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The human immunodeficiency virus type 1 polyadenylylation signal: A 3′ long terminal repeat element upstream of the AAUAAA necessary for efficient polyadenylylation

(RNA processing/upstream element)

ALEXANDRA VALSAMAKIS*, STEVEN ZEICHNER†, SUSAN CARSWELL*, AND JAMES C. ALWINE*

*Department of Microbiology, Graduate Group of Molecular Biology, School of Medicine, 560 Clinical Research Building, 422 Curie Boulevard, University of Pennsylvania, Philadelphia, PA 19104-6142; and †Division of Infectious Diseases, Children's Hospital of Philadelphia, Department of Pediatrics, University of Pennsylvania, Philadelphia, PA 19104

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ABSTRACT **Several polyadenylylation (PA) signals containing elements upstream of the AAUAAA have recently been characterized. Similar to PA elements found downstream of the AAUAAA, the upstream elements function to increase efficiency of AAUAAA use as a signal for cleavage and PA. Using deletion and linker scanning mutations we show that the PA signal of human immunodeficiency virus type 1 contains upstream elements transcribed from the U3 region of the 3′ long terminal repeat. The element that has the greatest effect on PA site use lies 77 to 94 nucleotides upstream of the AAUAAA, between the TATA element and the transcriptional initiation site. Mutations in the adjacent region, between 59 and 76 nucleotides upstream of the AAUAAA, have a smaller effect on PA efficiency. Mutations in a region further upstream, between 141 and 176 nucleotides upstream of the AAUAAA, also affected PA modestly. Functional similarity between upstream elements was indicated by the ability of the human immunodeficiency virus upstream region to replace the upstream region of the simian virus 40 late PA signal. The sequence of the major upstream element of human immunodeficiency virus is uracil-rich, analogous to many defined downstream PA elements. This fact may imply that upstream and downstream elements have similar mechanisms of action.**

Most higher eukaryotic mRNAs are posttranscriptionally processed at their 3′ ends by cleavage and polyadenylylation (PA). Most processing signals characterized are comprised of two elements: AAUAAA and an element lying 5–60 nucleotides (nt) downstream of the AAUAAA (1, 2). These downstream elements lack distinct consensus sequences but appear to be either G- or G+U-rich (1, 3, 4), and function to increase efficiency of PA specified by the adjacent AAUAAA.

Recently a second class of PA signals has been identified. These signals have elements upstream and downstream of AAUAAA. Examples of this class of signals include the simian virus 40 (SV40) late (5), adenovirus L1 (6), and ground squirrel hepatitis virus (7) PA signals. We have recently characterized the upstream element of the SV40 late PA signal; it lies 15–47 nt upstream of AAUAAA and, similar to its downstream counterpart, regulates efficiency of PA signal use (5). We are interested in identifying other PA signals with upstream elements. PA signals of retroviral transcripts are likely candidates, and we have studied the human immunodeficiency virus (HIV) as a model system.

Fig. 1 diagrams the proviral form of HIV with the long terminal repeats (LTRs) highlighted in relation to transcription and PA. In the 5′ LTR, the AAUAAA and downstream element (8) lie downstream of the transcriptional start site. Were these elements efficiently used, newly initiated RNAs would be processed at a site only 77 nt from the 5′ end. However, this result would not occur if the PA signal in the 5′ LTR was inefficient, due either to its proximity to the transcription start site, as has been recently suggested for the cauliflower mosaic virus (9) or to the lack of a U3-encoded upstream element. In the latter mechanism, a putative upstream element would appear in precursor RNAs only through transcription of the 3′ LTR. Existence of an upstream element in HIV has been suggested by analysis of chimeric PA signals (7). In the following study we have used deletion and linker scanning mutations to show that sequences upstream of the HIV-1 PA signal affect efficiency of PA. Mutation of U3 sequences 77–94 nt upstream of the AAUAAA greatly affected PA. Mutation of the adjacent region, 59–77 nt upstream, had a smaller effect. Additionally, mutation of 141–176 nt upstream had a modest effect. Lastly, the HIV upstream region can functionally replace the upstream region of the SV40 late PA signal, indicating that the two different upstream elements share similar functions.

MATERIALS AND METHODS

Construction of Transient Expression Plasmids Using Wild-Type and Deleted HIV-1 PA Signals. Two wild-type HIV PA signals were constructed from 3′ and 5′ LTRs; each showed equivalent PA efficiency. For simplicity they are designated pL-HIVPA in the text. Nucleotide numbering is according to the 3′ LTR. The 3′ -LTR-bearing plasmid was constructed by insertion of the 661-base-pair (bp) *Ban* I–*Xba* I (9068–9727) fragment from pU3R+39 (strain HXB3; ref. 8) into the pL-0 *Sma* I site (Fig. 2; ref. 5). The 5′ LTR was subcloned by replacement of the 3′ -LTR pL-HIVPA *Xba* I–*Bgl* II fragment with the equivalent fragment from p5′LTR-CAT (gift of L. Bachelor, DuPont; infectious proviral clone pHXB2gpt *Hpa* II–*Hin*dIII fragment subcloned into pSV0-CAT; CAT is chloramphenicol acetyltransferase). pL-ΔBglII resulted from deletion of the 507-bp *Bgl* II fragment (9105–9611) from 3′-LTR pL-HIVPA. pL-Δ9209, -Δ9264, and -Δ9371 were created by subcloning 5′-U3-region deletions constructed from NXS (*Nde* I–*Xho* I–*Sal* I) linker scanning mutants (see below).

Construction of Linker Scanning Mutants Through the Upstream PA Region. NXS linker scanning mutations were made by a PCR-directed technique (10). NXS mutants were first constructed in p5′LTR-CAT, then placed downstream of the CAT gene by replacing an analogous fragment of pL-HIVPA with the 550-bp linker substitution-bearing *Xba* I–*Bgl* II fragment of p5′LTR-CAT.

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Abbreviations: HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; nt, nucleotide(s); PA, polyadenylylation; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40.

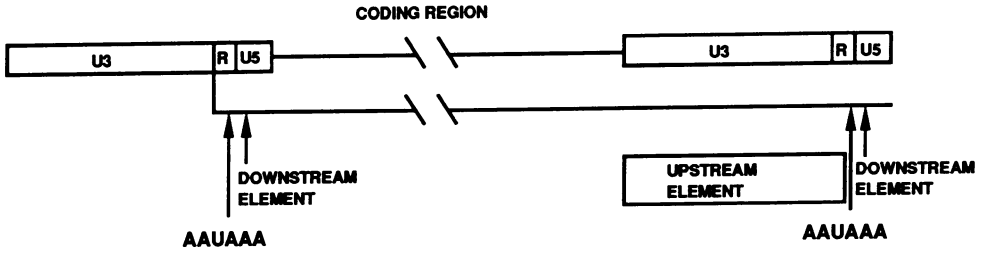

FIG. 1. Proviral form of HIV-1. The diagram highlights transcription that initiates in the 5′ LTR and reads through AAUAAA and downstream element of the PA signal within the 5′ LTR and again in the 3′ LTR where PA occurs. The suspected region of an upstream element that may increase the efficiency of use of the 3′ LTR PA signal is indicated.

Construction of Chimeric PA Signals. Plasmid pL-LPA contains the wild-type SV40 late PA signal (SV40 2533–2770) downstream from the CAT gene (5). pL-NUS was created by deletion of 110 bp upstream of AAUAAA, between *Bam*HI and *Xba* I, from pL-LPA (5). The chimeric PA signal containing the HIV upstream element and the SV40 late AAUAAA and downstream element, pL-HIVUS/LPA, was constructed by inserting the 236-bp *Ava* I–*Hin*dIII (9433–9669) fragment into pL-NUS at *Xba* I. Subcloning removed the last adenine of the HIV AAUAAA and thus destroyed the HIV PA signal.

Transfections and Nucleic Acid Analyses. COS cells (5×10^5 cells per 100-mm plate) were transfected by $CaPO_4$ technique with 10 μg of plasmid DNA (4, 5). Forty hours after transfection cells were harvested, and total nucleic acid was extracted. Total RNA was prepared from part of the extract (4, 5); 10 μg was fractionated on 1.5% agarose-formaldehyde gels and analyzed by Northern (RNA) blot using a ^{32}P-labeled CAT-specific RNA probe (4, 5). Plasmid DNA was prepared from a second sample of total nucleic acid and quantitated by dot blot. Variations in transfection efficiency were corrected through standardization to the relative amount of plasmid DNA in transfected cells (11).

RNase protection analyses were performed on total RNA prepared and analyzed by Northern blot as described above. Three-microgram samples were hybridized to an HIV–LTR-

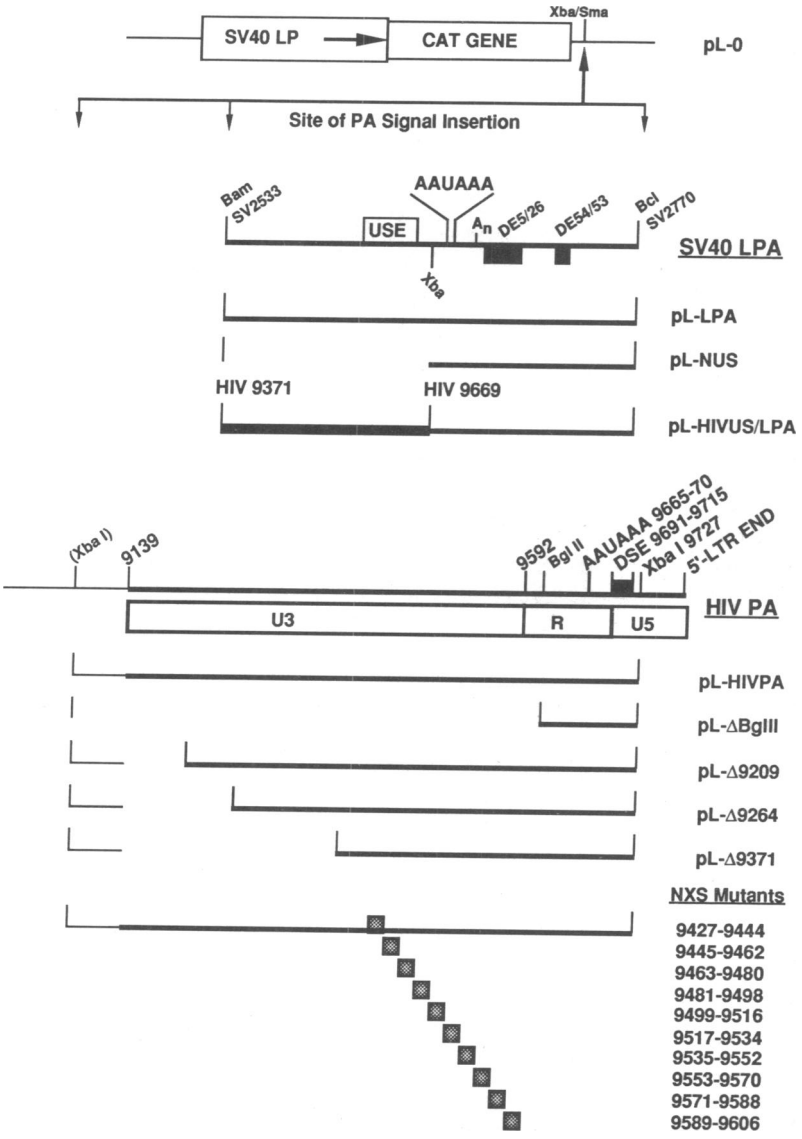

FIG. 2. Diagram of the transient expression plasmids used in these studies. The parent CAT expression vector pL-0 is shown with its polylinker region for insertion of PA signals. The CAT gene is under control of the large tumor antigen-responsive SV40 late promoter. High levels of late promoter activity are obtained using pL-0-based constructions because SV40 large tumor antigen mediates both plasmid amplification and transcriptional activation of the SV40 late promoter (10, 11). Diagrams of the SV40 late and the HIV PA signals (LPA and HIV PA, respectively) are shown; in each case positions of the AAUAAA and downstream element(s) are indicated. For the SV40 late PA signal the AAUAAA (SV40 nt 2657–2662) is flanked by an upstream element (5) as well as by two downstream elements (DSEs). One DSE is a guanine-rich region between 5 and 26 nt downstream of the AAUAAA (DE5/26) defined in the *Xenopus* oocyte system (12). The other element lies between 45 and 53 nt downstream of the AAUAAA (DE54/53). It is G+U-rich and was defined by using transfection analyses in COS cells (4). The definition of two elements by using different systems may indicate redundancy of downstream elements or different elements responsive to variable growth or developmental conditions of the cell. Shown under the diagrams of each PA signal are the specific mutations or constructions made in these regions and inserted into pL-0. Open spaces indicate regions deleted. The linker scanning mutations (NXS mutants) consecutively replaced 18-bp regions with an *Nba* I–*Xba* I–*Sal* I polylinker. Positions of the linkers in the context of the HIV PA insert are indicated by shaded boxes; the numeric nomenclature denotes the first and last base in the 3′-LTR replaced by the linker.

specific probe and digested with RNase A (5). The probe was prepared from pGEMHIVRU5, constructed by inserting the 144-bp *Bgl* II–*Eco*RI fragment from pLΔBglIII into pGEM3Zf$^-$. Nucleotides 1–116 of this fragment are HIV nt 9611–9727; this region lies downstream of all mutations described above and encodes the AAUAAA, cleavage site, and downstream region (8). The 5′-terminal 18 nt of the probe will hybridize to unprocessed transcripts because they are pL-0 polylinker sequences present in all HIV plasmids. Probes were synthesized with T7 RNA polymerase, 0.2 μg of gel-purified *Pvu* II–*Pvu* II template fragment (485 bp) from pGEMHIVRU5, and [^{32}P]UTP (5).

RESULTS

Activity of the SV40 Late and HIV PA Signals in Transient Expression Analysis. Fig. 2 shows the set of transient expression plasmids used in these studies; the parent plasmid pL-0 contains the SV40 late promoter and origin of replication upstream of the CAT gene. This is followed by a polylinker into which PA signals were inserted. In pL-LPA (Fig. 2), the SV40 late PA signal (LPA) was inserted (5). Using transient transfection of COS cells followed by Northern analysis (lane 1, Fig. 3*A* and ref. 5) and RNase protection of CAT-specific RNA (5), we have shown that pL-LPA produces high levels of properly polyadenylylated CAT RNA. Deletion of sequences upstream of AAUAAA in the SV40 LPA, pL-NUS, dramatically lowers utilization of the PA signal (Fig. 3*A*, lane 2).

The efficiency of the HIV PA signal was tested by insertion of 3′ LTR sequences (9139–9727) into the pL-0 polylinker. This plasmid, pL-HIVPA (Fig. 2), contains the HIV AAUAAA, downstream element (8), and putative upstream element(s). COS cells transfected with pL-HIVPA produced high levels of CAT-specific 1300-nt RNA. This RNA initiated at the SV40 late promoter and was cleaved and polyadenylylated at the HIV PA signal (Fig. 3*A*, lane 3; *B*, *C*, and *D*, lanes 1).

The HIV fragment inserted into pL-HIVPA might cause aberrant activation or occlusion of the SV40 late promoter because it contains an HIV-1 promoter and enhancer as well as a PA signal. However, we used the SV40 late promoter in our plasmids to avoid such interference. This promoter does not respond to the presence of enhancer elements. Rather, it is specifically and efficiently activated by SV40 large tumor (T)-antigen-mediated effects (13, 14). Hence, LTR-mediated activation of the late promoter was neither expected nor observed: comparison of the amount of CAT RNA produced by pL-LPA and pL-HIVPA shows nearly equivalent levels of properly initiated and processed CAT RNA (Fig. 3*A*, lanes 1 and 3; and data not shown), suggesting equal late promoter activity in each case. Additionally, transcription from the LTR was not detected. Hence occlusion of the late promoter is also unlikely. In transfections with pL-HIVPA, for example, we were unable to detect large RNAs corresponding to transcripts that initiate in the LTR and read through the late promoter and CAT gene. Hence, LTR-initiated transcription is either insignificant or terminates before the late promoter and CAT gene. Lastly, the LTR should not be active under our experimental conditions because the HIV trans-activator *tat* is not present and SV40 large tumor antigen, though a promiscuous trans-activator, has no more than a 2- to 3-fold effect on basal transcription from the HIV LTR (J.C.A., unpublished observation).

Deletion Analysis of the Upstream Region of the HIV PA Signal. To determine whether the HIV PA contains an upstream element we introduced a set of deletions into pL-HIVPA. Deletion to 9612, pL-ΔBglII (Fig. 2), reduced the level of properly polyadenylylated RNA to 9% of wild type (Fig. 3*B*, lane 2; Table 1), indicating an upstream element. To further define the upstream region, progressive deletions were analyzed (Fig. 2). All deletions, pL-Δ9220, pL-Δ9274, and pL-Δ9382, yielded wild-type levels of properly processed CAT RNA (Fig. 3*B*, lanes 3, 4, and 5). These data suggest that an element lies between nt 9372 and 9612.

Definition of the Upstream Element of the HIV PA Signal by Linker Scanning Analysis. Analysis of the region from 9372 to 9612 was accomplished with a set of linker scanning mutants that replaced consecutive 18-bp regions with an *Nde* I–*Xho* I–*Sal* I polylinker (NXS mutants). These mutants are shown in Fig. 2; their numeric designation indicates the first and last HIV nucleotide replaced by the linker.

Mutation NXS 9517–9534 (Fig. 3*D*, lane 3) consistently showed a modest decrease in properly polyadenylylated RNA to 80% wild type (Table 1). However, NXS 9571–9588 (Fig. 3*D*, lane 6) consistently caused a marked decrease in properly processed RNA to ≈30% of the wild-type level (Table 1). In addition, the flanking mutant NXS 9589–9606 (Fig. 3*D*, lane 7) caused a 50% decrease in the level of properly processed RNA.

NXS mutations between nt 9427 and 9516 (Fig. 3*C*, lanes 2–6; Table 1) showed no negative effect on production of properly processed RNA. However, a slight increase in levels of properly processed RNA was noted using mutations

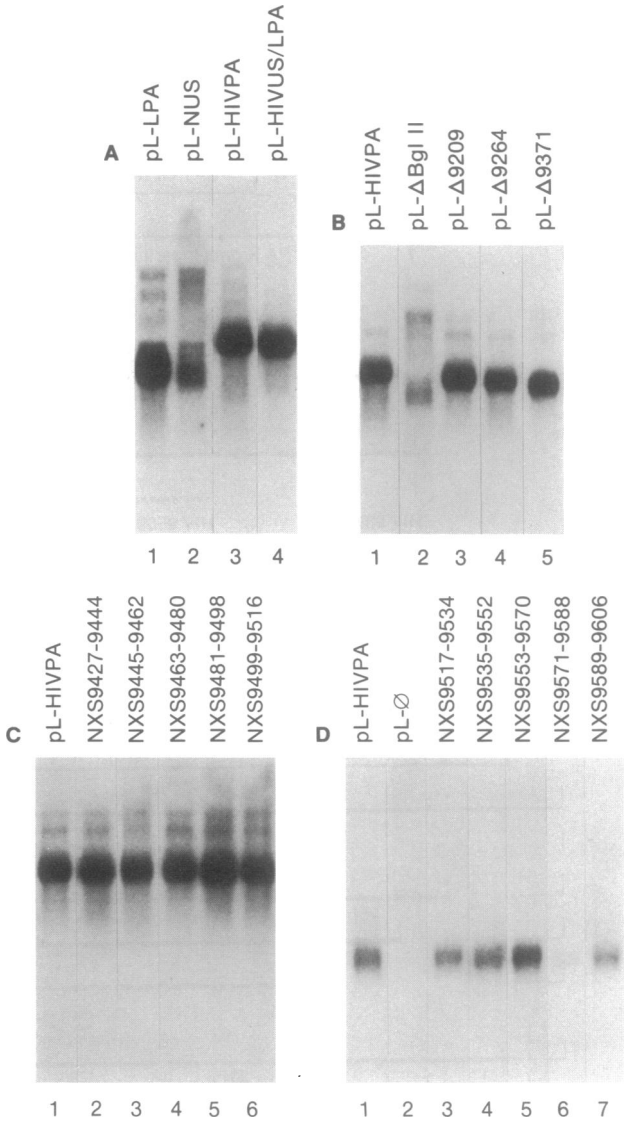

FIG. 3. Northern analysis of CAT-specific RNA produced in COS cells transfected with the transient expression constructions described in Fig. 2. Electrophoresis for the experiment of *D* was longer than in other experiments, and a 1% gel was used; hence the different appearance.

between 9463 and 9498 (NXS 9463–9480 and NXS 9481–9498; Table 1). Such data suggest that elements affecting RNA stability may have been mutated.

Nuclease Protection Analysis of the 3′ Ends of the RNAs Produced by the Linker Scanning Mutants. Nuclease-protection studies were used to demonstrate use of the HIV PA signal-cleavage site (Fig. 4). The region of the HIV LTR encoded by the 385-nt probe was downstream of all deletion and linker scanning mutations. RNAs processed at the HIV PA signal protect a 79-nt probe fragment. Extended RNAs protect a 144-nt fragment. The wild-type pL-HIVPA showed an abundant 79-nt band, whereas pL-ΔBgl II dramatically diminished this band. After correction for transfection efficiency (data not shown), NXS mutations between nt 9499 and 9606 produced levels of the 79-nt band in agreement with Northern blot quantitation. Mutant NXS 9499–9516 was lower than expected from the Northern analyses because less total cell RNA was present in the hybridization reaction. Overall, the data showed use of the HIV cleavage site in wild-type and mutant constructions.

The Upstream Element of the HIV PA Signal Can Functionally Replace the SV40 Upstream Element. To further test whether the HIV upstream region could function as an upstream PA element we asked whether it could replace the upstream element of the SV40 late PA signal. The use of the late PA signal, pL-LPA (Fig. 2), is reduced by >10-fold by removal of the upstream element, pL-NUS (Fig. 3*A*, compare lanes 1 and 2; ref. 5). However, insertion of HIV sequences 9433–9669, pL-HIVUS/LPA (Fig. 2), restored utilization of the late signal (Fig. 3*A*, lane 4). Similar rescue of the ground squirrel hepatitis virus PA signal by an HIV U3 fragment has been reported (7).

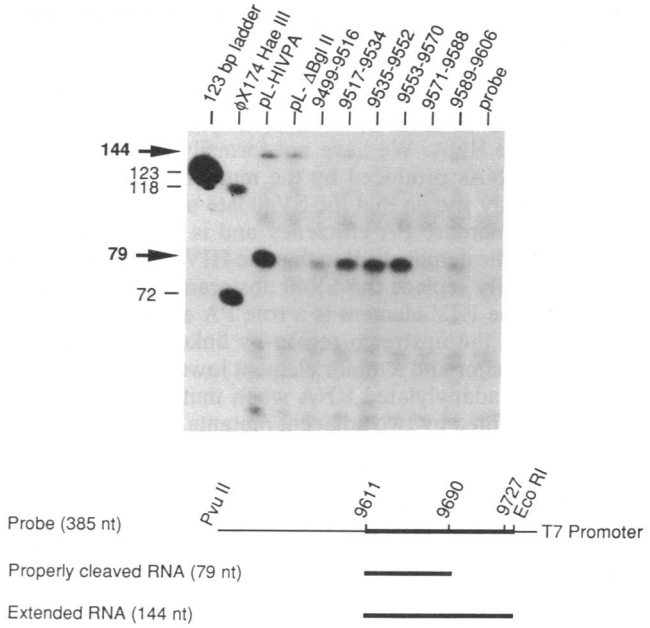

FIG. 4. (*Upper*) Ribonuclease-protection analysis to determine the position of the 3′ end of RNAs using the wild-type or mutant HIV PA signals. Three micrograms of total cellular RNA was hybridized with 2×10^5 cpm of ^{32}P-labeled RNA probe (see text). After RNase digestion the protected bands were separated on a 10% polyacrylamide/7 M urea gel. (*Lower*) The 79- and 144-nt bands are produced by the properly processed and extended transcripts, respectively. The remainder of the probe, thin lines, are pGEM3Zf$^-$ sequences not complementary to the transcripts.

DISCUSSION

The data suggest that HIV RNA processing occurs at the 3′ LTR PA signal because specific U3 sequences upstream of the AAUAAA mediate efficiency of signal use. This conclusion implies that the PA signal transcribed in the 5′ LTR cannot be efficiently used. However, other factors may also be important; for example, the PA signal in the 5′ LTR may

Table 1. Quantitation of properly processed CAT-specific RNA

	Data set 1				Data set 2				
	DNA	RNA	RNA/DNA	% WT	DNA	RNA	RNA/DNA	% WT	Mean ± SD
Wild type	2.22	9.77	4.40	100	2.00	4.76	2.38	100	100
pL-ΔBglII	3.57	1.40	0.39	9	3.95	0.89	0.22	9	9 ± 0
pL-Δ71	2.17	12.0	5.53	125	1.30	3.90	3.00	126	126 ± 1
pL-Δ125	2.12	8.80	4.15	94	1.60	3.58	2.24	94	94 ± 0
pL-Δ233	2.37	11.1	4.68	106	1.95	6.05	3.10	130	120 ± 14
Wild type	5.77	35.0	6.06	100	6.40	29.3	4.58	100	100
NXS									
9427–9444	5.45	36.9	6.77	111	4.33	22.4	5.17	112	112 ± 1
9445–9462	4.10	24.2	5.90	97	4.50	25.1	5.57	122	109 ± 17
9463–9480	2.05	18.5	9.02	149	4.25	29.5	6.96	152	150 ± 2
9481–9498	4.00	30.6	7.65	126	4.23	24.4	5.77	126	126 ± 0
Wild type	2.36	6.36	2.69	100	2.31	6.24	2.70	100	100
pL-ΔBglII	3.50	1.00	0.28	10			ND		10
NXS									
9499–9516	2.88	9.00	3.12	116	2.29	6.03	2.63	97	106 ± 13
9517–9534	1.55	3.35	2.16	80	2.72	6.28	2.30	85	82 ± 4
9535–9552	1.59	4.50	2.83	105	1.40	3.60	2.57	95	100 ± 7
9553–9570	2.44	8.10	3.31	123	1.70	4.41	2.59	96	110 ± 19
9571–9588	2.89	2.78	0.96	36	2.32	1.90	0.82	30	33 ± 4
9589–9606	2.26	3.24	1.43	53	2.91	3.84	1.32	49	51 ± 3

Total nucleic acid was harvested from transfected cells and divided into samples for RNA and plasmid DNA preparation. Northern blots of CAT RNA (similar to those shown in Fig. 3) and dot blots of the plasmid DNA were quantitated with a Molecular Dynamics PhosphorImager. PhosphorImager numerical readout varied with time of exposure of PhosphorImager screens to Northern and dot blots. Duplicate results of three representative experiments are shown. The signal from properly processed CAT bands was quantitated and then standardized for transfection efficiency by dividing by the plasmid DNA dot-blot signal value. From these numbers, the % of wild type (WT) properly processed CAT RNA was determined for each mutant. Mean % and SD for the duplicate samples are shown. ND, not determined.

be too close to the transcriptional start site for efficient use, as suggested for the PA signal of cauliflower mosaic virus (9).

It can be argued that the sequences defined as the HIV PA upstream element may be involved in stabilizing the transcript. Hence, deletion or mutation of the element may destabilize the RNA. We have not formally established the stability of RNAs produced by the mutants. However, we have previously shown that the SV40 late upstream element functions to increase PA efficiency and is not a stabilizing element (5). The demonstration that the HIV upstream region can functionally replace the SV40 upstream region strongly argues that the HIV element is a true PA element.

Analysis of the upstream region by linker scanning indicated that a major and a minor element lowered the levels of properly polyadenylylated RNA when mutated. The major element is defined by two adjacent mutants, NXS 9571–9588 and NXS 9589–9606. These mutations lower the level of properly processed RNA to 30% and 50% wild type, respectively. The minor element, which lowers the level of properly processed RNA to 80% of the wild-type level (Table 1), is indicated by mutation NXS 9517–9534. That each of these elements affects the level of properly processed RNA is suggested by the *Bgl* II deletion mutant pL-ΔBgl II, which removes both regions and lowers properly processed RNA to 9% of wild-type level. Interestingly, upstream elements of other retroid viruses, such as ground squirrel hepatitis virus (7), and DNA tumor viruses, such as SV40 (S.C., N. Schek, and J.C.A., unpublished observation), appear to be bipartite.

Fig. 5 shows the sequences of the major element between HIV nt 9571 and 9606. The region, beginning 59 nt upstream of the AAUAAA, is conserved among HIV-1 strains (15); the UUUUU sequence in this region is relatively highly conserved; a few isolates have UUUCU and UUUUC. The sequences giving the greatest effect when mutated are in the U3 region (underlined in Fig. 5, HIV nt 9571–9588) 77–94 nt upstream of the AAUAAA between the TATA box and the transcription initiation site of the LTR. The finding that mutation of the adjacent region, extending into the R region (nt 9589–9606), also had a significant effect on PA suggests that the element extends into this region. The overall region is uracil-rich (42% uracil, 25% guanine, 25% cytosine, 8% adenine); similar uracil-richness is seen in the SV40 upstream element (52% uracil; ref. 5). Such uracil-richness is reminiscent of the structure of downstream elements. In particular, the UUUUU element is also found in one of the downstream elements of the SV40 late PA signal (DE45/53; see Fig. 1 and legend; ref. 4). This SV40 downstream UUUUU element facilitates binding of C protein, an RNA binding protein found in heterogeneous nuclear ribonucleoprotein complexes (16, 17). In addition, this sequence will allow proper PA *in vitro* when coupled downstream of an AAUAAA element (17). This correlation between binding and PA is intriguing. Upstream and downstream elements may be binding sites for heterogeneous nuclear ribonucleoprotein proteins. PA signals with upstream and downstream elements would bracket AAUAAA with heterogeneous nuclear ribonucleoprotein-binding sites. This may greatly facilitate specific recognition of the AAUAAA by the PA complex and result in highly efficient PA.

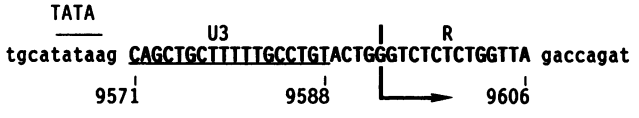

FIG. 5. Sequence of the major upstream element of the HIV-1 PA signal as defined by linker scanning analyses. The defined sequences between nt 9571 and 9606 are highlighted; the underlined region indicates the sequences replaced by mutant NXS 9571–9588 that had the greatest effect on PA. The upstream element is indicated in relationship to the TATA box and the transcriptional start site of the LTR. The AAUAAA is located 58 nt downstream.

PA appears to be a much more significant level of gene expression control than previously thought. Regulation via PA has been reported to occur in a tissue-specific and developmental manner (18–26). In addition, utilization of a PA signal has been reported to vary due to the growth state of the cell (27–30) and during viral infection (31–33). It seems likely that such regulation will be mediated through both upstream and downstream elements.

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