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An RNA Secondary Structure Juxtaposes Two Remote Genetic Signals for Human T-Cell Leukemia Virus Type I RNA 3′-End Processing

ANAT BAR-SHIRA,* AMOS PANET, AND ALIK HONIGMAN

Departments of Molecular Genetics and Virology, The Hebrew University-Hadassah Medical School, Jerusalem, 91010 Israel

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Sequence analysis of the human T-cell leukemia virus type I (HTLV-I) long terminal repeat (LTR) does not reveal a polyadenylation consensus sequence, AAUAAA, close to the polyadenylation site at the 3′ end of the viral RNA. Using site-directed mutagenesis, we demonstrated that two *cis*-acting signals are required for efficient RNA processing in HTLV-I LTR: (i) a remote AAUAAA hexamer at a distance of 276 nucleotides upstream of the polyadenylation site, and (ii) the 20-nucleotide GU-rich sequence immediately downstream from the poly(A) site. It has been postulated that the folding of RNA into a secondary structure juxtaposes the AAUAAA sequence, in a noncontiguous manner, to within 14 nucleotides of the polyadenylation site. To test this hypothesis, we introduced deletions and point mutations within the U3 and R regions of the LTR. RNA 3′-end processing occurred efficiently at the authentic HTLV-I poly(A) site after deletion of the sequences predicted to form the secondary structure. Thus, the genetic analysis supports the hypothesis that folding of the HTLV-I RNA in the U3 and R regions juxtaposes the AAUAAA sequence and the poly(A) site to the correct functional distance. This unique arrangement of RNA-processing signals is also found in the related retroviruses HTLV-II and bovine leukemia virus.

Human T-cell leukemia virus type I (HTLV-I) is the etiologic agent associated with adult T-cell leukemia (8, 38, 42). The HTLV-I provirus contains the structural retroviral genes *gag*, *pol*, and *env* and additional regulatory genes coding for Tax, a transcriptional activator (6), and Rex, a posttranscriptional regulator (15). Transcription of the retrovirus starts at the border of the U3 and R regions in the 5′ long terminal repeat (LTR). The RNA 3′ end is generated at the R and U5 border of the 3′ LTR (17). In the majority of cases studied so far, the genetic signals and the mechanisms for generation of retroviral RNA 3′ ends are similar to those described for most eukaryotic genes.

A functional poly(A) site is characterized by *cis*-acting RNA sequences, including the well-conserved AAUAAA hexamer, located 10 to 30 nucleotides (nt) upstream of the cleavage site [poly(A) site] and a highly variable downstream GU- or U-rich element (2, 7, 9, 10, 19, 20, 22, 25, 27, 28, 39, 44). The spacing between the AAUAAA sequence and the downstream element is critical for efficient poly(A) site functioning (10, 14, 19, 39). Careful examination of the HTLV-I LTR sequences proximal to the poly(A) site (R and U5 junction) did not reveal any AATAAA sequence at the consensus distance of 10 to 30 nt. The first and only AATAAA sequence in the LTR is found in the U3 region, 276 nt upstream of the poly(A) site. The location of the AATAAA sequence relative to the promoter and its distance from the poly(A) site vary in the different retrovirus groups (Table 1). In the first two groups, the poly(A) signal, located at either the R or the U3 regions as indicated in Table 1, is situated at the consensus 16 to 22 nt upstream of the poly(A) site. In the third group of retroviruses, which includes HTLV-I, an AATAAA sequence is found 258 to 288 nt upstream of the junction of the R and U5 regions. Since AATAAA sequences that do not function as poly(A) signals are found randomly along genomes, it is not clear that the AATAAA sequence found in the HTLV-I LTR is part of the RNA 3′-end generation system. Several hypotheses could be raised to explain the generation of the HTLV-I RNA 3′ end. (i) The RNA 3′ end is not generated by processing but by an as yet unknown mechanism such as transcription termination. (ii) The RNA 3′-end processing is controlled by a novel *cis*-acting genetic signal. (iii) Folding of the RNA brings the AAUAAA sequence sufficiently close to the poly(A) site to facilitate RNA processing. In the present work, we determined the *cis* signals involved in the generation of the HTLV-I RNA 3′ end and the possible mechanism which enables processing.

MATERIALS AND METHODS

Construction of recombinant plasmids. The *Hpa*I cleavage site is located in the pSV2CAT expression vector (11) downstream of the *cat* gene and about 80 nt upstream of the simian virus (SV40) poly(A) site. The 3′ end of the chloramphenicol acetyltransferase (CAT) mRNA is generated at this poly(A) site. The different HTLV-I sequences were cloned into the *Hpa*I site to generate the various recombinant plasmids. An 820-bp *Sma*I restriction fragment spanning from base 31 in U3 of the LTR to base 850 within the beginning of the *gag* gene (32) was inserted at the *Hpa*I cleavage site to give rise to plasmid pHT8 (Fig. 1A). Sequence numbers of HTLV-I relate to the first nucleotide of the U3 region (32). Conversion of the HTLV-I AATAAA (8584 to 8589) to GAGAAA by site-directed mutagenesis generated plasmid pHT5. Deletion of the SV40 poly(A) site in plasmids pHT8 and pHT5 (from the *Nhe*I cleavage site, position 826 in the HTLV-I *gag* DNA, to the *Bam*HI site in pSV2CAT, Fig. 1) yielded plasmids pHT14 and pHT17, respectively.

* Corresponding author.

TABLE 1. 3'-end-processing signals in retroviruses[a]

Retrovirus	Location of AAUAAA in LTR	Distance of AAUAAA from poly(A) site (nt)
Murine leukemia virus	R	16
Human immunodeficiency virus	R	18
Rous sarcoma virus	U3	22
Murine mammary tumor virus	U3	18
HTLV-I	U3	276
HTLV-II	U3	288
Bovine leukemia virus	U3	258

[a] The region in the LTR where the polyadenylation signal AATAAA is located and the distance to the polyadenylation site in various retroviruses (23, 26, 29, 31, 32, 35, 36).

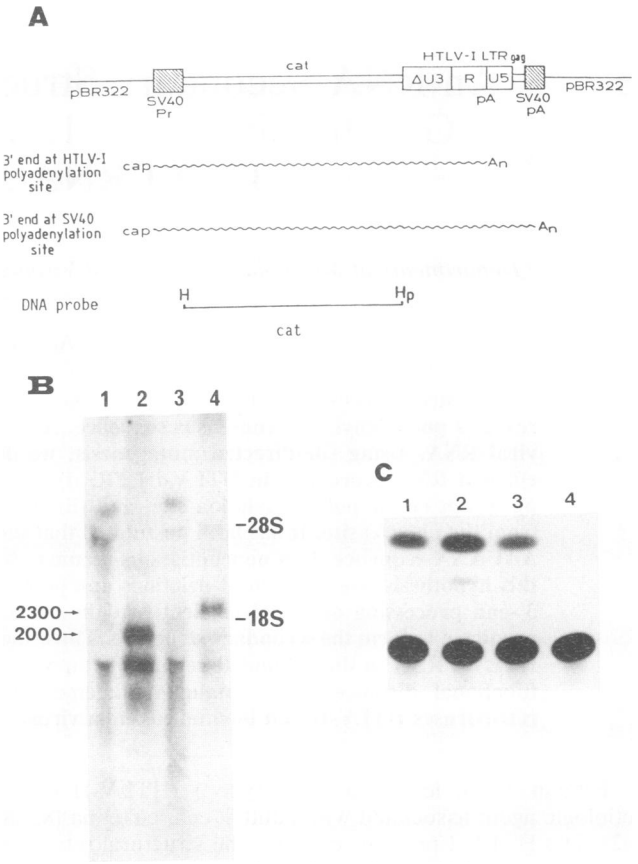


FIG. 1. Analysis of RNA 3'-end generation by vectors containing HTLV-I LTR. (A) Linear illustration of the recombinant vector pHT8. The HTLV-I LTR and the beginning of the *gag* gene (nt 31 to 850 according to Seiki et al. [33]) were cloned in the unique *Hpa*I restriction site of pSV2CAT (11), between the *cat* gene and the SV40 poly(A) signal (pA). The putative 3' ends of the RNA transcripts (wavy lines) initiated at the SV40 promoter (Pr) and processed at either of the two possible poly(A) sites are indicated. The DNA probe used to identify mRNA includes the *cat* gene sequences between the *Hin*dIII (H) and *Hpa*I (Hp) restriction sites. (B) Northern blot analysis of RNA isolated from human 293 cells (12) transiently expressing the pHT8 vector and derivative. Poly(A)$^+$ RNA was separated on an oligo(dT)-cellulose column. RNA was subjected to electrophoresis in glyoxal-agarose gel (21), transferred to GeneScreen filters, and hybridized to ^{32}P-labeled *cat* DNA probe by standard hybridization methods (43). Lanes 1 and 3, poly(A)$^-$ RNA; lanes 2 and 4, poly(A)$^+$ RNA. Lanes 1 and 2, RNA derived from plasmid pHT8; lanes 3 and 4, pHT5 [pHT8 mutated in the putative HTLV-I poly(A) signal, AATAAA to GAGAAA, by site-directed mutagenesis]. RNA size in nucleotides (2,000) was determined by reference to 18S and 28S rRNA size markers. (C) Transient *cat* gene expression of plasmids pHT8 (lane 1), pHT5 (lane 2), pHT14 (lane 3), and pHT17 (lane 4). CAT enzyme activity was determined on equal amounts of cellular protein.

pHT4 is missing all the HTLV-I sequences from 11 bases downstream to the AATAAA at positions 8584 to 8589. This plasmid was constructed by insertion of a *Stu*I-*Hin*fI HTLV-I DNA fragment (8150 to 8600 on the HTLV-I sequence) into the pSV2CAT *Hpa*I site. Conversion of the HTLV-I AATA AA sequence to GAGAAA yielded plasmid pHT19. An artificial *Bam*HI site was created by site-directed mutagenesis at the junction between the 3' end of the HTLV-I and the SV40 sequences in pHT4 and pHT19. Deletion of the *Bam*HI DNA fragment generated plasmids pHT20 and pHT21, respectively. These plasmids are missing the SV40 poly(A) site.

Cloning of HTLV-I sequences from *Stu*I located at the end of the *env* gene to *Sau*3A in the LTR U5 region (positions 8150 and 8893, respectively) into the pSV2CAT *Hpa*I site generated plasmid pHT1. In this plasmid, only 35 bases of the HTLV-I LTR U5 region are present. Mutation in the putative poly(A) signal yielded plasmid pHT3. Deletion of the SV40 poly(A) site in these two plasmids was done as described above (generating pHT11 and pHT13, respectively). Plasmid pHT10 was derived from pHT1 by deletion of the HTLV-I sequences downstream from the *Bsu*36I site located in the LTR R region (base 8667). The SV40 poly(A) site was deleted as described above (plasmid pHT12). To construct pHT22 (see Fig. 3), two synthetic complementary oligonucleotides composed of 50 bases of HTLV-I sequences (8574 to 8591, 8847 to 8878) and 7 additional bases at the ends (generating an *Hpa*I restriction site at the 5' end after cloning and adding a *Bam*HI site at the 3' end) were amended and ligated at the *Hpa*I site of pSV2CAT. The sequence of the synthetic oligonucleotide is 5'-AACATATG GCTCAATAAACTTGCTCAACTCTACGTCTTTGTTT CGTTTTCTGGATCC-3'. The SV40 poly(A) site was deleted by omitting the 135-bp DNA fragment located between the two *Bam*HI cleavage sites (pHT27). An *Eco*RV cleavage site was created in the 50-bp oligonucleotide at the postulated position for the beginning of the secondary structure. A 190-bp DNA fragment purified from pBR322 (*Nhe*I-*Eco*RV) was inserted at this position (plasmid pHT23). The SV40 poly(A) sequences were deleted as described above (pHT28). Introduction of point mutations at the GU-rich sequence downstream from the HTLV-I poly(A) site in plasmid pHT8 gave rise to pHT24 and pHT25. The point mutations in plasmid pHT25 generated a *Sal*I cleavage site. Deletion of the sequence from the *Sal*I site and downstream from *Nhe*I in *gag* yielded plasmid pHT26.

Site-directed mutagenesis. Conversion of the HTLV-I AA TAAA putative poly(A) signal (nucleotides 8584 to 8589) to GAGAAA within plasmids pHT8, pHT1, and pHT4 (Fig. 2) was performed by the method of Morinaga et al. (24). A 25-mer oligonucleotide, 5'-GCATATGGCTCGAGAAACTA GCAGG-3', was utilized for mutagenesis. The *Xho*I restriction site generated by the mutation facilitated verification of the mutated clones after colony hybridization. The mutations were further confirmed by nucleotide sequencing (30). To generate an additional *Bam*HI cleavage site in plasmids pHT1, pHT4, and pHT10, we utilized the oligonucleotide 5'-

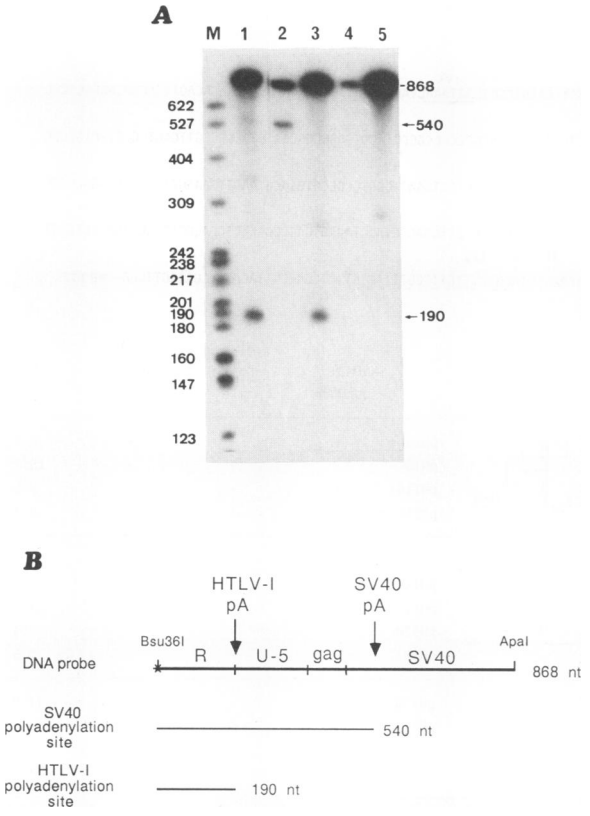


FIG. 2. S1 nuclease protection mapping of HTLV-I poly(A) site and signal. (A) Purified poly(A)$^+$ RNA from transfected 293 cells was hybridized to a ^{32}P-3'-end-labeled 868-bp DNA fragment (from the *Bsu*36I cleavage site, position 8689 within the HTLV-I LTR, to the *Apa*I site in pSV2CAT). The 3' end of RNA made of pHT8 harboring a complete HTLV-I LTR (lane 1) was mapped at the R-U5 junction, 190 bases from the *Bsu*36I cleavage site. The 3' end of RNA made of pHT5, harboring a mutation at the HTLV-I AAUAAA (lane 2), mapped at the SV40 poly(A) site (540-base protected fragment). Deletion of the SV40 poly(A) site from pHT8 (pHT14, lane 3) did not affect cleavage at the HTLV-I poly(A) site. No mRNA was detected when the two processing signals were missing (pHT17, lane 4). Full-length DNA probe can be seen in lane 5, and DNA markers are shown in lane M. Sizes of fragments (right) and markers (left) are in nucleotides. (B) Structures of plasmid and DNA probe and sizes of S1 nuclease DNA fragments protected by the different RNAs.

ACTTGTTTATTGGATCCTATAATGGTTAC-3'. To introduce mutations in the GU-rich sequence in plasmid pHT8, we utilized two oligonucleotides: 5'-ACTCTACGTCAGTA CTTCGTTTTCTGT-3' for the poly(A) site proximal region, generating a *Sca*I restriction site (pHT24), and 5'-TACGTC TTTGTGTCGACATCTGTTCTGC-3' for the distal region, generating a *Sal*I restriction site (pHT25).

Transfection of cells. Human 293 cells (12) were grown in Dulbecco's modified Eagle medium (4.5 g of glucose per liter), supplemented with 10% fetal calf serum and 2 mM glutamine. Transfections were done by the calcium phosphate precipitation method (41). Cells (2×10^6) in a 100-mm plate were transfected with 10 μg of DNA.

RNA analysis. RNA was isolated 30 h after transfection by the guanidium isothiocyanate method and centrifugation through cesium chloride cushion (4). The poly(A)$^+$ RNA was separated on oligo(dT)-cellulose columns by the method of Maniatis et al. (18), resolved by 1.2% agarose-glyoxal gel electrophoresis, and transferred to a GeneScreen filter (Dupont, NEN Research Products, Boston, Mass.) (21). A *Hin*dIII-*Hpa*I restriction DNA fragment of pSV2CAT (*cat* probe, Fig. 1A) was ^{32}P labeled by nick translation and used as a probe for hybridization. Northern (RNA) blot hybridization was done by the DEAE-dextran method (43).

S1 nuclease protection analysis. Poly(A)$^+$ RNA was prepared as described above. DNA probes (see Fig. 2 and 4) were 3' end labeled by using the Klenow fragment of *Escherichia coli* DNA polymerase I and the appropriate [^{32}P] deoxynucleoside triphosphate (dCTP for the pHT8, pHT24, pHT25, and pHT26 probes and dTTP for the pHT22 probe). Hybridization conditions and S1 nuclease digestion were essentially as described previously (17). Hybridization reactions included 0.1 to 1 μg of poly(A)$^+$ RNA and 3×10^4 cpm of 3'-end-labeled probe in a mixture of 80% formamide, 40 mM PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (pH 6.4), 400 mM NaCl, and 1 mM EDTA. RNA from pHT8, pHT5, pHT14, pHT24, pH25, and pHT26 was hybridized for 3 h at 57°C. RNA purified from pHT22- and pHT27-transfected cells was hybridized at 46°C for 3 h. RNA-DNA hybrids were digested for 30 min at 37°C with 200 U of S1 nuclease (Boehringer) in 280 mM NaCl–50 mM sodium acetate–4.5 mM $ZnSO_4$–20 μg of denatured salmon sperm DNA per ml. After phenol-chloroform extraction and ethanol precipitation, the protected fragments were separated on 4% acrylamide–8 M urea denaturing gels alongside appropriate size markers. Autoradiographic exposures were made at −70°C for 6 to 18 h with Curix intensifying screens.

CAT enzyme assay. Cells were harvested 30 h after transfection, and CAT enzyme activity was determined on equal amounts of cellular protein (11). Efficiency of transfection was determined by hybridization of low-molecular-weight DNA (16) with a ^{32}P-labeled DNA *cat* probe. The copy number of the episomal DNA was determined by comparison of the hybridization values with those obtained by hybridization of the same probe to calibrated standard dilutions of the plasmids used for the transfection. CAT activity was corrected accordingly.

RESULTS

Mapping of HTLV-I RNA 3' end. To study the genetic signals controlling the generation of the 3' end of HTLV-I RNA, we expressed the relevant LTR sequences of the virus in a specific eukaryotic vector. This expression vector was designed to identify signals involved in the generation of RNA 3' ends. The HTLV-I DNA fragment, purified from plasmid pATM31 (33), includes most of the 5' LTR and the beginning of the *gag* gene (nt 31 to 850 on the HTLV-I sequence). This fragment was inserted into the unique *Hpa*I site of pSV2CAT (11) between the bacterial reporter gene *cat* and the SV40 poly(A) site (pHT8, Fig. 1A). Transcription of the *cat* gene in pHT8 is expected to initiate at the SV40 early promoter and extend through the *cat* gene into the LTR sequence. RNA 3'-end processing may occur either at the HTLV-I LTR sequences, generating RNA about 2,000 bases long, or downstream at the SV40 poly(A) site, giving rise to RNA of about 2,300 nt. In both cases, functional *cat* mRNA should be expressed (Fig. 1A).

This recombinant plasmid, containing two poly(A) sites in tandem, facilitates isolation of stable polyadenylated RNA after inactivation of the putative HTLV-I RNA-processing signals. In addition, generation of stable RNA expression may be confirmed by monitoring CAT activity. To test the

utility of the recombinant plasmid pHT8, human 293 cells were transfected and RNA was purified after 30 h. Total cellular RNA was separated on an oligo(dT)-cellulose column. The polyadenylated [poly(A)$^+$] and the non-poly(A)$^+$ RNA were subjected to Northern blot analysis. A well-defined poly(A)$^+$ RNA species of about 2,000 nucleotides did hybridize to the ^{32}P-labeled DNA *cat* probe (Fig. 1B, lane 2). This RNA corresponded in size to the sequence between the SV40 promoter and the LTR in pHT8. No such RNA species was detected in the poly(A)$^-$ RNA fraction, indicating that the 3′ end generated in the HTLV-I LTR is polyadenylated. We cannot account for the lowest RNA band in the Northern blot. The size of this band, which is present in both the poly(A)$^+$ and poly(A)$^-$ RNA fractions, did not change after inactivation of the HTLV-I poly(A) site as described below (plasmid pHT14, Fig. 1B, lane 4).

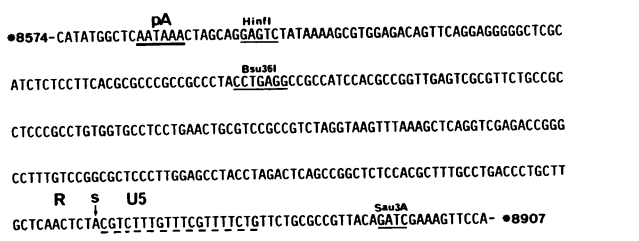
To precisely map the RNA 3′-end processing site, we subjected the purified poly(A)$^+$ RNA to S1 nuclease analysis after hybridization to a ^{32}P-3′-end-labeled 868-bp DNA probe. This DNA fragment, purified from pHT8, spans the R, U5, and SV40 sequences (Fig. 2B). As evident from Fig. 2, lane 1, a discrete DNA band 190 bases long was protected from S1 digestion. This DNA corresponds to the distance between the 3′ end of the DNA probe to the HTLV-I LTR R-U5 junction. This result indicates that the 3′ end of the *cat* RNA expressed from pHT8 is processed at the HTLV-I authentic poly(A) site. The HTLV-I poly(A) site is preferentially used in pHT8 as no poly(A) RNA with a 3′ end processed at the SV40 poly(A) site was detected (expected size of the protected DNA is 540 bases).

To verify that 3′-end RNA processing at the HTLV-I LTR in pHT8 is not dependent on the SV40 processing signals located downstream, we deleted the SV40 poly(A) signal (pHT14). S1 nuclease mapping of the poly(A)$^+$ RNA made in pHT14-transfected cells demonstrated that the 3′ end of *cat* RNA is generated efficiently and at the same position as in the presence of the SV40 poly(A) signal (Fig. 2, lane 3). CAT activity in cells transfected with pHT14 was also similar to that determined in pHT8-transfected cells (Fig. 1C, lanes 3 and 1, respectively). Thus, the HTLV-I LTR appears to provide all the essential signals for RNA 3′-end processing.

Identification of HTLV-I poly(A) signal. Examination of the R and U5 regions of the HTLV-I LTR does not reveal any AAUAAA hexamer at the expected distance from the RNA 3′ end. The first AATAAA sequence is found in the LTR U3 region, 276 nt upstream of the poly(A) site. To determine whether this sequence participates in processing of the HTLV-I RNA 3′ end, we mutated the sequence AATAAA in plasmids pHT8 and pHT14 to GAGAAA (plasmids pHT5 and pHT17, respectively). This sequence is not supposed to function as a polyadenylation signal (40).

The poly(A)$^+$ RNA made in pHT5-transfected cells was longer than that transcribed off the parental plasmid pHT8 (Fig. 1B, compare lane 4 with lane 2). The size difference suggests that the 3′ end of pHT5 RNA is generated at the SV40 poly(A) site rather than at the LTR sequences. The observation that pHT5 RNA protects a DNA fragment of 540 bases in the S1 analysis further indicates that the 3′ end of these RNA molecules is generated at the SV40 poly(A) site (Fig. 2A, lane 2). Since rescue of a 190-base DNA fragment is not detected, it follows that the conversion of the AAUAAA sequence to GAGAAA abolishes processing at the HTLV-I poly(A) site. It is not clear why CAT is expressed in the mutated plasmid pHT5 at a higher rate than in the parental plasmid pHT8 (Fig. 1C, lane 2, and Fig. 3).

Neither CAT activity nor polyadenylated RNA were de-



B

Plasmids		HTLV-I AAUAAA	HTLV-I poly A site	SV40 -poly A signal	CAT activity
R, U3, U5, pA, S	pHT8	+	+	+	8.0
	pHT5	-	+	+	15.3
	pHT14	+	+	-	9.5
	pHT17	-	+	-	0.7
	pHT4	+	-	+	25.9
	pHT19	-	-	+	13.2
	pHT20	+	-	-	0.4
	pHT21	-	-	-	0.5
	pHT10	+	-	+	11.8
	pHT12	+	-	-	0.7
	pHT1	+	+	+	13.5
	pHT3	-	+	+	6.3
	pHT11	+	+	-	6.4
	pHT13	-	+	-	0.9
	pHT22	+	+	+	13.1
	pHT27	+	+	-	15.1
	pHT23	+	+	+	13.2
	pHT28	+	+	-	0.5

FIG. 3. Genetic analysis of HTLV-I RNA 3′-end-processing signals by the CAT assay. (A) DNA sequence from the HTLV-I LTR including the poly(A) signal, sequences participating in the generation of the proposed secondary structure (8592 to 8846), the poly(A) site, and the putative processing signal downstream from the poly(A) site (s). Restriction endonuclease cleavage sites used are indicated. (B) Various plasmids were used for transfection followed by CAT assays as described in the legend to Fig. 1C. Efficiency of transfection was determined for each culture by hybridization of the low-molecular-weight DNA (16) with ^{32}P-labeled *cat* probe and compared with hybridization values (in counts per minute) of standard dilutions of the input plasmids. CAT activity (given in counts per minute per microgram of protein per minute of reaction) was corrected for concentration of plasmid in each sample. A schematic representation of HTLV-I sequences of the various plasmids is illustrated as a stem-and-loop structure (32). The solid line represents sequences that exist in the RNA of the designated plasmid, and the broken line represents deleted sequences (for details, see Materials and Methods). The U3, R, and U5 regions of the LTR as well as the poly(A) signal (pA) and poly(A) site (S) are indicated. + and − represent the presence and absence, respectively, of the poly(A) genetic signals. The results presented are of one representative experiment of several.

tected after transfection with a plasmid deleted of the SV40 poly(A) consensus signal and mutated in the HTLV-I AATAAA sequence (plasmid pHT17, Fig. 1C, lane 4; Fig. 2A, lane 4). This result further indicates that the AAUAAA sequence in the U3 region of the HTLV-I LTR is part of the RNA 3'-end-processing signal.

Role of RNA secondary structure in the polyadenylation process. The surprisingly long distance from the AATAAA poly(A) signal to the poly(A) site raises the question of how this remote signal participates in the processing event. Two possible mechanisms may be postulated. (i) The distance between the two genetic elements is circumvented by additional novel signals, and (ii) folding of the RNA juxtaposes the poly(A) signal to the consensus distance from the poly(A) site (32) (Fig. 2).

To examine these possibilities, we constructed several mutant plasmids that were expressed in 293 cells. CAT enzyme activity was assayed in equal amounts of cellular proteins, and the activity was adjusted to the efficiency of cell transfections. The results presented in Fig. 3 indicate that mutations or deletions of HTLV-I processing signals affect the stability of CAT mRNA and subsequent enzyme activity. In these experiments, control plasmids containing the SV40 poly(A) signal were included to avoid the possibility that the changes within the HTLV-I LTR might affect expression of the CAT mRNA through mechanisms unrelated to the polyadenylation reaction.

The plasmid pHT4 is deleted of all HTLV-I sequences immediately following the AATAAA sequence, and pHT19 is mutated in addition in the HTLV-I AATAAA sequence (Fig. 3). These two plasmids harboring the SV40 poly(A) signal expressed high CAT activity. The same plasmids deleted of the SV40 signals produced no CAT enzyme (pHT20 and pHT21, Fig. 3). Based on the correlation between the RNA analysis and CAT assays described in the previous section, these results suggest that sequences downstream of the AATAAA sequence are required for the generation of stable CAT mRNA.

To further delineate the entire HTLV-I polyadenylation signal, we constructed two more deletion mutants: pHT10, missing the HTLV-I sequences starting at the loop of the secondary structure and downstream (from base 8667), and pHT1, which includes the entire stem-and-loop structure and 35 nt downstream of the poly(A) site. This plasmid lacks most of the U5 region of the LTR. No CAT expression was detected after deletion of the SV40 poly(A) signal from pHT10 (pHT12), while normal levels of CAT were detected after transfection with pHT11 [pHT1 deleted of the SV40 poly(A)]. All the control plasmids harboring the SV40 poly(A) signal exhibited high CAT expression, indicating that the deletions in the HTLV-I sequences were not deleterious to CAT gene expression in the tested plasmids.

Taken together, these results indicate that proper processing of the HTLV-I RNA 3' end requires the presence of at least two elements: the AATAAA sequence and additional sequences residing in the R region and 35 bases of the U5 region. Some of the sequences needed for the polyadenylation reaction must reside at least 70 bases away from the AAUAAA sequence since plasmid pHT12, which contains the AAUAAA sequence and the adjacent downstream 70 bases, failed to produce CAT enzyme.

Requirement of poly(A) site downstream signal for efficient processing. RNA 3'-end-processing sites require the AAUAAA poly(A) signal and an additional *cis*-acting signal located downstream from the cleavage site. Sequence analysis of the HTLV-I region reveals a 16-nucleotide sequence,

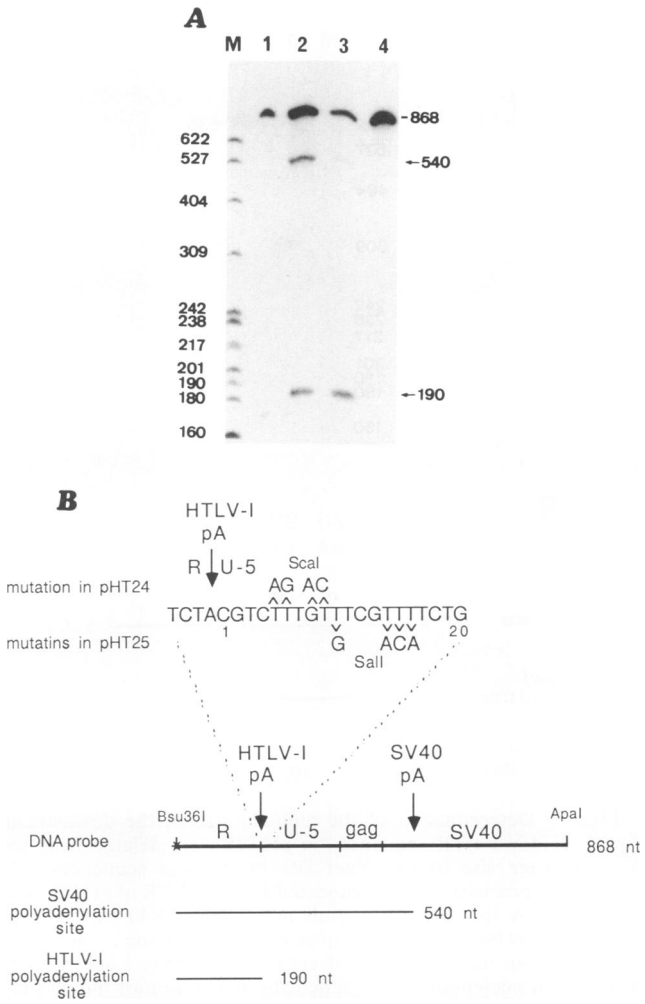


FIG. 4. Effect of poly(A) site downstream mutations on processing efficiency. Purified RNA from 293 cells transfected with either pHT24 or pHT25 harboring mutations in the downstream processing signal was subjected to S1 nuclease analysis. (A) RNA made of pHT24 and pHT25 with a 3' end generated at the HTLV-I poly(A) site rescued a 190-bp DNA fragment (lanes 2 and 3, respectively), while cleavage at the SV40 poly(A) site protected a 540-bp DNA fragment (lane 3). Mock-transfected RNA served as a control (lane 1). Size markers are presented in nucleotides (lane M). (B) Twenty bases of the DNA sequence of the HTLV-I U5 LTR region starting at the poly(A) site are presented. In pHT24, bases 5, 6, 8, and 9 were changed as indicated, generating a *ScaI* cleavage site. In pHT25, bases 10, 14, 15, and 16 were substituted as indicated to generate a *SalI* cleavage site. The structures of plasmids and DNA probe and the predicted sizes of S1 nuclease-resistant DNA fragments are illustrated.

TTTGTTTCGTTTTCTG, starting at the fifth base downstream from the R-U5 junction, the poly(A) site. This sequence resembles the U- or GU-rich element found to be part of functional poly(A) sites. To determine whether this sequence is part of the processing signals, we mutated plasmid pHT8, harboring the complete HTLV-I LTR, giving rise to two modified plasmids. In plasmid pHT24, four base substitutions were made in the proximal 5' end of the GU-rich sequence (bases 5, 6, 8, and 9 of the U5). In plasmid pHT25, thymidine residues 10, 14, 15, and 16 of the U5 region were mutated (Fig. 4B). The efficiency of processing

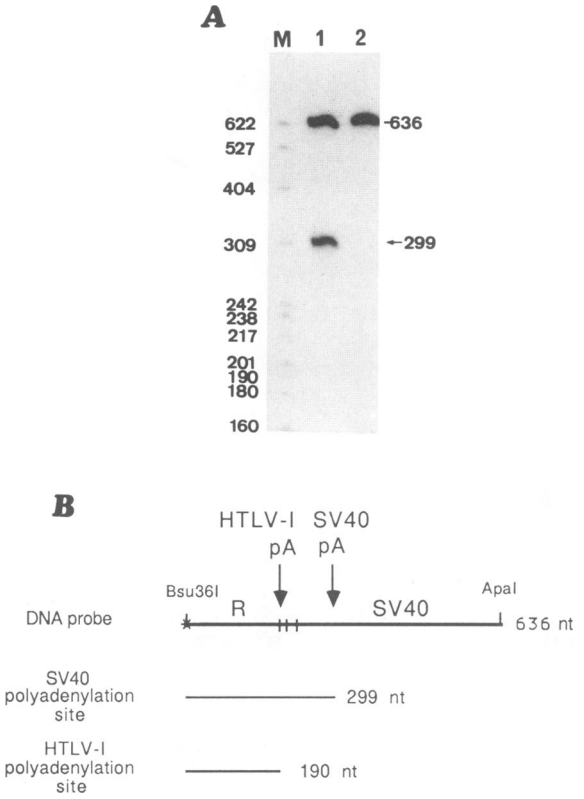


FIG. 5. Determination of the minimal size of the downstream signal. HTLV-I LTR sequences in pHT25 were deleted from the *Sal*I cleavage site to the *Nhe*I site in the *gag* sequences. (A) Absence of processing at the truncated HTLV-I LTR of pHT26 gave yield to RNA species with 3′ ends only at the SV40 poly(A) site, protecting 299 bases of the 637-bp probe (lane 1). Lane 2, protection by RNA from mock-transfected cells as a control. Lane M, size markers (in nucleotides). (B) Schematic illustration of the plasmid and DNA probe and expected sizes of S1-resistant DNA fragments.

was reduced only in pHT24, giving rise to two RNA species in S1 analysis of the RNA 3′ ends. One RNA species rescued a 190-nt DNA fragment, mapping the 3′ end of this RNA at the HTLV-I poly(A) site, and the other protected a 540-nt fragment corresponding to the location of the SV40 poly(A) site. RNA made from plasmid pHT25 protected only one DNA fragment of 190 bases, indicating that the efficiency of processing at the HTLV-I poly(A) site was not affected by the distal mutations (Fig. 4A, lane 3; compare with Fig. 2A, lane 1). Quantitative analysis by CAT assay demonstrated a reduction of about 70% in CAT activity in pHT24 compared with pHT25 or pHT8 (data not shown).

To further delineate the requirement for additional *cis*-acting processing signals, we deleted the HTLV-I U5 sequences, starting at base 15 downstream from the poly(A) site, from plasmid pHT25. The remaining U5 sequence harbors three base substitutions at positions 10, 14, and 15 (plasmid pHT26, Fig. 5B). Only one DNA fragment of 299 nt was rescued in the S1 nuclease assay (Fig. 5A). The size of this protected DNA band corresponds to the distance from the ^{32}P-labeled 3′ end of the probe to the SV40 poly(A) site. This result indicates clearly that some bases downstream of base 15 of the U5 region are essential for the 3′-end processing at the HTLV-I poly(A) site. Efficient processing occurs at the HTLV-I poly(A) site in plasmid pHT22 harboring only 20 nt of the U5 region (see below). It may be concluded that the nucleotides between bases 15 and 20 of the U5 are required for the functioning of the HTLV-I poly(A) site. From the results presented in this section, we suggest that a genetic signal 20 nt long is located immediately downstream from the HTLV-I poly(A) site. In this case, since mutations in bases 10, 14, 15, and 16 did not affect processing of the RNA, it may be concluded that the base composition of this signal is variable. While base substitution at the proximal part of this signal only reduced the efficiency of processing (pHT24), deletion of the distal part abolished processing completely (pHT26).

Requirement for correct physical distance between the processing *cis* signals. We demonstrated above that the poly(A) signal AAUAAA is located 276 bases upstream of the R-U5 junction, the poly(A) site. Based on sequence analysis, it was postulated that folding of the RNA structure could bring the poly(A) site to within 14 bases of the AAUAAA signal (32). This spacing between the processing signals is in accordance with the spacing found in other genes. If indeed the folding of the RNA affords the 3′-end RNA processing in the HTLV-I, deletion of this structure should not interfere with the generation of HTLV-I 3′-end RNA at the authentic site. However, if essential *cis*-acting RNA processing signals reside within these 255 nt, the deletion may interfere with the 3′-end processing. To test this hypothesis, we synthesized a 50-bp synthetic oligonucleotide composed of 18 bp homologous to the region 5′ to the putative secondary structure, including the AATAAA sequence, and 32 bp homologous to the region 3′ to the putative stem, including the poly(A) site. The synthetic DNA fragment was cloned into pSV2CAT at the 3′ end of the *cat* gene (pHT22). As a control, a similar plasmid but deleted of the SV40 poly(A) signal was constructed (pHT27). CAT assays indicated that functional *cat* RNA is made after transfection of cells with either pHT22 or pHT27 (Fig. 3). To determine whether the 3′ end of the RNA maps to the HTLV-I authentic site, we subjected RNA purified from these cells to S1 nuclease protection mapping. The probe in this experiment was a 599-bp DNA fragment isolated from pHT22, extending from within the *cat* gene to beyond the SV40 poly(A) site (Fig. 6B). Regardless of the presence or absence of the SV40 poly(A) signal, only one discrete band of 164 bases was rescued in the S1 nuclease mapping (Fig. 6A, lanes 1 and 2). The size of this band corresponds to the distance between the 3′ end of the ^{32}P-labeled DNA probe and the R and U5 junction. This result indicates that the RNA 3′ end is generated within the 50-bp HTLV-I sequence independent of sequences predicted to form the secondary RNA structure. Furthermore, U5 sequences 20 bp downstream from the poly(A) site are not essential for the polyadenylation reaction. Since an S1-protected DNA fragment extending up to the SV40 poly(A) site (expected size, 260 bases) was not observed, the processing at the synthetic oligonucleotide was as efficient as in the complete HTLV-I LTR (compare Fig. 2A, lane 1, with Fig. 6A, lane 1). Based on these observations, it is concluded that under our experimental conditions, all the essential *cis*-acting signals for efficient and accurate processing of HTLV-I RNA are present within the 50-base oligonucleotide. The fact that generation of 3′ ends occurs at the authentic HTLV-I processing site in the deletion mutants pHT27 and pHT22 supports the hypothesis that the 276 intervening nucleotides fold into a secondary structure that juxtaposes the AAUAAA sequence to the downstream processing signals. To further test this conclusion, we introduced a 190-bp DNA

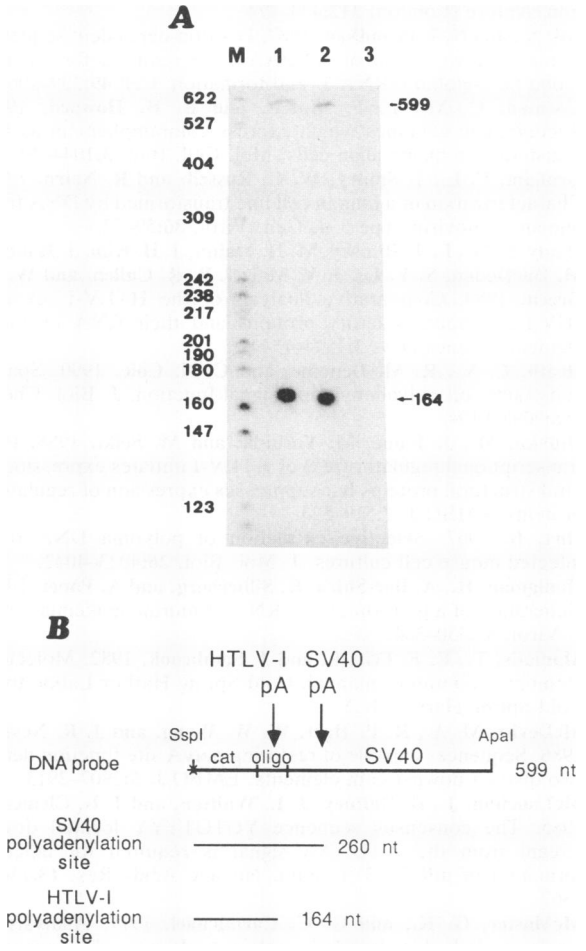


FIG. 6. Minimal sequence requirement for functional poly(A) site. RNA purified from pHT22- and pHT27-transfected 293 cells was hybridized to a ^{32}P-labeled 599-bp DNA probe and subjected to an S1 nuclease protection assay. The rescued probe fragments were resolved by polyacrylamide gel electrophoresis. (A) Lane 1, DNA fragment protected by pHT22 RNA. Lane 2, probe rescued by pHT27 RNA protection. Lane 3, traces of full-length probe rescued after hybridization with mock-transfected RNA. DNA markers are presented in lane M (in nucleotides). (B) Structures of plasmid and DNA probe and expected sizes of probes rescued by RNA polyadenylated at the HTLV-I poly(A) site (164 nt) or the SV40 poly(A) site (260 nt).

restriction fragment from pBR322 (3) between the poly(A) signal and the poly(A) site of the deletion mutants pHT22 and pHT27 (plasmids pHT23 and pHT28, respectively). Based on sequence analysis, the RNA made from this DNA fragment is not expected to fold into a secondary structure. Moving the two processing elements apart abolished *cat* expression (compare pHT28 with pHT27, Fig. 3B). This result further indicates that the spacing between the AAUAAA hexamer and the downstream elements is critical for efficient poly(A) function.

DISCUSSION

Generation of polyadenylated 3' ends of eukaryotic RNA depends on at least three *cis*-acting signals: the poly(A) signal AAUAAA; the poly(A) site, located 10 to 30 nt downstream from the poly(A) signal; and further downstream, GU-rich or U-rich signals. The spacing between the AAUAAA element and the downstream signals is critical for efficient poly(A) site function. Sequence analysis of the HTLV-I LTR did not reveal a poly(A) signal consensus sequence at the expected distances from the R-U5 junction. This observation might have suggested that the 3' end of HTLV-I RNA is not generated by endonucleotic cleavage and subsequent polyadenylation. However, the fact that the stable RNA with 3' ends residing at the R-U5 junction of the HTLV-I LTR was found only in the poly(A)$^+$ fraction of the purified RNA suggests that the 3' end of HTLV-I RNA is generated by processing and subsequent polyadenylation (Fig. 1B and 2).

Only one AATAAA sequence is found in the HTLV-I LTR. This sequence is located 276 nt upstream of the R-U5 junction. To analyze whether this remote sequence indeed functions as a *cis* signal for RNA processing, we mutated it to GAGAAA. S1 nuclease analysis of RNA expressed from plasmids harboring the HTLV-I LTR 3' to the *cat* gene mapped the poly(A) site at the R-U5 junction. Polyadenylation by the HTLV-I LTR was completely abolished in the GAGAAA mutant. This result proves that the poly(A) signal is located in the HTLV-I LTR at an unusually remote distance from the poly(A) site. The various LTR deletion mutants devoid of sequences downstream of the poly(A) signal demonstrated that (i) some *cis*-acting signals downstream from the AAUAAA sequence are required for HTLV-I RNA 3' processing (pHT10, pHT4) and that (ii) these downstream *cis*-acting processing signals reside within the LTR, down to 20 nt within the U5 region (pHT22, pHT26). Weiss et al. (39) have demonstrated that the stability of the ternary processing complex depends on the nature of the U- or GU-rich downstream sequence element. HTLV-I sequence analysis revealed GU-rich sequence extending down to 20 nt from the poly(A) site: 5'-CGTCTTTGTTTC GTTTTCTG-3'. Point mutation and deletion analysis revealed that while four base substitutions within the nine proximal bases (positions 5, 6, 8, and 9) reduced the efficiency of RNA 3'-end processing (pHT24), deletion of bases 15 to 20, TTTCTG, abolished processing completely (pHT26). However, point mutations which substituted the thymidine residues at positions 10, 14, 15, and 16 did not affect the processing at the HTLV-I poly(A) site (pHT25). These results suggest that the downstream processing signal of HTLV-I is composed of two sequence boxes: the proximal CGTCTTT GT and, 5 nt downstream, a TTTCTG box. Another possibility is that the complete 20 nt serve as a recognition site for the processing complex but that base requirement in the middle of the signal is not stringent.

Previous studies have demonstrated that the spacing (10 to 30 nt) between the AAUAAA element and the downstream element is critical for efficient RNA 3'-end processing. Our finding that the functional HTLV-I poly(A) site is controlled by a remote AAUAAA hexamer raises the question of how the correct spacing is achieved. The intervening 276-base RNA may fold into a secondary structure to bring the AAUAAA poly(A) signal sufficiently close to the cleavage site for generation of HTLV-I 3'-end RNA. We synthesized a 50-bp oligonucleotide which included the poly(A) signal, the poly(A) site 14 bases away, and 20 more nucleotides downstream. In this sequence, the spacing of the various *cis* elements controlling HTLV-I RNA 3'-end processing mimicked the predicted distances after folding of the RNA. The fact that the RNA 3' end was processed efficiently at the authentic HTLV-I poly(A) site suggests that the hypothesis described above is correct. This conclusion is supported by

the fact that CAT expression is abolished when the processing signals are separated by 190 bases of RNA which cannot fold into a secondary structure.

The physical distances between the AAUAAA element and the downstream signals in the synthetic oligonucleotide were designed according to the theoretically calculated secondary structure. The spacing between the processing elements may differ in reality from the calculated distances. In this case, efficiency of processing might be different and might also require additional *cis* signals. However, the fact that the LTR poly(A) signal was dominant in all plasmids that contained a consecutive SV40 poly(A) signal suggests that the efficiency of polyadenylation is not dependent on sequences within the stem-and-loop structure.

Sequences within the HTLV-I RNA secondary structure at the R region contain the Rex-responsive element (5, 13, 34, 37). The *rex* gene product (p21) was found to be a *trans*-regulator of RNA splicing that enables expression of unspliced *gag-pol* and *env* mRNAs. It is interesting that formation of the highly stable secondary structure controls both the generation of HTLV-I unspliced RNA and the RNA 3′ end. However, while splicing is regulated by a *trans*-acting viral protein, cellular proteins appear to be sufficient for HTLV-I 3′-end RNA processing using the unique viral signals.

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ADDENDUM

During preparation of the manuscript, an article by Ahmed et al. (1) dealing with the same subject was published.

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