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Sequences Regulating Poly(A) Site Selection within the Adenovirus Major Late Transcription Unit Influence the Interaction of Constitutive Processing Factors with the Pre-mRNA

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The adenovirus major late transcription unit (MLTU) encodes five families of mRNAs, L1 to L5, each distinguished by a unique poly(A) site. Use of the promoter-proximal L1 poly(A) site predominates during early infection, whereas poly(A) site choice shifts to the promoter-distal sites during late infection. A mini-MLTU containing only the L1 and L3 poly(A) sites has been shown to reproduce this processing switch. In vivo analysis has revealed that sequences extending 5′ and 3′ of the L1 core poly(A) site are required for efficient processing as well as for regulated expression. By replacement of the L1 core poly(A) site with that of the ground squirrel hepatitis virus poly(A) site, we now demonstrate that the L1 flanking sequences can enhance the processing of a heterologous poly(A) site. Upon recombination of the chimeric L1-ground squirrel hepatitis virus poly(A) site onto the viral chromosome, the L1 flanking sequences were also found to be sufficient to reproduce the processing switch during the course of viral infection. Subsequent in vitro analysis has shown that the L1 flanking sequences function to enhance the stability of binding of cleavage and polyadenylation specificity factor to the core poly(A) site. The impact of L1 flanking sequences on the binding of cleavage and polyadenylation specificity factor suggests that the regulation of the MLTU poly(A) site selection is mediated by the interaction of constitutive processing factors.

In eukaryotic cells, the 3′ ends of most mRNAs are generated by the endonucleolytic cleavage of the nascent transcript followed by rapid addition of poly(A) to the newly formed 3′ end (for reviews, see references 24, 46, and 47). In mammalian cells the cleavage event is directed by two RNA sequence elements: the highly conserved hexanucleotide AAUAAA, found 10 to 35 nucleotides 5′ of the cleavage site, and a less well-conserved GU- or U-rich downstream element, found 20 to 50 nucleotides 3′ of the cleavage site. These sequences, which constitute the core poly(A) site, are sufficient in many cases for cleavage and polyadenylation of the pre-mRNA. Additional elements that influence 3′ processing have been identified upstream and downstream of the core poly(A) site in several genes, including adenovirus L1 and L3 (10, 11, 35), simian virus 40 (SV40) late (6, 38), *Drosophila* doublesex (20), and retroelements (4, 12, 37, 44). Previous studies have demonstrated the requirement for multiple factors in the recognition and processing of mammalian poly(A) sites (for reviews, see references 24, 46, and 47). Cleavage and polyadenylation specificity factor (CPSF), which is responsible for the recognition of the AAUAAA hexamer, is required for both cleavage and polyadenylation (3, 9, 16, 17, 21, 29, 41). Cleavage stimulatory factor (CstF) interacts with the CPSF-RNA complex to form a stable CPSF-CstF-RNA complex, dependent upon the presence of the downstream element (17, 18, 40, 50). The stability of these protein-RNA complexes plays a major role in determining the processing efficiency of a poly(A) site (15, 48). Other factors involved in pre-mRNA 3′ processing include poly(A) polymerase, cleavage factors CF1 and CF2, and poly(A) binding protein II (9, 16, 41, 42, 45).

Although the majority of transcription units possess a single poly(A) site, there are numerous examples of complex transcription units that contain multiple poly(A) sites. One such example is the adenovirus major late transcription unit (MLTU), which encodes five families of mRNAs, L1 to L5 (Fig. 1; for a review, see reference 30). The members of each family share a common poly(A) site but differ with respect to their splicing pattern. Differential poly(A) site and splice site choice leads to the production of approximately 20 mRNA species from the MLTU pre-mRNA. The regulation of alternative splicing and polyadenylation is therefore critical to a productive viral infection. The MLTU is transcribed both early and late in the infection (i.e., before and after the onset of viral DNA synthesis). During the early phase of infection, the L1 poly(A) site is chosen predominantly over the downstream sites (1, 32, 39). With the onset of DNA replication, a shift in poly(A) site selection occurs such that each of the four downstream sites is used slightly more than the L1 poly(A) site (31). A miniature MLTU containing only the L1 and L3 poly(A) sites in tandem has been shown to reproduce this processing switch, indicating that the 3′ processing switch is independent of splice site choice (13). Thus, the MLTU is one of the few examples of regulation of RNA processing at the level of poly(A) site choice.

An analysis of the efficiency of the individual poly(A) sites suggests that the MLTU is composed of a balanced set of processing sites. Poly(A) site selection appears to be dictated by both the relative processing efficiency of each site as well as the position of each site within the transcription unit. The

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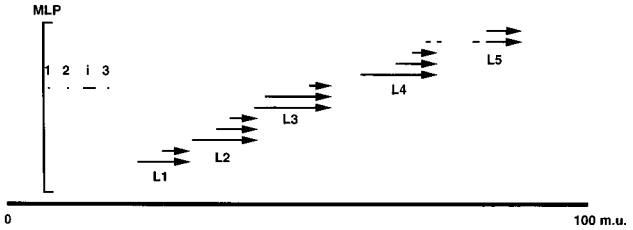


FIG. 1. Structure of the MLTU of adenovirus. Diagram of the adenovirus chromosome (thick line) indicating the positions of the L1 to L5 mRNA families. The exons designated 1, 2, and 3 are common to all mRNAs; the i exon is present in some early mRNAs. The arrows indicate the positions of the protein-coding exons. The bracket indicates the major late promoter (MLP) of the MLTU. m.u., map units.

promoter-proximal L1 poly(A) site is a relatively weak site (35, 36), as would be required to allow for the use of the four downstream processing sites. The sequence context of the L1 AAUAAA hexamer appears to be primarily responsible for the inefficient processing of the L1 poly(A) site (35). Mann et al. have shown that the CPSF-CstF-RNA complex formed at the L1 poly(A) site is less stable than that formed at the stronger downstream L3 poly(A) site (25). The relative instability of the CPSF-CstF-RNA complex at the L1 poly(A) site suggests that selection of this site within the context of a complex transcription unit may be particularly sensitive to the concentration of CPSF and/or CstF. Intriguingly, these investigators have also shown that there is a decrease in the level of CstF activity during the course of viral infection (25). Thus, a rate-limiting concentration of CstF during the late phase of infection may shift the balance of processing from the predominant use of the promoter-proximal L1 site to the use of the more efficient downstream poly(A) sites.

The sequences responsible for the regulation of poly(A) site choice within the MLTU have been analyzed in considerable detail (10, 11, 49). Differential poly(A) site selection within the MLTU has been shown to rely principally upon sequences flanking the L1 core poly(A) site. Sequences from −113 to −50 and +52 to +170 with respect to the cleavage site are required both for efficient processing at the L1 poly(A) site during early infection as well as for the switch in poly(A) site choice during the late phase of infection (10). We have now shown that both the enhancement of processing as well as regulated expression can be transferred to a heterologous core poly(A) site. The core poly(A) site of the ground squirrel hepatitis virus (GSHV) was placed within the context of the L1 upstream and/or downstream flanking sequences and assayed for processing efficiency in a *cis*-competition assay with a downstream L3 poly(A) site. The GSHV core poly(A) site is a weak signal which has previously been shown to require an upstream element for efficient processing (37). In transient transfection assays, the L1 flanking sequences enhanced the use of the heterologous GSHV core poly(A) site. In addition, when the chimeric L1-GSHV poly(A) site was placed in the context of the adenovirus chromosome, the L1 flanking sequences were found to be sufficient to reproduce the processing switch during the course of viral infection. An in vitro analysis demonstrated that the L1 flanking sequences stabilized the binding of CPSF at the L1 core poly(A) site. These results suggest that the sequences flanking the L1 poly(A) site are discrete regulatory elements which participate in the control of poly(A) site selection during viral infection by modulating the interaction of the L1 poly(A) site with the constitutive 3′-end processing machinery.

MATERIALS AND METHODS

Cell cultures and virus strains. Human 293 monolayer cells were propagated in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. Suspension cultures of human 293 cells and HeLa cells were propagated in Joklik-modified minimum essential medium plus 5% calf serum. Adenovirus strains sub360 and vML1+170-L3 were described previously (10, 23).

DNA transfection, virus infection, and RNA isolation. Transient-transfection assays were performed by the calcium phosphate coprecipitation procedure as previously described (11). For viral infections, 293 monolayer cells were infected either at a multiplicity of 50 PFU per cell for 6 h in the presence of 5 μg of cytosine arabinoside (early infection) per ml or at a multiplicity of 20 PFU per cell for 22 to 24 h (late infection). RNA from transfected or infected cells was obtained by the acid guanidinium thiocyanate-phenol-chloroform extraction method (8), and poly(A)$^+$ RNAs were isolated on oligo(dT)-cellulose columns (2). Aliquots of 120 and 5 μg of RNA were applied to the column for early and late infection, respectively. To examine mRNA stability, actinomycin D was added to cells 6 h (early) or 22 h (late) after infection to a final concentration of 10 μg/ml to inhibit all new RNA polymerase II-dependent transcription. RNAs were then isolated 2 or 4 h (early) and 8 or 16 h (late) after the addition of actinomycin D. To determine the effectiveness of the drug, control dishes were pulse-labeled for 1 h with [^{3}H]uridine at each time point and RNA was obtained as described above. The transcription block was greater than 99% at each time point tested.

Plasmid and virus constructions. The L1-L3 plasmids pML1+170-L3 and pML1+52Δ63-L3, containing either wild-type or mutant L1 poly(A) sites, respectively, have been described previously (10). The chimeric GSHV-L1 poly(A) sites were constructed in a stepwise manner from cassettes containing the various components. The GSHV core poly(A) signal is on a *Taq*I-*Nsi*I fragment derived from plasmid Δ11/SL1 (or Δ11/SL1.A) (provided by R. Russnak), and contains sequences from −62 to +51 with respect to the GSHV cleavage site (Fig. 2). Plasmid Δ11/SL1.A is the same as Δ11/SL1 except for a single point mutation converting the wild-type GSHV hexanucleotide UAUAAA to the consensus AAUAAA signal. These two plasmids, Δ11/SL1 and Δ11/SL1.A, which contain a unique *Xho*I site at −62 with respect to the GSHV cleavage site, were derived from pGSpA.1 and pGSpA.1/A (37), respectively. Wild-type and mutant L1 upstream regulatory elements from pEGC-D and pEGCΔ63-D (11), respectively, were generated by PCR and cloned 5′ of the GSHV core as *Xba*I-*Cla*I fragments. An *Xba*I site was incorporated into the 5′ end of PCR products for cloning convenience. The L1 downstream regulatory element (+44 to +170), derived from plasmid pML1+170-L3 (10), was also generated by PCR and cloned 3′ of the GSHV core as an *Nsi*I-*Kpn*I fragment. The PCR primers included *Nsi*I or *Kpn*I sites for cloning purposes. The *Xba*I-*Kpn*I fragment of pML1+170-L3, containing the wild-type L1 poly(A) site, was replaced with *Xba*I-*Kpn*I fragments containing the various L1-GSHV chimeric poly(A) sites. The structure of all chimeric sites was confirmed by restriction mapping and sequence analysis. The transcription units in these expression vectors are diagrammed in Fig. 2. One set of chimeric constructs (pL1GL1-L3, pL1G-L3, pGL1-L3, and pG-L3) contains the GSHV wild-type UAUAAA signal. Another set (pL1GAL1-L3, pL1GA-L3, pGAL1-L3, and pGA-L3) has the point mutation converting UAUAAA to the consensus AAUAAA signal. Each plasmid was designated with a G to identify it as containing the poly(A) core element of GSHV, followed by L3, indicating the presence of the L3 poly(A) site. The L1 before and after the G (or GA) indicates the presence of upstream (nucleotide [nt] −50 to −113 with respect to the L1 cleavage site) and downstream (nt +44 to +170 with respect to the L1 cleavage site) regulatory elements, respectively. Plasmids pL1SV (10) and pL1(X)-SV are transfection control plasmids for L1-L3 constructs and G-L3 constructs, respectively. pL1(X)-SV is similar to pGL1-L3, except that it contains the SV40 early poly(A) site in place of the GSHV and L3 poly(A) sites.

Four recombinant viruses, vL1GL1-L3, vG-L3, vL1GAL1-L3, and vGA-L3, were generated by the method of overlap recombination as described previously (7) and were named after their parental plasmids, pL1GL1-L3, pG-L3, pL1GAL1-L3, and pGA-L3, respectively. Viruses were plaque purified and isolated from infected 293 cells by CsCl gradient centrifugation (19).

S1 nuclease analysis. Poly(A)$^+$ RNA from transfections or infections of each construct was analyzed with a DNA probe made from the corresponding plasmid. An *Xba*I-*Eco*RV fragment spanning the two poly(A) sites in each plasmid was purified. Each DNA fragment was specifically 3′ end labeled at the *Xba*I site upstream of the L1 poly(A) site by using the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dCTP. Hybridizations and S1 digestions were performed as previously described (10). Results were quantitated with a Molecular Dynamics PhosphorImager after the products were resolved on a 6% polyacrylamide–8 M urea gel.

Preparation of RNA substrates for in vitro analysis. The RNAs used for the analysis of processing complexes were generated by in vitro transcription with SP6 polymerase in the presence of [α-^{32}P]UTP. The DNAs that served as templates for in vitro transcription were pGL1+170-L3 and pGL1+52Δ63-L3. These plasmids were constructed by cloning a poly(A) site-containing *Xba*I fragment from pML1+170-L3 or pML1+52Δ63-L3, respectively, into pGEM3Zf. The plasmids were linearized with *Acc*65I before transcription to generate a pre-mRNA containing only the L1 poly(A) site. The L3 and human immunodefi-

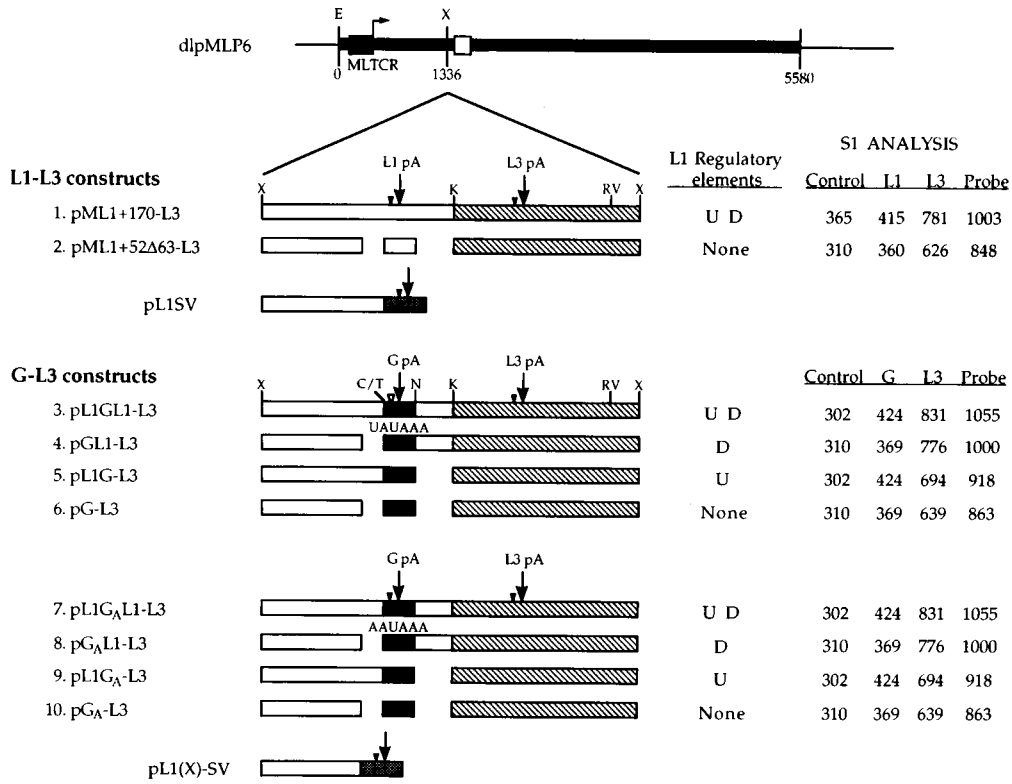


FIG. 2. Plasmid maps of mini-MLTUs. The expression vector dlpMLP6 (13) was adapted for both transient expression of the mini-MLTUs and their recombination onto the adenovirus genome. It contains the leftmost 5,580 bp of the adenovirus type 5 sub360 genome (solid bars), except that the adenovirus type 2 major late transcription control region (MLTCR) replaces the E1A promoter. In addition, the E1B promoter and E1A poly(A) site are deleted (open box). Assay poly(A) sites were inserted into the *Xba*I site of dlpMLP6. Shown are sequences of adenovirus L1 (open boxes) and L3 (hatched boxes), GSHV (solid boxes), and SV40 (solid boxes in SV constructs). Also shown are cleavage sites (arrows), AAUAAA signals (filled triangles), and a GSHV UAUAAA signal (open triangle). Abbreviations for restriction sites: C, *Cla*I; E, *Eco*RI; K, *Kpn*I; N, *Nsi*I; T, *Taq*I; X, *Xba*I; and RV, *Eco*RV. Plasmids pL1SV and pL1(X)-SV are transfection control plasmids for L1-L3 constructs and G-L3 constructs, respectively. The presence of L1 regulatory elements for each construct is indicated as follows: U, upstream element; and D, downstream element. Probe and protected fragment sizes (in nucleotides) for each S1 nuclease protection analysis are shown at the right.

ciency virus type 1 (HIV-1) (*use*/CPS) pre-mRNAs were prepared as previously described (14, 17). The 121-nt non-poly(A) site-containing Rep3 RNA was transcribed from a template containing a sequence from the first intron of the hamster Rep3 gene cloned into pGEM9Zf(−) at *Eco*RI and *Sac*I. The template was linearized with *Msc*I to yield an RNA containing 80 nt of Rep3 sequence and 41 nt of vector sequence.

Analysis of 3′ processing complexes. CPSF and CstF were prepared from HeLa cell nuclear extracts as previously described (17, 18). CPSF was fractionated by DEAE-Sepharose, MonoS, and Blue Sepharose to yield a Blue Sepharose 1 M fraction (17). CstF was fractionated by DEAE-Sepharose and MonoS chromatography to yield a MonoS 200 mM fraction (18). Competition assays were performed by the simultaneous addition of 2.7 fmol of ^{32}P-labeled L3 pre-mRNA (17) and increasing amounts of unlabeled L1 (wild type) or ΔL1 (mutant) pre-mRNA to a 20-μl reaction mixture containing 5 μl of CPSF Blue Sepharose fraction (0.31 mg/ml) (16), 2 μl of CstF MonoS fraction (0.27 mg/ml) (18), 83 mM KCl, 0.6 μg of tRNA, 1% polyvinyl alcohol, 16.5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 8.3% glycerol, 0.17 mM EDTA, 0.08 mM phenylmethylsulfonyl fluoride, and 0.41 mM dithiothreitol. The reaction mixtures were incubated for 10 min at 30°C. The reaction mixtures were then placed on ice, and heparin was added to 5 mg/ml. The reaction mixtures were electrophoresed on a nondenaturing 3% polyacrylamide (100:1) gel in 25 mM Tris–25 mM boric acid–1 mM EDTA (pH 8.0) at 300 V for 2 h at 4°C. The gel was preelectrophoresed for 30 min prior to loading.

The complex stability assays were initiated by the incubation of 1 pmol of L1, ΔL1, HIV-1 (positive control), or Rep3 [negative control, non-poly(A) site containing RNA] pre-mRNA in a 25-μl reaction mixture containing 14 μl of CPSF Blue Sepharose fraction (0.06 mg/ml) (17), 83 mM KCl, 0.6 μg of tRNA, 1% polyvinyl alcohol, 16.5 mM HEPES (pH 7.9), 8.3% glycerol, 0.17 mM EDTA, 0.08 mM phenylmethylsulfonyl fluoride, and 0.41 mM dithiothreitol. The reaction mixtures were incubated for 10 min at 30°C. To the reaction mixture was then added 20 fmol (in 1 μl) of a ^{32}P-labeled HIV-1 poly(A) site-containing pre-mRNA (*use*/CPS) (14). The incubation was continued at 30°C, and 5.2-μl aliquots were taken of each sample at 5-, 10-, 20-, and 30-min time points, placed on ice, and treated with heparin (5 mg/ml). The reaction mixtures were electrophoresed on a nondenaturing 3% polyacrylamide (100:1) gel in 25 mM Tris–25 mM boric acid–1 mM EDTA (pH 8.0) at 300 V for 2 h at 4°C. The gel was preelectrophoresed for 30 min prior to loading.

RESULTS

Sequences flanking the L1 core poly(A) site can enhance the processing of a heterologous core poly(A) site. The ability of sequences flanking the L1 core poly(A) site to enhance the processing of a heterologous core poly(A) site was investigated through the use of the chimeric GSHV-L1 poly(A) sites shown in Fig. 2. The L1 upstream (nt −50 to −113 with respect to the L1 cleavage site) and/or downstream (nt +44 to +170 with respect to the L1 cleavage site) sequences were placed such that they flanked the GSHV core poly(A) site. Both the L1 and GSHV core poly(A) sites are relatively weak processing signals (35–37). The wild-type GSHV poly(A) site contains a variant hexamer (UAUAAA) which is responsible, at least in part, for the relatively weak activity of this processing site. We also assayed a mutant GSHV poly(A) site in which processing efficiency has been increased by the conversion of the variant hexamer to the consensus AAUAAA sequence. The relative use of each of the GSHV poly(A) sites was determined in a *cis*-competition assay with the downstream L3 poly(A) site in a mini-MLTU. Each mini-MLTU plasmid was cotransfected into human 293 cells with a control plasmid that contained the SV40 early poly(A) site in place of the assay poly(A) sites. Total cellular poly(A)$^+$ RNA was isolated 48 h after transfec-

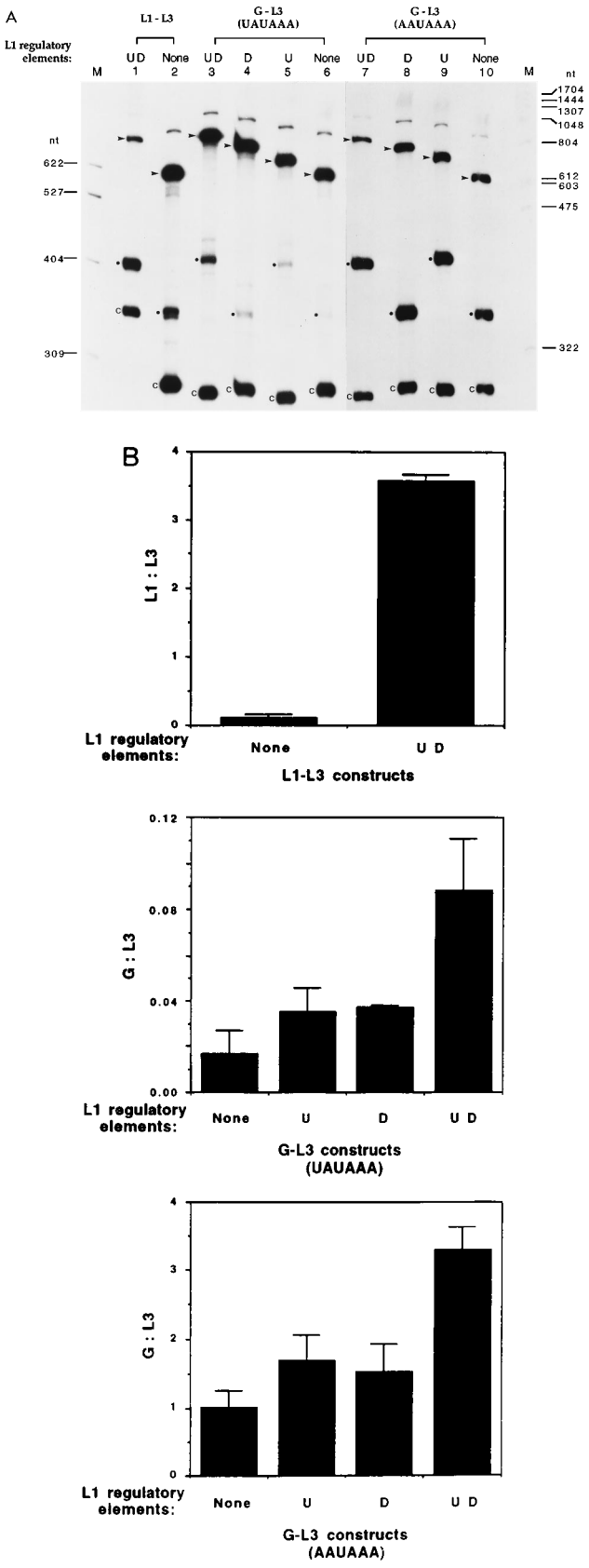


FIG. 3. Relative poly(A) site use in tandem constructs transfected into 293 cells. (A) S1 analysis. Probes were hybridized to poly(A)$^+$ transcripts and analyzed by S1 nuclease protection as described in Materials and Methods. Protected fragments are indicated as follows: C, poly(A)$^+$ transcripts from the control plasmid; filled circles, poly(A)$^+$ transcripts from assay plasmids cleaved at the first poly(A) site (L1 or GSHV); and arrowheads, poly(A)$^+$ transcripts cleaved at the L3 poly(A) site. The band at the top of each lane represents undigested probe. Sizes of markers (lanes M) are indicated at the left and right. The presence of upstream (U) or downstream (D) elements for each construct is indicated above the lane number. Lanes: 1, pML1+170-L3; 2, pML1+52Δ63-L3; 3, pL1GL1-L3; 4, pGL1-L3; 5, pL1G-L3; 6, pG-L3; 7, pL1GAL1-L3; 8, pGAL1-L3; 9, pL1GA-L3; and 10, pGA-L3. (B) Quantitation of results. The relative use of two poly(A) sites (L1/L3 or G/L3 ratio) is plotted for each construct. Each value represents the average of two independent experiments.

tion and analyzed with a specific 3′-end-labeled probe spanning the two poly(A) sites. The probes used for pML1+170-L3 and pML1+52Δ63-L3 are complementary to sequences upstream of the SV40 poly(A) site in control RNA produced from pL1SV, allowing for the simultaneous resolution of control and experimental products. Similarly, probes from the G-L3 constructs protect sequences upstream of the SV40 poly(A) site in plasmid pL1(X)-SV. Figure 2 shows the sizes of the probe and protected bands for each construct in the S1 protection analysis.

The S1 analysis of poly(A)$^+$ RNAs from the transfected chimeric constructs is shown in Fig. 3. The relative use of the poly(A) sites is determined by the ratio of poly(A)$^+$ transcripts processed at the two poly(A) sites (L1/L3 or G/L3 ratio). As we have shown previously (10, 11), L1 sequences upstream and downstream of the L1 core element enhance the relative use of the L1 poly(A) site in mini-MLTUs (Fig. 3A, compare lanes 1 and 2). The L1 flanking sequences in the L1-L3 constructs increased the relative use of the L1 site from 0.1 to 3.6 (Fig. 3B). In constructs containing a wild-type (UAUAAA) or mutant (AAUAAA) GSHV core poly(A) site, the presence of both upstream and downstream L1 flanking sequences also increased GSHV processing relative to the L3 poly(A) site by three- to fivefold (Fig. 3B). The presence of only the upstream or downstream L1 sequence gave an intermediate level of enhancement in each case. We have demonstrated previously that these effects are not due to changes in spacing caused by the deletions (10, 11). These results indicate that the sequences flanking the L1 core poly(A) site can enhance the processing efficiency of a heterologous core poly(A) site when expressed transiently in 293 cells.

Construction of recombinant viruses containing mini-MLTUs encoding GSHV and L3 poly(A) sites. Having shown that the L1 flanking sequences enhanced the 3′ processing of a heterologous core poly(A) site in transfected cells, we then examined the ability of these sequences to regulate the use of a heterologous poly(A) site in the context of an adenovirus infection. Four chimeric constructs (pL1GL1-L3, pG-L3, pL1GAL1-L3, and pGA-L3) were recombined onto the adenovirus genome. Recombinant viruses were isolated and designated vL1GL1-L3, vG-L3, vL1GAL1-L3, and vGA-L3. The mini-MLTUs were inserted into the E1 region of the adenovirus chromosome; therefore, each recombinant virus carries both the mini-MLTU and the endogenous MLTU. The virus vML1+170-L3 was used as a positive control for the processing switch (10, 13). The probes used for the G-L3 viruses cross-hybridize with the endogenous L1 transcripts. This signal can be distinguished from that of the G-L3 mini-MLTU by the sizes of the protected fragments. In this way, the endogenous L1 RNA served as an internal control for the G-L3 viruses.

Poly(A) site selection in viral mini-MLTUs containing G-L3 constructs during infection. Human 293 monolayer cells were infected with the five recombinant adenoviruses for 6 h in the presence of cytosine arabinoside to obtain early RNA, or for

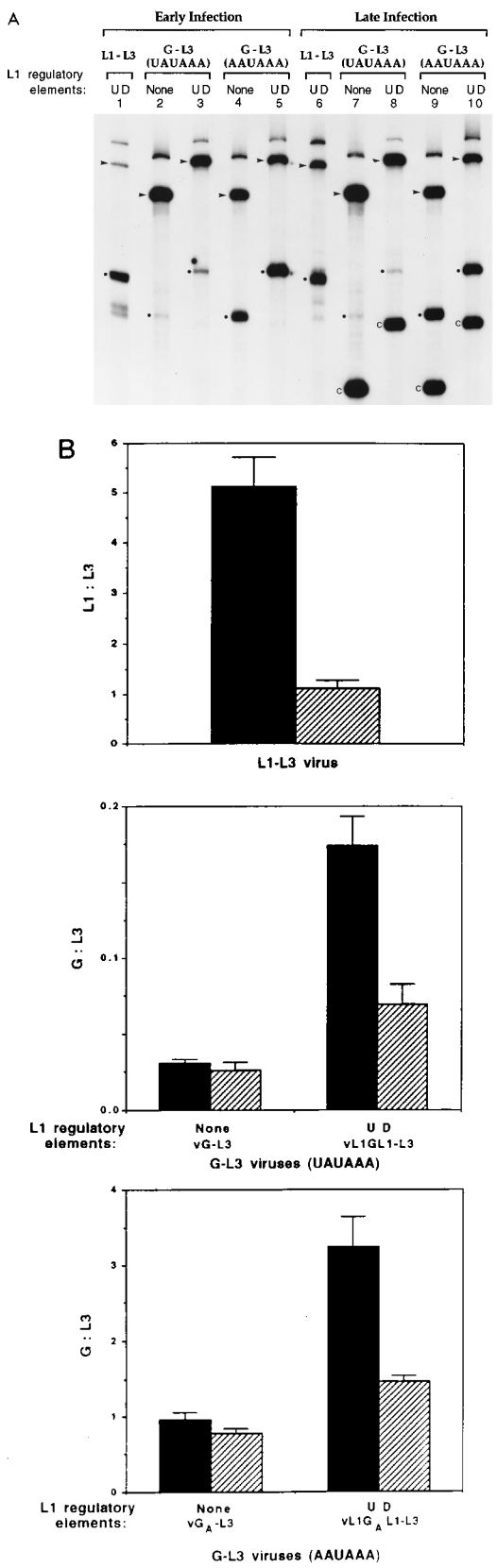


FIG. 4. Poly(A) site selection in viral mini-MLTUs during infection. (A) S1 analysis. Probes were hybridized to poly(A)$^+$ RNA from early or late infections and analyzed by S1 nuclease protection. S1 products are designated as described in the legend to Fig. 3, except that C indicates poly(A)$^+$ transcripts cleaved at the endogenous viral L1 poly(A) site. RNAs expressed from early (lanes 1 to 5) and late (lanes 6 to 10) infected cells are shown. RNAs are from vML1+170-L3 (lanes 1 and 6), vG-L3 (lanes 2 and 7), vL1GL1-L3 (lanes 3 and 8), vGA-L3 (lanes 4 and 9), and vL1GAL1-L3 (lanes 5 and 10). (B) Quantitation of results from viral infections. The L1/L3 or G/L3 ratio for each recombinant virus during early (solid bars) and late (hatched bars) infection is shown. Each value represents the average of at least two independent experiments.

22 to 24 h without cytosine arabinoside to obtain late RNA. Cytosine arabinoside blocks viral DNA replication and is included to ensure that the infection remains in the early stage. RNA from infected cells was harvested and analyzed by S1 nuclease protection as described for transfected cells. The results are shown in Fig. 4. The efficiency of poly(A) site switching in each viral mini-MLTU was determined by the differences in the ratios of L1 (or GSHV) and L3 poly(A) site use (L1/L3 or G/L3 ratio) early and late. Switching of the L1 poly(A) site was efficient in the wild-type control vML1+170-L3, with processing ratios of L1 to L3 shifting from 5.13 (early) to 1.12 (late) (Fig. 4B, top panel). With the GSHV constructs, there was no difference between the G/L3 ratios during early and late infection for recombinant viruses in the absence of L1 flanking sequences (Fig. 4B). In the presence of L1 flanking sequences, however, mini-MLTUs with both the wild-type (UAUAAA) and mutant (AAUAAA) GSHV core poly(A) sites exhibited the processing switch during the course of viral infection: for the vL1GL1-L3 virus, the ratio changed from 0.17 to 0.07, and for vL1GAL1-L3, the ratio changed from 3.25 to 1.47 (Fig. 4B). Thus, the transfection and infection analyses of the chimeric constructs demonstrate that sequences flanking the L1 poly(A) site can both enhance the efficiency of a heterologous poly(A) site as well as confer regulated expression in the context of the viral chromosome.

Stability of mini-MLTU transcripts. Several lines of evidence argue against a role for differential RNA stability in the temporal regulation of mini-MLTU RNAs. First, the early-to-late processing switch in vML1+170-L3 is seen with nascent pulse-labeled RNAs (13). Second, L1 sequences upstream of AAUAAA do not influence the stability or transport of L1 RNAs expressed from plasmids transfected into 293 cells (11). Third, L1 sequences upstream or downstream of AAUAAA do not affect RNA stability in late infected cells or transfected cells (10). Since we were using chimeric sites, however, we wished to look directly at the stability of the processed RNAs during the course of the infection. To do this, actinomycin D was added to cells 6 h (early) or 22 h (late) after infection to inhibit all new RNA polymerase II-dependent transcription. Poly(A)$^+$ RNA was isolated at various time points after the addition of actinomycin D and analyzed by S1 nuclease protection. During early infection, RNA transcripts processed at the GSHV and L3 poly(A) sites had a half-life of less than 4 h, whereas late transcripts were very stable. During late infection, the G/L3 ratio for each recombinant remained constant at each time point following inhibition of transcription (Fig. 5). As shown previously for L1-L3 constructs (10), this result suggests that the relative stabilities of RNAs processed at the GSHV and L3 poly(A) sites for each recombinant were similar in late infected cells. Similarly, the G/L3 ratios during early infection remained constant for recombinant viruses vL1GL1-L3 and vL1GAL1-L3, both of which contain the L1 regulatory elements (Fig. 5b and d). This suggests that the stabilities of RNAs which contain the L1 regulatory elements were similar during early infection and therefore supports the conclusion that the change in ratios is due to differential poly(A) site choice. The G/L3 ratios increased slightly over time for vG-L3

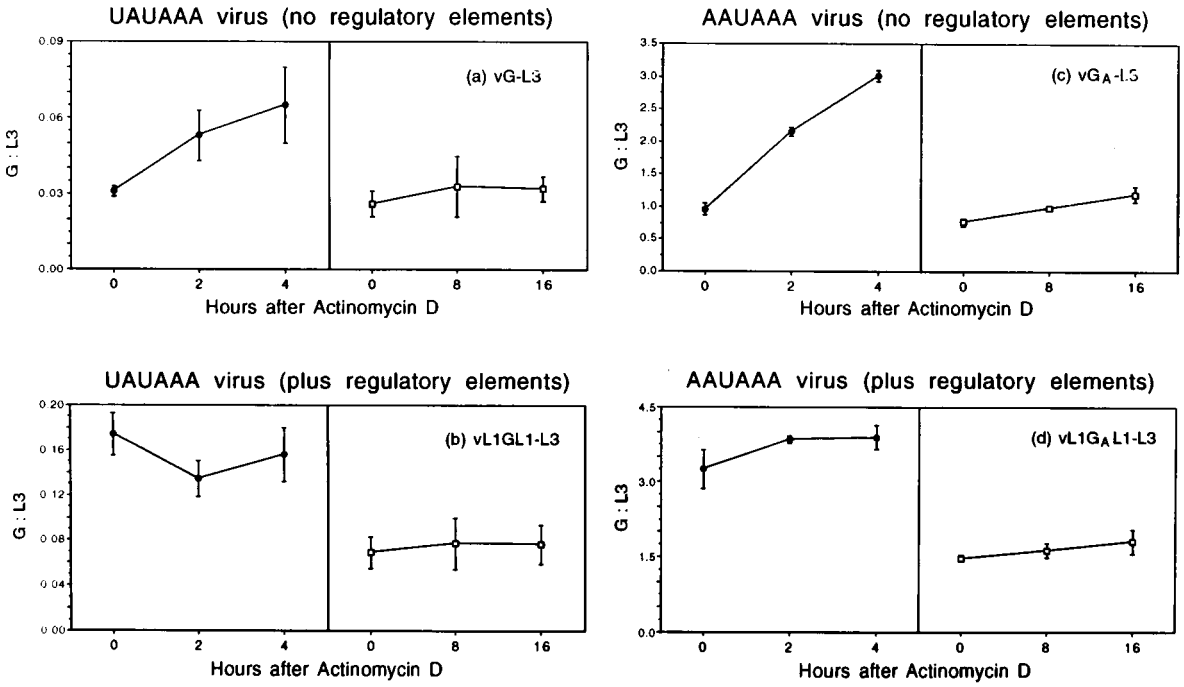


FIG. 5. Stability of mini-MLTU transcripts from early (●) or late (□) viral infections. Actinomycin D was added to a final concentration of 10 μg/ml at 6 h (early) or 22 h (late) after infection. The G/L3 ratios for each recombinant virus during early and late infection at various time points after the addition of actinomycin D are shown.

and vGA-L3 during early infection (Fig. 5a and c), suggesting that in the absence of L1 regulatory elements, RNAs processed at the GSHV site were more stable than RNAs processed at the L3 site. Since the steady-state level of the GSHV transcripts is determined by the rate of synthesis minus the rate of degradation, this indicated that the relative use of the GSHV poly(A) site for these two viruses was actually lower than that indicated by the steady-state analysis, since RNAs processed at this site are turned over less rapidly. These results support our conclusion that these constructs do not undergo the processing switch. Together these studies therefore suggest that mini-MLTUs containing the GSHV poly(A) site flanked by L1 upstream and downstream regulatory sequences can reproduce the switch in poly(A) site choice during the course of viral infection.

Sequences flanking the L1 core poly(A) site enhance the stability of the CPSF-RNA complex. In order to understand the mechanism by which the L1 flanking sequences enhance the processing of a core poly(A) site, we have examined their impact on the interaction of processing factors with the pre-mRNA in vitro. Previous work has shown that the stability of processing complexes formed on the pre-mRNA directly correlated with the efficiency of processing (14, 48). We therefore examined the influence of the L1 flanking sequences on the ability of the L1 core poly(A) site to compete for processing factors. Increasing concentrations of either unlabeled wild-type L1 or mutant (ΔL1, lacking the L1 regulatory sequences) pre-mRNA were used in a competition assay with a ^{32}P-labeled pre-mRNA containing the efficient adenovirus L3 poly(A) site. The L1 or ΔL1 competitor RNA was added simultaneously with the ^{32}P-labeled L3 pre-mRNA to a reaction mixture containing partially purified CPSF and CstF. Following a 10 min incubation at 30°C, the CPSF-CstF-RNA complexes were resolved on a nondenaturing polyacrylamide gel, and the amount of specific complex was quantitated on a PhosphorImager. The data presented in Fig. 6 indicate that the L1 flanking sequences enable the L1 core poly(A) site to compete more effectively for CPSF-CstF binding.

The sequence context of the AAUAAA hexamer had pre-

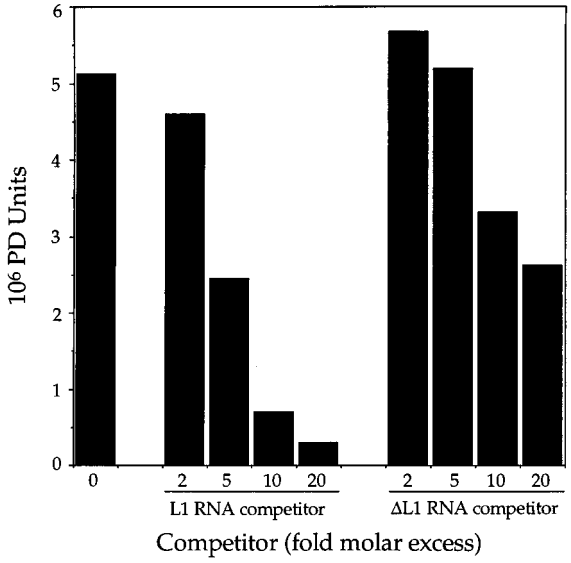
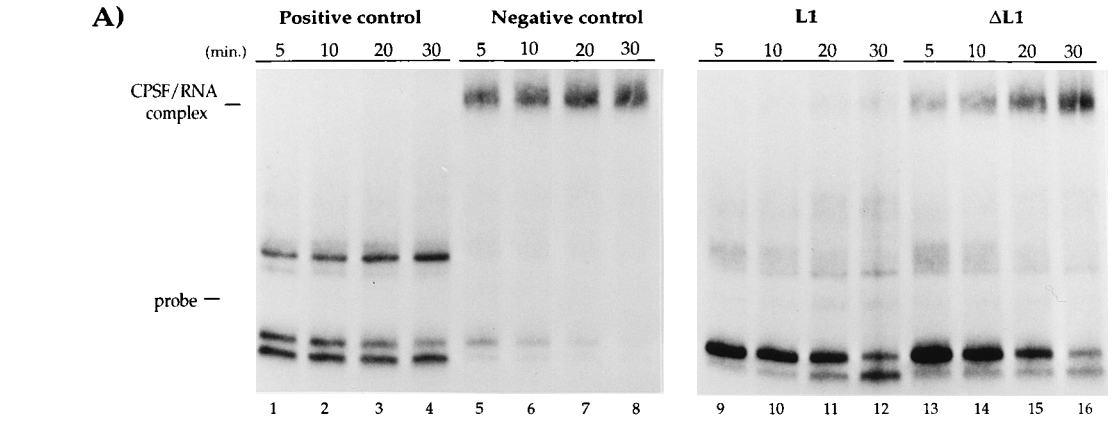


FIG. 6. Impact of L1 flanking sequences on poly(A) site competition for CPSF-CstF binding. 2.7 fmol of ^{32}P-labeled L3 pre-mRNA along with the specified amount of unlabeled competitor L1 or ΔL1 pre-mRNA was incubated with partially purified CPSF and CstF for 10 min at 30°C. The reactions were treated with heparin at 0°C, and the protein-RNA complexes were resolved on a nondenaturing 3% polyacrylamide gel. Specific CPSF-CstF-RNA complexes were quantitated by PhosphorImager. The *y* axis is in arbitrary phosphor density (PD) units.



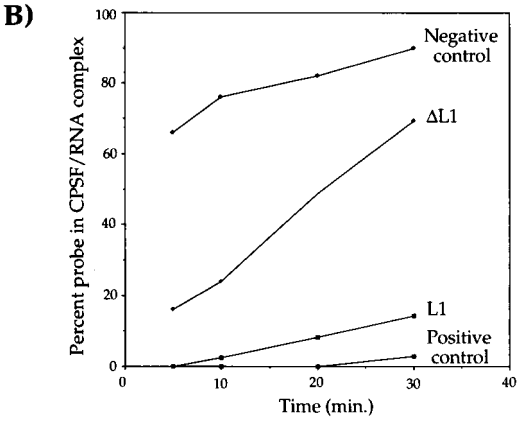


FIG. 7. Impact of L1 flanking sequences on the stability of the CPSF-RNA complex. (A) 1 pmol of unlabeled RNA was incubated with partially purified CPSF for 10 min at 30°C. ^{32}P-labeled HIV-1 pre-mRNA (20 fmol) was added, and aliquots were removed after an additional incubation for 5, 10, 20, or 30 min at 30°C. Each aliquot was treated with heparin at 0°C, and the RNA-protein complexes were resolved on a nondenaturing 3% polyacrylamide gel. (B) PhosphorImager quantitation of the data in panel A.

viously been shown to be suboptimal for processing at the L1 poly(A) site (35). We therefore investigated the impact of L1 flanking sequences on the binding of CPSF, the factor responsible for recognition of the AAUAAA hexamer. Since the in vivo-defined sequences that contribute to enhanced processing at the L1 core poly(A) site extend a considerable distance upstream and downstream of the L1 core poly(A) site (10), the RNAs we have used for the in vitro analysis exceed 600 nt. The large size of these RNAs makes the direct quantitative analysis of the CPSF-L1 RNA complex by gel mobility shift impractical, as a significant amount of the complex remains in the well (data not shown). We have therefore used the following indirect assay to examine the stability of the binding of CPSF to the L1 pre-mRNA. The unlabeled L1 or ΔL1 pre-mRNA was incubated with extensively purified CPSF for 10 min at 30°C to allow for the formation of the CPSF-RNA complex. The stability of this complex was then assayed by the addition of a ^{32}P-labeled HIV-1 pre-mRNA. The HIV-1 pre-mRNA contains both a core poly(A) site and an upstream 3′ processing enhancer and thus forms a relatively stable CPSF-RNA complex (15). The rate of the formation of the CPSF-HIV-1 RNA complex is therefore a reflection of the dissociation rate of the initial CPSF-L1 RNA complex. As seen in Fig. 7A (lanes 1 to 4), preincubation with an excess of unlabeled HIV-1 pre-mRNA (positive control) sequesters CPSF, thereby blocking the subsequent formation of a CPSF-^{32}P-labeled HIV-1 RNA complex. Conversely, preincubation with an equivalent amount of a non-poly(A) site-containing RNA (negative control) allows for the rapid formation of a CPSF-^{32}P-labeled HIV-1 RNA complex (Fig. 7A, lanes 5 to 8). Preincubation of wild-type L1 pre-mRNA with CPSF resulted in the slow accumulation of a CPSF-^{32}P-labeled HIV-1 RNA complex (Fig. 7A, lanes 9 to 12), while preincubation with the ΔL1 pre-mRNA [L1 core poly(A) site in the absence of flanking sequences] resulted in the more rapid accumulation of a CPSF-^{32}P-labeled HIV-1 RNA complex. These results demonstrate that sequences flanking the L1 core poly(A) site enhance the stability of the binding of CPSF at the L1 core poly(A) site.

DISCUSSION

The adenovirus MLTU directs the synthesis of a complex array of alternatively processed mRNAs (for a review, see reference 30). Five families of messages, encoding distinct gene products, are defined by the use of five alternative poly(A) sites. Poly(A) site choice, and hence the relative expression of each transcript family, is regulated during the course of viral infection. The in vivo analysis of the promoter-proximal L1 poly(A) site has revealed that sequences extending 5′ and 3′ of the core poly(A) are required for both efficient processing and regulated expression (10). We now show that these sequences are capable of enhancing the processing of a heterologous poly(A) site as well as conferring regulated site selection in the context of a complex transcription unit. These sequences enhanced the efficiency of processing at the GSHV core poly(A) site when expressed in transiently transfected cells and allowed for its proper regulation during the course of infection when expressed from the adenovirus chromosome. Thus, these sequences represent discrete regulatory elements. Furthermore, in vitro analysis demonstrated that the L1 poly(A) site regulatory sequences serve to stabilize the initial binding of CPSF at the core poly(A) site. This is the first example in which sequences that serve to regulate poly(A) site selection within a complex transcription unit have been shown to influence the interaction of constitutive processing factors at the core poly(A) site.

Elements that enhance 3′-end processing have been found to reside within the sequences flanking several core poly(A) sites (4, 6, 10–12, 35, 37, 38, 44). These elements appear to function in an orientation-dependent manner (37) but exhibit little sequence or apparent structural similarity. The functional interchangeability of several elements (37, 44) suggests that

they participate in a common step in mRNA 3′-end processing. Our finding that sequences required for efficient processing at the L1 core poly(A) site enhance the processing of the heterologous weak GSHV core poly(A) site lends additional support to this view of 3′ processing enhancers. The ability of the L1 flanking sequences to stabilize the binding of CPSF, the factor responsible for the initial recognition of the AAUAAA hexamer, suggests that these sequences function in a manner similar to that of the HIV-1 mRNA 3′-end processing enhancer (15). Sequences upstream of the HIV-1 core poly(A) site have been shown to increase the stability of CPSF binding through a direct interaction with the CPSF 160-kDa subunit. These results suggest that these sequences may provide additional CPSF contact sites and/or may serve to present the core poly(A) site in a structure conducive to stable CPSF binding (19a). As shown by Prescott and Falck-Pedersen (35), processing at the L1 core poly(A) site is inefficient as a consequence of sequences directly adjacent to the L1 AAUAAA hexamer. Thus, processing at the L1 poly(A) site, when placed in competition with a more efficient downstream poly(A) site, requires the presence of additional sequences that enhance its efficiency. 3′ processing efficiencies within the MLTU, however, must be carefully balanced so as not to completely prevent the use of the four downstream poly(A) sites late in the infection.

The ability of the L1 flanking sequences to facilitate the binding of CPSF to the pre-mRNA is consistent with their role in enhancing processing efficiency of the L1 core poly(A) site. The key question to be addressed is the mechanism by which these sequences promote the predominant use of this poly(A) site during early adenoviral infection and yet allow for it to be bypassed in favor of downstream poly(A) sites during late infection. Mann et al. (25) have recently shown that the activity of CstF [which binds the downstream element of the core poly(A) site] decreases during late infection. Coupled with our demonstration of the impact of L1 flanking sequences on CPSF binding, these results suggest a mechanism by which processing efficiency may be regulated by the interaction of constitutive processing factors. In the early stage of the infection, the L1 flanking sequences promote the formation of the initial CPSF-RNA complex, which is then converted to a "committed" processing complex (48) by the binding of CstF. As CstF activity becomes limiting during late infection, however, processing at downstream sites that form a more stable CPSF-CstF-RNA complex would be favored. The L1 flanking sequences therefore appear to contribute to the regulation of processing at L1 through their ability to facilitate the formation of the CPSF-RNA complex, thereby assuring that the conversion of the CPSF-RNA complex to the CPSF-CstF-RNA complex is indeed the rate-limiting step in processing at L1. In the absence of the L1 regulatory elements, binding of CPSF at the L1 core poly(A) site is unstable and the formation of the CPSF-RNA complex may therefore become rate limiting. In this case, the decline in CstF activity that accompanies the late phase would be expected to have little impact on the already low level of processing. This prediction is borne out by the loss of L1 regulation upon deletion of the sequences flanking the core poly(A) site (10) as well as by the unregulated use of the GSHV core poly(A) site alone (this work). These data support a model for the 3′ processing of MLTU pre-mRNAs in which poly(A) site selection is dictated by the relative processing efficiencies of the poly(A) sites within the transcription unit and is modulated by the interaction of constitutive 3′ processing factors.

The regulation of 3′ processing within the MLTU must require a balance of processing efficiencies among all five poly(A) sites. We suggest that small changes in the efficiency of processing at the promoter-proximal L1 site may be sufficient to significantly alter the distribution of mRNA 3′ ends during the course of viral infection. Consistent with this view, the impact of the L1 regulatory sequences is seen only in the context of a complex transcription unit composed of competing processing sites (11). When assayed as the only poly(A) site in the transcription unit, the sequences flanking the L1 core poly(A) site had no apparent effect on steady-state levels of correctly processed mRNAs (35). In addition, the influence of these sequences upon the regulation of 3′ processing at L1 is a consequence of changes unique to the late phase of viral replication. In the presence of the L1 flanking sequences, processing efficiencies in uninfected and early virus-infected cells are equivalent (compare Fig. 3B and 4B), suggesting that constitutive processing factors are sufficient to direct the predominant use of the L1 poly(A) site. The regulation of poly(A) site choice is unlikely to result from the induction of an L1-specific *trans*-acting regulatory factor in late-phase cells. Superinfection of virus or transfection of plasmids carrying a mini-MLTU into late virus-infected cells results in the early phase processing phenotype (13). As proposed by DeZazzo et al. (10), the restriction of late-phase regulation to the nascent transcripts of replicated viral chromosomes may be a consequence of their compartmentalization within the nucleus. In the late phase, viral DNA has been shown to reside in multiple foci localized near the periphery of the nucleus (27, 28) and in stable association with the nuclear matrix (51). Upon progression from the early to late phase of infection, the transcription rate of the MLTU increases 400- to 1,000-fold (39), greatly increasing the local demand for 3′ processing factors. A decrease in the local concentration of processing factors might therefore be expected as the infection progresses, leading to the observed alterations in the pattern of 3′ processing of the MLTU pre-mRNAs.

Changes in the levels or activities of constitutive processing factors have also been suggested to account for the regulation of poly(A) site selection within the immunoglobulin heavy-chain gene (33). Alternative processing of the immunoglobulin heavy-chain pre-mRNA involves competition between mutually exclusive splicing and polyadenylation reactions (34). The key to the regulation of immunoglobulin pre-mRNA processing during the course of B-cell development has been shown to be the balanced efficiency of competing processing sites (33). Developmental regulation can be maintained upon replacement of the immunoglobulin processing sites with heterologous sites of comparable efficiency. Regulation therefore does not appear to be directed by gene-specific *trans*-acting factors but rather may be mediated by changes in the abundance or activity of constitutive processing factors during the course of B-cell development. A similar regulatory mechanism has emerged from the investigation of alternative splicing. The concentrations of the constitutive factors ASF/SF2, hnRNPA1, PTB, U2AF, and SR proteins have been implicated in the control of splice site choice in a variety of systems (5, 22, 26, 43). We suggest that the regulation of 3′ processing within the MLTU may represent yet another example of a rather simple strategy for the regulation of competing processing events through the modulation of the activities of constitutive processing factors.

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