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In Vitro Cleavage of the Simian Virus 40 Early Polyadenylation Site Adjacent to a Required Downstream TG Sequence

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Exogenous RNA containing the simian virus 40 early polyadenylation site was efficiently and accurately polyadenylated in in vitro nuclear extracts. Correct cleavage required ATP. In the absence of ATP, nonpoly(A)$^+$ products accumulated which were 18 to 20 nucleotides longer than the RNA generated by correct cleavage; the longer RNA terminated adjacent to the downstream TG element required for polyadenylation. In the presence of ATP analogs, alternate cleavage was not observed; instead, correct cleavage without poly(A) addition occurred. ATP-independent cleavage of simian virus 40 early RNA had many of the same properties as correct cleavage including requirements for an intact AAUAAA element, a proximal 3′ terminus, and extract small nuclear ribonucleoproteins. This similarity in reaction parameters suggested that ATP-independent cleavage is an activity of the normal polyadenylation machinery. The ATP-independent cleavage product, however, did not behave as an intermediate in polyadenylation. The alternate RNA did not preferentially chase into correctly cleaved material upon readdition of ATP; instead, poly(A) was added to the 3′ terminus of the cleaved RNA during a chase. Purified ATP-independent cleavage RNA, however, was a substrate for correct cleavage when reintroduced into the nuclear extract. Thus, alternate cleavage of polyadenylation sites adjacent to a required downstream sequence element is directed by the polyadenylation machinery in the absence of ATP.

Polyadenylation of nascent pre-m RNAs occurs rapidly after synthesis of nascent transcripts (5, 23, 31). The mechanism of polyadenylation is thought to involve endonucleolytic cleavage followed by poly(A) addition at or near the site of cleavage. Two concensus elements, an AAUAAA upstream element (7, 13, 20, 26, 29, 30a) and a downstream TG box (4, 9, 12, 17, 25, 27, 30), are required for polyadenylation in vivo and in vitro. The identity of the catalytic factors which recognize these consensus elements and are responsible for cleavage are unknown, although the participation of small nuclear ribonucleoproteins (snRNPs) has been suggested (1, 12a). Cleavage and polyadenylation are inhibited in vitro by anti-Sm antibodies directed against the family of snRNPs involved in splicing of pre-m RNAs (21, 22). Polyadenylation is also inhibited by antisera directed against U1 snRNPs. Antibodies directed against U1 and U2 snRNPs, however, do not immunoprecipitate polyadenylation complexes constituted in vitro, whereas anti-Sm antibodies do (12). Furthermore, oligonucleotide-directed cleavage of U1, U2, or U4 RNAs in in vitro polyadenylation extracts has no effect on polyadenylation (1a, 12a).

In vitro nuclear extracts accurately polyadenylate precursor RNAs (11, 21, 30); different precursor RNAs containing different polyadenylation signals are processed with different efficiency. Consensus sequence elements required for polyadenylation in vivo are also necessary for the in vitro reaction. Cleavage occurs at the site of poly(A) addition, and poly(A) addition rapidly follows cleavage. When ATP is removed or replaced with nonincorporatable ATP analogs, cleavage in the absence of A addition is observed.

In this report, we demonstrate efficient and accurate cleavage and polyadenylation of simian virus 40 (SV40) early RNA in nuclear extracts. The properties of the polyadenylation reaction with SV40 early RNA were compared to those with a precursor RNA containing the adenovirus L3 poly(A) site (21, 22). SV40 RNA displayed a different ATP dependence than that previously reported for L3 RNA. In the absence of ATP, no correct cleavage of SV40 early RNA was observed; instead, downstream cleavage adjacent to the TG box occurred. Both ATP-dependent and ATP-independent cleavage required an intact AAUAAA element, a proximal 3′ end, and extract snRNPs. The longer cleavage product generated in the absence of ATP did not preferentially chase into product RNA, however, suggesting that it is not a normal intermediate in polyadenylation but is, instead, the product of an alternate activity of the polyadenylation machinery.

MATERIALS AND METHODS

RNA substrates. In vitro transcription was performed as described previously (18) except that the GTP concentration was 300 μM and 1 mM GpppG was included to produce capped transcripts. The template used to produce the adenovirus L3 precursor was a gift of C. Moore. The template used to produce the SV40 early RNA (pSVE) contained the *Bam*HI-*Pst*I fragment of SV40 DNA inserted downstream from the Sp6 promoter in the Sp64 vector. M13 DNA complementary to the Sp6 transcripts was used to map cleavage of the substrates in the in vitro reactions. The adenovirus M13, pADH12, was a genomic adenovirus type 2 fragment and a gift of C. Moore. The SV40 M13, MSVL, contained the *Bam*HI-*Bcl*I fragment of SV40 DNA.

Nuclear extract. Nuclear extract was prepared by the method of Dignam et al. (6). A 5- to 10-ng sample of radiolabeled Sp6 transcript was added to a typical reaction mixture in a final volume of 0.025 ml. Reaction conditions were essentially as described by Moore and Sharp (22) except that a final concentration of 3% polyvinyl alcohol was required for maximal activity with SV40 early precursor RNA substrates. The reaction contained 1 mM ATP, 20 mM creatine phosphate, 44 mM KCl, 3% polyvinyl alcohol, and 44% nuclear extract. In experiments including ATP analogs,

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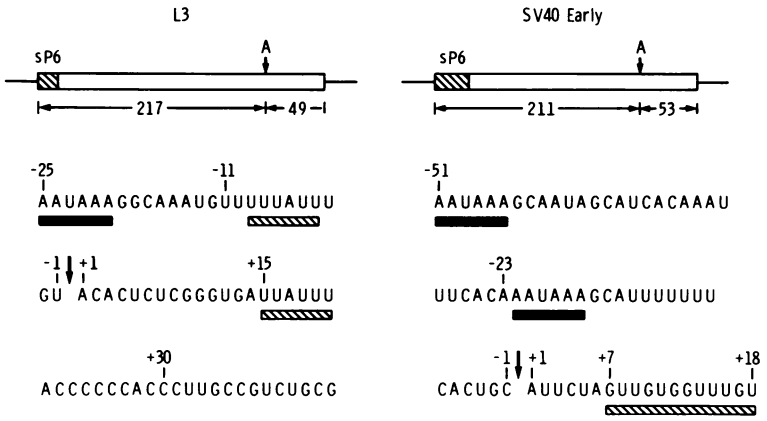

FIG. 1. Structure of the SV40 and L3 precursor RNAs. Upstream consensus hexonucleotide sequences are indicated with dark boxes; putative downstream elements are shown by hatched boxes.

ATP was replaced with the appropriate concentration of analog. Experiments done in the absence of ATP also eliminated creatine phosphate from the reaction. Cleavage activity varied from extract preparation to extract preparation for reasons that we do not yet understand. Cleavage of L3 and SV40 substrate varied from 30 to 90% and 20 to 60% of input, respectively. Incubations were at 30°C. RNA was prepared and analyzed as described by Moore and Sharp (22).

To map RNA cleavage sites, we hybridized RNA to M13 DNA containing a DNA insert complementary to the substrate RNA. After hybridization, T2 RNase was added to 2 U/ml, and the hybrids were digested at 37°C for 30 min. After protease K digestion, isolated RNA was subjected to gel electrophoresis in denaturing acrylamide gels. This assay served to distinguish RNAs which had undergone A addition at the 3′ end of the substrate RNA from those which had undergone cleavage and A addition.

−20 A mutation. A point mutation within the SV40 early polyadenylation site was created by oligonucleotide-directed point mutagenesis (32). The cleavage-proximal AAUAAA element was altered to AAAAAA in the mutation. The identity of the mutation was confirmed by direct DNA sequencing.

RESULTS

In vitro polyadenylation of SV40 early RNA. Nuclear extracts similar to those described by Dignam et al. (6) were used to examine polyadenylation of exogenously provided radiolabeled RNAs containing polyadenylation sites from the SV40 early and adenovirus L3 transcription units. Both precursors were of approximately the same size (Fig. 1). The Sp6 transcription template used to generate SV40 precursor RNA contained the Sp6 promoter fused to 237 nucleotides of SV40 DNA containing the early polyadenylation site. Truncation at the *Bam*HI site at the end of the SV40 insert followed by transcription produced a 264-nucleotide precursor RNA including 53 nucleotides downstream of the cleavage site. The L3 template was composed of 217 nucleotides of adenovirus type 2 DNA; truncation with *Dra*I produced a precursor RNA including 49 nucleotides downstream of the cleavage site (22).

RNA products of incubation in the extracts were analyzed either directly on denaturing gels or after hybridization to complementary genomic single-stranded DNA and treatment with RNase T2 to digest unhybridized sequences (22). The latter analysis served to distinguish those RNAs which had been correctly cleaved and polyadenylated from those which had undergone poly(A) addition at the 3′ end of the precursor RNA. Direct gel examination of the reaction products indicated that both substrates were highly competent to direct poly(A) addition (Fig. 2, ATP lanes). However, when the two reactions were analyzed with the hybridization assay (Fig. 2, HYB lanes), L3 precursor RNA was correctly cleaved with higher efficiency than was SV40 precursor RNA.

A total of 10 to 30% of the input SV40 substrate RNA was cleaved in 2 h as compared with 60 to 70% of the L3 RNA. The SV40 cleavage activity was accurate; comparison of in vitro-cleaved and -polyadenylated RNA with in vivo-produced early SV40 mRNA indicated similar length T2-resistant bands after hybridization to complementary M13 DNA (data not shown). Thus, although the SV40 site was correctly cleaved and polyadenylated in vitro, end polyadenylation rather than cleavage and A addition was the preferential reaction.

Despite the comparative low efficiency of SV40 early polyadenylation in vitro, the observed activity was still considerably higher than that observed by other investigators using comparable extracts (12, 16). The reason for this discrepancy in activity is not clear. Our extracts required inclusion of 3% polyvinyl alcohol for high efficiency with SV40 but not L3 templates; this addition may provide a necessary concentration of polyadenylation factors. Interestingly, splicing proceeded at high efficiency in the same extracts in the absence of polyvinyl alcohol (1a). The identity of the factors responsible for the difference in activity of splicing and polyadenylation templates and for the difference between the activity of different polyadenylation templates is unknown.

Polyadenylation was also examined with SV40 substrates which included more sequence information beyond the cleavage site. Two precursors containing 327 and 597 nucleotides of SV40 sequence downstream of the cleavage site were accurately cleaved in vitro but with markedly lower efficiency than observed for the shorter precursors (data not shown). Other investigators have also noticed a dependence on the proximity of a 3′ terminus to the cleavage site for maximal in vitro activity (16, 30a).

Cleavage at the site of poly(A) addition should release a downstream RNA fragment. We were unable to detect this RNA during normal reactions, presumably because of the presence of active 5′ → 3′ exonucleases that digest RNAs

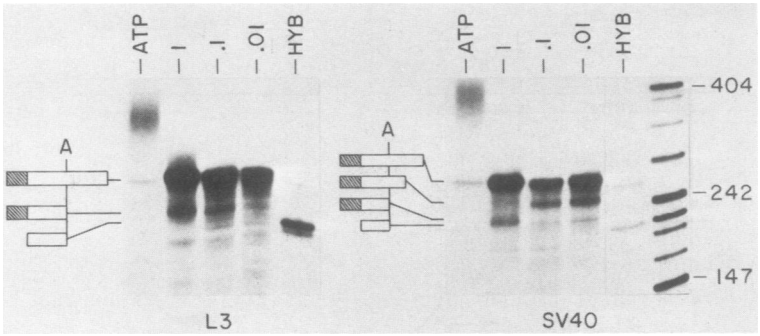

FIG. 2. In vitro polyadenylation of L3 and SV40 early RNA in the presence of ATP or ATP analogs. L3 and SV40 RNA precursor RNAs were incubated for 2 h in nuclear extracts containing ATP (lanes ATP and HYB) or in which ATP had been replaced with the indicated concentration (1, 0.1, or 0.01 mM) of AMP(CH_2)PP. RNA from reactions was directly analyzed (lanes ATP, 1, 0.1, and 0.01) or analyzed after hybridization to complementary M13 DNA and treatment with T2 RNase (lane HYB) to remove unhybridized RNA sequences. Schematics at the side of the diagram indicate the structure of the RNA in each band. Both the poly(A) tail and the Sp6 leader (▩) were unhybridized to M13 DNA and were removed by T2 digestion. A marks the site of in vivo cleavage. Precursor (▩), poly(A)$^+$ product (⟆ A_n), T2-resistant product from hybridization to precursor or end-adenylated RNA (□), and T2-resistant product from hybridization to cleaved RNA (□) are indicated. Poly(A)$^+$ product contains RNAs which were end adenylated and RNAs which were correctly cleaved and adenylated. Note that the mapped cleavage product is shorter than the unmapped correct cleavage product because of the T2 removal of the Sp6 leader on the precursor RNA. Numbers to the right show nucleotides.

with unprotected 5′ termini. Other investigators have utilized EDTA to stabilize such downstream fragments (12, 22, 30a). In our hands the presence of EDTA shifted the site of cleavage from the normal site of A addition to multiple upstream positions. Therefore, we could not use this approach to detect the downstream cleavage product. Other workers have, however, detected such a fragment after cleavage of SV40 early RNA in their extracts (12a). Therefore, it seems likely that cleavage of SV40 RNA in our extracts is occurring at the site of A addition and that the inability to detect a released downstream fragment is a result of extract nucleases rather than a fundamental difference in reaction properties.

Removal of ATP causes alternate cleavage of polyadenylation sites in vitro. Replacement of extract ATP with the nonincorporatable analogs AMP(CH_2)PP and cordycepin triphosphate (3′ dATP) results in the accumulation of correctly cleaved but nonpoly(A)$^+$ products when using L3 or SV40 late precursor RNAs (22, 30a). Inclusion of these two analogs in our extracts produced similar results with L3 RNA but not with SV40 early RNA (Fig. 2). At final concentrations of 0.01 M, 0.1 M, and 1 mM AMP(CH_2)PP, cleavage of SV40 early precursor RNA yielded different products at different final concentrations of analog. At 0.01 and 0.1 mM AMP(CH_2)PP, the major product of incubation was an RNA of 230 nucleotides, suggesting downstream cleavage approximately 19 nucleotides past the poly(A) addition site. A trace amount of correct cleavage product was also observed. At 1 mM AMP(CH_2)PP, the longer RNA was not observed; the predominant product under these conditions was a band equivalent in length to the authentic cleavage RNA. The appearance of the alternate longer product RNA was not dependent on inclusion of analog. In the absence of analog, ATP, and creatine phosphate, only the alternate cleavage product was produced (Fig. 3). The kinetics of accumulation of ATP-independent and ATP-dependent cleavage products were comparable. Both occurred slower than A addition at the 3′ end of the precursor RNA and both required the presence of polyvinyl alcohol for maximum activity (data not shown).

We were unable to visualize a small (35-nucleotide) fragment representing the downstream RNA released upon ATP-independent cleavage. This is not surprising considering our inability to visualize the downstream cleavage product during a normal reaction. Our best present evidence suggests that the 230-nucleotide ATP-independent RNA is a linear RNA product of a downstream cleavage reaction. Preparation of a 5′-truncated polyadenylation site (containing 135 nucleotides of SV40 DNA from the *Hpa*I-*Bam*HI sites) produced an ATP-independent cleavage RNA of 126 nucleotides and placed the alternate cleavage site 18 to 20 nucleotides downstream of the A addition site (data not shown). Furthermore, the cleavage product behaved as a linear RNA. Its migration on polyacrylamide gels was not dependent on the percentage of acrylamide in the gels, and the length of the T2-resistant band produced after hybridization to complementary M13 DNA was consistent with cleavage at the indicated position. Thus, the RNA had none of the properties normally associated with RNA lariats.

Similar requirements for ATP-dependent and ATP-independent cleavage. Polyadenylation is dependent upon an intact AAUAAA element (7, 12a, 20, 26, 29, 30a). To examine the importance of this sequence for ATP-dependent and ATP-independent cleavage of SV40 early RNA, we created a point mutation within the proximal AAUAAA element of the SV40 early polyadenylation site by site-directed mutagene-

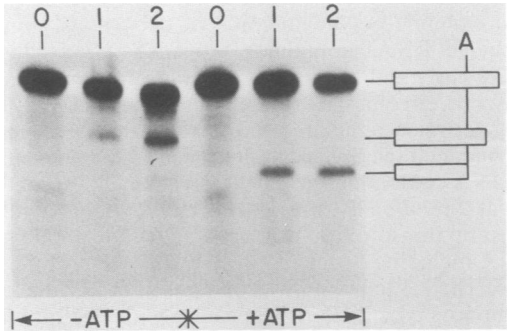

FIG. 3. Time course of ATP-dependent and ATP-independent cleavage of SV40 early RNA. SV40 early RNA was incubated in the extracts in the presence or absence of ATP for the indicated time (in hours). RNA was prepared and analyzed by gel electrophoresis after hybridization to complementary M13 DNA and treatment with T2 RNase. Symbols are described in the legend to Fig. 2.

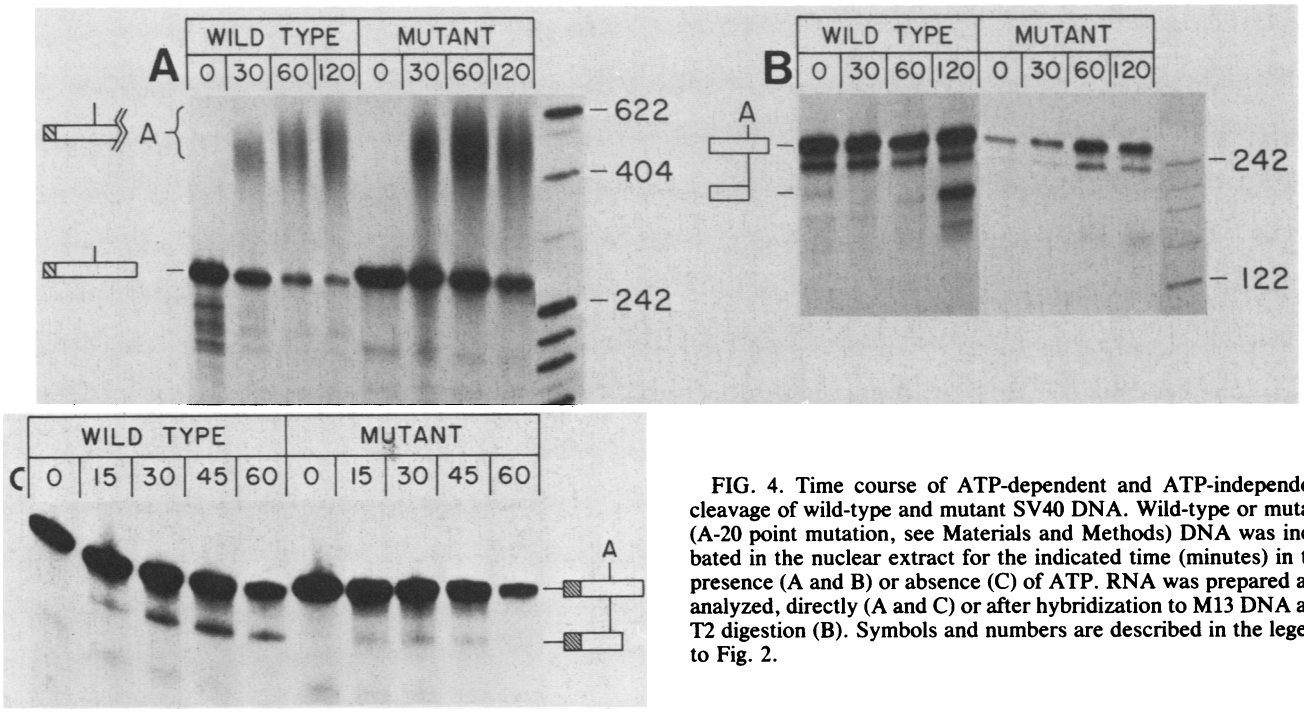

FIG. 4. Time course of ATP-dependent and ATP-independent cleavage of wild-type and mutant SV40 DNA. Wild-type or mutant (A-20 point mutation, see Materials and Methods) DNA was incubated in the nuclear extract for the indicated time (minutes) in the presence (A and B) or absence (C) of ATP. RNA was prepared and analyzed, directly (A and C) or after hybridization to M13 DNA and T2 digestion (B). Symbols and numbers are described in the legend to Fig. 2.

sis. The SV40 polyadenylation site contains two copies of the AAUAAA element. Only the downstream copy is thought to direct polyadenylation in vivo. The internal U in this sequence was altered to an A, a modification which has been shown to abolish polyadenylation in other sites (29). Mutant RNA directed end poly(A) addition with the same efficiency as did wild-type RNA (Fig. 4A); cleavage activity, however, was considerably reduced (Fig. 4B). A reduction was also observed in the ATP-independent cleavage activity (Fig. 4C). The observed residual cleavage with the mutant may be directed by the remaining unaltered upstream copy of the AAUAAA element. Observation of a reduction in both ATP-dependent and ATP-independent cleavage in the absence of a required polyadenylation consensus sequence element suggested that both cleavage activities are associated with the normal polyadenylation machinery.

The sequence requirements for ATP-independent cleavage was further examined by incubating RNA precursors that did not contain polyadenylation sites in the extract in the absence of ATP (data not shown). Three alternate RNAs were employed: a 464-nucleotide adenovirus type 2 active splicing precursor RNA containing a portion of exon 1, an internally deleted intron 1, and the entire second exon of the major late transcription unit (24); a 467-nucleotide SV40 RNA containing antisense sequences from the SV40 late transcription unit; and a 250-nucleotide RNA derived from pSP64 and containing no eucaryotic sequences. None of these templates was cleaved in vitro in the absence of ATP. Thus, only precursor RNAs containing valid polyadenylation consensus sequences were substrates for ATP-independent cleavage. The stability of the nonpolyadenylation substrate RNAs in the extract further argues that the alternate product band observed with the SV40 early poly(A) precursor RNA in the absence of ATP is the product of endonucleolytic cleavage and is not the result of exonuclease digestion back to a stable secondary structure or protein-protected domain within the precursor RNA. If such exonucleases were active in the extract, some degree of digestion of alternate substrates would have been expected. Therefore, we suggest that the observed ATP-independent cleavage is caused by an extract endonuclease.

The effect of substrate length on ATP-independent cleavage was also examined (Fig. 5). Longer substrates containing 323 and 594 nucleotides of SV40 sequence downstream of the correct cleavage site were incubated in the extracts in the absence of ATP. Both templates were substrates for the alternate cleavage reaction although at very low efficiency. The majority of the longer precursor RNAs, however, remained uncleaved during incubation. The extent of the reduction of ATP-independent cleavage with longer substrates was comparable to the reduction in activity of correct cleavage with the same substrates (data not shown). Thus, ATP-independent cleavage exhibited a dependence on the close proximity of the cleavage site and the 3′ terminus of the precursor RNA, and this dependence was similar to that of the correct cleavage reaction. Furthermore, no other RNase-sensitive sites were strongly activated in these RNAs in the absence of ATP.

Polyadenylation of L3 precursor RNA in vitro is inhibited by anti-snRNP antibodies (22). Both anti-Sm antibodies directed against the extended U snRNP family and anti-RNP antibodies specific for U1 snRNPs inhibited in vitro cleavage. ATP-dependent cleavage of SV40 early RNA in vitro was also inhibited by anti-Sm and anti-RNP antibodies (Fig. 6). The antibodies used for this experiment were high-titered patient sera. The anti-Sm serum reacted only with the snRNP B and D polypeptides on Western blots and was therefore judged to have little anti-RNP character. Similarly, the anti-RNP antibody selectively immunoprecipitated U1 snRNPs and reacted specifically with the 67,000-dalton U1 snRNP-specific polypeptide on Western blots and was judged to have little anti-Sm character. Inclusion of either antibody in in vitro polyadenylation reactions inhibited both normal polyadenylation and ATP-independent cleavage. With comparable amounts of antibody, however, the observed inhibition of ATP-independent cleavage was not as striking as the inhibition of correct cleavage, suggesting that although ATP-independent cleavage required snRNPs, its

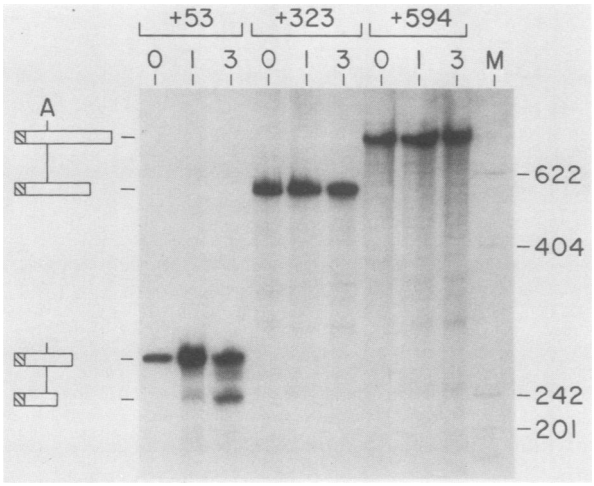

FIG. 5. ATP-independent cleavage of SV40 precursor RNAs with different amounts of downstream sequence. Sp6 transcripts containing 53, 323, or 594 nucleotides of SV40 sequence downstream of the in vivo cleavage site were generated by cleavage of pSVE with *Bam*HI, *Hae*II, or *Pst*I, respectively. Each RNA was incubated in the nuclear extract without ATP for the indicated time. RNA was prepared and analyzed directly by gel electrophoresis. Symbols and numbers are described in the legend to Fig. 2. Lane M, Molecular weight markers are *Hpa*II-digested pBR322.

dependence on snRNPs was somewhat altered as compared with the dependence of correct polyadenylation.

ATP-independent cleavage product RNA does not behave as an intermediate in polyadenylation. Polyadenylation is thought to involve cleavage at the site of poly(A) addition. Released downstream cleavage fragments have been observed for several different precursor RNAs including SV40 early RNA (12, 12a, 22, 30a). Therefore, the SV40 RNA produced upon incubation in the absence of ATP does not have properties suggestive of its being an intermediate in normal polyadenylation. To further examine this possibility, products produced in the absence of ATP were chased by the addition of either ATP or ATP plus fresh extract to reactions set up in the absence of ATP (Fig. 7). Extract was incubated with SV40 early precursor RNA for 2 h in the absence of ATP to permit accumulation of the alternate

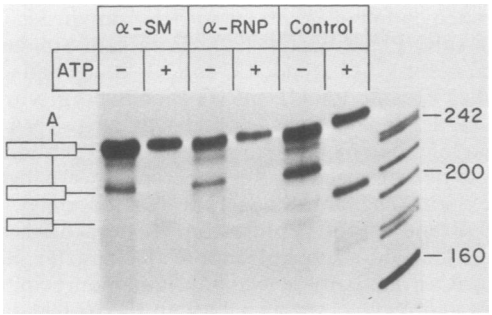

FIG. 6. Inhibition of ATP-dependent and ATP-independent cleavage by anti-snRNP antibodies. Extract was preincubated with U1-specific (α-RNP), snRNP-specific (α-Sm), or control human (control) antibodies (see text) for 30 min on ice. The rest of the reactants were added, and incubation with (+) or without (−) ATP was continued at 30°C for 2 h. RNA was prepared and analyzed by gel electrophoresis after hybridization to M13 DNA and T2 RNase treatment. Symbols and numbers are described in the legend to Fig. 2.

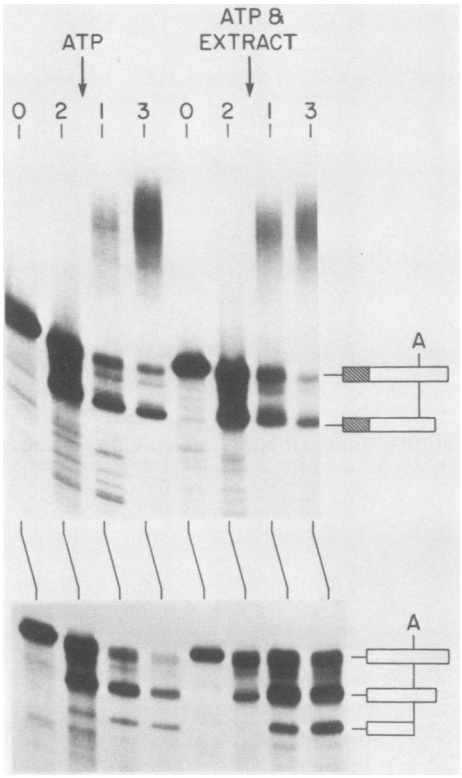

FIG. 7. ATP-independent cleavage product is preferentially end polyadenylated after an ATP chase. SV40 precursor RNA was incubated for 2 h in nuclear extract in the absence of ATP. ATP or ATP and fresh extract were added (arrow), and the incubation was continued. RNA was removed at 1 and 3 h after reintroduction of ATP. RNA was analyzed directly (top) or after hybridization to M13 DNA and T2 RNase treatment (bottom). Symbols are described in the legend to Fig. 2.

cleavage product RNA. ATP was added, and incubation was continued for an additional 3 h. Poly(A)$^+$ products accumulated after the addition of ATP as evidenced by the variable-length material migrating above precursor RNA in the polyacrylamide gel of reaction products shown in Fig. 7. Concomitant with the appearance of poly(A)$^+$ product during the chase, the amounts of both precursor and alternate cleavage RNAs decreased. This decrease was more apparent when fresh extract was added with the ATP. Appearance of poly(A)$^+$ RNA after introduction of ATP could have been due to end-A addition or to further cleavage and A addition. To distinguish these two possibilities, we subjected reaction products to hybridization to complementary M13 DNA and treatment with RNase T2 (Fig. 7). Correctly cleaved RNA accumulated during the chase as evidenced by the production of a 211-nucleotide band. The ATP-independent cleavage band, however, did not correspondingly decrease in intensity during the chase reaction. Instead, a decrease in intensity of unreacted precursor RNA was noted during the chase, suggesting that the correct cleavage product RNA created during the chase came from cleavage of precursor RNA rather than from further cleavage of the ATP-independent cleavage RNA. Thus, the ATP-independent cleavage product RNA served as a preferential substrate for end polyadenylation during the chase; it did not serve as a substrate for cleavage at the normal polyadenylation site.

Removal of ATP did not irreversibly alter the ability of the extract to carry out correct cleavage. Addition of SV40

precursor RNA with the ATP at the beginning of the chase resulted in correct cleavage only (data not shown). No aberrantly cleaved RNA was observed. Thus, our best interpretation of the continued observation of the alternate cleavage product during the chase experiment in Fig. 7 is that alternate cleavage RNA is neither created nor further cleaved during the chase, but is end adenylated.

The ATP-independent cleavage product RNA contains roughly 19 nucleotides of sequence downstream from the poly(A) addition site. Deletion mutations in the SV40 early polyadenylation site suggest that this should be enough information to direct accurate polyadenylation both in vivo and in vitro (12). Therefore, the 229 to 231-nucleotide ATP-independent cleavage product was gel purified from a reaction without ATP to test for its ability to direct correct cleavage when reintroduced into the nuclear extract. Under these conditions, approximately 50% of the product of the second incubation was correctly cleaved RNA (Fig. 8); a similar percent conversion was observed when precursor RNA was subjected to the same protocol. Therefore, ATP-independent cleavage RNA contained all the sequence information necessary to direct polyadenylation. The inability of the ATP-independent cleavage RNA to compete with precursor RNA for cleavage after a reintroduction of ATP suggests that this RNA is not a normal intermediate during polyadenylation. Rather, this product has characteristics suggesting that alternate cleavage occurs in the absence of ATP.

DISCUSSION

In this communication we report the ability of nuclear processing extracts to accurately polyadenylate exogenously provided precursor RNAs containing the SV40 early polyadenylation site; the activity was compared with that observed with a precursor RNA containing the polyadenylation site from the adenovirus L3 transcription unit. Polyadenylation of SV40 RNA behaved similarly to that of L3 RNA with respect to some features of the reaction but not others. A addition and cleavage were uncoupled by the replacement of ATP with nonincorporatable analogs such as cordycepin, AMPP(CH_2)P, or AMP(CH_2)PP with both SV40 and L3 substrate RNAs. In the total absence of ATP or analogs, however, the two substrates behaved differently. Whereas correctly cleaved but not poly(A)$^+$ RNA was the preferred product with L3 RNA, an alternate product accumulated in reactions with SV40 RNA. This product had properties consistent with its being a linear RNA produced via alternate cleavage 18 to 20 nucleotides downstream of the site of A addition and adjacent to the downstream TG box.

The switch from alternate to correct cleavage of SV40 precursor RNAs as the concentration of either ATP or analog is raised suggests that an ATP-dependent conformational change occurs in some component of the polyadenylation machinery at high ATP concentrations permitting recognition of the correct cleavage site. The ability of ATP analogs to affect this change indicates that neither cleavage nor incorporation of the ATP is required for the transition. Instead, ATP behaves as an allosteric effector.

ATP-independent cleavage of SV40 early RNA required many of the same factors as did ATP-dependent cleavage including an intact AAUAAA element, a proximal 3′ terminus, and extract snRNPs. Thus, the observed alternate cleavage would appear to be an activity associated with the normal polyadenylation machinery. ATP-independent cleavage did not appear to be an intermediate in the normal polyadenylation reaction. The structure of the alternate cleavage RNA was inconsistent with such a role. Furthermore, it did not preferentially chase into correctly cleaved and poly(A)$^+$ RNA upon reintroduction of ATP into the extracts. When isolated from a gel and reintroduced into the extract, however, the ATP-independent cleavage RNA was converted into correct product RNA, indicating that it contained all the sequences necessary for correct polyadenylation. Thus, the polyadenylation machinery has the capacity to alternatively cleave polyadenylation precursor RNAs in the absence of ATP.

The ATP-independent cleavage RNA was not converted to correctly cleaved RNA even when fresh extract was added with the ATP in chase experiments. This resistance suggests both that the alternate cleavage RNA was sequestered within processing complexes and unavailable to fresh extract components and that incubation in the absence of ATP irreversibly altered an extract component essential for cleavage. One of the prominent alterations that occurs in the extract in the absence of ATP is the conversion of U1 RNA to a foreshortened form, U1*. We are currently investigating whether the cleavage of U1 RNA is related to the ATP-independent cleavage of SV40 early RNA.

Sequences downstream of the A addition site have been implicated as being required for correct polyadenylation (4, 9, 12, 17, 18, 25, 27, 30). SV40 RNA contains such a TG box from 7 to 18 nucleotides downstream of the cleavage site. Deletion of this sequence inhibits polyadenylation in vivo (12); inhibition can be reversed by the replacement of this sequence with a similar sequence from another RNA. The ATP-independent cleavage site within SV40 early RNA lies adjacent to the TG box. Finer mapping studies are in progress to further locate the precise site of the 3′ terminus of the ATP-independent product RNA. The function of the TG box is unknown. Models have been proposed which suggest that the TG sequences are recognized via hybridization to U4 RNA sequences (27). The U4 sequences utilized in this scheme include nucleotides 63 to 72. However, removal of nucleotides 66 to 85 within U4 RNA by oligonucleotide-directed cleavage did not impair in vitro polyaden-

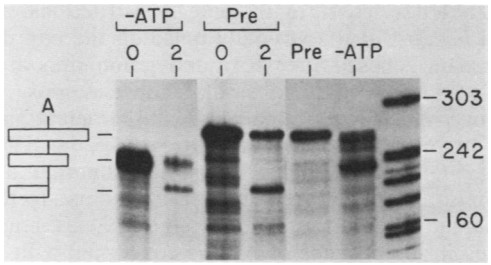

FIG. 8. Reintroduction into the nuclear extract of purified ATP-independent cleavage RNA produces correctly cleaved and adenylated RNA. The RNA products from a reaction in the absence of ATP were isolated from polyacrylamide gels. Both precursor RNA and the ATP-independent cleavage RNA were isolated. Each RNA was then reintroduced into a normal polyadenylation reaction containing ATP. RNA products were isolated after incubation for 2 h and analyzed for correct cleavage by hybridization to M13 DNA and T2 RNase digestion. −ATP, Gel-purified ATP-independent cleavage RNA after 0 or 2 h of incubation in extract containing ATP; Pre, gel-purified precursor RNA after 0 or 2 h of incubation in extract containing ATP. Pre, −ATP, Reaction products from a normal reaction after 0 (Pre) or 2 (−ATP) h of incubation in the absence of ATP. Symbols and numbers are described in the legend to Fig. 2.

ylation (1a), suggesting that this model of hybridization-recognition is not adequate to explain the function of the TG box.

RNase protection studies have indicated that downstream sequences within the SV40 early polyadenylation site are protected from digestion by anti-Sm immunoprecipitable extract components (12a). In contrast, only sequences upstream of the cleavage site were protected in similar experiments utilizing L3 RNA. Interestingly, the L3 precursor RNA does not contain a very good version of the TG sequence, suggesting that downstream protection might require TG sequences. Instead of a TG box, L3 RNA contains extensive self-complementarity; two perfect complements to the required AAUAAA hexanucleotide are present in the L3 sequence, one abutting the cleavage site and one further downstream.

The presence of a TG box within a precursor RNA correlates with several features of in vitro polyadenylation including susceptibility to ATP-independent cleavage. Thus, the SV40 early and the L3 polyadenylation sites may be representatives of two different classes of polyadenylation sites. The L3 class works efficiently in in vitro extracts, contains regions complementary to AAUAAA, does not contain a TG box, has no downstream sequences protected by snRNPs, and is not a substrate for ATP-independent cleavage. SV40-like polyadenylation sites work weakly in vitro, have no sequences complementary to AAUAAA, contain good TG boxes, have downstream sequences which are protected by snRNPS, and are substrates for ATP-independent cleavage.

The relevance of the ATP-independent cleavage reaction observed in vitro to polyadenylation in vivo is unclear. ATP is required for several reactions during in vitro splicing, including bond breaking and assembly of the spliceosome (3, 8, 10, 24). Furthermore, ATP-dependent conformational changes within snRNPs in the extract have been observed (1a, 2). Thus, the cleavage we observed may be the result of an altered conformation of one of the components of the processing complex.

It is also possible that the ATP-independent cleavage is related not to productive polyadenylation but to other RNA-processing events. Two such events come to mind. The SV40 early polyadenylation site is frequently used as a polyadenylation cassette in gene constructions. It is so utilized because it is extremely polar on the expression of downstream genes. Other polyadenylation sites are not as effective in this regard in similar constructions. Furthermore, in vivo, it is extremely difficult to detect significant steady-state nuclear RNA downstream of the SV40 early polyadenylation site. Thus, it is possible that a site of transcription termination is located very close to the SV40 early polyadenylation site and that the cleavage reaction we observed in the absence of ATP is concerned with an RNA cleavage involved in transcription termination rather than with polyadenylation.

Alternatively, the observed cleavage could be related to some other type of nuclear RNA processing. Some fraction of nuclear RNA turns over very rapidly after its synthesis in nuclei (5, 31). In addition, certain RNAs have an increased instability during certain stages of the cell cycle, suggesting that posttranscriptional processing occurs to turn over unwanted sequences. Both thymidine kinase (11) and dihydrofolate reductase (15) pre-mRNAs demonstrate this kind of conditional instability. In constructs containing the dihydrofolate reductase gene (14), the sequences required for this control have been positioned near the polyadenylation site. Thus, an aberrant cleavage at or near a polyadenylation site might target an RNA for degradation rather than for processing. We are presently looking to see whether the SV40 early polyadenylation site ever directs in vivo cleavage of the type we observed in vitro.

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