Regulation of adenovirus major late transcription unit (MLTU) mRNA biosynthesis involves poly(A) site selection between five available sites, L1 through L5. The 5' proximal site completely dominates during early infection, whereas all five sites are used during late infection with L3 being favored slightly over the others. Previous studies have shown this early to late poly(A) switch will occur in the absence of MLTU-specific splicing patterns and hinges in large part on the character of the first poly(A) site. We have used in vitro assays to characterize basic features of the L1 and L3 pre-mRNAs which may help define how processing at poly(A) sites is controlled. We have found that L1 is 5-10-fold less efficient than L3 as a substrate for RNA cleavage. A primary difference between the L1 and L3 sites lies in the kinetics of their use, with cleavage at L3 occurring at twice the rate of cleavage at L1. In addition, L1 is 20-fold less effective than L3 in competing for processing factors. To investigate the sequence elements that contribute to poly(A) site efficiency, we have used competition assays in which the competitor RNAs lack upstream or downstream elements.

Generation of a 3' end is an essential step in the maturation of all cellular RNA products. In higher eukaryotes, the 3' end of most messenger RNAs is formed by endonucleolytic cleavage of a precursor RNA, followed by the addition of 200-250 AMP residues to the newly generated 3' OH terminus (for reviews, see Refs. 1-3). For the majority of mRNAs produced, the efficiency of the cleavage/polyadenylation reaction is unknown, since mRNAs which do not follow the normal path of processing are assumed to undergo rapid degradation in the nucleus. For simple transcription units that contain a single polyadenylation site, the efficiency of 3' end formation could have a major impact on the amount of nuclear pre-mRNA that ends up in the cytoplasm as mature mRNA.

Processing efficiency plays a critical role in controlling mRNA biosynthesis in complex transcription units, where multiple processing sites are available. The adenovirus MLTU, which contains five poly(A) sites (L1 through L5), is a good example of one such complex transcription unit (4-8; for review, see Ref. 9). In an early adenovirus infection, the 5' proximal L1 site is used almost exclusively, although transcription continues well past the downstream L2 and L3 poly(A) sites (6, 10, 11). The onset of a late viral infection brings on a dramatic change in the transcription and processing pattern of the MLTU. High levels of transcription through all five poly(A) sites occurs, with each site being used productively (5, 6). The determinants involved in controlling this early to late poly(A) switch are not known at present. In vitro experiments using a minigene containing the L1 and L3 poly(A) sites inserted in tandem into the E1a transcription unit reproduced the processing switch (12), indicating that the poly(A) switch will occur independent of MLTU-specific splicing patterns. Deletion analyses of the L1 poly(A) site suggest that sequences 5' and 3' of the core AAUAAA play a role in allowing it to dominate over a downstream poly(A) site in early infections as well as in transfections (13, 14).

Two sequence determinants of poly(A) site processing activity are the well-characterized core AAUAAA and a GU/U-rich region located 20-50 bases 3' to the AAUAAA. Cleavage occurs between these two elements. Deletion or mutation of either element results in decreased levels of steady state mRNA (15-28). In vitro, the AAUAAA core has been shown to bind to specificity factors that are required for both accurate cleavage of the pre-mRNA and for AAUAAA-dependent polyadenylation of a precleaved substrate (29, 30). The downstream GU/U-rich region has been shown to interact with cleavage stimulatory factors that are required for efficient cleavage in vitro, but unnecessary for efficient polyadenylation once cleavage has taken place (29-32). Interaction of cleavage factors with the GU/U-rich region has been shown to stabilize the interaction between specificity factor and the AAUAAA (29-31). This enhanced stabilization is reflected in a more efficient cleavage/polyadenylation reaction (31). Deletion of the GU/U-rich region from a number of poly(A) sites, including L1 and L3, has shown that this region is required for efficient cleavage. At this point, it is not completely clear how cis elements and trans factors combine to form a processing complex, or what determinants affect processing efficiency at the poly(A) site.

An important step toward understanding how poly(A) site selection and regulation occurs is to characterize the pre-mRNA substrates involved and their interaction with poly(A) site processing factors. Using an in vitro processing reaction, we have made a direct comparison of the MLTU L1 and L3 poly(A) sites. These studies show that the proximal L1 poly(A) site acts as a low affinity substrate and that its processing efficiency is quite sensitive to sequence manipulations. The L3 poly(A) site acts as a high affinity substrate which is less sensitive to sequence manipulations.

Varied Poly(A) Site Efficiency in the Adenovirus Major Late Transcription Unit

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MATERIALS AND METHODS

Plasmids—The two wild type poly(A) signal clones (pL1 and pL3) were constructed by amplifying ~200-bp fragments (206 bp of L1 and 207 bp of L3), extending ~100-bp 5' and 3' of the cleavage site, by the PCR technique. Previously described plasmids were used as PCR templates (12). These DNA fragments were subcloned into the pGEM 2 vector (Promega) at the XbaI/EcoRI restriction endonuclease sites such that transcription by T7 RNA polymerase generated the pre-mRNA. An NcoI restriction endonuclease site was introduced at the cleavage site of pL3 using a PCR-directed technique (33) to generate pL3/NcoI, the plasmid used to transcribe precleaved L3 pre-mRNA. The wild type L1 poly(A) clone (pL1) naturally contains an NcoI restriction endonuclease site at the poly(A) cleavage site.

Upstream deletion poly(A) site constructs were made in a two-step process. First, an Apal restriction site was introduced 10 bp upstream of the cleavage site by the PCR technique. Following this, the pGEM 2 polylinker, and Apal to remove all adenovirus sequences were blunted with T4 DNA polymerase, and the plasmids recircularized with T4 DNA ligase. All constructs were sequenced (and shown to be correct) before being used.

Cells and Extracts—HeLa cells were grown in suspension at 3-7 × 10^6 cells/ml in Joklik's modified minimal essential medium containing 10% fetal calf serum. The nuclear extracts were prepared as described by Gilmartin et al. (54). The extracts were dialyzed against 20 mM HEPES, pH 7.9, 150 mM KCl, 0.2 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride, and aliquots were frozen in liquid N2 and stored at -70 °C for up to 6 months.

Preparation of 32P-Labeled Pre-mRNA—Transcription by T7 RNA polymerase was performed according to manufacturer's suggestions on DNA templates linearized with EcoRI (full-length) or NcoI (precleaved). Transcription reactions included 0.5 mM 3'-O-methylGppp(5')G (Pharmacia LKB Biotechnology Inc.), 0.1 mM ATP, CTP, UTP, and GTP (Pharmacia), and 0.8 μM [32P]UTP (3000 Ci/mM from Du Pont-New England Nuclear). Pre-mRNAs were electrophoresed on a preparative 3.5% polyacrylamide, 8 M urea gel. The pre-mRNA was excised from the gel and the gel slice homogenized in an extraction buffer containing guanidinium thiocyanate and phenol (35). RNA was purified from the homogenate according to Chomczynski and Sacchi (35).

Preparation of Unlabeled Competitor Pre-mRNA—Transcription by T7 RNA polymerase was performed as described above in the presence of 0.5 mM 3'-O-methylGppp(5')G, 0.5 mM ATP, CTP, UTP, and GTP, 0.1 mM GTP, and 80 μM [32P]UTP (3000 Ci/mM). Aliquots were quantitated by scintillation counting and visualized on a denaturing acrylamide gel followed by autoradiography. By this criterion, >99% of the competitor RNA was the expected length.

In Vitro Cleavage and Polyadenylation Assays—Unless stated otherwise, in vitro processing reactions were carried out at 30 °C for 30 min in the presence of 1 nM substrate RNA and 40% HeLa nuclear extract (2 mg/ml final protein concentration). Cleavage reactions contained 0.5 mM 3'-dATP; polyadenylation reactions contained 1 mM ATP and 0.7 mM MgCl2. All reactions contained 5 mM creatine phosphate and 2.5% polyvinyl alcohol. Reactions were stopped with guanidinium thiocyanate and phenol and RNAs were purified according to the method of Chomczynski and Sacchi (35). RNA reaction products were separated on denaturing acrylamide gels and visualized by autoradiography.

Quantitation of Reaction Products—Reaction products were quantitated either by excision of the labeled RNA from the gel followed by scintillation counting or by laser densitometric analysis of the autoradiogram (each autoradiogram contained a set of counts/min standards). Counts/min were converted to femtomoles based on the specific activity of the [32P]UTP used during transcription by T7 RNA polymerase and the number of UMP residues in the RNA product. The L1 5' cleavage products, which are 142 nt long and include 42 nt of polylinker sequences, contain 22 UMP residues. The L3 5' cleavage products, which are 154 nt long and include 42 nt of polylinker sequences, contain 40 UMP residues.

RESULTS

L1 Is a Weaker Poly(A) Site Than L3—Previous use of in vitro processing assays has shown that both the L1 and L3 poly(A) sites are cleaved and polyadenylated in HeLa nuclear extracts (36-39). The L3 poly(A) site is the substrate used originally to develop the in vitro cleavage/polyadenylation system and has been used since in many laboratories as a substrate for the fractionation and purification of cleavage/ polyadenylation factors (29, 34, 40-45). In Fig. 1, we compare processing of the L1 and L3 pre-mRNAs in the HeLa nuclear extract. The DNA templates that were used for transcription by T7 RNA polymerase include ~200 bp of adenovirus DNA containing either the L1 or the L3 poly(A) site. The run-off transcription products generated contain ~100 nucleotides of adenovirus-specific RNA on either side of the cleavage site (Fig. 1A). The processing reactions were performed in two sets of conditions: 1) in the presence of 3'-dATP and EDTA (cleavage conditions), and 2) in the presence of ATP and MgCl2 (polyadenylation conditions). While both conditions allow for cleavage of the substrate RNA, only the polyadenylation conditions allow for adenylation of the 5' cleavage products. The reaction products were run directly on a polyacrylamide, 8 M urea gel and visualized by autoradiography.

Analysis of the reaction products from the in vitro cleavage reactions (Fig. 1B, lanes 2 and 8) showed that a portion of both L1 and L3 RNA transcripts were cleaved to products of ~150-nt (5' cleavage product) and ~100-nt (3' cleavage product) lengths. The L3 products were seen to be more abundant than the L1 products, indicating that the L3 poly(A) site is more efficiently cleaved than the L1 poly(A) site.

FIG. 1. In vitro cleavage and polyadenylation assays of adenovirus L1 and L3 pre-mRNAs. Panel A, a schematic of the template DNA transcribed by T7 RNA polymerase to generate substrate pre-mRNAs. The known cis processing elements (AATAAAA, GT/T-rich region, and cleavage site) are indicated on each template. The full-length pre-mRNAs contain ~150 nucleotides ~100 nt adenovirus and ~50-nt polylinker) and ~100 nucleotides 3' of the normal cleavage sites. Linearizing the template DNA at the NcoI site followed by transcription with T7 RNA polymerase generates run-off products which end two nucleotides past the normal cleavage site. These precleaved RNA products are ~150 nucleotides in length. The L3 wild type plasmid was modified to introduce an NcoI restriction endonuclease site at the cleavage site (L3/NcoI) for the generation of precleaved L3 mRNA (see "Materials and Methods"). Panel B, RNA processing reactions were carried out with full-length L1 (lanes 1-3) or L3 (lanes 7-9) and precleaved L1 (lanes 4-6) or L3 (lanes 10-12) pre-mRNAs under cleavage conditions (lanes 2, 5, 8, and 11) or polyadenylation conditions (lanes 3, 6, 9, and 12). Control assays replacing the nuclear extract with an equal volume of reaction buffer (lanes 1, 4, 7, and 10) indicate the size of each unprocessed pre-mRNA. Reaction products were electrophoresed on a 3.5% polyacrylamide, 8 M urea gel. Cleavage products, polyadenylated products, and unprocessed pre-mRNA are indicated.
uct). Cleavage at the L1 poly(A) site occurred at two to three sites, as seen by the size heterogeneity in the L1 5′ cleavage product. Cleavage at the L3 poly(A) site occurred at two locations separated by ~6 nt. This heterogeneity in cleavage site at L1 and L3 has also been seen by other laboratories (34, 36). Quantification of the 5′ cleavage products, either by analysis of the autoradiogram with a laser densitometer or scintillation counting the RNA after excision from the gel, showed a consistent 5–10-fold molar excess of L3 5′ cleavage product over L1 5′ cleavage product.

When ATP and MgCl₂ were substituted for 3′-dATP and EDTA in the polyadenylation reactions, the 5′ cleavage products were lengthened by 100–200 nt, indicating that they had been polyadenylated (Fig. 1B, lanes 3 and 9). While it is difficult to quantitate the polyadenylated material, it is clear that the relative strengths of L1 and L3 seen in the cleavage assay is maintained in the polyadenylation assay (Fig. 1B, compare lanes 2 and 8 to lanes 3 and 9).

Precleavage Increases the Efficiency of L1 Polyadenylation—As a first step toward understanding what makes L3 a stronger poly(A) site, in the next section, we have assayed the polyadenylation efficiency of the two sites once they have been precleaved. Multiple protein components are required for the accurate and efficient cleavage/polyadenylation of a precursor mRNA that spans the cleavage site (29, 34, 40–46). However, if the precursor mRNA has been precleaved (i.e. if the 3′ end of the precursor mRNA lies at or within several nucleotides of the normal cleavage site), only a subset of these protein components are required for its accurate and efficient polyadenylation (29, 40–42, 44, 45). For this reason, comparison of the processing efficiencies with and without precleavage at L1 and L3 may give an indication of what step(s) in the processing reaction give rise to the difference in poly(A) site strength between L1 and L3.

The precleaved L1 transcript was made by linearizing the DNA template at the Neol site before transcription with T7 RNA polymerase. The precleaved L3 transcript was made in the same way, using a DNA template in which a four-base pair substitution has introduced an Neol site at the L3 cleavage site (Fig. 1A). These precleaved RNAs were then processed in a HeLa nuclear extract under either cleavage or polyadenylation conditions. Incubation under cleavage conditions resulted in no apparent change in the RNA substrates, as would be expected if only one 3′-dATP molecule were added to the 3′ end of the substrate RNA (Fig. 1B, lanes 5 and 11). Incubation under polyadenylation conditions resulted in conversion of a portion of the input RNA to a product that was 100–200 nt longer, indicating that these RNAs had been polyadenylated (Fig. 1B, lanes 6 and 12). The efficiencies of polyadenylation of the precleaved L1 and L3 RNAs appear to be roughly equivalent. This result shows that L1 is able to efficiently utilize those factors required for polyadenylation (SF and poly(A) polymerase) once the need for those additional factors required for cleavage (CFI, CFII, and CstF) has been obliterated by precleavage. Since these additional factors play an important role in stabilizing the cleavage complex at an intact poly(A) site (29–31), it seems likely that L1 is limited in its ability to form a stable cleavage complex. This possibility will be addressed directly in Fig. 4.

L1 Is Cleaved at a Slower Rate Than L3—To further characterize the disparity between L1 and L3 in vitro cleavage efficiency, we have examined the kinetics of cleavage at these two sites. In this experiment, 300-μl cleavage reactions were set up in 30% HeLa nuclear extract, and 25-μl aliquots were taken at various time points. The reaction products, separated on a denaturing acrylamide gel, are shown in Fig. 2A. Product yield reached a plateau at approximately 30 min, at which time 7% of the input L1 and 16% of the input L3 had been cleaved. Longer time points showed a nominal loss of product and substrate which is probably due to ribonuclease activity.

The data, taken from the linear range of product yield and summarized graphically in Fig. 2B, show that the L3 poly(A) site is cleaved at twice the rate of the L1 poly(A) site.

L1 and L3 Processing Are Dependent on Protein and RNA Concentration—Enzyme and substrate concentration are components which are likely to affect the efficiency/rate of polyadenylation both in vitro and in vivo. During the course of adenovirus infection, the concentration of viral transcripts and potentially of cleavage/polyadenylation factors is altered. Nuclear viral RNA concentrations increase during late infection due to an increased number of viral DNA templates and an increased level of transcription initiated on the major late promoter (7, 47–50). The concentration of cleavage/polyadenylation factors is also likely to change in the late infected nucleus due to the blocked translation of host mRNAs (51, 52). Altering the concentration of substrate and/or enzyme in the in vitro processing system is a potential means of regulating poly(A) site use during the transition from early to late infection. For these reasons, we have varied the concentrations of both nuclear extract and pre-mRNA in the in vitro cleavage assay.

Increasing the extract concentration from 0 to 40% (0–2 mg/ml final protein concentration) resulted in increasing the cleavage of both L1 and L3, with L3 remaining the stronger poly(A) site throughout (Fig. 3A, lanes 1–8, 12–18). Both L1 and L3 showed maximal processing activity at 40% extract concentration. At greater concentrations of extract, both L1 and L3 show decreased yields of 5′ cleavage product, probably due to the increased rate of degradation at these high concentrations of extract.

Using 40% nuclear extract, we next varied the concentration of the substrate RNAs (Fig. 3B). In this experiment, all reactions contain 0.1 nM 5MelppppG-capped [52P]UTP-labeled pre-mRNA. Unlabeled capped pre-mRNAs containing the appropriate poly(A) site were then added to increase the RNA concentration to either 1 nM (lanes 2 and 5) or 10 nM (lanes 3 and 6). Quantification of the cleavage products showed that while L3 processing is saturated at some point between 1 and 10 nM RNA, L1 processing is not yet saturated at 10 nM RNA. As the substrate concentration for the weaker L1 pre-mRNA was increased, the final product yield rose to levels that are comparable to the stronger L3 pre-mRNA. At 100 nM input pre-mRNA (2,500 fmol in a 25-ml reaction), 21 fmol of L1 cleavage product were generated as compared to 38 fmol of L3 cleavage product.

L3 Competes for Cleavage/Polyadenylation Factors More Efficiently Than L1—Specificity factor has been shown to bind precursor mRNA containing an AAUAAA in vitro (29, 30). The interaction between SF and the AAUAAA containing pre-mRNA is relatively unstable but can be stabilized by the addition of cleavage stimulatory factors (CstF), and this stabilization is dependent on a downstream GU/U-rich element (29–31). Finally, the ability to form a stable ternary complex at the poly(A) site with SF, CstF, and RNA, has been correlated with increased poly(A) site strength in vitro (31). In order to test the abilities of L1 and L3 to build stable cleavage complexes, we have assayed the abilities of L1 and L3 to compete for a limited amount of processing factors.

In this experiment, 1 nM capped 32P-labeled RNA containing either the L1 or the L3 poly(A) site was incubated under cleavage conditions in 20% HeLa nuclear extract in the presence of a 1–100-fold excess of capped unlabeled RNA containing either the L1 or the L3 poly(A) site. The extent to which
the labeled pre-mRNA is processed in the cleavage reaction is a measure of its affinity for cleavage factors and the stability of the cleavage complex.

Cleavage of \(^3\)P-labeled L3 pre-mRNA in the presence of unlabeled L1 or L3 competitor clearly showed a difference between these two poly(A) sites (Fig. 4A). L3 processing is resistant to competition with excess RNA containing the L1 poly(A) site (Fig. 4A, lanes 3–9). However, L3 is susceptible to competition when the competitor RNA contains the L3 poly(A) site (Fig. 4A, lanes 10–16), indicating that processing factors are near limiting in these reactions. The amount of L3 cleavage product produced showed that a 20–50-fold molar excess of L1 pre-mRNA is required to achieve 50% inhibition of L3 processing. The same inhibition was achieved by the addition of an equimolar amount of L3 pre-mRNA (Fig. 4B). This result indicates that L3 is 20–50-fold more efficient than L1 at stably binding cleavage factors.

The converse experiment, where \(^3\)P-labeled L1 pre-mRNA is processed under cleavage conditions in the presence of unlabeled competitor, is consistent with L1 acting as a low affinity substrate. In this experiment, L1 processing was nearly completely inhibited by an equimolar amount of RNA containing the L3 poly(A) site (Fig. 4A, lanes 26–32). However, a 5–10-fold molar excess of unlabeled L1 pre-mRNA was needed to see 50% inhibition of L1 processing (Fig. 4A, lanes 19–25). These results agree with the earlier results (Fig. 3B), showing that in order to saturate an extract for cleavage/polyadenylation activity, more L1 pre-mRNA is needed than L3 pre-mRNA. The competition experiments define L1 as a low affinity substrate and L3 as a high affinity substrate for the assembly of a cleavage/polyadenylation complex.

**Sequences Upstream and Downstream of the Core AAUAAA Are Required for Efficient Competition**—Using the competition assay, we have addressed which sequence elements in L1 and L3 are important for stably binding cleavage/polyadenylation factors. To this end, we have used competitor pre-mRNAs that lack either sequences downstream of the cleavage site or upstream of the AAUAAA. The downstream deletion (precleaved) competitors contain adenovirus sequences extending from ~100 nt upstream of the cleavage site to the cleavage site (Fig. 5A). The upstream deletion competitors contain adenovirus sequences extending from 10 nt upstream of the AAUAAA to ~100 nt downstream of the cleavage site. Based on our limited current knowledge of the formation of a
cleavage complex formed at the poly(A) site, so that deleting this region allows for more efficient competition for SF and/or poly(A) polymerase. The second explanation is that precleaved L1 competes efficiently for a limiting amount of SF and/or poly(A) polymerase, which is required for both the cleavage and the polyadenylation reactions (29, 34, 40–46). The latter possibility seems more likely since we have already shown that precleaved L1 is much more efficiently polyadenylated than full-length L1 (Fig. 1, lanes 3 and 6). Deletion of upstream sequences from the L1 competitor RNA had no effect on its ability to compete with L3 for processing factors (Fig. 5B). The L1 competition results indicate neither the upstream nor the downstream elements of L1 enhance the ability of the core AAUAAA to bind processing factors.

Competition assays using all or part of the L3 poly(A) site against full-length L3 processing are shown in Fig. 5C. In contrast to the L1 results, deletion of the downstream GU/U-rich element from the L3 competitor reduced its ability to compete with an intact L3 poly(A) site for processing activity. As with the deletion of the GU/U-rich region from the L1 poly(A) site, there are two possible explanations for this decreased competition. Either the GU/U-rich element is stabilizing the cleavage complex formed at the L3 poly(A) site, or the precleaved L3 competitor has lost the ability to build a stable cleavage complex, but has retained the ability to compete for poly(A) polymerase. Precleaved L3 competes for processing factors ~5-fold more efficiently than precleaved L1 (Fig. 5, B and C) while they are polyadenylated with near equal efficiencies (Fig. 1). These data suggest that precleaved L3 is able to compete for those factors required for polyadenylation (Fig. 1) as well as those additional factors required for cleavage (Fig. 5) of the intact L3 poly(A) site. The deletion of sequences upstream of the L3 poly(A) site from the competitor RNA had the same affect as the downstream deletion, a reduced ability to compete for processing factors. The com-

**Fig. 4.** Comparison of the L1 and L3 pre-mRNAs in a processing competition. Panel A, processing of L3 (lanes 1–16) or L1 (lanes 17–32) 32P-labeled pre-mRNA at 1.0 nM in the presence of increasing concentrations of unlabeled L1 (lanes 3–9 and 19–25) or unlabeled L3 (lanes 10–16 and 26–32) competitor pre-mRNA. Assay conditions were as previously described, using 36% nuclear extract. Control assays containing no nuclear extract (lanes 1 and 17) or no competitor RNA (lanes 2 and 18) were included. Following RNA purification and electrophoresis on a 3.5% polyacrylamide, 8 M urea gel, 5' cleavage products were quantified by both laser scanning densitometry and scintillation counting of excised gel fragments. Panel B, histogram showing the amount of full-length 32P-labeled L3 cleavage product from each competition assay as a percentage of that amount produced in the absence of competitor RNA. A transcript containing ~200 nucleotides of RNA lacking AAUAAA or GU/U-rich regions (CAT, from a portion of the chloramphenicol acetyltransferase gene) was included as a nonspecific control. The concentrations of competitor pre-mRNAs which caused a 50% decrease in the yield of 32P-labeled 5' L3 cleavage product are indicated above the histogram.

stable ternary complex at a poly(A) site, we would expect both the AAUAAA and the downstream GU/U-rich element to be required for the assembly of a stable cleavage complex and therefore required to compete efficiently for processing factors.

Precleavage of the L1 competitor RNA (Fig. 5B) allowed it to compete slightly more efficiently than full-length L1 against L3 for processing activity. This result shows that the downstream GU/U-rich region of L1 does not enhance the stability of the cleavage complex. There are two possible explanations for the slight increase in competition. The first is that the downstream region of L1 actually destabilizes the
bined results from these experiments lead us to believe that L1 shows little cooperation between the core AAUAAA and surrounding elements in binding processing factors. On the other hand, the L3 pre-mRNAs have multiple and potentially redundant sequence elements which contribute to the binding of cleavage/polyadenylation factors.

**DISCUSSION**

Regulation of mRNA biosynthesis in the adenovirus MLTU is an example of RNA processing control which directly involves alternative poly(A) site selection. During early infection, the promoter proximal site (L1) is used exclusively while all five sites (L1-L5) are used during late infection. Previous work has shown that this early to late poly(A) switch can be reproduced using a minigene construct containing the first (L1) and the third (L3) sites in tandem, indicating that the poly(A) sites themselves contain all of the cis acting elements needed for the switch (12). As a step toward understanding the early to late switch, we have examined the in vitro processing characteristics of these two poly(A) sites. We have shown that L1, the promoter proximal site that dominates during early infection, is a much weaker poly(A) site than the downstream L3 site. This difference in strength is seen in the yield of cleavage products, the rate of cleavage (L3 cleaves twice as fast as L1), and the ability to compete for cleavage factors (L3 competes for cleavage factors 20-50-fold more efficiently than L1). We have also shown that multiple sequence elements contribute to the formation of an efficient poly(A) site such as L3, that these elements lie upstream and downstream of the AAUAAA, and that these elements cooperate to direct the assembly of a stable cleavage complex.

We have shown that sequences upstream of the L3 AAUAAA contribute to the formation of a stable cleavage complex. L3 is not unique in having an upstream enhancing element. Both the SV40 late (53) and HIV (54) poly(A) sites have been shown to contain upstream enhancing elements. It is worth noting that the upstream elements of the L3, SV40 late, and HIV poly(A) sites are all U-rich. While U-rich regions have been shown to be binding sites for heterogeneous nuclear ribonucleoprotein C proteins, this binding has yet to be correlated with poly(A) site strength (55-57). It has also been suggested that the U-rich nature of these upstream elements may indicate that they are functionally similar to downstream GU/U-rich elements (54). The fact that removal of either the upstream or downstream elements from L3 has a similar effect on the ability to form a stable cleavage complex supports this suggestion. These data suggest that adenovirus has evolved multiple, and potentially redundant, means of insuring that the L3 is a strong poly(A) site.

There are many known complex transcription units that use alternate poly(A) sites to generate different gene products. One well-studied example is the IGm heavy chain transcription unit. The production of secreted (μs) or membrane bound (μm) Ig heavy chains in B cells is the result of differential splicing and polyadenylation of a common precursor RNA molecule. This differential processing is tightly regulated, with B cells producing both μs and μm and plasma cells producing solely μm. Like L1 in the MLTU, the upstream IgM poly(A) site (μs) is weaker than the downstream site (μm) (58). In addition, it has recently been shown that during the transition from B cell to plasma cell, the in vivo cleavage and polyadenylation efficiency at the μs poly(A) site increases while the efficiencies of the alternative splice donors remains unchanged (59). However, this difference in poly(A) site strength is not reflected in different in vitro rates of cleavage, regardless of whether the nuclear extracts are prepared from HeLa cells, B cells, or plasmacytoma cells (60). This is similar to the L1/L3 situation where the relative efficiencies of the two poly(A) sites are the same whether the nuclear extracts are made from uninfected, early infected, or late infected HeLa cells (data not shown).

In the IgM system, the position of the weak poly(A) site is important. When the μm site is placed in the upstream position, the downstream μs site is not utilized (61). We have seen similar results with the adenovirus L13 minigene constructs. When L3 is the upstream position (L31 minigene), the L1 site is utilized very inefficiently, both early and late in infection. As is the case with the IgM transcription unit, the poly(A) switch in the adenovirus MLTU depends on both strength and relative position of the poly(A) sites. Recently, DeZazzo and Imperiale (13, 14) have demonstrated that sequences upstream and downstream of the L1 core AAUAAA are required for proper in vivo regulation of MLTU poly(A) site usage. When these elements were removed, the dominance of the upstream L1 site was lost, resulting in an increased use of the downstream site. These deletions had no affect on the utilization of the single poly(A) site in a simple transcription unit, suggesting that it acts to supress downstream site usage in transcripts containing multiple poly(A) sites. Based on our data, the selector element does not affect the ability of L1 to compete for processing factors. It is not yet clear how this selector element is functioning in vivo.

One question that remains to be answered is how the weak upstream poly(A) site is able to completely dominate in an early infection. One possibility is that in an early infected cell, the poly(A) site closest to the 'MeGppGpG cap has an advantage over downstream poly(A) sites. This preference for the 5' poly(A) site would be at least partially lost during the transition from early to late infection, when poly(A) site selection would be based primarily on site strength. There are many possible models which could account for poly(A) site selection based on cap proximity. One obvious model is that polyadenylation in an early infected cell is coupled to transcription, which has an inherent 5' to 3' directionality. Alternatively, recognition of a poly(A) site in an early infected cell could involve a 5' to 3' scanning mechanism without being coupled to transcription. While these are attractive models, they are difficult to address experimentally.

The polarity in poly(A) site preference could also be explained by a model that does not invoke any sort of 5' to 3' directionality. For example, in an early infected cell, a low concentration of viral pre-mRNA and a high concentration of cleavage/polyadenylation factors could allow for the use of all poly(A) sites on a pre-mRNA transcript. In this case, only those transcripts that are capped (i.e. those that end at the 5' L1 poly(A) site) would be stable. The transition from early to late infection is accompanied by an increase in the amount of viral pre-mRNA and perhaps a change in the amounts of various cleavage/polyadenylation factors. This increase in the amount of pre-mRNA to be processed and/or alteration in the concentrations of cleavage/polyadenylation factors could result in the ability to process only a fraction of the poly(A) sites (ideally, only one site/transcript). In this case, every 3' processing event would give rise to a mature mRNA. This model requires that any alteration in RNA or factor concentration be localized to discrete regions in the infected nucleus since superinfection experiments have shown that when a late infected cell is infected with a second adenovirus, the superinfecting virus uses the L1 poly(A) site exclusively (12).

Observations made by several laboratories suggest that the relative amounts of pre-mRNA and cleavage/polyadenylation

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2. Falck-Pedersen, unpublished data.
factors may change during the transition from early to late infection and that these changes are likely to be constrained to discrete nuclear locations. First, the 50- and 64-kDa polypeptide subunits of the CstF complex are localized in fine granules distributed diffusely throughout the nucleus (32). In addition, adenovirus DNA has been shown to be tightly associated with the nuclear matrix of infected cells (62). Finally, the rate of transcription of the major late promoter has been shown to increase 400-1000-fold during the transition from early to late infection (7). Taken together, these observations suggest that the transition from early to late infection is likely to be accompanied by a localized depletion of CstF (and possibly of other cleavage/polyadenylation factors as well) in the regions surrounding the viral transcription template. Of course, proof of this will have to wait until antibodies to more of the cleavage/polyadenylation factors have been generated.

Acknowledgments—We thank Gretchen Edwards-Gilbert, Michael O’Donnell, and Stewart Shuman for helpful suggestions during the preparation of this manuscript.

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