SP6 or T7 RNA polymerase in the presence of 0.5 mM m⁷GpppG (Pharmacia), 15 μ M GTP and 0.4 μ M [α -³²P]GTP. The transcripts were gel-purified prior to use. The DNAs that served as the templates for the *in vitro* transcription of the pre-mRNA substrates were assembled from synthetic oligonucleotides and the plasmids pGEM-1 and pGEM-9Zf(-) (Promega). pGEM-9Zf(-) was digested with *Sac*I and the 3' overhang was removed by treatment with T4 DNA polymerase. The vector was then digested with *Eco*RI and synthetic oligonucleotides containing the HIV-1 sequence were inserted. The resulting plasmid, pCPS, was digested with *Eco*RI and transcribed with T7 RNA polymerase. Transcripts from this template initiated at +1 of the HIV-1 sequence and extended through position +130 (Ratner *et al.*, 1987). An *Xho*I site was introduced at the poly(A) cleavage site of pCPS by a three nucleotide substitution in order to facilitate the synthesis of both cleavage substrates and pre-cleaved RNA polyadenylation substrates. The *Xho*I site was also used for the construction of chimeric poly(A) sites, as discussed below. A restriction site has previously been introduced at the poly(A) cleavage site of the adenovirus L3 poly(A) site without a detectable effect on processing (Gilmartin and Nevins, 1989). The plasmid pCPS/AACAAA is identical to pCPS except for the single T to C change within the AATAAA hexamer. The construct pCPS/ Δ dse, in which the downstream element (dse) was replaced by polylinker sequences from pGEM-2, was constructed by digestion of pCPS with *Xho*I, which was then blunt-ended with DNA polymerase I (Klenow fragment). The plasmid was then digested with *Sac*I. The fragment containing the HIV-1 sequence was ligated to a *Sac*I-*Sac*I fragment from pGEM-9Zf(-) and a pGEM-2 fragment (containing the polylinker sequence) that had been digested with *Eco*RI and blunt-ended, and then recut with *Sac*I. pCPS/ Δ dse was digested with *Hind*III prior to T7 RNA polymerase transcription.

The plasmids *puse*/CPS, *puse*/CPS/AACAAA, *puse*/CPS/ Δ dse and *p Δ use*/CPS were assembled from synthetic oligonucleotides containing HIV-1 U3 sequences from positions 9538 to 9591 (numbering according to Valsamakis *et al.*, 1991) and R/U5 sequences identical to those present in pCPS. The oligonucleotides were inserted into *Hind*III/*Eco*RI digested pGEM-1. These plasmids were digested with *Eco*RI prior to SP6 RNA polymerase transcription. The transcripts synthesized from these constructs are illustrated in Figure 2A. *puse*/CPS/AACAAA contains a T to C change in the AATAAA hexamer; *puse*/CPS/ Δ dse contains the same substitution of the downstream element (dse) with pGEM-2 polylinker sequences as in pCPS/ Δ dse; *p Δ use*/CPS contains the NXS 9571-9588 mutation described by Valsamakis *et al.* (1991).

Transcripts containing the adenovirus 5 L3 poly(A) site were generated by SP6 RNA polymerase transcription of pL3 (previously termed pAAU+ds, Gilmartin and Nevins, 1989). The chimeric plasmids pHIV/L3 and pL3/HIV were constructed as follows. pCPS was digested with *Xho*I and treated with Klenow polymerase followed by *Bgl*II digestion. pL3 was digested with *Acc*I, which cuts at the L3 poly(A) cleavage site, and treated with Klenow polymerase followed by *Bgl*II digestion. The fragment containing the upstream sequence of the HIV-1 poly(A) site was then ligated to the fragment containing the downstream sequence of the L3 poly(A) site to generate pHIV/L3. Conversely, the upstream sequence of the L3 poly(A) site was blunt-end ligated to the downstream sequence of the HIV-1 poly(A) site to generate pL3/HIV.

The plasmid p Δ Bgl/CPS was constructed by digestion of pCPS with *Bgl*II and *Bgl*II. The fragment containing HIV-1 sequences was then ligated to a *Bam*HI-*Bgl*I pGEM-1 fragment. Transcription of p Δ Bgl/CPS yielded an RNA that contained 34 nucleotides of 5' sequence derived from the pGEM-1 polylinker fused to HIV-1 sequences at position +21. Plasmid p Δ L3 was constructed by digestion of pL3 with *Bam*HI, which cuts 25 bp 5' of the AATAAA hexamer, and *Eco*RI. The fragment containing adenovirus sequences was then ligated to *Bam*HI/*Eco*RI digested pGEM-1. Plasmid pTAR/L3 was constructed by insertion of an oligonucleotide

containing HIV-1 sequences from +1 to +65 into *Hind*III/*Bam*HI digested p Δ L3.

The TAR deletion and stem disruption mutants depicted in Figure 5A were assembled from synthetic oligonucleotides. The constructs *puse*/ Δ TAR and *p Δ use*/ Δ TAR were constructed by deletion of the nucleotides from positions 9593 to 9649 (numbering according to Valsamakis *et al.*, 1991) from *puse*/CPS and *p Δ use*/CPS respectively. To construct *puse*/stem1, the nucleotides involved in TAR RNA base-pairing from positions 9592 to 5613 were substituted with their complementary base in the TAR stem. The two bulged nucleotides at positions 9596 and 9608 were unaltered. The same approach was taken in constructing *puse*/stem2, in which positions 9631 to 9650 were substituted with their complementary base in the TAR stem. Thus, in *puse*/stem1/2, which combines the mutations of *puse*/stem1 and *puse*/stem2, the base-pairing potential of the TAR stem is identical to the wild-type, only the sequence of the two sides of the stem are reversed.

3'-end labeling, RNase T1 and alkaline hydrolysis ladders

Capped RNAs were 3'-end labeled using T4 RNA ligase (Pharmacia) and [³²P]pCp as described by England *et al.* (1980), and subsequently gel-purified. RNase T1 and alkaline hydrolysis ladders of ³²P-3'-end labeled *use*/CPS RNA were generated as described by Query *et al.* (1989).

In vitro 3' processing

Cleavage reactions were carried out essentially as described by Gilmartin *et al.* (1988). 2.9 $\times 10^{-14}$ moles of ³²P-labeled pre-mRNA were incubated in a total reaction vol of 25 μ l containing 72% nuclear extract, 54 mM KCl, 14.4 mM HEPES (pH 7.9), 7.2% glycerol, 0.5 mM 3'dATP, 2% polyvinylalcohol, 0.14 mM EDTA, 0.07 mM PMSF and 0.36 mM DTT for 90 min at 30°C. The reactions were stopped by the addition of SDS to 0.5%, treated with proteinase K, phenolized and ethanol precipitated.

Processing complex formation

Processing complexes were formed in a 20 μ l reaction containing 40% nuclear extract, 87 mM KCl, 0.5 mM MgCl₂, 16.6 mM HEPES (pH 7.9), 8.3% glycerol, 1% polyvinylalcohol, 0.17 mM EDTA, 0.08 mM PMSF and 0.41 mM DTT. The reactions were pre-incubated with 10 μ g of tRNA for 10 min at 30°C prior to the addition of 1.7 $\times 10^{-14}$ moles of ³²P-labeled pre-mRNA. The reactions were incubated with the pre-mRNA for 10 min at 30°C. The reactions were then placed on ice and heparin was added to 5 mg/ml. After at least 5 min on ice the heparin-treated reactions were electrophoresed on a non-denaturing 3% polyacrylamide gel [acrylamide:bisacrylamide = 100:1, in 25 mM Tris base, 25 mM boric acid and 1 mM EDTA (pH 8.0)] at 300 V for 2 h at 4°C. The gel was pre-electrophoresed for 30 min prior to loading.

Complex stability assays were carried out in a 35 μ l reaction containing 40% nuclear extract, 87 mM KCl, 0.5 mM MgCl₂, 16.6 mM HEPES (pH 7.9), 8.3% glycerol, 1% polyvinylalcohol, 0.17 mM EDTA, 0.08 mM PMSF and 0.41 mM DTT. The reactions were pre-incubated with 10 μ g of tRNA for 10 min at 30°C prior to the addition of 2.9 $\times 10^{-14}$ moles of ³²P-labeled pre-mRNA. Following the addition of the pre-mRNA, the reaction was incubated for 10 min at 30°C. A 10 μ l aliquot of each reaction was removed and placed on ice, followed by the addition of heparin to 5 mg/ml. To the remaining reaction was added $\sim 3 \times 10^{-12}$ moles of an unlabeled *use*/CPS pre-mRNA in 2 μ l. The incubation was continued at 30°C, and 10.8 μ l aliquots were removed at 10 and 20 min, placed on ice and treated with heparin. The reactions were then electrophoresed on a non-denaturing 3% polyacrylamide gel as described above.

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