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Inhibition of polyadenylation by stable RNA secondary structure

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ABSTRACT

The presence of a polyadenylation signal in the repeat (R) region of the HIV-1 genome, which is located at both the 5' and 3' ends of the viral transcripts, requires differential regulation of polyadenylation. The HIV-1 poly(A) site can fold in a stable stem–loop structure that is well-conserved among different human and simian immunodeficiency viruses. In this study, we tested the effect of this hairpin on polyadenylation by introducing mutations that either stabilize or destabilize the RNA structure. The HIV-1 sequences were inserted into the pSV2CAT reporter plasmid upstream of the SV40 early poly(A) site. These constructs were transfected into COS cells and transcripts were analyzed for the usage of the HIV-1 versus SV40 poly(A) site. The wild-type HIV-1 poly(A) site was used efficiently in this context and destabilization of the poly(A) hairpin did not affect the polyadenylation efficiency. In contrast, further stabilization of the hairpin severely inhibited HIV-1 polyadenylation. Additional mutations that repair the thermodynamic stability of this mutant hairpin restored the polyadenylation activity. These results indicate that the mechanism of polyadenylation can be repressed by stable RNA structure encompassing the poly(A) signal. Experiments performed at reduced temperatures also suggest an inverse correlation between the stability of the RNA structure and the efficiency of polyadenylation.

INTRODUCTION

The 3' end of almost all eukaryotic RNA polymerase II transcripts is processed to produce mature polyadenylated mRNA molecules. The polyadenylation reaction involves the site-specific endonucleolytic cleavage at the 3' end of the precursor transcript, followed by the addition of ~250 adenosine ribonucleotides (1–5). The important polyadenylation signal is the AAUAAA sequence, which is located ~10–30 nucleotides (nt) upstream of the site of poly(A) addition in most mRNAs. Upstream and downstream U- and GU-rich enhancer elements have been described, but these elements lack a conserved sequence motif (6–8). The AAUAAA hexamer and the downstream enhancer elements compose the core poly(A) site, which appears to be sufficient, at least in some cases, to direct polyadenylation in vivo.

The cellular factors that implement the first step of the polyadenylation reaction are the cleavage and polyadenylation specificity factor (CPSF) and the cleavage stimulation factor (CstF), which recognize the AAUAAA hexamer and the GU-rich downstream sequence element, respectively (9–13). Two additional factors (CFI and CFII) are required for cleavage, although their exact roles have not yet been established. Poly(A) polymerase (PAP), the enzyme that generates the poly(A) tail, is usually also needed for the cleavage step. The poly(A) addition step depends on CPSF, PAP and the poly(A) binding protein PABII. Cooperative binding of these factors to the precursor mRNA is required for efficient polyadenylation (1,9).

Polyadenylation is a constitutive process for most cellular transcripts (14), but regulated polyadenylation has been described for some genes. The immunoglobulin (IgM) heavy chain gene encodes for both a membrane-associated and secreted form of this protein (15,16). The mRNA precursor contains two polyadenylation signals, and the choice of the polyadenylation site determines the type of IgM molecule produced. The relative usage of these polyadenylation signals, and thus the ratio of the two mRNAs produced, is regulated during B-cell maturation. The production of the membrane-bound form is favored in pre-B and B cells, whereas the production of the secreted form is favored in mature plasma cells.

Regulated polyadenylation is essential for HIV-1 and other retroviruses that have the poly(A) signal encoded within the R region, because this sequence is present at both the 5' and 3' ends of all viral transcripts. It is obvious that the 5' poly(A) site must be occluded, whereas the 3' poly(A) site is used efficiently. Numerous mechanisms have been proposed to explain regulated polyadenylation for these retroviruses. (i) The promoter proximity model. There may be a minimum distance that the RNA polymerase II complex must transcribe to reach a conformation and/or composition that is competent to recognize the poly(A) signal (17,18). According to this model, the HIV-1 core poly(A) signals are sufficient for efficient polyadenylation, but are inhibited when positioned too close to the mRNA start site. (ii) The upstream activator model. The core HIV-1 poly(A) signals are postulated to be inefficient, but enhanced by sequences within the U3 region that are present exclusively upstream of the 3' poly(A) site (19–24). (iii) The splice donor inhibition model.

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The major HIV-1 splice donor site, which is located downstream of the 5’ poly(A) site only, has been suggested to interfere with efficient polyadenylation (25,26).

The HIV-1 repeat (R) region adopts an RNA structure consisting of two stable hairpins. The upstream hairpin structure is termed TAR, and is essential for virus replication as it forms the binding site for the viral Tat transcriptional activator protein (27). The downstream structure is called the poly(A) hairpin (28–30). This hairpin structure is conserved in a variety of human and simian immunodeficiency viruses (29), and contains several poly(A) signals; the AAUAAA hexamer, the CA cleavage site and part of the GU-rich downstream enhancer element. HIV-1 mutants with either a stabilized or destabilized hairpin structure demonstrate a severe replication defect (30), suggesting a critical role for this RNA structure in viral gene expression. In this study, we tested the effect of the RNA hairpin on the polyadenylation efficiency. The HIV-1 poly(A) signal was used efficiently for the wild-type and the destabilized mutants. In contrast, stabilization of the stem-loop structure strongly inhibited polyadenylation, indicating that RNA secondary structure may provide a manner to regulate the accessibility of poly(A) signals.

**MATERIALS AND METHODS**

**Plasmid constructs**

HIV-1 sequences used in this study were derived from the infectious molecular clone pLAI (31). The poly(A) hairpin mutations were introduced in the Blue-5’LTR subclone as described previously (30). Revertant viruses were obtained in prolonged culture experiments (32), and part of their genome was subcloned in the Blue-5’LTR plasmid. Structure prediction and free-energy minimization were performed with the MFOLD program in the GCG package (33). The PvuII–HindII blunt-ended DNA fragment containing the HIV-1 polyadenylation signal was subsequently inserted into the unique HindIII site of the pSV2-CAT vector (34), in between the CAT open reading frame and the SV40 early polyadenylation signal. These plasmid constructs are referred to as pSV2CAT–HIV. The PvuII site is present in the HIV-1 sequences of the U3 region (position –22), and the HindII site is present in the Bluescript polylinker immediately downstream of the HIV-1 encoded Ctd site, which is located within the Gag coding sequences (position +374). The B mutant contains an additional PvuII site at position +57. Thus, insertion of the PvuII–HindII fragment of mutant B into pSV2-CAT leads to the loss of a 78 nt U3-R fragment (position –22/+57), including the U3 upstream enhancer element.

**Transfection procedures**

COS cells were grown at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium containing 8% fetal calf serum (FCS), 100 U of penicillin and 100 µg/ml of streptomycin. The cells were grown to 60% confluency in 90 mm dishes and transfected with 12.5 µg DNA by the DEAE–dextran method (35). The medium was refreshed after 24 h incubation. The cells were incubated for another 48 h at 37°C in the standard protocol, or at 21, 25 and 37°C for the temperature study.

**RNA analysis**

Total cellular RNA was isolated by the hot phenol method (35) and dissolved in 25 µl water. The RNA was analyzed by northern blotting (36). RNase protection and RT–PCR. Northern blots were probed with a CAT-specific DNA probe (1599 bp HindIII–Hpal fragment of pSV2-CAT).

An RNA probe for RNase protection was synthesized as follows. The Blue-5’LTR plasmid of mutant E was linearized with PvuII and transcribed by T3-RNA polymerase in 10 µl transcription buffer (40 mM Tris pH 7.5, 2 mM spermidine, 10 mM DTT and 12 mM MgCl2) containing 1 µl plasmid, 10 U T3-RNA polymerase (Boehringer Mannheim), 20 µCi [γ-32P]UTP (3000 Ci/mmol), 0.06 µmol each of ATP, GTP and CTP, 0.02 µmol UTP and 20 U RNase Inhibitor (Boehringer Mannheim) for 2 h at 37°C. After DNase I (Boehringer Mannheim) treatment, this radiolabeled transcript was run on a 6% acrylamide–bisacrylamide/7 M urea gel (acrylamide:bisacrylamide ratio, 19:1). The gel fragments containing the full-size transcripts were isolated and incubated in 400 µl elution buffer (0.5 M NaHAc, 0.1% SDS, 0.1 mM EDTA) at 37°C for 10 h. The eluted product was precipitated with ethanol to remove SDS. The first precipitation was performed by the addition of 1 ml ethanol (96%). The RNA was dissolved in 300 µl water and the second precipitation was performed by the addition of 30 µl of 3 M NaAc pH 5.2 and 600 µl ethanol. The transcript was dissolved in 100 µl water. For each RNase protection assay, we mixed 2 µl probe, 2.5 µl total cellular RNA and 20 µg τRNA (Escherichia coli; Boehringer Mannheim). The RNAs were vacuum-dried and dissolved in 10 µl hybridization buffer (80% formamide, 40 mM PIPES pH 6.7, 0.4 M NaCl, 1 mM EDTA). The mix was covered with two drops of paraffin, denatured for 5 min at 90°C and incubated overnight at 50°C. After slowly cooling to room temperature, we added 250 µl RNase buffer (10 mM Tris pH 7.5, 0.5 M NaCl), 10 µg RNase A and 200 U RNase T1, followed by incubation at 30°C for 30 min. One microgram of carrier τRNA was added before phenol-extraction and ethanol precipitation. The sample was dissolved in formamide loading buffer, denatured for 2 min at 90°C and analyzed on a 6% acrylamide–bisacrylamide/7 M urea gel (acrylamide:bisacrylamide ratio, 19:1).

The RT–PCR protocol consisted of an initial reverse transcription reaction that was performed on total cellular RNA (2.5 µl) with a GT19 primer (100 ng). Annealing of this primer to the RNA was performed in 7.5 µl annealing buffer (133 mM Tris–HCl pH 8.5, 21.3 mM MgCl2, 80 mM KCl, 2.7 mM DDT) by heating for 10 min at 65°C, and slowly cooling to room temperature. After annealing, 8 U RNase Inhibitor (Boehringer Mannheim) and 12.5 U AMV-RT enzyme (Boehringer Mannheim) were added and reverse transcription was performed in 20 µl RT buffer (50 mM Tris–HCl pH 8.3, 8 mM MgCl2, 30 mM KCl and 2 mM DTT) with 0.3 mM of each dNTP. The cDNA product (1 µl) was amplified by PCR with 100 ng GT19 antisense primer and 100 ng CATX sense primer (TAGTGCCCTTGACTAGAGATC) in 100 µl PCR buffer (20 mM Tris–HCl pH 8.3, 2 mM MgCl2, 50 mM KCl, 0.1 mg/ml BSA), 10 mM of each dNTP and 1.5 U Taq polymerase (35 cycles; 1 min at 95°C, 1 min at 55°C and 2 min at 72°C). The PCR material was precipitated and dissolved in 6 µl water. The CATX primer (1.5 µg) was 5’-end labeled in 20 µl kinase buffer (50 mM Tris–HCl pH 7.5, 10 mM MgCl2, 5 mM DTT) with T4 polynucleotide kinase (Boehringer Mannheim) and 25 µCi [γ-32P]ATP (3000 Ci/mmol, Amersham) for 1 h at 37°C.
Three additional PCR cycles were performed with 1 µl of the labeled CATX primer (75 ng), 100 ng GT19 primer and 2 µl of the non-radiolabeled PCR material. The PCR sample was denatured in formamide buffer and analyzed on a 6% acrylamide–bisacrylamide/7 M urea gel (acrylamide:bisacrylamide ratio, 19:1). In the temperature study, the gel signals were quantitated with the PhosphorImager (Molecular Dynamics). The relative HIV-1 poly(A) site usage was calculated by comparison of the HIV-1 polyadenylated transcript with the read-through SV40-polyadenylated transcript. To do this, the signal of the short HIV-1 fragment (p) plus the read-through SV40 fragment (q) was set at 100% (p + q = 100%), and the relative HIV-1 poly(A) site usage (r) was calculated as follows: $r = \frac{p}{p+q} \times 100$.

DNA sequencing analysis

The PCR product obtained with the GT19/CATX primer set was phenol extracted, precipitated with ethanol and dissolved in 50 µl water. One microlitre of PCR product and 40 ng CATX primer were used for the Taq DyeDeoxy Terminator cycle sequencing protocol (ABI Biosystems). The sequencing sample was analyzed on an Applied Biosystem 373 DNA sequencer.

RESULTS

To study the role of the HIV-1 poly(A) hairpin in polyadenylation, several structure mutants were designed. Figure 1 shows the wild-type hairpin (wt, $\Delta G = -15.3$ kcal/mol) and the mutant hairpins with mutations that either stabilize (mutant A, $\Delta G = -25.7$ kcal/mol) or destabilize the structure (mutants B, $\Delta G = -8.8$ kcal/mol; mutants C and D, $\Delta G = -6.8$ kcal/mol). The CD double mutant contains the sequence alterations of mutants C and D, and folds a hairpin with a calculated thermodynamic stability that is slightly higher than that of the wild-type structure ($\Delta G = -17.0$ kcal/mol). The E deletion mutant provided a negative control in which the upper part of the hairpin, including the AAUAAA hexamer, was removed.

The wild-type and mutant poly(A) sites were cloned into the pSV2-CA T vector (34) downstream of the CA T gene and upstream of the SV40 early polyadenylation signal (Fig. 2). These pSV2CAT–HIV constructs were transiently transfected into COS cells and total cellular RNA was isolated at 3 days after transfection. Usage of the HIV-1 poly(A) signal will result in the formation of a 1673 nt transcript (Fig. 2). Inefficiency of the HIV-1 signal will result in read-through transcription and usage of the SV40 poly(A) site, yielding an extended 2047 nt transcript. We used RNase protection to measure the HIV-1 polyadenylation efficiency. An antisense RNA probe was made from the sequence of deletion mutant E (Fig. 2). Transcripts polyadenylated at the HIV-1 poly(A) site will protect a probe fragment of 77 or 88 nt, depending on the position of the mutations in the individual HIV-1 sequences (Table 1). The B mutant contains a U3-deletion (see Materials and Methods) and, therefore, produces a fragment of only 8 nt. Read-through transcription and polyadenylation at the SV40 poly(A) site results in the protection of two fragments, a 5′-fragment of the previously mentioned length and a 3′-fragment of 284, 287 or 375 nt, depending on the sequence of the template (Table 1). The polyadenylation signal of the wild-type HIV-1 sequence was used efficiently (Fig. 3, lanes 3 and 6). As expected, the E deletion mutant produced a read-through signal (Fig. 3, lane 9). Two read-through products were observed for the A mutant (Fig. 3, lane 4), which are probably caused by alternative splicing in the vector sequences (37). This result indicates that the HIV-1 poly(A) signal can be silenced by stable RNA structure. No read-through products were observed for the destabilized mutants B, C and D and the double mutant CD (Fig. 3 and results not shown). Because the B mutant lacks part of the U3 sequence, HIV-1 polyadenylation is apparently not dependent on the upstream U3 enhancer in the context of this plasmid construct. Likewise, partial mutation of the downstream GU-rich enhancer...
The two transcript forms generated by the pSV2CAT–HIV construct. The CAT gene is transcribed from the SV40 early promoter and polyadenylation takes place at either the HIV-1 poly(A) site or at the SV40 early poly(A) site. Polyadenylation at the HIV poly(A) site produces a transcript of 1673 nt, whereas read-through produces a transcript of 2047 nt. Usage of the HIV-1/SV40 poly(A) sites was analyzed by RNase protection and RT–PCR, and the probes/primers are indicated. The antisense RNA probe used for the RNase protection assay was derived from the sequence of the deletion mutant E. Because of the 31 nt deletion in mutant E (Δ), the probe yields a short fragment of 88 nt when the HIV-1 poly(A) site is used and two fragments of 88 and 287 nt when the SV40 poly(A) site is used. Polyadenylation at the HIV-1 and SV40 poly(A) sites results in PCR fragments of 256 and 629 bp, respectively.

**Table 1. Predicted fragments in the RNase protection and the RT–PCR assays**

<table>
<thead>
<tr>
<th>Constructs</th>
<th>RNase protection</th>
<th>RT–PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIV poly(A)</td>
<td>SV40 poly(A)</td>
</tr>
<tr>
<td>wt</td>
<td>88</td>
<td>88 + 287</td>
</tr>
<tr>
<td>wt−c</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>A</td>
<td>88</td>
<td>88 + 287</td>
</tr>
<tr>
<td>B</td>
<td>8</td>
<td>8 + 287</td>
</tr>
<tr>
<td>C</td>
<td>88</td>
<td>88 + 284</td>
</tr>
<tr>
<td>D</td>
<td>77</td>
<td>77 + 284</td>
</tr>
<tr>
<td>CD</td>
<td>77</td>
<td>77 + 284</td>
</tr>
<tr>
<td>E−c</td>
<td>–</td>
<td>375</td>
</tr>
<tr>
<td>pSV2-CATF</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>rev.A2</td>
<td>88</td>
<td>88 + 287</td>
</tr>
<tr>
<td>rev.A4</td>
<td>88</td>
<td>88 + 287</td>
</tr>
</tbody>
</table>

*a*Fragment length in nucleotides (nt).

*b*Fragment length in base pairs (bp).

*c*HIV-1 poly(A) signal is absent.

We also used RT–PCR to measure polyadenylation at the wild-type and mutant HIV-1 poly(A) sites. Reverse transcription was performed on total cellular RNA with a GT primer, as illustrated in Figure 2, and the cDNA was PCR-amplified with the same GT primer in combination with an upstream primer that is specific for transcripts generated by the pSV2CAT–HIV constructs. Polyadenylation at the wild-type HIV-1 poly(A) site will result in the formation of a 256 bp PCR product, whereas usage of the downstream SV40 signal will result in a 629 bp fragment (Fig. 2). The predicted lengths of the PCR fragments for the different mutants are listed in Table 1. The RT–PCR products were radiolabeled and analyzed on a denaturing gel. This analysis confirms that polyadenylation at the HIV-1 poly(A) site is efficient for the wild-type sequence and for mutants B, C and D with a destabilized hairpin, and the double mutant CD (Fig. 4).

The control constructs, wt and E, produced only the read-through signal. Read-through transcription was also observed for the A mutant, but a low level of HIV-1 polyadenylation can be detected with this sensitive PCR protocol.

The sequence changes in mutant C affect part of the downstream GU-rich enhancer element near the cleavage site (Fig. 1). To determine whether these mutations affect the actual site of cleavage, the PCR fragment of mutant C was sequenced. We found that the cleavage site used by this mutant is identical to that of the wild-type HIV-1 element, that is the CA dinucleotide at position 96–97 (Fig. 1).

The sequence changes in mutant C and CD did not affect the efficiency of polyadenylation. Similar results were obtained in northern blotting experiment (not shown).

It can be argued that the polyadenylation defect of mutant A is not caused by the stabilized RNA structure, but rather by the three nucleotide changes in this mutant (Fig. 1). In fact, the C nucleotide of the CA cleavage site is removed in this mutant, which may affect the polyadenylation efficiency and/or the site of cleavage. To test whether the observed inhibition of polyadenylation of the mutant A is structure-specific, we made additional pSV2CAT–HIV constructs with sequences that were derived from revertant viruses that restored the replication capacity of virus mutant A (30,32). Such revertant sequences contain additional mutations that reduce the thermodynamic stability of the mutant A hairpin. We tested two revertant elements A2 and A4, each with two additional mutations (marked by a black box in Fig. 5). The thermodynamic stability of the hairpin in the A2 and A4 revertants (ΔG = −17.1 and −14.9 kcal/mol, respectively) is significantly reduced compared with the A mutant (ΔG = −25.7 kcal/mol), and is similar to that of the wild-type HIV-1 structure (ΔG = −15.3 kcal/mol). The wild-type, mutant and revertant constructs were transfected into COS cells and the transcripts were analyzed by the RT–PCR assay (Fig. 6). Polyadenylation at the wild-type HIV-1 poly(A) site will result in the formation of a 256 bp PCR product, whereas usage of the downstream SV40 signal will result in a 629 bp fragment. These products will be 254 and 627 bp for the A mutant and the...
Figure 3. RNase protection analysis of the pSV2CAT–HIV transcripts. COS cells were transfected with the pSV2CAT–HIV constructs indicated on top of the panel. The 375 nt RNA probe is shown in lane 1. The mock sample (lane 2) represents RNase protection on total cellular RNA of mock-transfected cells. Both short and long transcripts [HIV-1 versus SV40 poly(A) site] produce the 5′ signal, only the long transcript yields a 3′ signal (indicated on the left). The predicted length of the protected fragments for the different mutants are listed in Table 1.

revertants (Table 1). The read-through product observed for the A mutant (Fig. 6, lane 1) was not observed for the two revertants (Fig. 6, lanes 3 and 4), indicating that polyadenylation was fully restored in both revertants. We subsequently analyzed the actual site of cleavage for the two revertants. Both revertant sequences were found to polyadenylate at the normal position. Thus, cleavage occurred at the UA dinucleotide, with the U residue replacing the deleted C96 residue.

Figure 4. RT–PCR analysis of the pSV2CAT–HIV transcripts. COS cells were transfected with the pSV2CAT–HIV constructs indicated on top of the panel. The mock sample (lane 1) represents an RT–PCR assay on total cellular RNA of mock-transfected cells. Polyadenylation at the wild-type HIV poly(A) site results in a short PCR fragment of 256 bp, polyadenylation at the SV40 early poly(A) site results in a long PCR fragment of 629 bp. The exact length of the PCR products of the different mutants are listed in Table 1 and indicated on the left.

Table 2. HIV-1 poly(A) site usage at different temperatures

<table>
<thead>
<tr>
<th>Constructs</th>
<th>21°C</th>
<th>25°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>87</td>
<td>92</td>
<td>96</td>
</tr>
<tr>
<td>A</td>
<td>6</td>
<td>6</td>
<td>44</td>
</tr>
<tr>
<td>B</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>C</td>
<td>99</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>D</td>
<td>96</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>CD</td>
<td>86</td>
<td>87</td>
<td>93</td>
</tr>
</tbody>
</table>

Temperature is a parameter that influences the thermodynamic stability of RNA structure. For instance, the calculated thermodynamic stability (in kcal/mol) of the wild-type hairpin changes as a function of the temperature: −15.3 (37°C), −20.2 (25°C) and −22.1 (21°C). By varying the assay temperature, we tried to obtain additional evidence for a repressive role of stable RNA structure on the process of polyadenylation. We therefore performed transfections with the pSV2CAT–HIV constructs and cultured the cells for 2 days at 21, 25 and 37°C. The RT–PCR protocol was used to determine the relative HIV-1 poly(A) site usage (Table 2). At 37°C, the relative polyadenylation efficiencies of the wild-type HIV-1 sequence and the CD mutant were 96 and 93%, respectively. Optimal polyadenylation (100%) was measured for all three destabilized mutants B, C and D. Reduced polyadenylation (44%) was measured for the stabilized A mutant. These findings confirm the previous results, but also indicate that a hairpin with wild-type thermodynamic stability (wild-type and the CD mutant) is slightly inhibitory to polyadenylation. Decreasing the temperature to 25 and 21°C resulted in a significant reduction of HIV-1 polyadenylation with the wild-type construct, the CD mutant and the stabilized A mutant. The polyadenylation efficiency of the destabilized mutants was not reduced (mutant B), or reduced only marginally (mutants C and D) at lower temperatures, indicating that the loss of polyadenylation observed for the other constructs is not a general phenomenon, but rather triggered by stable RNA structure.
temperature (32), in which the wild-type virus was demonstrated to be replication impaired (30), such revertants contain additional mutations that weaken the poly(A) hairpin, without removal of the original mutations. Indeed, opening of the stabilized poly(A) hairpin restored efficient usage of the HIV-1 poly(A) signal. To test whether this inhibition is induced by stable RNA structure, we also tested several revertant sequences that improve replication of the mutant virus (30,32). Such revertants contain additional mutations that weaken the poly(A) hairpin, without removal of the original mutations. Indeed, opening of the stabilized poly(A) hairpin restored efficient usage of the HIV-1 poly(A) signal. Because the revertants still contain the original mutations, it can be concluded that the inhibition of polyadenylation is caused by the presence of the upstream U3 enhancer may overcome the potential repressive effect of RNA structure. Thus, a complex interplay of positive and negative elements may be involved in regulated HIV-1 polyadenylation. It is obvious that experiments with full-length HIV-1 transcripts in infected cells are required to further elucidate the role of the poly(A) hairpin. Several literature reports are consistent with a negative role of stable RNA structure in polyadenylation. The poly(A) site of the bovine growth hormone gene has been suggested to lose activity when the AAUAAA hexamer and the site of endonucleolytic cleavage were positioned within an artificial RNA structure element (38). In vitro evolution studies were used to select for variant HIV-1 poly(A) sequences that are active in polyadenylation (39,40), and such sequences are characterized by the absence of strong secondary structure. RNA structure can also positively influence the process of polyadenylation. The HTLV-I retrovirus uses a complex RNA secondary structure to juxtapose the AAUAAA hexamer, the CA cleavage site and part of the downstream GU-enhancer are positioned within a stable RNA stem–loop structure (28–30). We called this structure the poly(A) hairpin. It has been demonstrated that this hairpin is important for viral replication, as mutants with a stabilized or destabilized hairpin are replication impaired (30). We now report that the stabilized hairpin exhibits a severe polyadenylation defect. Further studies are needed to test whether this inhibition is induced by stable RNA structure, we also tested several revertant sequences that improve replication of the mutant virus (30,32). Such revertants contain additional mutations that weaken the poly(A) hairpin, without removal of the original mutations. Indeed, opening of the stabilized poly(A) hairpin restored efficient usage of the HIV-1 poly(A) signal. Because the revertants still contain the original mutations, it can be concluded that the inhibition of polyadenylation is caused by the presence of the upstream U3 enhancer may overcome the potential repressive effect of RNA structure. Thus, a complex interplay of positive and negative elements may be involved in regulated HIV-1 polyadenylation. It is obvious that experiments with full-length HIV-1 transcripts in infected cells are required to further elucidate the role of the poly(A) hairpin.
AAUAAA hexamer and the cleavage site, which are separated by 274 nt in the linear sequence (41–43). A spacing role of RNA structure has also been suggested for the TAR hairpin, which may juxtapose the upstream enhancer and the poly(A) signal (22,44).

RNA structure has been suggested to play a regulatory role at several levels of gene expression. For instance, ample evidence has been provided for a regulatory role of template structure in RNA splicing and translation. Splicing was found to be repressed upon introduction of artificial hairpins within the 5′ splice donor site of Saccharomyces cerevisiae mRNA species (45) and several naturally occurring RNA secondary structures that modulate splicing efficiency have been documented (46,47). Likewise, many reports demonstrated the effects of RNA structure on initiation of translation. For instance, reduction of the thermodynamic stability of the hairpin at the ribosomal binding site of the coat protein cistron of coliphage MS2 led to an increase in translation in a predictable and calculable manner (48). This inverse correlation between RNA stability and translation activity was demonstrated to represent binding of ribosomes to the unfolded form of the ribosomal binding site. Similarly, expression of the polyprotein of hepatitis C virus was strongly inhibited when the hairpin containing the ribosomal binding site was systematically stabilized (49). These examples and the current finding that RNA structure can also play a regulatory role in mRNA polyadenylation, highlight the potential of RNA structure to fine-tune gene expression levels.

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