RNA Polymerase II-dependent Positional Effects on mRNA 3’ End Processing in the Adenovirus Major Late Transcription Unit

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During the early phase of adenovirus infection, the promoter-proximal L1 poly(A) site in the major late transcription unit is used preferentially despite the fact that the distal L3 poly(A) site is stronger (i.e. it competes better for processing factors and is cleaved at a faster rate, in vitro). Previous work had established that this was due at least in part to the stable binding of the processing factor, cleavage and polyadenylation specificity factor, to the L1 poly(A) site as mediated by specific regulatory sequences. It is now demonstrated that in addition, the L1 poly(A) site has a positional advantage because of its 5’ location in the transcription unit. We also show that preferential processing of a particular poly(A) site in a complex transcription unit is dependent on RNA polymerase II. Our results are consistent with recent reports demonstrating that the processing factors cleavage and polyadenylation specificity factor and cleavage stimulatory factor are associated with the RNA polymerase II holoenzyme; thus, processing at a weak poly(A) site like L1 can be enhanced by virtue of its being the first site to be transcribed.

The maturation of mRNA in eukaryotic cells is a complex process in which the pre-mRNA is spliced, cleaved, and polyadenylated. In most cases only one mRNA is produced from each transcription unit. In these simple transcription units, 3’ end processing (cleavage and polyadenylation) is largely dependent upon a series of cis-acting signals (for reviews see Refs. 1–3). These signals include a well conserved hexanucleotide sequence, AAUAAA, which is located 10–35 nucleotides upstream of the cleavage site, and a less conserved G/U- or U-rich region, which is found 20–50 nucleotides downstream of the cleavage site. Cleavage occurs between these two elements and is generally on the 3’ side of an A residue (4). Cleavage and polyadenylation specificity factor (CPSF)1 binds to the AAUAAA hexanucleotide, whereas cleavage stimulatory factor (CstF) interacts with the less conserved downstream sequence as well as with CPSF (5). CPSF is required for initial recognition of the substrate and for cleavage and polyadenylation (6–11), whereas CstF enhances processing efficiency by interacting with the CPSF-RNA complex and stabilizing it (8, 12–14). Together with other factors such as CF1, CF2, poly(A) polymerase, and poly(A)-binding protein II, the CPSF-CstF complex catalyzes cleavage of the pre-mRNA (7, 8, 11, 15, 16). Poly(A) polymerase then adds a poly(A) tail of roughly 200–250 residues to the newly processed 3’ end in a CPSF-dependent reaction (17). CPSF and CstF co-localize with sites of transcription in the nucleus (18), suggesting that pre-mRNA processing is closely linked to transcription. Indeed, CPSF has been shown to be recruited to the initiation complex by transcription factor IID (19). The integration of processing and transcription is supported further by results demonstrating that processing is dependent on the carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II (pol II) in vivo (20) and in vitro (21) and that both CPSF and CstF form a complex with the CTD (20).

Although most transcription units are simple and contain only one poly(A) site, others have multiple poly(A) sites and/or splice sites. These complex transcription units are capable of producing multiple mRNAs from a single promoter. Recent evidence suggests that promoter structure can modulate the recruitment of serine/arginine-rich (SR) proteins, which also associate with the CTD, and thus control splice site selection (22). Alternative RNA processing can be an important part of the regulation of gene expression in complex transcription units, resulting in tissue-specific and temporal regulation of transcripts (for review see Ref. 23). For example, sex-specific splicing of the Drosophila tra pre-mRNA controls sexual differentiation (24, 25), and tissue-specific alternative processing of the calcitonin pre-mRNA produces functionally different proteins (26). Moreover, alternative 3’ end processing determines whether the membrane-bound or the secreted form of the mouse immunoglobulin heavy chain is produced (27–29). Poly(A) site strength and position in the transcription unit is thought to be responsible at least in part for this differential processing (30, 31).

Complex transcription units are also found in adenovirus. Because the adenovirus DNA genome is only 36 kb, it needs to encode a large amount of information in a relatively small space. This was accomplished by the evolution of a number of differentially regulated complex transcription units, which increase the pool of possible transcripts adenovirus can express (32). The most well characterized of these is the major late transcription unit (MLTU), which encodes the structural proteins produced during the late phase of infection (for review see Ref. 33). The MLTU consists of five families of gene products, L1–L5, each with its own poly(A) site. The first poly(A) site, L1, is 8 kb from the promoter, whereas the last site, L5, is over 26 kb from the promoter. Through differential selection of a poly(A) site, followed by alternative splicing within each family (34), the MLTU can produce ~20 different transcripts.
Previous work has demonstrated that the L1 poly(A) site is used predominantly early in infection (prior to viral DNA replication), whereas, late in infection (after the onset of viral DNA replication), all of the poly(A) sites are used almost equivalently (34–37). This late processing pattern occurs despite the fact that a poly(A) site can be used as soon as it is transcribed (34). Indeed, because L1 is the first poly(A) site to be transcribed, one might expect that it would have a positional advantage and be processed preferentially at all times. One model to account for this contradiction is that the distal sites compete better for processing factors, compensating for their position in the transcription unit. Indeed, previous work has demonstrated that, in trans, the L3 poly(A) site is processed at a faster rate than the L1 site and is a stronger competitor for processing factors (38). Thus, it is conceivable that early in infection the L1 poly(A) site has a positional advantage and is used preferentially despite the presence of stronger sites downstream.

This hypothesis is supported by some evidence, but that evidence is not conclusive.

Although past work has examined processing rates and factor binding at the L1 and L3 poly(A) sites on separate transcription units, these studies did not examine the behavior of these poly(A) sites in cis on the same transcription unit. Therefore, to further characterize the mechanism of alternative 3’ end processing, we have examined the processing patterns of transcription units containing the L1 and L3 poly(A) sites in tandem. Our results indicate that in cis the 5’ L1 poly(A) site is favored or used roughly equivalently to the distal L3 poly(A) site, and that this effect is dependent upon ongoing transcription by RNA polymerase II. We conclude that the position of a poly(A) site in the transcription unit can play an important role in governing the use of that site.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Enzymes were purchased from New England Biolabs, Life Technologies, Inc., and Promega. Radiochemicals were obtained from Amersham Pharmacia Biotech.

**Cell Culture—**Human 293 cells were propagated as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. HeLa cells were grown in spinner flasks in Joklik-modified Dulbecco's modified Eagle's medium supplemented with 5% calf serum.

**Plasmids and Bacteria—**All plasmids were grown in DH5α, except for L3L1 plasmids, which were grown in JM109. pML1-L170, L3, which contains the L1 and L3 poly(A) sites in a eukaryotic transcription unit (39), was used in the transcription-processing reactions (40). The L1 and L3 poly(A) sites was isolated from pGL1 and pGL3-L1, respectively, except that the order of the Kpn I and Xba I restriction sites were used to amplify the now reversed poly(A) sites.

**Nuclear Extract—**Nuclear extract was prepared from exponentially growing HeLa cells as described (43) with the following modifications: 4-(2-aminoethyl)benzenesulfonyl fluoride was added to buffer C to 1 mM and to buffer D to 0.1 mM, and the extract was dialyzed for 2.5 h in a 200-fold excess volume of buffer D.

**RNA Substrates—**2 μg of linearized template DNA was incubated with 150 units of T7 RNA pol in a 50-μl reaction containing 40 μM Tris (pH 8.0), 25 mM NaCl, 8 mM MgCl2, 2 mM spermidine, 2.8 mM cap analog (m7Gp(5′)pppG), Amersham Pharmacia Biotech), 5 mM dithiothreitol, 5 μCi of [α-32P]dUTP, 100 μg GTP, and 1 mM each ATP, UTP, and CTP at 18 °C for 3 h. Reactions were stopped by addition of 2 μl of Promega RQ1 DNase (1000 units/ml), followed by incubation at 37 °C for 15 min. The RNA yield was determined by the amount of tritiated UTP incorporated into trichloroacetic acid-precipitable counts.

**In Vitro Reactions—**All in vitro reactions were performed under conditions in which the amount of product was directly proportional to the input DNA or RNA, i.e. all-trans-acting factors were in excess.

Transcription-processing reactions were performed by incubating 1 μg of supercoiled substrate DNA at 30 °C for 2 h in a 50 μl reaction containing 110 μg of nuclear extract, 3% polyvinyl alcohol, 20 mM creatine phosphate, 2 mM MgCl2, and 600 μl each GTP, ATP, UTP, and CTP. When T7 RNA pol was being assayed, the reaction mixture also contained 80 units of T7 RNA pol and 1 μg/ml a-amanitin. The α-amanitin chase experiment was carried out by setting up transcription-processing reactions as described above; however, after incubation at 30 °C for 1 h, 1 μg/ml α-amanitin was added and the reaction mixtures were incubated for an additional 1 or 2 h. As controls, transcription-processing reaction mixtures were incubated for 3 h at 30 °C in the absence or presence of 1 μg/ml α-amanitin. All RNA bands were hybridized with an equal volume of stop buffer (50 μM Tris, 50 mM EDTA, and 0.1% SDS) and 30 μg of proteinase K, and then incubated at 37 °C for 15 min. RNA was resuspended in TE buffer containing RNA (4 μg) isolated from pl1SV-transfected cells (to serve as an internal control) then loaded on oligo(dT)-cellulose columns to isolate poly(A) RNAs.

**In vitro processing of T7 RNA pol-transcribed pre-synthesized mRNAs was carried out for 2 h at 30 °C in 50-μl reaction mixtures, which contained 2 mM MgCl2, 3% polyvinyl alcohol, 20 mM creatine phosphate, 1 mM ATP, 110 μg of nuclear extract, and 2 nm (100 fmol) pre-mRNA. The reactions were quenched, and RNA was extracted as described above for the transcription-processing reactions.

**DNA Transfection and RNA Isolation—**Transient-transfection assays were performed as described previously (44). Cells in each 10 cm2 dish were transfected with 10 μg of test plasmid and 10 μg of sonicated salmon sperm DNA. RNA was isolated from the transfected cells either by the acid guanidinium thiocyanate-phenol-chloroform extraction method (45) or using the NucleoSpin RNA II kit from CLONTECH.

**S1 Nuclease Analysis—**Poly(A)1 RNA was linearized from nuclear extracts or from transfections was isolated by oligo(dT)-cellulose chromatography and analyzed with a DNA probe made from the corresponding plasmid. To make these probes, a fragment spanning the poly(A) site(s) from each plasmid was purified and 3′-end-labeled at the Xba I site upstream of the proximal poly(A) site using the Klenow fragment of DNA polymerase I and [γ-32P]dCTP. Hybridizations and S1 digestions were performed as described previously (44). ML1L2, L1L3, and L1LK3 RNA’s were hybridized at 60 °C and L3L1 RNAs at 58 °C. The predicted sizes of protected bands are indicated in Fig. 1. Results were quantitated with a Molecular Dynamics PhosphorImager after the reactions of the S1 digestion were resolved on 6% polyacrylamide, 8 % urea gels.
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RESULTS

A Promoter-proximal Weak Poly(A) Site Is Processed to a Greater Extent than a Distal, Strong Poly(A) Site—Our investigation of the effects of position on processing was conducted using constructs containing L1 and L3 poly(A) sites incubated in an uninfected HeLa cell nuclear extract. Previous experiments in which processing at these poly(A) sites was evaluated in trans demonstrated that the L3 poly(A) site competes better for processing factors and is cleaved at a faster rate than the L1 poly(A) site (38). To ensure that we could reproduce this observation in our system, we performed processing reactions in which either an L1 or an L3 pre-mRNA was used as a substrate (Fig. 2A, lanes 1 and 4). We also performed a competition assay in which equal amounts of L1 and L3 pre-mRNA were incubated in the same processing reaction (lanes 2 and 3). Because both probes in this experiment were end-labeled to the same specific activity, we could directly compare processing at the L1 and L3 sites. Whether the two sites were assayed alone or together, processing at the L3 poly(A) site was greater.

Previous results from coupled transcription-processing reactions in an uninfected HeLa cell nuclear extract, however, demonstrated that when the L1 and L3 poly(A) sites were in cis on the same transcription unit, the L1 site was used preferentially (40). This accurately reflects the behavior of these sites in early infected HeLa cells using an identical construct (41). One possible explanation for the apparent inconsistency is that, when the L1 and L3 poly(A) sites compete in cis, the L1 site has a positional advantage because it is the first site to be transcribed, and is therefore the first site available to the processing factors. If this explanation is correct and position is important, moving the distal L3 poly(A) site farther downstream should result in a relative increase in processing at the proximal L1 poly(A) site. This is because the increase in spacing would allow the proximal poly(A) site more time to interact with the processing factors before the distal site is transcribed. To evaluate this hypothesis, we designed mini-MLTUs (constructs containing the major late promoter (MLP) driving only the L1 and L3 poly(A) sites) in which the L3 poly(A) site was moved farther downstream by inserting a 429 nucleotide spacer between the L1 and L3 poly(A) sites. We performed transcription-processing reactions in a HeLa cell nuclear extract in the presence and absence of this spacer (L1KL3 and L1L3 mini-MLTUs, respectively; Fig. 1). Following incubation in the nuclear extract, poly(A)+ transcripts were isolated and analyzed by S1 nuclease protection assay (Fig. 2, B and C). In the absence of the spacer, the ratio of transcripts processed at the L1 poly(A) site to those processed at the L3 poly(A) site (L1:L3) was 2.5 (lane 1), whereas in its presence the ratio was 7.2 (lane 2). Thus, the presence of the spacer in the mini-MLTU caused preferential L1 poly(A) site use to increase almost 3-fold, supporting the hypothesis that a 5' poly(A) site has a positional advantage in the transcription unit. To confirm that processing in a nuclear extract reaction is representative of processing in intact cells, we transfected 293 cells with the L1L3 and L1KL3 mini-MLTUs, analyzed the isolated poly(A)+ RNA by S1 nuclease protection assay, and compared the results with those of the in vitro reactions (Fig. 2C). The ratio of L1:L3 poly(A) site use was similar in both the transfection and the transcription-processing assays using either L1L3 or L1KL3 mini-MLTU.

If the 5' poly(A) site has such an advantage, moving the L3 poly(A) site from a distal position to a proximal one should result in a relative increase in processing at the L3 site. To test this hypothesis, we reversed the poly(A) sites in the L1L3 construct, creating an L3L1 mini-MLTU, which we then used as a substrate in transcription-processing reactions (Fig. 2B). With the L1L3 construct, the ratio of L1:L3 RNA was 2.5 (lane 1); however, with the L3L1 construct, the ratio of L1:L3 RNA was only 0.023 (lane 3). Thus, there is a 100-fold increase in relative processing at the L3 site when it is moved from the distal position to the proximal one, probably reflecting the inherent strength of the L3 poly(A) site along with its 5' position. In 293 cells transfected with the L3L1 construct, processing was undetectable at the L1 poly(A) site and was observed only at the L3 site (data not shown).

The results described above indicate that the distal L3 poly(A) site is processed to a lower extent because it is transcribed after the L1 poly(A) site; however, it is possible that the low processing efficiency is the result of simple distance from the 5' end of the pre-mRNA. To examine this possibility, we introduced a mutation in the AATAAA hexanucleotide sequence at the L1 poly(A) site in an L1L3 construct (mL1L3) such that processing can no longer occur at the L1 site (42). We performed transcription-processing assays using L1L3 and mL1L3 substrates, and we measured relative processing efficiencies (Fig. 2D, lanes 3 and 4). We observed that the L3 site...
in mL1L3 is processed twice as much as much as the L3 site in L1L3, indicating that, in the absence of an upstream competing poly(A) site, the distal poly(A) site is processed to a greater extent. We noted, however, that the mutation in the L1 poly(A) site did not result in quantitative recovery of processing at L3, i.e. the total amount of processing of the mL1L3 construct was only 50–80% of the total processing of the L1L3 construct. A similar result was obtained when we analyzed RNA isolated from 293 cells that were transfected with either L1L3 or mL1L3 (lanes 1 and 2). These observations suggest that, although relative position in a construct is important, absolute distance from the 5' end also might play a role in affecting processing efficiency. In addition, incomplete recovery of processing at L3 in the mL1L3 construct could be caused by a repressor activity in the L1 sequence, which was shown to be independent of the AAUAAA hexanucleotide and is localized in repressor activity in the L1 sequence, which was shown to be essential before the 5' end processing event on a single pre-mRNA, i.e. during incubation in the extract pre-mRNAs were first processed at the distal poly(A) site and then processed again at the proximal site. To test this possibility, we examined processing of L1 and L3 poly(A) sites in a HeLa cell nuclear extract using an α-amanitin chase to inhibit further RNA pol II transcription (47); hence, in the presence of α-amanitin, only those transcripts that are already synthesized would be processed (Fig. 3A, compare lanes 1 and 5). If secondary processing events were taking place, then an increase in L1 processing would be seen relative to processing of the L3 poly(A) site after α-amanitin was added to the reaction mixture. Our results (Fig. 3, A and B) suggest that such secondary processing does not occur. The L1L3 construct was incubated in the nuclear extract for 1 h, following which either the reaction was stopped or α-amanitin was added is 1.88 ± 0.48 (after 1 h), whereas that of the L3 poly(A) site is 1.67 ± 0.25 (after 1 h). The same is true for the 2-h time point. The similarity in these ratios suggests that, once a transcript is made, it is processed only once. Additional evidence for this conclusion comes from the finding that the L1:L3 ratio is higher in the L1KL3 construct than in the L1L3 construct, and there is no reason to expect that increasing the distance between the two poly(A) sites would result in more secondary processing. Indeed, it appears that pre-mRNAs from complex transcription units are

<sup>2</sup> D. S. Karow, unpublished results.
DISCUSSION

The work presented in this report suggests that relative position in a complex transcription unit plays an important role in governing poly(A) site use. Analysis of poly(A) transcripts not processed more than once in the nuclear extract and that, consequently, the preferential processing of the proximal poly(A) site that we detect is a result of its position relative to the promoter.

The Processing Pattern Is Altered on T7 RNA Polymerase Transcribed Pre-mRNA—We next determined whether or not the positional effect might be related to association of processing factors with RNA pol II. To address this question, we compared processing efficiencies of poly(A) sites that had been transcribed by either RNA pol II or T7 RNA pol. We chose T7 RNA pol because this polymerase lacks a CTD and hence should be unable to bind processing factors CPSF and CstF (48). Trancription by T7 RNA pol alone was effected by the addition of α-amanitin to the nuclear extract to inactivate the endogenous RNA pol II. When the linearized T7 promoter-driven L1L3 construct (pGL1+170-L3) was incubated with nuclear extract in the absence of T7 RNA pol and α-amanitin, the L1 and L3 poly(A) sites were processed in the same ratio as the L1 and L3 poly(A) sites driven by the MLP in the pML1+170-L3 construct, i.e. processing at the proximal L1 site was 3-fold that at the L3 site (Fig. 4, compare lanes 2 and 4). This result indicates that the processing machinery interacts similarly with both L1L3 pre-mRNAs when transcribed by RNA pol II. Although we have not determined the 5′ end of the L1L3 pre-mRNA generated by RNA pol II from the pGL1+170-L3 construct, we assume that transcription was initiated from the T7 promoter based on previous reports (49, 50). In contrast, when T7 RNA pol was used to transcribe pGL1+170-L3 in an α-amanitin-treated nuclear extract, the transcripts were processed almost exclusively at the distal L3 site (lane 1). This result indicates that preferential processing at the 5′-proximal site is not a consequence of transcription per se, but is specific to transcription by the RNA pol II holoenzyme. Furthermore, the observed preferential processing of the L3 site in T7 RNA pol-synthesized pre-mRNAs in this experiment supports our earlier argument that each RNA gets processed only once. In addition to the bands corresponding to RNAs processed at the L1 and L3 sites, there are other bands that we have not as yet characterized fully (Fig. 4, lane 1), but which are consistent with products resulting from termination by T7 RNA pol as they can also be detected in the poly(A)− fraction (data not shown). The L1 and L3 bands do not appear to be termination products as they are not detected in the poly(A)− fraction.

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no affinity for processing factors, to synthesize transcripts in a HeLa cell nuclear extract, we observed a difference in the processing pattern. According to our results, in T7 RNA pol synthesized transcripts, the L3 poly(A) site is used preferentially over the proximal, but weaker, L1 site, suggesting that when the association between the polymerase and the processing machinery is attenuated (or nonexistent), the stronger poly(A) site is able to compete for processing factors even when it is farther away from the promoter. Although these results do not definitively establish the specific involvement of the CTD of RNA pol II, they demonstrate that an intrinsic property of the polymerase is linked to the predominant use of the L1 site.

Past work has provided the framework for a model that explains alternative 3' end processing in the adenovirus MLTu. For example, we and others have demonstrated that regulatory sequences upstream and downstream of the L1 poly(A) site are necessary for the predominant use of the L1 site early in infection (39, 44), that splice sites are not involved (41), and that binding of CPSF to the pre-mRNA is stabilized by these regulatory sequences (53). Our present results allow us to modify this model. Specifically, this work suggests that the L1 poly(A) site also has a positional advantage in the MLTu and that this advantage is dependent on RNA pol II. The distance between the poly(A) sites on the viral chromosome is much greater than that in our constructs, which would potentially give the L1 site an even greater positional advantage with respect to 3' end processing. In addition to effects related to RNA pol II, processing at the proximal poly(A) site may also be enhanced because of its proximity to the RNA 5' cap structure. Cooke and Alwine (54) have demonstrated that the processing efficiency of a pre-mRNA containing a single poly(A) site was augmented if that pre-mRNA had a 5' cap. Furthermore, they showed that excess 5' cap structures inhibited processing whereas cap analogs did not, presumably because the analogs could not titrate cap binding factors. It is conceivable that cap binding factors may interact with the proximal poly(A) site and/or the 3' processing machinery to enhance processing. We would note that it is possible that an interaction with cap binding factors may play a role in our system, because the T7-transcribed pre-mRNAs in the experiment shown in Fig. 4 are likely not capped. As a whole, then, we conclude that early in infection processing at the L1 site is favored because of the presence of the regulatory sequences and because of its positional advantage.

Although positional advantage may explain the preferential use of the L1 poly(A) site early in infection, it cannot account for the fact that the more distal sites are used roughly equally to the L1 site late in infection. One possible explanation for the decrease in processing at the L1 site is a change in the levels of processing factors present in the late infected nucleus. The transcription rate of the MLTu increases 400–1000-fold late in infection, at which time viral transcripts represent 20–40% of the mRNA in the cell (33). Conceivably, many of the processing factors could be titrated away by the abundant viral tran- scripts. In addition, translation of transcripts from cellular genes is inhibited during the late phase of the viral infection (55), making it possible that the expression of processing factors is decreased. Indeed, investigators have demonstrated that the activity of the processing factor CstF decreases slightly late in infection (56). We would note, however, that a simple titra- tion of processing factors in and of itself cannot account for the switch, as the processing pattern from a virus newly introduced into a late-infected cell is still early in nature (41). This indicates that factors are limiting, their access to unreplicated and replicated viral chromosomes is different. Increasing evidence suggests that mRNA 3' end processing is intimately coupled to transcription (for reviews, see Refs. 57 and 58); thus, altered poly(A) site use could be a reflection of a change in the association of processing factors with the RNA pol II holoenzyme. This hypothesis is supported by the difference we observed in the processing pattern when RNA pol II was replaced with T7 RNA pol. Although the system used here is only an approximation, our observation provides an insight into the mechanism by which the switch in processing efficiency during late infection might be accomplished.

We would speculate that other mechanisms might also be involved in the late processing switch. It is possible, for example, that unidentified 3' end processing repressors come into play during late infection, preventing use of the promoter-proximal sites. Alternatively, an alteration in the structure of the regulatory region of the L1 poly(A) site could affect binding of processing factors, thus causing an attenuation in processing efficiency at that site. Indeed, Graveley and co-workers (59) have shown that human immunodeficiency virus pre-mRNA structure plays an important role in poly(A) site recognition by CPSF. It is clear that in vitro systems such as the one we have used in the present work will be extremely useful in sorting out the relative contribution of factors potentially affecting poly(A) site choice.

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