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Formation of mRNA 3′ termini: stability and dissociation of a complex involving the AAUAAA sequence

David Zarkower and Marvin Wickens

Department of Biochemistry, College of Agriculture and Life Sciences, University of Wisconsin-Madison, Madison, WI 53706, USA

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Formation of the 3′ termini of mRNAs in animal cells involves endonucleolytic cleavage of a pre-mRNA, followed by polyadenylation of the newly formed end. Here we demonstrate that, during cleavage *in vitro*, the highly conserved AAUAAA sequence of the pre-mRNA forms a complex with a factor present in a crude nuclear extract. This complex is required for cleavage and polyadenylation. It normally is transient, but is very stable on cleaved RNA to which a single terminal cordycepin residue has been added. The complex can form either during the cleavage reaction, or on a synthetic RNA that ends at the polyadenylation site. Mutations which prevent cleavage also prevent complex formation. The complex dissociates during or after polyadenylation, enabling the released activities to catalyze a second round of cleavage.

Key words: polyadenylation/mRNA processing/snRNPs/AAUAAA

Introduction

In animal cells, messenger RNAs (mRNAs) are formed by extensive modification of primary transcripts (Darnell, 1982; Nevins, 1983; Birnstiel *et al.*, 1985). These maturation steps include cleavage to form a new 3′ terminus, polyadenylation, splicing, base methylation and transport from the nucleus to the cytoplasm. In this report we focus on the two steps that generate the 3′ end of most mRNAs: cleavage and polyadenylation.

These two steps are of special interest because they are universal and can be regulated. The 3′ termini of all mRNAs yet examined are formed by endonucleolytic cleavage of a longer RNA, not by transcription termination; similarly, most mRNAs — perhaps all but certain histone mRNAs — are polyadenylated (Nevins, 1983; Birnstiel *et al.*, 1985). Cleavage occurs soon after transcription (Nevins and Darnell, 1978), and so defines the structure of the RNA substrate for splicing and other subsequent maturation steps. Moreover, the site of cleavage can be regulated: a single gene can produce transcripts with different mRNA 3′ termini in different cell types or at different stages of viral infection (Nevins, 1983; Leff *et al.*, 1986). Polyadenylation also can be regulated. At fertilization, for example, certain maternal mRNAs are polyadenylated, while other lose their poly(A) (Colot and Rosbash, 1982; Rosenthal *et al.*, 1983; Dworkin and Dworkin-Rastl, 1985).

At least two sequences in the pre-mRNA, the AAUAAA sequence (Proudfoot and Brownlee, 1976; Fitzgerald and Shenk, 1981; Montell *et al.*, 1983; Wickens and Stephenson, 1984; Higgs *et al.*, 1984; Zarkower *et al.*, 1986) and the downstream element (Gil and Proudfoot, 1983; McDevitt *et al.*, 1984; Sadofsky and Alwine, 1984; Woychik *et al.*, 1984; Hart *et al.*, 1985; Sadofsky *et al.*, 1985; Conway and Wickens, 1985; McLaughlin *et al.*, 1985) are required for efficient cleavage and polyadenylation. The sequence AAUAAA is located 5–30 bases before the polyadenylation site of nearly all animal cell mRNAs. It is more highly conserved (Proudfoot and Brownlee, 1976; Fitzgerald and Shenk, 1981; McLaughlin *et al.*, 1985; Berget, 1984) and more strictly required for cleavage than is the downstream element, which lies beyond the polyadenylation site (e.g. Wickens and Stephenson, 1984; Conway and Wickens, 1985).

Mutations in either sequence reduce cleavage efficiency, but all RNA that is cleaved — mutant or not — is polyadenylated. For example, point mutations in AAUAAA reduced cleavage as much as 50-fold, but of the small amount of cleaved RNA that is produced, all is polyadenylated (Montell *et al.*, 1983; Wickens and Stephenson, 1984). In contrast, polyadenylation of synthetic RNAs that end at the poly(A) site ('pre-cleaved RNAs') strictly requires AAUAAA (Manley, 1983; Manley *et al.*, 1985; Zarkower *et al.*, 1986). To reconcile these results, it has been proposed that both the cleavage enzyme and the poly(A) polymerase may be associated with a factor that recognizes AAUAAA and that both activities require the formation of a complex with AAUAAA. Thus, once the factor that recognizes AAUAAA has bound to the pre-mRNA, cleavage occurs, and polyadenylation follows immediately. In contrast, polyadenylation of pre-cleaved RNA would require AAUAAA, since the complex would have to be formed *de novo* (Manley, 1983; Zarkower *et al.*, 1986).

The factors that cleave and polyadenylate mRNAs have not yet been isolated. Although a small nuclear ribonucleoprotein particle (snRNP) may be involved (Moore and Sharp, 1984, 1985; Hashimoto and Steitz, 1986), it has not yet been identified. snRNPs clearly are critical not only for splicing, but also for the formation of histone mRNA 3′ termini (Galli *et al.*, 1983; Birchmeier *et al.*, 1984; Strub *et al.*, 1984; Birnstiel *et al.*, 1985). However, since histone mRNAs generally lack poly(A) and AAUAAA, their cleavage may be exceptional.

The analysis of interactions between snRNPs and pre-mRNAs *in vitro* has proved invaluable in studying mRNA splicing and has led to a pathway for the assembly of a functional splicing complex (Brody and Abelson, 1985; Frendewey and Keller, 1985; Grabowski *et al.*, 1985; Bindereif and Green, 1986; Padgett *et al.*, 1986). Several different snRNPs are required; each binds at or near a conserved sequence in the precursor (Mount *et al.*, 1983; Krämer *et al.*, 1984; Black *et al.*, 1985; Chabot *et al.*, 1985; Krainer and Maniatis, 1985).

In this report, we identify a complex between the highly conserved AAUAAA sequence of a viral pre-mRNA and a factor present in a crude nuclear extract. We examine the function of this complex, its stability, and its dissociation from the precursor after cleavage and polyadenylation.

Results

Hybridization of an oligonucleotide to AAUAAA prevents cleavage

If cleavage requires recognition of the AAUAAA sequence, then blocking AAUAAA by base-pairing with a synthetic oligonucleotide might prevent cleavage. To test this possibility, we prepared

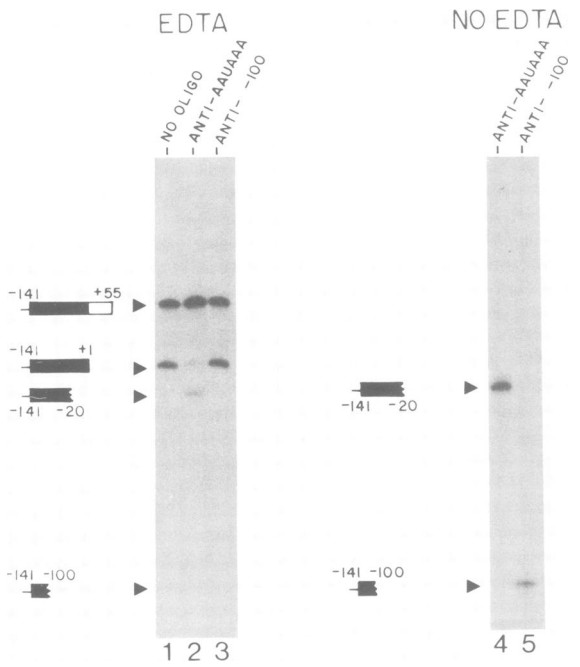

Fig. 1. Hybridization of an oligonucleotide to AAUAAA prevents cleavage. −141/+55 RNA was incubated for 1 h in nuclear extract containing 1 mM EDTA and 0.5 mM 3′ dATP (cordycepin triphosphate), either with no oligonucleotide **(lane 1)**, with anti-AAUAAA oligo **(lane 2)** or with anti-−100 oligo **(lane 3)**. Control reactions were prepared identically, but without EDTA, and contained either anti-AAUAAA oligo **(lane 4)**, or anti-−100 oligo **(lane 5)**. After the reactions, RNA was deproteinized and analyzed by gel electrophoresis.

two 16 nucleotide, single-stranded DNA molecules. The first is complementary to AAUAAA (and 10 adjacent nucleotides) ('anti-AAUAAA oligo'). The second — a control — is complementary to a region not involved in cleavage and polyadenylation, from 85 to 100 nucleotides before the cleavage site ('anti- −100 oligo'). Each oligonucleotide was added to a crude extract of HeLa cell nuclei (Dignam *et al.*, 1983), together with a synthetic SV40 late pre-mRNA containing 141 bases before the polyadenylation site and 55 bases beyond (referred to as −141/+55 RNA). The reactions contained 0.5 mM 3′ dATP (cordycepin 5′ triphosphate) to inhibit polyadenylation (Moore and Sharp, 1985) and 1 mM EDTA to inhibit endogenous RNase H. After 60 min, RNA was isolated and analyzed by polyacrylamide gel electrophoresis (Figure 1). −141/+55 RNA contains 225 nucleotides whereas cleaved RNA (−141/+1 RNA) contains only 170.

RNA incubated with the anti-AAUAAA oligo (lane 2) is cleaved less efficiently than RNA incubated either with no oligo (lane 1) or with the anti- −100 oligo (lane 3). To confirm that the oligonucleotides had annealed to the RNA, identical reactions were performed without EDTA (lanes 4 and 5). Under these conditions, RNase H is active. In the presence of either oligonucleotide, the RNA is digested by RNase H into smaller RNA fragments (lanes 4 and 5; see below), thereby demonstrating that RNA:DNA hybrids had formed. We conclude that cleavage is prevented when AAUAAA is base-paired to a DNA oligonucleotide, and suggest that a factor must bind to AAUAAA in order for cleavage to occur.

A complex forms at AAUAAA

To detect factors associated with AAUAAA, we have used the oligonucleotide/RNase H protection method of Ruskin and Green (1985), as shown in Figure 2A. Uniformly labelled −141/+55 RNA is incubated in the nuclear extract. One hour later, after processing is complete, the anti-AAUAAA oligonucleotide is added. If no factor is bound to AAUAAA, then the oligonucleotide will hybridize. As a result, the endogenous RNase H activity of the extract will digest the RNA at the AAUAAA sequence, producing two fragments. Of these, only the capped, 5′ terminal fragment (−141/−20 RNA) is stable; the 3′ terminal fragment is rapidly degraded in the extract. On the other hand, if a factor has bound stably to AAUAAA, then the RNA will be inaccessible to the oligonucleotide, and so be 'protected' from RNase H digestion. Gel electrophoresis is used to quantitate the proportion of RNA that is protected.

Figure 2B presents the results of such an experiment. −141/+55 RNA was incubated in extract containing 3′ dATP. In the absence of any oligonucleotide, 60% of the RNA is cleaved (Figure 2B, lane 1). Addition of the anti-AAUAAA oligo to this reaction mixture results in RNase H digestion of the precursor, but not of the cleaved product (lane 4). In contrast, addition of the control, anti- −100 oligo, results in the digestion of both species (lane 5). At the start of the incubation, the input −141/+55 RNA is fully accessible to both oligonucleotides (lanes 2 and 3). From these results, we conclude that during the incubation, a complex forms between the AAUAAA sequence and a factor in the extract.

An alternative explanation is that the secondary structure of the RNA changes during cleavage so as to make the AAUAAA sequence inaccessible to the oligonucleotide. This is unlikely, however, since cleaved RNA purified from one reaction, when reincubated in a second, is initially fully accessible to the anti-AAUAAA oligo (see Figure 5).

Complexes are readily detected on cleaved RNA, but not on precursor: <5% of the −141/+55 RNA remaining after a 1-h incubation is resistant to the anti-AAUAAA oligo (compare lanes 1 and 4). Our interpretation of these results, presented in detail in the Discussion, is that although the complex actually forms on the precursor, that precursor is then immediated cleaved, leaving the complex stably associated with cleaved RNA.

Complex formation requires AAUAAA

Single base changes in AAUAAA greatly reduce cleavage efficiency (Montell *et al.*, 1983; Higgs *et al.*, 1984; Wickens and Stephenson, 1984; Zarkower *et al.*, 1986). To test whether a point mutation in AAUAAA also affects formation of the complex at AAUAAA, we prepared a pre-mRNA identical to −141/+55 RNA except for a mutation to AAUACA. The mutant RNA is cleaved 5−10% as efficiently as wild-type RNA (Figure 2B, compare lanes 1 and 6). The AAUACA sequence in this precursor remains accessible after incubation (lane 7), as does its −100 sequence (lane 8). Thus the AAUACA mutation prevents both cleavage of the pre-mRNA and formation of a stable complex at AAUAAA.

Time course of complex formation

Wild-type −141/+55 RNA was incubated in extract containing 3′ dATP (Figure 3). At 10-min intervals, either the anti-AAUAAA oligo (lanes 1−6) or the anti- −100 oligo (lanes 7−12) was added and the reaction stopped 10 min later. By 10−20 min (lanes 1−3) most of the RNA is processed and has formed a complex at AAUAAA. In contrast, <5% of the RNA is protected from the anti- −100 oligo at any time (lanes 7−12). The fact that protection appears at approximately the same time as cleavage and requires a wild-type AAUAAA sequence suggests that complex formation and cleavage are related.

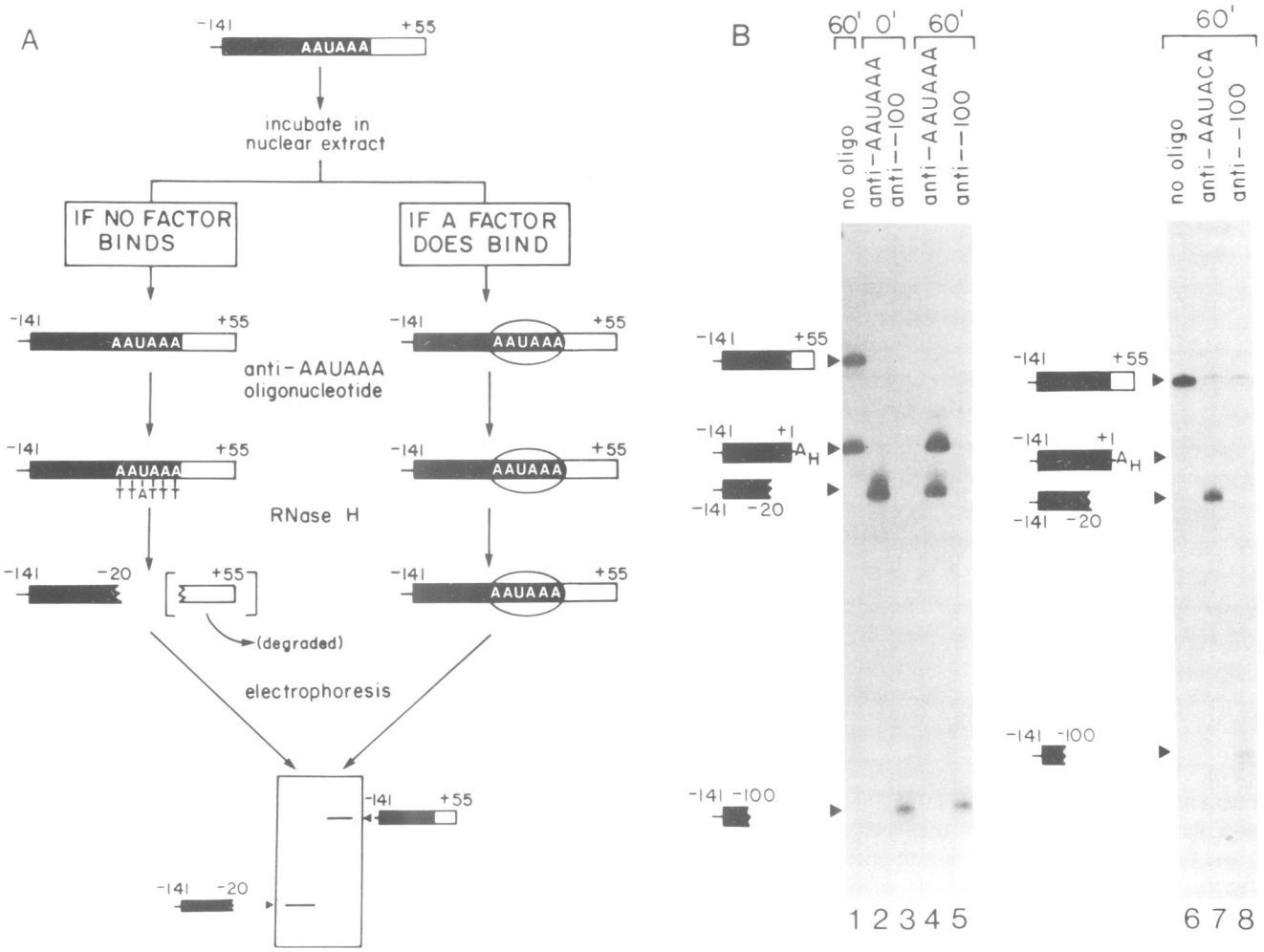

Fig. 2. A complex forms at AAUAAA. (**A**) Diagram of oligonucleotide/RNase H protection assay. (**B**) Experimental results. **Lanes 1–5:** uniformly labelled −141/+55 RNA (1 ng) was added to nuclear extract containing 0.5 mM 3′ dATP. **Lane 1:** incubation for 75 min, no oligo added. **lane 2:** anti-AAUAAA oligo added at 0 min; **lane 3:** anti- −100 oligo added at 0 min; **lane 4:** anti-AAUAAA oligo added after 60 min; **lane 5:** anti- −100 oligo added after 60 min. **Lanes 6–8:** incubation as above, except using −141/+55 RNA containing AAUACA. **Lane 6:** incubation for 75 min, no oligo added; **lane 7:** anti-AAUAAA oligo added after 60 min; **lane 8:** anti- −100 oligo added after 60 min. In all cases, incubations were continued for 15 min after addition of the oligonucleotides, then RNA was prepared. An A_H in the diagram indicates a single cordycepin residue.

Complexes are stable in 3′ dATP containing reactions

The previous experiment demonstrates that AAUAAA is inaccessible during a 10-min incubation. To examine the stability of the complex more stringently, we allowed the anti-AAUAAA oligo to remain in the extract with the complex for 2 h, rather than 10 min. If the complex dissociates at any time during that period, then the AAUAAA sequence should be digested by RNase H.

−141/+55 RNA was incubated in extract containing 3′ dATP. In a control 2-h reaction to which no oligo was added, 80% of the RNA has been processed (Figure 4, lane 1). To a parallel reaction, anti-AAUAAA oligo was added after 15 min and the incubation then continued for either 15 min (lane 2) or 2 h (lane 3). Extending the time spent in the presence of the oligonucleotide from 15 min (lane 2) to 2 h (lane 3) does not significantly decrease the fraction of RNA that is protected. This strongly suggests that, once formed, complexes are stable.

This interpretation assumes that both RNase H activity and the oligonucleotide remain throughout the 2-h incubation. To test this assumption, labelled −141/+159 RNA (lane 5) was added, after 2 h, to a reaction identical to that in lane 3. It is completely digested by RNase H in 15 min (lane 4), demonstrating that RNase H and the oligonucleotide are present.

To summarize the data presented in Figures 2–4, a complex forms at AAUAAA during cleavage *in vitro*. Formation of the complex, like cleavage, requires an intact AAUAAA sequence. Once formed in the presence of 3′ dATP, it is stable.

Pre-cleaved RNAs form complexes

'Pre-cleaved RNAs' [RNAs with 3′ termini at the polyadenylation site (+1), but without poly(A)] serve as substrates for efficient polyadenylation *in vitro*, as long as they contain an intact AAUAAA sequence (Zarkower *et al.*, 1986). In the following experiment (Figure 5), we test whether these RNAs also form complexes at AAUAAA.

Pre-cleaved RNA was prepared by incubating −141/+55 RNA in nuclear extract reactions containing EDTA to prevent polyadenylation (Moore and Sharp, 1985) and eluting the cleaved product from a polyacrylamide gel. The purified RNA was then added to nuclear extract containing 3′ dATP (Figure 5A, lane 1).

At the beginning of the reaction, the RNA is accessible both at AAUAAA and at −100 (lanes 2 and 3), as expected. After the incubation, however, most of the RNA is protected at

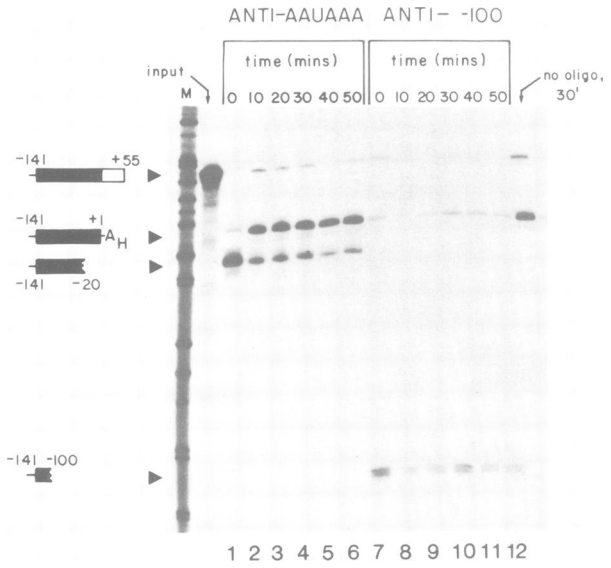

Fig. 3. Time course of complex formation. 7.5 ng −141/+55 RNA was incubated in the nuclear extract in a total volume of 175 μl. The reaction contained 0.5 mM 3′ dATP. At the times indicated above each lane, 12.5 μl of this mixture was added to anti-AAUAAA oligo **(lanes 1–6)** or anti-−100 oligo **(lanes 7–12)** and incubation continued for 10 min. Lane 'input': −141/+55 RNA; lane 'no oligo', 30'': RNA isolated after 30 min, no oligo aded; lane 'M': end-labelled *Msp*I-pBR322.

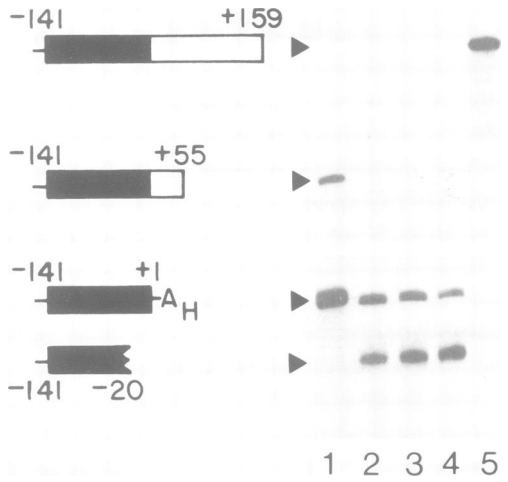

Fig. 4. Stability of complexes. −141/+55 RNA was incubated in the extract. **Lane 1:** incubation for 2 h, no oligo added. **lane 2:** anti-AAUAAA oligo added after 15 min, incubation continued for 15 min; **lane 3:** anti-AAUAAA oligo added after 15 min, incubation continued for 2 h; **lane 4:** anti-AAUAAA oligo added after 15 min, incubation continued for 2 h, then labelled −141/+159 RNA added, incubation continued for 15 min; **lane 5:** labelled −141/+159 RNA, no incubation (same amount as added to incubation in **lane 4**).

AAUAAA (lane 4). It remains accessible at −100 (lane 5). These data demonstrate that information sufficient for complex formation is contained in cleaved RNA (between positions −141 and +1) and that cleavage *per se* is not required for complex formation.

The AAUAAA sequence is required for complex formation on pre-cleaved RNA. Pre-cleaved RNA containing AAUACA, prepared as described above, was incubated in the extract. Complexes do not form on this RNA (lanes 6–10); rather, its AAUACA sequence remains accessible (lanes 7 and 9), as does its −100 sequence (lanes 8 and 10).

We conclude that complexes form with the AAUAAA sequence of pre-cleaved RNA, but only if that sequence is intact. This complex is likely to play a role in polyadenylation of pre-cleaved RNAs, since that reaction also requires AAUAAA (Manley, 1983; Manley *et al.*, 1985; Zarkower *et al.*, 1986).

A terminal cordycepin residue is required to stabilize the complex

The non-hydrolyzable ATP analog, α,β-methyleneadenosine 5′-triphosphate (AMPCPP), permits cleavage but blocks polyadenylation, presumably because it cannot be polymerized (Moore and Sharp, 1985). Surprisingly, RNA cleaved in the presence of AMPCPP does not form a stable complex at AAUAAA (not shown). Either AMPCPP prevents complex formation or a 3′ terminal 3′ dATP residue, as is added to cleaved RNA in cordycepin-containing reactions, stabilizes the complex.

To distinguish between these possibilities, two pre-cleaved RNAs were prepared which differ only in whether they have a terminal cordycepin residue. One (called −141/+1 RNA) ends at position +1 with a 3′ hydroxyl group. It was purified from a cleavage reaction carried out in the presence of EDTA. The other (called −141/+1:A_H RNA) is the same, except that, after +1, a single adenosine residue lacking a 3′ hydroxyl group (cordycepin) has been added. This RNA was isolated from a cleavage reaction containing 3′ dATP. The identity of these RNAs, including their 3′ termini, was confirmed by RNase T1 fingerprinting and by gel electrophoresis of the terminal oligonucleotides (M.Sheets and M.Wickens, in preparation).

The two pre-cleaved RNAs were incubated in the nuclear extract in the presence of AMPCPP. −141/+1 RNA does not form a complex at AAUAAA (Figure 5, lanes 1–3); −141/+1:A_H RNA does (lanes 4–6).

We conclude that a terminal 3′ deoxyadenosine residue prevents dissociation of the complex formed at AAUAAA. This may reflect a stable interaction of cordycepin-terminated RNA with the presumed poly(A) polymerase.

Competition for cleavage factors

In 3′ dATP-containing reactions, since complexes are stable, any RNA that can form complexes should be able to compete for cleavage. To test this prediction, increasing amounts of different unlabelled RNAs were mixed with a trace amount of labelled −141/+55 RNA. These mixtures were incubated in the extract with 3′ dATP, and cleavage of the labelled RNA analyzed by electrophoresis. Representative results are shown in Figure 6A and illustrated in Figure 6B and C. The structure of each competitor is drawn in Figure 6B and C.

−141/+55 RNA competes efficiently, demonstrating that cleavage is saturable by substrate. Six picomoles of unlabelled −141/+55 RNA reduces cleavage of 6 fmol of labelled −141/+55 RNA more than 100-fold (Figure 6A, B). As controls, neither a −141/−20 RNA (which lacks the AAUAAA sequence and downstream element), nor an RNA which contains 45 bases of prokaryotic sequences (designated 'prokaryotic control' in Figure 6A) competes significantly, even at the highest concentrations tested (Figure 6A and B).

Mutations that prevent complex formation also prevent competition, as shown by the following two observations. First, −141/+55 RNA containing AAUACA is an inefficient competitor: 6 pmol of the mutant RNA achieve the same extent of inhibition as 0.35 pmol of wild-type RNA. Second, pre-cleaved RNA competes efficiently if and only if it contains both an intact AAUAAA and a nearby 3′ end (Figure 6C). Cleavage is reduced 98% by 5 pmol of −141/+1 RNA, but only 15% by 5 pmol of the same RNA containing AACAAA, not AAUAAA. Similarly, a −141/+1 RNA to which an additional 222 bases of pro-

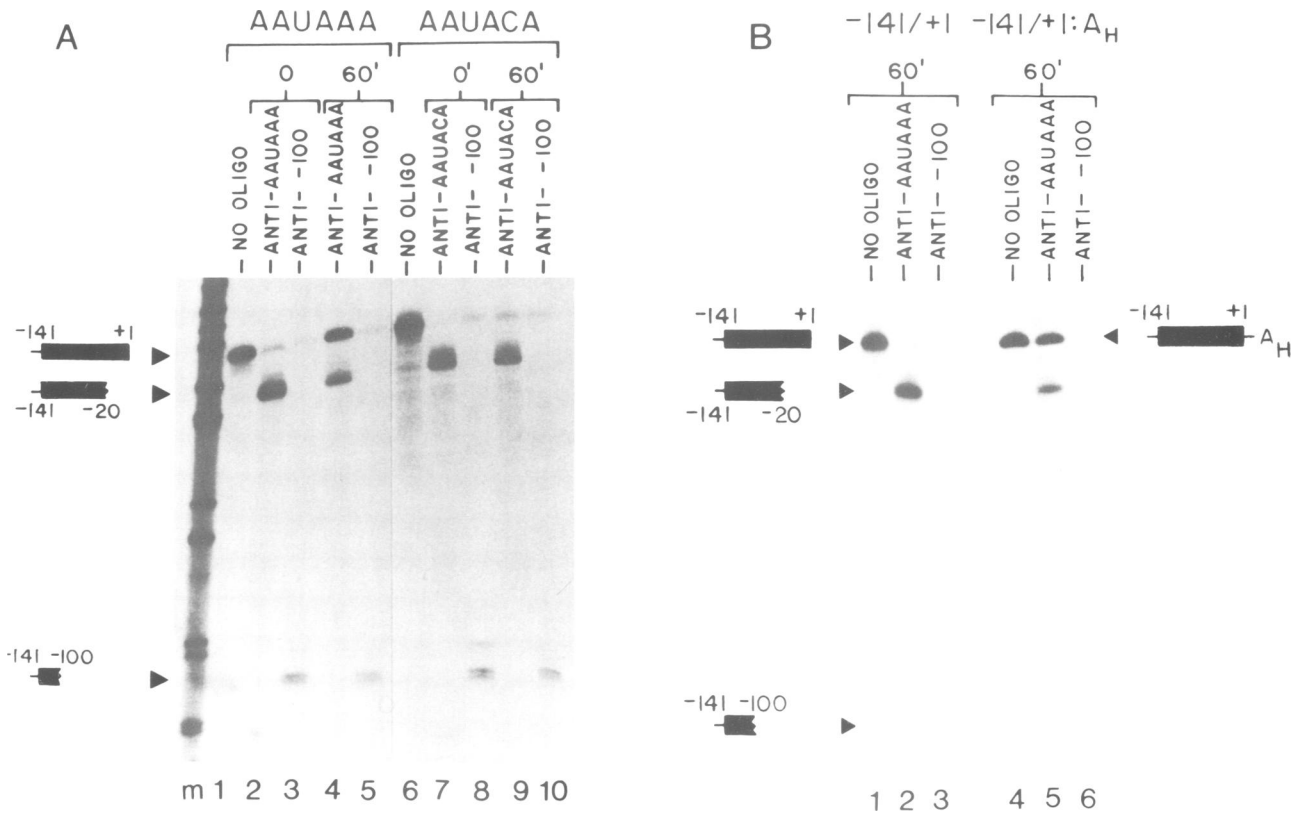

Fig. 5. Pre-cleaved RNAs form complexes, and a terminal cordycepin is required to stabilize the complex. **(A)** Pre-cleaved RNAs form complexes. −141/+1 RNAs, containing either AAUAAA **(lanes 1–5)** or AAUACA **(lanes 6–10)**, were purified from reactions containing EDTA and incubated in extract containing 0.5 mM 3′ dATP. Under these conditions, a single cordycepin residue is added to the 3′ end of the wild-type −141/+1 RNA, blocking the addition of poly(A). Mutant −141/+55 RNA is cleaved inefficiently (Figure 2; Zarkower *et al.*, 1986) but yields enough product to make this experiment possible. **Lanes 1** and **6:** incubation for 75 min, no oligo added; **lanes 2** and **7:** anti-AAUAAA oligo at 0 min. **lanes 3** and **8:** anti- −100 oligo added at 0 min; **lanes 4** and **9:** anti-AAUAAA oligo added after 60 min; **lanes 5** and **10:** anti- −100 oligo added after 60 min. **(B)** A terminal cordycepin is required to stabilize the complex. −141/+1 RNA **(lanes 1–3)** or −141/+1:A_H RNA **(lanes 4–6)**, prepared from EDTA- or 3′ dATP-containing reactions, was incubated in extract containing 4 mM AMPCPP. This ATP analog inhibits polyadenylation (Moore and Sharp, 1985). After 60 min, the indicated oligonucleotides were added. **Lanes 1** and **4:** no oligo added; **lanes 2** and **5:** anti-AAUAAA oligo added; **lanes 3** and **6:** anti- −100 oligo added. In all cases, incubation was continued for 10 min after the oligonucleotide was added.

karyotic sequence has been added competes less efficiently than −141/+1 RNA. It also is cleaved inefficiently and forms few complexes (D.Zarkower and M.Wickens, in preparation).

In summary, the competition experiments (Figure 6) permit the following conclusions. To compete efficiently, an RNA must contain AAUAAA and a nearby 3′ terminus. The terminus can be generated by cleavage during the incubation (as with −141/+55 RNA), or by transcription *in vitro* (as with synthetic −141/+1 RNA). Importantly, −141/+1 RNA [which is a substrate for polyadenylation, but not for cleavage (Zarkower *et al.*, 1986)] competes efficiently for cleavage. This suggests that the same factor recognizes AAUAAA in both reactions. The data are consistent with, but do not prove, the existence of stable complexes.

Polyadenylated RNA does not form complexes

To determine whether the complex at AAUAAA is present after polyadenylation, −141/+55 RNA was incubated in the nuclear extract without 3′ dATP. Under these conditions, the RNA is cleaved and then polyadenylated, giving rise to a collection of products of heterogeneous size, ~200 bases longer than cleaved RNA (Figure 7, lane 1). The polyadenylated RNA is fully accessible to the anti-AAUAAA oligo (lane 4), as is the precursor at the start of the reaction (lane 2). In contrast, in a parallel control experiment to which 3′ dATP was added, 65% of the cleaved RNA was protected (not shown).

In principle, lack of protection of polyadenylated RNA could be due to the depletion of some extract component in the absence of 3′ dATP. This is not the case, however: pre-cleaved RNA carrying a 3′ terminal cordycepin residue (i.e. −141/+1:A_H RNA), which cannot be extended into poly(A), becomes protected at AAUAAA during incubation under polyadenylation conditions (Figure 7, lanes 6–8). We conclude that complexes do not remain after polyadenylation.

Polyadenylated RNA cannot form a complex *de novo*. Cleaved and polyadenylated RNA containing ~100 A residues was prepared by incubating −141/+55 RNA in the nuclear extract and eluting the polyadenylated product from a polyacrylamide gel. The purified, polyadenylated RNA was incubated in extract containing 3′ dATP (Figure 7, lanes 9–11). As a control, a small amount of pre-cleaved RNA was added to the same reaction. After incubation, the polyadenylated RNA is not protected at AAUAAA (lane 10) or at −100 (lane 11). This inhibition of complex formation is not specific for poly(A): vector sequences added onto a −141/+1 RNA also inhibit complex formation (not shown).

Cleavage factors recycle

Complexes dissociate by the time polyadenylation is complete (Figure 7). After processing factors dissociate from one precursor, can they form a complex on another, and then catalyze a second round of cleavage? The following experiment (Figure 8) addresses this question.

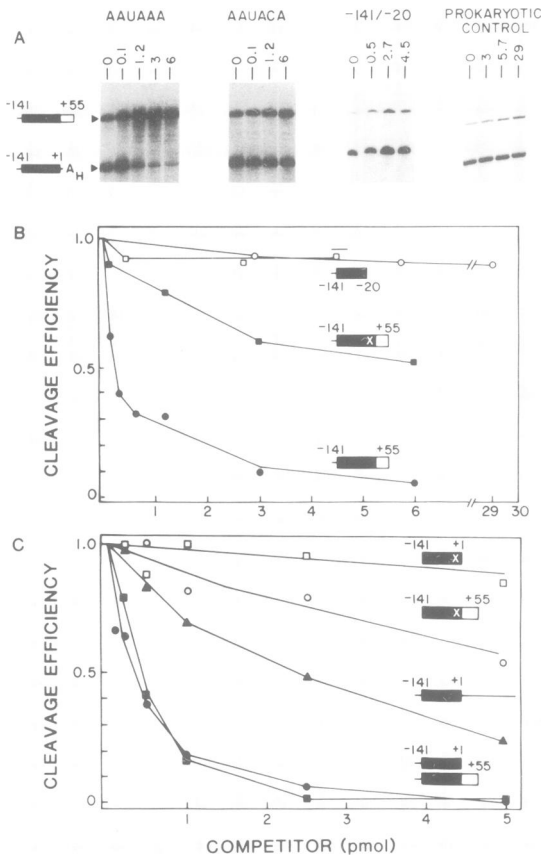

Fig. 6. Competition for cleavage factors. **(A)** Labelled −141/+55 RNA (6 fmol; 0.5 ng) was mixed with increasing amounts (indicated above each lane) of unlabelled −141/+55 RNA ('AAUAAA'), −141/+55 RNA with a mutation to AAUACA ('AAUACA'), −141/−20 RNA, or a 45-base long RNA containing only vector-derived sequences ('prokaryotic control'). These RNA mixtures were incubated in extract containing 3′ dATP for 60 min. RNA was then prepared and cleavage quantitated by gel electrophoresis. **(B,C)** Cleavage efficiency of labelled −141/+55 RNA versus added unlabelled competitor RNA. Reactions were performed as described in **(A)**. Each contained 6 fmol of labelled RNA and varying amounts of different competitor RNAs, as indicated on the abscissa. The structure of each RNA is illustrated and discussed in the text; a white X in the diagram indicates a mutation to AACAAA. The synthesis, quantitation and detailed structure of the competitor RNAs is described in Materials and methods. Note that the −141/+1 RNA used here was transcribed *in vitro*, not purified from processing reactions.

The protocol consists of two sequential incubations (Figure 8A). In the first, unlabelled competitor RNA is incubated with extract, either with or without 3′ dATP. In the second, labelled RNA is added and both reactions receive 3′ dATP to block polyadenylation. Cleavage of the labelled RNA is assayed by electrophoresis, and is compared with that observed when no competitor is added.

When the first incubation contains 3′ dATP, stable complexes should form and sequester the cleavage activity, preventing cleavage of the labelled RNA during the second incubation. Conversely, when the first incubation lacks 3′ dATP, polyadenylation will occur, complexes will dissociate, and processing components should be released. If, after their release, they can catalyze a second reaction, then the labelled RNA added in the incubation will be cleaved.

The results of such an experiment are presented in Figure 8B. Pre-incubating the extract without any competitor RNA only slightly reduces cleavage efficiency relative to a standard reaction with no pre-incubation [lanes 1 (65%) and 2 (60%) versus the standard, lane 6 (95%)]. This is true whether 3′ dATP is present (lane 1) or not (lane 2). In contrast, if the first incubation contains 3′ dATP and −141/+55 RNA, then cleavage during the second incubation is reduced an additional 4-fold, to 15% (lane 3). This inhibition is not observed if the competitor RNA carries an AAUAAA point mutation (lane 4). Most importantly, it also is not observed if 3′ dATP is omitted from the first incubation (lane 5); rather, cleavage of the labelled RNA is just as efficient as it is when no competitor RNA is added at all (lane 2).

Taken together, these results demonstrate that dissociation of the complex enables processing components to participate in a second round of cleavage. Presumably, this second round of cleavage involves a second round of complex formation as well.

Summary of results and a working model

During mRNA 3′ end formation, a complex forms between the highly conserved AAUAAA sequence and a factor present in a crude nuclear extract. The complex can form either during cleavage of an mRNA precursor, or on a purified cleaved RNA. In both cases, a wild-type AAUAAA sequence is necessary. The complex is stable on cleaved RNAs to which a single 3′ terminal cordycepin residue has been added. It is not detected after polyadenylation, nor can purified, polyadenylated RNA form a complex *de novo*. Dissociation of the complex after or during polyadenylation enables cleavage of a second precursor to occur.

Based on these findings and on recently published work (Manley *et al.*, 1985; Hashimoto and Steitz, 1986; Zarkower *et al.*, 1986), we propose a working model of cleavage and polyadenylation. Its strengths and limitations will be considered in the Discussion. (i) The factor that recognizes AAUAAA is physically associated with, and may itself possess, poly(A) polymerase activity. It may also be stably associated with the endonucleolytic activity, though this is unproven. (ii) The factor that recognizes AAUAAA interacts with the mRNA precursor prior to cleavage, forming a complex. This interaction requires AAUAAA and the downstream element. (iii) Cleavage occurs rapidly after complex formation. (iv) Under physiological conditions, the complex dissociates either during or after polyadenylation. Dissociation is prevented by a terminal 3′ deoxyadenosine. (v) Dissociation of the complex from one pre-mRNA enables the processing components that are released to form a complex with another pre-mRNA, beginning a second cycle of cleavage and polyadenylation.

Discussion

The fundamental conclusion that a complex forms at AAUAAA is based on oligonucleotide/RNase H protection assays (Figure 2A; Ruskin and Green, 1985): we interpret protection of AAUAAA from RNase H digestion to be a result of a complex formed between AAUAAA and a factor required for processing. Six control experiments support this interpretation and eliminate possible artefacts in the assay. First, protection is not an inherent property of the precursor; rather, it is acquired only after incubation in the extract (Figures 2−5). Second, protection is sequence specific; a region of the precursor not involved in processing (−100 to −85) is not protected (Figures 2−5,7). Third, protection is prevented by mutations in AAUAAA, as is cleavage (Figure 2). Fourth, protection is not due to non-specific binding of a factor to RNA 3′ termini, since pre-cleaved RNAs form complexes only if they contain a wild-type AAUAAA sequence (Figure 5). Fifth, although we routinely assay protection in 3′ dATP-containing reactions (Figures 2−7), protection is

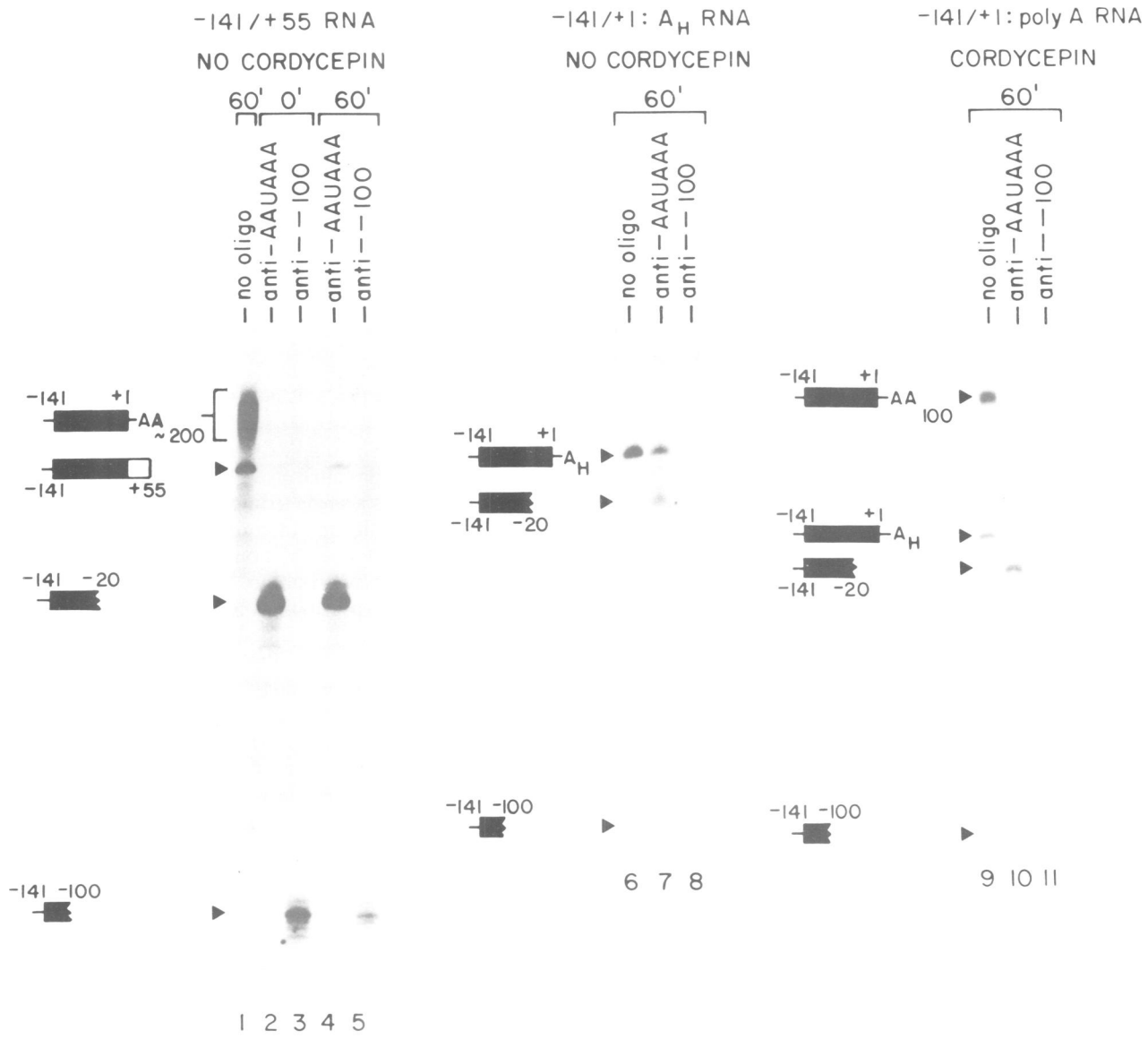

Fig. 7. Polyadenylated RNA does not form complexes. Labelled RNAs were added to extract with or without 3′ dATP, as indicated. **Lanes 1–5:** −141/+55 RNA, no 3′ dATP. **Lane 1:** incubation for 70 min, no oligo added; **lane 2:** anti-AAUAAA oligo added at 0 min; **lane 3:** anti- −100 oligo added at 0 min; **lane 4:** anti-AAUAAA oligo added after 60 min; **lane 5:** anti- −100 oligo added after 60 min. **Lanes 6–8:** −141/+1:A_H RNA, no 3′ dATP. **Lane 6:** incubation for 70 min, no oligo added; **lane 7:** anti-AAUAAA oligo added after 60 min; **lane 8:** anti- −100 oligo added after 60 min. **Lanes 9–11:** −141/+1:poly(A) RNA (mixed with a small amount of −141/+1:A_H RNA), 3′ dATP added. **Lane 9:** incubation for 70 min, no oligo added; **lane 10:** anti-AAUAAA oligo added after 60 min; **lane 11:** anti- −100 oligo added after 60 min.

not due to inhibition of some extract component by this ATP analog: a suitable RNA forms complexes in AMPCPP (Figure 5), in AMPPCP (not shown), and without any analog (Figure 7). Finally, protection is probably not due to a change in RNA secondary structure after cleavage, since purified, cleaved RNA is not inherently resistant (Figure 5).

Competition experiments demonstrate that cleavage is saturable by substrate (Figure 6). More importantly, without exception, the effectiveness of an RNA as a competitor parallels the efficiency with which that RNA forms a complex at AAUAAA. For example, RNAs carrying AAUAAA mutations or lacking a downstream element are cleaved inefficiently, form few complexes, and compete poorly (Figures 2, 5, 6). Similarly, after an RNA has been polyadenylated, it forms no complex (Figure 7) and competes less effectively than it did before polyadenylation (not shown).

Because an RNA's ability to form a stable complex is always correlated with its ability to compete, we infer that competition is due to titration of the complex, and that complex formation is required for cleavage. Although this interpretation — which we favor — is simple and is consistent with all the data, we cannot prove that it is the complex that is titrated by excess RNA. In principle, formation of complexes at AAUAAA might stoichiometrically inactivate some other extract component that is required for cleavage. This alternative explanation requires that inactivation of the hypothetical component occur during formation of the complex (as opposed to cleavage *per se*), since pre-cleaved RNA competes for cleavage.

We propose that complexes initially form on the precursor, before cleavage, for the following reasons. The AAUAAA sequence of the precursor must be recognized, since cleavage is blocked by AAUAAA mutants and by an anti-AAUAAA oligo-

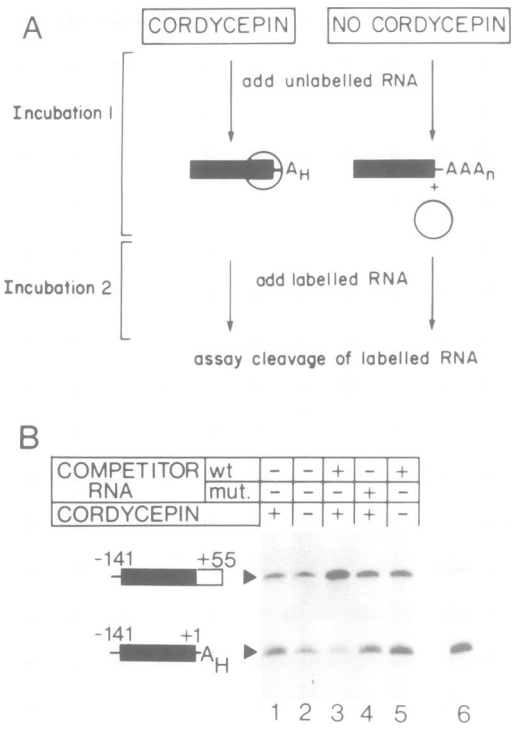

Fig. 8. Cleavage factors recycle. **(A)** Diagram of experimental protocol. 0.5 mM 3′ dATP is present during the second incubation, whether or not the first incubation contained 3′ dATP. **(B)** Unlabelled RNA (0.3 pmol) was added to extract either with or without 0.5 mM 3′ dATP, and incubated for 45 min. The components present during this first incubation are given above each lane. After the first incubation, labelled −141/+55 RNA (6 fmol) was added to the same reaction mixes, and 3′ dATP was added to adjust all reactions to 0.5 mM. Incubation was then continued for 45 min. Cleavage of the labelled RNA was assayed by gel electrophoresis and autoradiography. First incubation contained: **lane 1**, no RNA, 3′ dATP present; **lane 2**, no RNA, no 3′ dATP; **lane 3**, −141/+55 RNA, 3′ dATP present; **lane 4**, −141/+55 RNA with AACAAA mutation, 3′ dATP present; **lane 5**, −141/+55 RNA, no 3′ dATP. In **lane 6** only a single, standard incubation was performed, using 1 ng labelled −141/+55 RNA and 0.5 mM 3′ dATP. The slight variation in band intensity is due to differential loss during RNA isolation, not to differential stability in the extract.

nucleotide. Furthermore, a small fraction of precursor is reproducibly protected in the presence or absence of 3′ dATP (for example, see Figures 3 or 7). Similarly, Hashimoto and Steitz (1986) have recently detected a complex which protects both the AAUAAA sequence and downstream element of the SV40 late mRNA precursor from ribonuclease T1 digestion. Presumably, in both their experiments and ours, these protected precursors have formed a complex but have not yet undergone cleavage. From the fact that such precursor complexes are rare, we deduce that cleavage occurs quickly once the complex has formed. Indeed, the fraction of precursor protected appears highest when cleavage activity is maximal, from ~10 to 20 min after the start of incubation (Figure 3).

Proof that the complex forms initially on the precursor might be obtained using conditions in which complexes form, but cleavage is blocked. Such conditions have not yet been found: the ATP analog AMPCPP permits both cleavage and complex formation (Figure 5), while a different ATP analog, AMPPCP, or heat treatment, prevents both (D.Zarkower, not shown).

The same component recognizes AAUAAA in cleavage and in polyadenylation. We draw this conclusion because pre-cleaved RNA — which is a substrate only for polyadenylation, not for cleavage (Zarkower *et al.*, 1986) — competes for cleavage (Figure 6). It is therefore likely that, under physiological conditions, the complex first forms before cleavage has occurred and remains until after polyadenylation has begun. Precisely when the complex dissociates is not yet clear, but presumably this occurs after the addition of one (Figure 5) but before the addition of 100 (Figure 7) A residues to the cleaved RNA.

The complex is required for cleavage, as inferred from competition experiments (Figure 6), and is also likely to be required for polyadenylation, since polyadenylation and complex formation exhibit the same requirements; namely, an intact AAUAAA (Figures 2, 5 and 6; Manley, 1983; Zarkower *et al.*, 1986) and a 3′ terminus near AAUAAA (Figure 6; Manley, 1983). Although the proximal 3′ end normally is generated during the cleavage reaction, cleavage *per se* is not necessary to form a complex — purified, cleaved RNAs do so efficiently.

To form a stable complex, the RNA must contain not only AAUAAA and a proximal end, but also a 3′ terminal cordycepin residue. How does a single terminal cordycepin stabilize the complex? The critical feature of cordycepin-terminated RNA could be either its having one more nucleotide than cleaved RNA, or its lack of a 3′ hydroxyl group. It should be possible to decide between these two possibilities by preparing pre-cleaved RNAs that have been suitably modified at their 3′ ends. In either case, the terminal cordycepin may trap the complex by stabilizing a normally transient interaction between the poly(A) polymerase and its substrate.

After polyadenylation, few or no complexes persist; similarly, polyadenylated RNA cannot form a complex *de novo* (Figure 7). The inhibitory effect of a 3′ terminal poly(A) tract is not specific for poly(A), since a prokaryotic sequence also inhibits (Figure 6C, and D.Zarkower, unpublished). We have not yet determined the minimum length necessary for inhibition.

After dissociation of the complex, the released components can participate in cleavage of a second pre-mRNA. Pre-incubation of the extract with excess RNA substrate prevents cleavage of an RNA added later only if the pre-incubation contains 3′ dATP; if 3′ dATP is omitted, polyadenylation occurs, and inhibition is abolished (Figure 8). Our interpretation of these results — that processing components present in the complex recycle — depends on competition being due to titration of the component that recognizes AAUAAA.

A snRNP may form the complex with AAUAAA, since anti-Sm antibodies prevent cleavage (Moore and Sharp, 1984, 1985) and precipitate the region of the precursor containing AAUAAA (Hashimoto and Steitz, 1986). The results presented here suggest that if a snRNP is involved, it must participate in more than one cleavage event. It is reasonable to expect that such recycling also occurs during splicing. However, after splicing *in vitro*, snRNPs only gradually dissociate from excised introns (Brody and Abelson, 1985; Frendewey and Keller, 1985; Grabowski *et al.*, 1985; Bindereif and Green, 1986), indicating that lariat formation and intron excision are not sufficient for rapid disassembly. Perhaps cleavage and polyadenylation will provide a simpler system with which to examine the formation and dissociation of complexes between mRNA precursors and processing components.

The data we have presented establish that a complex forms at AAUAAA, and that it plays a critical role in cleavage and polyadenylation. Ultimately, to understand mRNA 3′ end formation, the enzymes and structural components that participate must be purified. The description of conditions which stabilize the complex, as reported here, may facilitate such purifications.

Materials and methods

Plasmid construction and the synthesis of RNA substrates

The region of SV40 from 141 bases before to 159 bases past the late region polyadenylation site (from the natural *Bam*HI site to a synthetic *Hin*dIII site) was cloned into pSP65 (Melton *et al.*, 1984), generating pSP −141/+159. This template was cleaved with *Dra*I 55 bases past the polyadenylation site, and transcribed with SP6 polymerase to produce −141/+55 RNA. −141/+55 RNA contains 29 bases of vector-derived sequence followed by 196 bases of SV40 sequence.

To generate the AAUACA mutation, oligodeoxynucleotide-directed mutagenesis was performed as described by Bauer *et al.* (1985). Mutants were confirmed by dideoxynucleotide sequencing (Sanger *et al.*, 1977). The mutant template, pSP −141/+70:AAUACA, contains a *Bam*HI/*Hin*dIII fragment of SV40, from 141 bases before the polyadenylation site to 70 bases after. Transcription of this template after cleavage with *Dra*I yields −141/+55 RNA containing AAUACA rather than AAUAAA.

To prepare substrates for RNA processing, SP6 polymerase was incubated with cleaved DNA in the presence of [^{32}P]UTP and GpppG (Melton *et al.*, 1984; Konarska *et al.*, 1984). Full length transcripts were eluted from a polyacrylamide gel slice by soaking overnight in 0.5 M ammonium acetate, 0.1% SDS, 0.5 mM EDTA at 37°C. The eluate, contaning 50−95% of the total radioactivity, was centrifuged at 13 000 r.p.m. to remove debris and precipitated twice with ethanol.

Preparation of nuclear extract and processing in vitro

Nuclear extract was prepared from HeLa cells by the method of Dignam *et al.* (1983). Standard reactions contained 11 μl nuclear extract, 40 mM KCl, 20 mM phosphocreatine (Sigma), 0.1 mM ATP, 2.8% polyvinyl alcohol and 0.5−1 ng of labelled pre-mRNA in a total volume of 25 μl. Reactions were incubated at 30°C. Certain reactions, as indicated in the text, received either 0.5 mM cordycepin triphosphate (Sigma) or 4 mM AMPCPP (Sigma) or 4 mM AMPPCP (Sigma), or 0.5 mM EDTA. The ATP analogs were added to reactions that still contained 0.1 mM ATP.

RNA was isolated by addition of 6 volumes of 100 mM Tris−HCl (pH 7.9), 12.5 mM EDTA, 150 mM NaCl and 1% SDS (Konarska *et al.*, 1984), phenol/$CHCl_3$ extraction and ethanol precipitation. Electrophoresis was performed through a 6% polyacrylamide, 7 M urea sequencing gel (Sanger and Coulson, 1978). Gels were dried and exposed to X-ray film for 1−4 days. For quantitation, autoradiograms were scanned using a laser microdensitometer (Ricoh). Several different exposures of each autoradiogram were used to ensure accuracy.

Oligonucleotide/RNase H protection assay

Synthetic DNA oligonucleotides were added to nuclear extract reactions at the times indicated in the text. (The sequences of the DNA oligonucleotides are: anti-AAUAAA oligo, 3′GACGTTATTTGTTCAA5′; anti-AAUACA oligo, 3′GACGTTATGTGTTCAA5′; anti- −100/−85 oligo, 3′TTGATCTTACGTCACT5′. Regions complementary to AAUAAA or AAUACA are underlined.) Thirty-five nanograms of oligonucleotide was added; this represents a 500-fold molar excess over the RNA substrate. In some experiments, 1 unit of RNase H (P/L) and 3 mM $MgCl_2$ were added together with the oligo. RNase H digestion of DNA/RNA hybrids is complete in 5 min (not shown). Degradation of the non-capped 3′ fragments requires 5 min more (not shown). Therefore, incubations were continued for 10 min after adding oligos. For zero time points, oligonucleotides and RNA were added simultaneously to extract and incubated for 10 min.

Competition experiments

−141/+1 RNAs for use as competitors (Figure 6) were transcribed from pSPSVL: AAUAAA or pSPSVL:AACAAA (Wickens and Stephenson, 1984), linearized with *Hin*dIII. These templates contain *Eco*RI and *Hin*dIII synthetic linker sequences on either side of AAUAAA. −141/−20 RNA was transcribed from pSPSVL: AAUAAA linearized at the *Eco*RI site before AAUAAA. The 'prokaryotic control' RNA was transcribed from a pSP65 derivative containing a 26-bp *Xba*I/*Pst*I insert of bacteriophage M13 sequence and linearized at the *Pst*I site. The −141/+1 RNA with 222 bases of prokaryotic sequence was transcribed from pSV −141/+1:X. This template contains SV40 sequence from −141 to +1 with a synthetic *Xba*I linker inserted at +1, followed by pBR322 sequences from 29 to 3609. This template was linearized at its *Ssp*I site (position 4170 of pBR322).

To quantitate the mass of competitor RNA, transcripts were synthesized in the presence of [^{3}H]UTP. Full-length transcripts were eluted from a polyacrylamide gel after u.v.-shadowing. Aliquots of the reaction mix and of the purified RNA were counted in a liquid scintillation counter. From the specific radioactivity of UTP, the mass of purified RNA was calculated. In any one set of competition experiments (Figure 6B, or Figure 8), all competitor RNAs were prepared from a single reaction mix containing [^{3}H]UTP, ensuring that the relative concentrations of each RNA are accurate, even if there are small errors in the absolute mass.

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