Alternative Polyadenylation of Adeno-associated Virus Type 5 RNA within an Internal Intron Is Governed by the Distance between the Promoter and the Intron and Is Inhibited by U1 Small Nuclear RNP Binding to the Intervening Donor

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Adeno-associated virus type 5 is unique among adeno-associated virus serotypes in that it uses a polyadenylation site in the center of the genome. The great majority of transcripts generated from the upstream P7 and P19 promoters are polyadenylated at a site in the central intron ((pA)p); however, most of the viral transcripts generated by the proximal P41 promoter are polyadenylated at the distal polyadenylation site at the 3′ end of the genome ((pA)d) and subsequently spliced. Polyadenylation at (pA)p increases as the distance between the RNA initiation site and the (pA)p site is increased. The steady-state level of RNAs polyadenylated at (pA)p is independent of the promoter used or of the intervening sequence but is dependent upon competition with splicing, inhibition by U1 snRNP binding to the intron donor, and the intrinsic efficiency of the cleavage/polyadenylation reaction. Each of these determinants shows a marked dependence on the distance between the RNA initiation site and the intron and (pA)p. Finally, unlike other reported systems, inhibition of (pA)p by U1 snRNP binding to the intron donor is decreased as the distance between the donor and (pA)p is increased.

The human adeno-associated viruses are small, nonenveloped, single-stranded DNA paroviruses that replicate in mammalian cells best in the presence of larger helper DNA viruses, such as adenovirus and herpes virus (1). Although transmission of adeno-associated virus serotype 5 (AAV5) appears to follow acquisition of herpesviruses rather than adenovirus (2), it has been shown that adenoassociated virus can provide full helper function for AAV5 genome replication (3). The AAV5 genome is transcribed using three primary promoters (P7, P19, and P41, designated by their map unit location; see Fig. 1) and a weak initiator that lies within the inverted terminal repeat (4). The P7- and P19-generated RNAs encode the large and small Rep proteins, respectively, from the large open reading frame in the 5′ half of the genome. These RNAs utilize a polyadenylation site ((pA)p) within the single intron in the center of the genome at high efficiency. P7- and P19-generated RNAs polyadenylated at (pA)p are not spliced, and so the polyadenylation site choice for these AAV5 RNAs is independent of an upstream 3′ splice site. In contrast, RNAs generated from the P41 promoter, which lies only 78 nt upstream of the AAV5 intron, utilize (pA)p at significantly reduced efficiency. The P41-generated RNAs primarily read through (pA)p, are polyadenylated at a site at the 3′ end of the genome ((pA)d), and accumulate as spliced mRNAs. These mRNAs encode the viral capsid proteins from the large open reading frame in the 3′ half of the genome. Unspliced P41-generated RNAs that utilize either (pA)p or (pA)d are present in the cytoplasm of infected cells; however, they have not been shown to encode functional protein products. Polyadenylation of AAV5 RNA at (pA)p depends upon a consensus AAUAAA signal at nt 2177, which is immediately upstream of the first intron acceptor A1, which lies at nt 2204 (see Fig. 1). RNA cleavage and polyadenylation occurs 11–14 nt downstream of the first AAUAAA motif (4). Efficient polyadenylation at (pA)p requires both a downstream element that lies within the A2 3′ splice site and an upstream element that is located within the P7- and P19-generated RNAs but upstream of the P41 promoter (5).

There are a number of examples in which the binding of splicing factors affect 3′-terminal cleavage and/or poly(A) addition. Perhaps the best studied examples involve U1 snRNP interactions at 5′ splice donor sites. There are at least two well characterized systems in which 5′ splice sites have been shown to have an inhibitory effect on polyadenylation. The first case involves the regulation of expression of papillomavirus late genes, which is governed at least partially by the regulation of polyadenylation. In undifferentiated epithelial cells, a cryptic splice donor site upstream of the late polyadenylation site inhibits poly(A) addition but not pre-mRNA cleavage (6). Following U1snRNP binding to the cryptic donor site, the U1 70-kDa protein, when bound to U1snRNA, interacts with and inhibits the poly(A) polymerase (7). In the second example, read-through of the proximal polyadenylation signal in the upstream long terminal repeat (LTR) of human immunodeficiency virus (HIV) is due to inhibition of that site by a downstream intron donor (8). The mechanism of this inhibition is not fully clear. It has been proposed that U1 snRNP binding directly to this 5′ donor sequence inhibits recognition of the pre-mRNA by the polyadenylation machinery (9). Consistent
with this, U1 snRNP was found to inhibit cleavage when bound to a 5′ splice site downstream of the poly(A) site in HIV LTR (8), and the stem-loop structure of U1 snRNA, which binds U1 70-kDa, was required for poly(A) site occlusion (10). The requirement for interaction between U1A, U1 70-kDa, and poly(A) polymerase could not, however, be demonstrated in vitro (11). Alternatively, it has also been proposed that U1 snRNP prevents 5′ exon definition by interfering with the cap-binding complex (8, 9).

Conversely, U1 snRNP has also been shown to enhance polyadenylation. The U1A protein has been shown to interact with and stabilize cleavage and polyadenylation stimulating factor binding, which stimulates simian virus 40 late RNA 3′ end formation (12, 13). In another example, efficient recognition of the polyadenylation site in exon 4 of the CTCGRP gene is dependent on the U1snRNP binding to a pseudo 5′ splice site downstream of this poly(A) site; it is likely that the U1A protein is involved in this process (14).

In this manuscript we show that the alternative polyadenylation at (pA)p of AAV5 RNAs depends upon the distance between the RNA initiation site and the central intron and (pA)p site. The steady-state levels of RNAs polyadenylated at (pA)p was independent of the nature of the promoter or the intervening sequences; however, it was dependent upon competition with splicing, inhibition by U1 snRNP binding to the intervening donor, and the intrinsic efficiency of the cleavage/polyadenylation reaction. Each of these determinants of (pA)p usage showed a marked distance effect. Unlike other reported systems, inhibition of (pA)p by U1 snRNP was reduced when the distance between the U1 snRNP-binding donor and (pA)p was increased.

EXPERIMENTAL PROCEDURES

Cells

293 cells were maintained in Dulbecco’s modified Eagle’s medium with 5% fetal calf serum in 5% CO2 at 37 °C.

Plasmid Constructions

Mutations in P41Cap Minimal Construct and the U1 RNA Mutants—The 5′ splice site (donor) mutations in P41Cap minimal construct and the U1 RNA mutants were constructed as follows. In the P41CapDms, P41CapDm1, and P41CapDm10 constructs, the donor site was mutated to as shown in Fig. 2. Those donor mutations were also moved into the P41CapA2CAA plasmid, in which the cleavage site of the A2′ 3′ splice site (CAG) was changed to be CAA, which inhibits splicing of the AAV5 intron to either acceptor 1 or 2 (thus creating the P41CapA2CAdams, P41CapA2CAA dm1, and P41CapA2CAA dm10 plasmids.

U1 snRNA Mutants—To construct U1 snRNA mutants, the U1 donor binding sequence (3′-GUCCAUUCA-5′) in U1 RNA-expressing pUC13U1 (a gift from Alan Weiner, University of Washington, Seattle (15)) was mutated to GUUACUCG, GGCAUGCU, UGGCAUACU, and UGGCAUUACU to generate, respectively, U1S, U1mA, U1m1, and U1m10.

Distance Constructs—P7Cap, P7Rep530Cap, P7Rep720Cap, P7Rep890Cap, P7Rep1260Cap, and P7Rep1630Cap plasmids were constructed by deletion, from the parent pAV5RepCap plasmid (4), of P7Repn890Cap, P7Repn1260Cap, and P7Repn1630Cap plasmids were created by insertion of PCR DNA from the constructs that contained the mutations (5) in P7Cap, P7J700Cap, P7J1250Cap, and P7JCap. P7J170CapSm and P7J530CapSm were constructed by insertion of prokaryotic prB322 sequence (6, 7) in P7Cap, P7J700Cap, P7J1250Cap, and P7JCapSm. P7J170CapSm was created by insertion of prokaryotic prB322 sequence (6, 7) in the donor site in P41CapA2CAA. P41DJ700CapA2CAA and P41DJ300CapA2CAA were constructed by insertion of prB322 DNA (nt 1380–1468 and 1114–1245).

(pA)p Mutation Constructs—P7CapSmA, P7J700CapSmA, P7J1250CapSmA, and P7J1650CapSmA were constructed by silent mutation of the (pA)p site (5) in P7Cap, P7J700Cap, P7J1250Cap, and P7JCap.

Polyadenylation at (pA)p of AAV5 RNAs depends upon the distance between the U1 snRNP-binding donor and (pA)p site. P41Cap,A2CAADms, P41J170CapA2CAA, and P41J1650CapA2CAA were generated by replacement of the P7 promoter and the intron in P41CapA2CAA dm1 to create P41J-330CapA2CAA dm1, P7J530CapA2CAA dm1, P7J700CapA2CAA dm1, P7J1250CapA2CAA dm1, and P7J1650CapA2CAA dm1.

To create plasmids P41DJ330CapA2CAA, P41DJ700CapA2CAA, P41DJ1250CapA2CAA, P41DJ530CapSmA, P41DJ700CapSmA, P41DJ1250CapSmA, P41DJ530CapSmA, and P41DJ1650CapSmA were generated by replacement of the (pA)p site in the plasmids described above.

Probe Constructs—All of the probes used for RNase protection assays were homologous to the reporter construct being tested. The DH probe, which covers the (pA)p site and intron acceptors, was described previously (14). All of the homologous DH probe clones were created by insertion of PCR DNA from the constructs that contained the mutations in the DH probe region (nt 1994–2341) into pGEM3Z using BamHI and HindIII linkers. The restriction fragments used to reintroduce PCR-generated mutations back into the parent plasmids were completely sequenced at the DNA Core Facility at the University of Missouri-Columbia to confirm that only desired mutations were present.

RNase Protection Assay

Plasmid DNA (2 μg/60 mm plate) was transiently transfected into 60–80% confluent 293 cells using LipofectAMINE and the Plus reagent (Invitrogen) as described previously (16). Where mentioned either the wild-type U1 or the U1 variant plasmids were co-transfected. Total RNA was isolated 36–48 h later by using guanidine isothiocyanate as described previously (17). RNase protections were performed as described before (17–19). All of the DH probe templates were generated by EcoRI linearization of the pGEM3ZDH plasmids. The probes were produced by in vitro transcription by using SP6 polymerase. RPA signal was quantified with the Molecular Image FX machine and Quantity one (version 4.2.2) images software. The relative mol percentages of individual species of RNAs were determined after adjusting for the number of 32P-labeled uridine in each protected band as described previously (17). The percentage of (pA)p was calculated by dividing the adjusted signal from the (pA)p band with the adjusted signals from all of the bands (5).

RESULTS

Polyadenylation of P41-driven Transcripts at (pA)p Is Inhibited by U1snRNP Binding to the Intervening Donor—During AAV5 infection, greater than 95% of P7- and P19-derived transcripts polyadenylate at (pA)p, which lies within the AAV5 intron (Fig. 1). In contrast, ~75% of transcripts generated by the P41 promoter, which lies only 265 nt upstream of (pA)p, are polyadenylated at the 3′ end of the genome at (pA)d and subsequently spliced (4, 5). Greater than 95% of P41-generated transcripts produced from nonrepeating constructs read through (pA)p (4, 5).

Because (pA)p lies within a functional intron and because in other systems U1snRNP interaction with RNA has been shown to influence polyadenylation at nearby sites, we investigated whether U1 snRNP binding to the AAV5 donor site influences polyadenylation at (pA)p. In minimal constructs expressing only the AAV5 P41 transcription unit, mutations of the intron donor, predicted to decrease the binding of endogenous U1
snRNA to that site, increased the relative amount of P41-driven RNA that was polyadenylated at (pA)p. This was true either when these mutations were introduced into an otherwise functional intron (P41CapDms, P41CapDm1, and P41CapDm10; Fig. 2, lanes 2–4) or within the background of an additional mutation of the intron 3′ A2 acceptor site, which has been shown (5) to abolish splicing of the intron to either acceptor (P41CapA2CAADm10; Fig. 2, lane 5). Interestingly, for P41CapDms, which has a wild-type intron splice acceptor, co-transfection of U1S snRNP resulted in an increase in the relative amount of spliced product (Fig. 2, lane 16) to levels similar to P41Cap (Fig. 2, lane 1), indicating that the U1S snRNP bound to the shifted binding site in a functionally competent manner.

In the second experiment, individual mutant U1 snRNA genes, each made complementary to a donor mutation and so predicted to regain binding, were transfected with that mutant donor construct. In each of these cases the increased (pA)p usage caused by the original donor mutation was significantly suppressed by addition of the complementary U1snRNA gene (Fig. 2, lanes 12–14). Transfection of the wild-type U1 snRNA gene had no suppressing effect in these experiments (Fig. 2, lane 15). We conclude that U1snRNP binding to the intron donor inhibits polyadenylation of P41-generated transcripts at (pA)p.

The Level of Polyadenylation at (pA)p of P7-driven RNAs Is Directly Proportional to the Distance between the Promoter and the Intron and Is Determined by Both Competition with Splicing and a Splicing-independent Mechanism—As mentioned above, P7-generated RNAs utilize (pA)p at high efficiency, whereas RNAs generated by the proximal P41 promoter primarily read through. To investigate this distance effect further, we generated two sets of constructs. In the first, various portions of the rep gene were deleted to move the P7 promoter closer to the intron donor site (Fig. 3, left panel). In the second set of constructs, the P7 promoter was placed at various distances upstream from the intron, using increasingly larger pieces of bacterial plasmid DNA as intervening sequence (Fig. 3, right panel). As the P7 promoter was positioned incrementally further upstream of the intron (and also (pA)p), with either native AAV5 (P7ReppCap) series or heterologous DNA of prokaryotic origin (P7JCap series) serving as the intervening sequences, polyadenylation at (pA)p increased, and the splicing of P7-generated RNA decreased (Fig. 3). Similar results were also obtained when the intervening sequence was either an inverted copy of the same prokaryotic heterologous sequence used in the P7JCap series of plasmids or sequence from phage lambda and also with a set of constructs analogous to the P7JCap series in which the P41 promoter rather than P7 was used (data not shown). These results indicated that the efficiency of polyadenylation at (pA)p was determined by the distance between the promoter and the intron rather than the promoter context or the nature of the intervening sequence (i.e. a cryptic 3′ splice site).

The accumulation of AAV5 RNA polyadenylated at (pA)p at steady-state conditions should be a consequence of the intrinsic efficiency of cleavage and polyadenylation at (pA)p, the strength of inhibition by the intervening intron donor, and competition for precursor molecules by the splicing process. Because our previous experiments with AAV2 demonstrated that excision of the AAV2 intron was inversely proportional to the distance between the RNA start site and the intron donor (16), we examined the effect on excision of the AAV5 intron in the absence of competition from polyadenylation at (pA)p.

Similar to what was seen when both splicing and polyadenylation were allowed (Fig. 3), splicing of either P7 or P41 RNAs generated from a construct in which the (pA)p AUAAAA was destroyed was inversely proportional to the distance between the RNA initiation site and the intron (Fig. 4). When either the P7 or P41 promoters were close to the 5′ splice site (P41CapSMpA and P7CapSMpA in Fig. 4, lanes 1 and 7), splicing of those RNAs was strong; however, when the promoters were moved to the position of the native P7 (P41J1650CapSMpA and P7J1650CapSMpA in Fig. 4, lanes 6 and 12), splicing was poor. These results, which may reflect the efficiency of definition of the 5′ exon, indicated that, as for AAV2, the distance between the promoter and the intron also directly affected the splicing efficiency of the AAV5 intron in vivo. Therefore, the increased polyadenylation at AAV5 (pA)p, seen as the promoter is moved further upstream, may have been at least partially a consequence of reduced competition for precursor molecules because of a decrease in the splicing of transcripts generated from P7 at a distance.

The same distance variants shown in Fig. 3 were then introduced in the background in which splicing was disabled by the
A2 splice acceptor mutation. In these experiments, polyadenylation at (pA)p was also increased as the distance between the cap site and the intron donor and (pA)p was increased (Fig. 5), indicating that even when splicing was abolished, polyadenylation at (pA)p was governed by the distance between the cap site and the intron donor and (pA)p. Similar results were obtained with analogous constructs in which the P7 promoter was replaced by P41 (Fig. 6, left panel). One component, therefore, of the distance dependence of (pA)p usage in these experiments was splicing-independent and was either a direct distance dependence on the efficiency of (pA)p utilization or, alternatively, a reduction in binding of U1 snRNP to the donor as the distance between the promoter and the intron (and (pA)p) was increased. However, because in the absence of splicing, polyadenylation at (pA)p occurred at relatively closer distances than when splicing was permitted (compare Fig. 5 with Fig. 3), the distance effect on (pA)p usage was at least partially because of the decrease in splicing efficiency of RNAs generated from AAV5 promoters at farther distances upstream of the cap site and the intron and (pA)p. This latter distance effect could be either due to an intrinsic increase in efficiency of the polyadenylation process as the polymerase complex traverses a longer distance or due to a decrease in U1 snRNP inhibition of (pA)p as this distance increases.

Alternative Polyadenylation of AAV5 RNA

Polyadenylation at (pA)p Is Subject to a Modest Distance Effect Even When Mutation Abolishes U1 snRNP Binding to the Intron Donor—The results described above indicated that in the presence of a functional intron there was competition for precursor AAV5 pre-mRNAs between polyadenylation at (pA)p and splicing that was governed by the distance between the cap site and the intron donor and (pA)p. However, when splicing was abolished, polyadenylation at (pA)p was still governed by the distance between the cap site and the intron and (pA)p. This latter distance effect could be either due to an intrinsic increase in efficiency of the polyadenylation process as the polymerase complex traverses a longer distance or due to a decrease in U1 snRNP inhibition of (pA)p as this distance increases.

The major component that determines the balance of correctly processed AAV5 mRNAs appears to be the interplay between splicing, which requires U1 binding to the donor, and inhibition of polyadenylation at (pA)p. Polyadenylation at (pA)p is inhibited by the U1 snRNP binding to the donor site. The observation that ~75% of RNAs generated by AAV5 P41 at its normal position are polyadenylated at (pA)p when U1 snRNP binding to the donor site is abolished (Fig. 2, lanes 6–8) suggested that any direct effect on the efficiency of cleavage and polyadenylation at (pA)p conferred by the distance between the cap site and the polyadenylation site must be small. However, such an effect could be demonstrated
FIG. 3. As the distance between the promoter and the (pA)p site increases, the level of polyadenylation of P7-generated (and P19-generated) RNA at (pA)p is increased, whereas the level of splicing is decreased. Plasmids with either different size deletions between the P7 promoter and the intron, in the pAV5RepCap backbone (lanes 1–6), or plasmids with different size insertions of pBR322 DNA between the P7 promoter and the intron in the P7Cap (lanes 7–12) backgrounds (diagrammed on the right), were transfected into 293 cells. The distance between the P7 start site and the intron donor is indicated. Total RNAs were protected with the DH probe, which is depicted under each diagram. Bands protected by the DH probe from a representative experiment are shown and labeled on the left. Quantifications of the ratio of P7-generated (and P19-generated) RNAs polyadenylated at (pA)p relative to the total P7-generated (and P19-generated) RNAs are shown as averages with standard deviations and are the results of at least three separate experiments.

FIG. 4. Splicing of P7- and P41-generated RNAs is distance-dependent in the absence of polyadenylation at (pA)p. Plasmids with different size insertions of pBR322 DNA between the P41 promoter and the intron in the P41CapSMpA background (lanes 1–6) or between the P7 promoter and the intron in the P7CapSMpA background (lanes 7–12) (diagrammed on the right) were transfected into 293 cells. Total RNAs were protected with the homologous DHSMpA probe, which is depicted under each diagram. Bands protected by the DHSMpA probe from a representative experiment are shown and labeled on the left. Quantifications of the ratio of spliced (Spl) to unspliced (Unspl) P7-generated (or P41-generated) RNAs are shown as the averages of at least three experiments and include standard deviations.
using constructs in which the U1 snRNP-binding site (donor site) was mutated in the group of P41CapCAADm1 distance constructs (Fig. 7). As seen before, when both acceptor and donor sites were abolished by mutation (P41CapA2CAADm1), RNAs generated from the P41 at its native position were polyadenylated efficiently (77%) at (pA)p (Figs. 2, lane 7, and 7).

However, as the P41 promoter was positioned incrementally upstream to the position of native P7 promoter, polyadenylation (pA)p was increased by 110–112%. These results, which measured the efficiency of the cleavage and polyadenylation at (pA)p in the absence of competition for splicing or inhibition by U1 snRNP, demonstrated that the efficiency of polyadenylation at (pA)p itself decreased, albeit modestly, as the distance between the RNA initiation site and (pA)p was increased.

Inhibition of Polyadenylation at (pA)p Is Decreased as the Distance between the U1 snRNP-binding Donor Site and the (pA)p AAUAAA Is Increased—Isn’t the efficiency of U1 snRNP inhibition of (pA)p dependent upon the distance between the donor and the AAUAAA? For the HIV LTR and papillomavirus late poly(α), inhibitory U1-binding sites, either functional or cryptic, are very close to the AAUAAA sites of the targeted polyadenylation signals (7, 8). It has been reported, however, that the inhibition of polyadenylation within the HIV LTR is relatively unaffected by the distance between the U1-binding site and the polyadenylation site (8). Recently it has been suggested that that U1 70-kDa inhibition of polyadenylation is a more general phenomenon, and in these test systems, inhibition was also shown to be distance-independent (22).

For AAV5, the distance between the U1-binding site (donor site) and the AAUAAA signal is increased by the addition of heterologous sequence, polyadenylation of P41-generated RNAs at (pA)p increased from −28% to 79% (Fig. 8, lanes 1–8). These results indicated that as these two signals were separated, inhibition of (pA)p by U1 snRNP was decreased, suggesting that the distance between the donor and (pA)p was an important determinant governing U1 inhibition. However, because the levels of polyadenylation achieved in these experiments were still less than seen for the analogous construct in which the donor site was abolished (compare Fig. 8, lane 8, with Fig. 7, lane 6), it is likely that U1 binding retains some inhibitory activity even at this distance from (pA)p (1842 nt).

DISCUSSION

Under normal circumstances, the great majority of AAV5 transcripts generated from the upstream P7 and P19 promoters are polyadenylated at (pA)p within the viral intron; however, most transcripts generated by the proximal P41 promoter read through (pA)p. They are polyadenylated at the distal polyadenylation site at the 3′ end of the genome (pA)d and subsequently spliced. What determines the distance-related efficiency at which (pA)p is used? We demonstrate that the efficiency of polyadenylation at (pA)p is independent of the promoter type or the intervening sequence, and the steady-state levels of alternatively polyadenylated RNAs are controlled at multiple levels. It is a consequence of the efficiency of cleavage and polyadenylation, the strength of inhibition by the donor, and competition for precursor molecules by the splicing process. All three of these determinants are influenced by the distance between the RNA initiation site and the intron and (pA)p. For AAV5 P7- and P41-generated RNAs, polyadenylation and/or splicing at the intron represents processing of the 5′-terminal exon.
The distance dependence of polyadenylation at (pA)p is independent of the nature of the promoter and the intervening sequences. Plasmids with different size insertions of pBR322 DNA between the P41 promoter and the intron in the P41CapA2CAA (lanes 1–6) backgrounds and plasmids with heterologous promoters (HIV LTR, AAV2 P5, and CMV IE) either in the P41CapCAA or P41J1650CapCAA backbone (diagrammed on the right) were transfected into 293 cells. 36–40 h later, total RNAs were protected with the DHA2CAA probe, which is depicted under each diagram. Bands protected by the DHA2CAA probe from a representative experiment are shown and labeled on the left. Quantifications of the ratio of RNAs polyadenylated at (pA)p relative to the total RNAs are shown as averages with standard deviations and are the results of at least three separate experiments.

Polyadenylation at the (pA)p site is subject to a minor distance effect even when mutation abolishes U1 snRNP binding to the intervening donor site. Plasmids with different size insertions of pBR322 DNA between the P41 promoter and the intron in the P41CapA2CAADm1 background (lanes 1–6), in which both donor and acceptor sites have been destroyed, were transfected into 293 cells. Total RNAs were protected with the homologous DHA2CAA probe, which is depicted under each diagram. Bands protected by the DHA2CAA probe from a representative experiment are shown and labeled on the left. Quantifications of the ratio of P41-generated RNAs polyadenylated at (pA)p relative to the total P41-generated RNAs are shown as averages with standard deviations and are the results of at least three separate experiments.
Polyadenylation of P41-generated RNAs at (pA)p is inhibited by U1 snRNP binding to the intervening donor site. There are at least two well characterized examples of regulation of polyadenylation by splice donors in viral systems, both mediated by U1 snRNP. In the papillomavirus system, in which U1 snRNP binds to a cryptic binding site upstream of the affected poly(A) site, the U1 70-kDa protein has been shown to inhibit polyadenylation by not the preceding cleavage reaction (7, 23). In the case of HIV, where inhibition is due to U1 binding to a downstream donor site, both cleavage and polyadenylation are inhibited (8, 9).

Polyadenylation of the IgM μ gene, which is regulated during B-cell development, occurs at a polyadenylation site upstream of a functional donor (24, 25). In this case, however, inhibition of polyadenylation at the μ poly(A) site in the intron between the Cμ4 and M1 exons in plasma cells is not mediated by the full U1 snRNP particle (26) but rather by binding of the U1 A protein to sites downstream from the 5′ splice site and upstream of the secretory poly(A) site (27).

As either the P7 or P41 promoter was positioned incrementally further upstream of (pA)p and the intron donor, with either AAV5 (P7RepnCap series) or heterologous DNA (P7JCap series) serving as the intervening sequences, polyadenylation at (pA)p increased, and the splicing of P7-generated RNA decreased (Fig. 3). Previous experiments with AAV2 demonstrated that excision of the single AAV2 intron was inversely proportional to the distance between the RNA start site and the intron donor (16). The experiments described here, in which the AAV5 (pA)p AAUAAA signal was destroyed, showed that splicing of the AAV5 intron was similarly affected. These results suggested that increased polyadenylation at (pA)p of RNAs generated from a distance (P7), may have been, at least to some extent, because of diminished competition for splicing.

When the P7 promoter was positioned at various distances from the intron and (pA)p in a background in which splicing was disabled by the A2 acceptor mutation, polyadenylation at (pA)p was also increased, suggesting that even when splicing was abolished, polyadenylation at (pA)p was governed by the distance between the cap site and the intron and (pA)p. This will be discussed further below. However, in these constructs polyadenylation at (pA)p was increased at relatively closer distances (Fig. 5) than when splicing was permitted (Fig. 3). This observation suggested that the distance effect on (pA)p usage seen is at least partially due to the decrease in splicing efficiency of RNAs generated from promoters at farther distances upstream of the intron and (pA)p site, perhaps because splicing of P7-driven transcripts might be less able to compete with polyadenylation at (pA)p.

The simplest model to explain the interdependence of splicing and polyadenylation in our system is that strong binding of U1 to the donor facilitates both splicing and inhibition of (pA)p and that perhaps as a consequence of 5′ exon definition, U1 binding is stronger when the promoter is close (e.g. P41-generated RNAs, which are inhibited for polyadenylation at (pA)p and splice) and is weaker when the distance is large (e.g. P7-generated transcripts, which splice poorly, are not inhibited for polyadenylation at (pA)p and are efficiently polyadenylated there).

P7- and P19-initiated RNAs that are polyadenylated at (pA)p are not spliced, and so in contrast to some RNAs with distinct 3′-terminal exons, polyadenylation site choice for these AAV5 RNAs is independent of a functional upstream 3′ splice site. It is also unlikely that the AAV5 region upstream of (pA)p contains a cryptic 3′ splice site that affects (pA)p usage; similar levels of (pA)p usage are detected when the region between the P7 promoter and the intron are replaced with either heterologous prokaryotic sequence from a bacterial plasmid (Figs. 3 and 5), an inverted copy of that sequence (data not shown), or sequence from phage lambda (data not shown). Furthermore, when a functional 3′ splice site from the related parovirus B19 or a synthetic consensus 3′ splice site was inserted –100 nt upstream of (pA)p, no difference in the levels of (pA)p usage was detected (data not shown). Polyadenylation of unspliced AAV5 P7- and P19-initiated RNAs are therefore governed primarily by determinants other than an upstream 3′ splice site.
5′-Terminal exon definition typically requires that factors binding at or near the cap site interact with and stabilize the binding of factors (usually U1 snRNP) to the proximal 5′ splice site, and this interaction may be more critical when the proximal 5′ donor is nonconsensus (28). The cap structure, via binding to the cap-binding complex (CBC) (29–31) has been suggested to affect both splicing and polyadenylation. The CBC has been shown to stimulate U1 snRNP binding to the cap-proximal 5′ splice site (32–34). Although the interaction between the CBC and U1 snRNP is not thought to be direct (28), a mediator of this interaction has not yet been found. It has been suggested that a nuclear CBC facilitates association of U1 snRNP with the cap-proximal 5′ splice site (28), and this interaction was shown not to be dependent on a strict spacing between the cap and the affected 5′ splice site, over a distance of 383 nt. It seems feasible that for AAV5, CBC binding to the cap site may stabilize factors binding to the nonconsensus AAV5 donor, however, only when the cap site is very close (78 nt for P41) and not at a distance (1668 nt for P7 and 1088 nt for P19). This is also consistent with our observation that splicing is stronger when the cap site close to the donor site than at a distance of 1650 nt (Fig. 4). It is possible that if interactions between CBC and U1 snRNP mediate the distance-related donor inhibition seen for AAV5, it is due to the strengthening of the association of U1 snRNP to the nonconsensus donor site, which would lead to the enhancement of the inhibitory activity of U1.

Even in the absence of competing splicing, however, (pA)p usage was seen to be distance-dependent. This difference could be attributed to at least two reasons. As discussed above, binding and hence inhibition by U1 snRNP is likely to be distance-dependent in our system; however, experiments in which this donor was destroyed also showed that there was an intrinsic, although modest, decrease in polyadenylation efficiency as the size of the transcription unit was increased (Fig. 7). That polyadenylation at (pA)p is distance-dependent in the absence of competing splicing or donor inhibition suggests a model of co-transcriptional modulation of polyadenylation at (pA)p. It may be that the elongating transcription complex is more efficient at polyadenylation when it has a longer distance to traverse rather than when it is close to the donor. This could feasibly be due to an increased opportunity to load the 3′ end processing factors onto an elongating transcription complex via interaction with the phosphorylated carboxyl-terminal domain of RNA polymerase II (20), because the complex traverses longer distances (as would be the case for P7 and P19), as compared with when the promoter is very close to the inhibiting donor site and (pA)p (as it would be for P41). In the yeast system, it has been demonstrated that 3′ end processing factors start to load onto RNA polymerase II transcription complexes at the promotor and are recruited progressively as polymerase II travels along the gene (21). Perhaps greater concentrations of 3′ end processing factors on polymerase complexes elongating from the more remote P7 or P19 promoter could override U1 snRNP inhibition of polyadenylation at (pA)p, whereas the levels of these factors recruited when transcription is from the closer P41 promoter may not provide this effect. Our results do suggest, however, that in the AAV5 system, such a co-transcriptional model is not the major determinant of alternative polyadenylation at (pA)p. As shown in Fig. 7, 77% of P41-generated RNAs produced from constructs in which both the 5′ and 3′ splice sites were destroyed polyadenylate at (pA)p, and increasing the distance to 1655 nt only increased polyadenylation efficiency at (pA)p to 59%. This observation suggests that co-transcriptional recruitment of polyadenylation factors over even a short distance (265 nt) is sufficient for efficient polyadenylation, and an increase in polyadenylation efficiency caused by co-transcriptional effects over a long distance was a minor effect.

We also show that the distance between the U1 snRNP-binding site (the donor site) and the AAVAA poly(A) signal is critical for U1 snRNP inhibition of polyadenylation at pAp. As the distance between these two elements was increased from 187 nt to 1642 nt, polyadenylation at (pA)p was increased from 28 to 79%. Polyadenylation was increased nearly to its maximal level at an intermediate distance of ~800 nt (Fig. 8, lane 5). In previously described cases of U1 snRNP inhibition of polyadenylation, the distance between the U1-binding site and the poly(A) site is quite close; 11 nt in the case of papillomavirus (7), 197 nt in HIV (8), and less than 45 nt in U1A auto-regulation (35). These results in other systems were interpreted as suggesting that a tight physical proximity was needed for either U1A or U1 70-kDa to interact with poly(A) polymerase directly. Recently, it has been proposed that inhibition of polyadenylation by U1 is perhaps a more general phenomenon (22, 36). These investigators found that U1 snRNA inhibited gene expression even when placed far upstream (up to 1190 nt) of the AAUAAA poly(A) site. This inhibition could reflect a direct, but “long distance” interaction between U1 snRNP and 3′ end poly(A) signal; however, a model of disruption of the terminal exon was proposed (22). That the spacing between U1 snRNP and the (pA)p AAUAAA signal in the AAV5 system is constrained suggests a more direct interaction of U1 RNA-binding proteins, such as U1 A or U1 70-kDa with the polyadenylation machinery.

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Alternative Polyadenylation of Adeno-associated Virus Type 5 RNA within an Internal Intron Is Governed by the Distance between the Promoter and the Intron and Is Inhibited by U1 Small Nuclear RNP Binding to the Intervening Donor

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