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Cleavage and polyadenylation of messenger RNA precursors *in vitro* occurs within large and specific 3′ processing complexes

Tim Humphrey[1], Gerhard Christofori[2], Vlatka Lucijanic and Walter Keller[2]

Division of Molecular Biology, Institute of Cell and Tumor Biology, German Cancer Research Center, D-6900 Heidelberg, FRG

[1]Present address: Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK
[2]Present address: Department of Cell Biology, Biocenter of the University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

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We have investigated the assembly of complexes associated with *in vitro* cleavage and polyadenylation of synthetic pre-mRNAs by native gel electrophoresis. Incubation of SP6-generated pre-mRNA containing the adenovirus L3 polyadenylation site in HeLa cell nuclear extract results in the rapid assembly of specific complexes. Formation of these complexes precedes the appearance of cleaved intermediates and polyadenylated products and is dependent on an intact polyadenylation signal within the pre-mRNA. The specific complexes do not form on RNAs with point mutations in the AAUAAA sequence upstream of the L3 polyadenylation site. Furthermore, such mutant RNAs cannot compete for factors involved in the assembly of specific complexes on wild-type pre-mRNA. Upon complex formation a 67-nucleotide region of the L3 pre-mRNA is protected from RNase T1 digestion. This region contains both the upstream AAUAAA signal and the GU-rich downstream sequences. Cleavage and polyadenylation occur within the specific complexes and the processed RNA is subsequently released. We propose that the assembly of specific complexes represents an essential step during pre-mRNA 3′ end formation *in vitro*.

Key words: adenovirus/mRNA 3′ end formation/RNA processing/HeLa cell nuclear extract

Introduction

Polyadenylation of the primary transcript is required for the maturation of most eukaryotic mRNAs, and the poly(A) tail is thought to confer stability on the RNA molecule (reviewed by Birnstiel *et al.*, 1985; Proudfoot and Whitelaw, 1987). Furthermore, the polyadenylation process may play a regulatory role in gene expression since the utilization of different polyadenylation sites within a gene can result in the production of alternative forms of protein (Alt *et al.*, 1980; Early *et al.*, 1980; Rogers *et al.*, 1980; Amara *et al.*, 1982). In most eukaryotic genes studied so far, RNA polymerase II transcription terminates at an ill-defined region downstream of the polyadenylation site. Processing signals on the primary transcript specify removal of these downstream sequences by precise endonucleolytic cleavage at the polyadenylation site and subsequent addition of poly(A) sequences to the newly formed 3′ terminus. Histone pre-mRNAs are usually not polyadenylated, although their 3′ termini are also generated by endonucleolytic cleavage of the RNA precursor (Birnstiel *et al.*, 1985; Gick *et al.*, 1986).

Two sequence elements define the site of cleavage and polyadenylation on the pre-mRNA. A highly conserved AAUAAA signal which is located 10–30 nucleotides upstream of the poly(A) site (Proudfoot and Brownlee, 1976; Fitzgerald and Shenk, 1981; Higgs *et al.*, 1983; Montell *et al.*, 1983; Wickens and Stephenson, 1984; Moore *et al.*, 1986; Zarkower *et al.*, 1986), together with GU-rich sequences downstream of the cleavage site (Gil and Proudfoot, 1983; McDevitt *et al.*, 1984; Sadofsky and Alwine, 1984; Woychik *et al.*, 1984; Conway and Wickens, 1985; Hart *et al.*, 1985; McLauchlan *et al.*, 1985; Sadofsky *et al.*, 1985; Sperry and Berget, 1986; Gil and Proudfoot, 1987) are crucial for accurate and efficient 3′ end formation of pre-mRNA both *in vivo* and *in vitro*.

The biochemistry of the cleavage and polyadenylation reaction is not yet understood since the components involved have not been identified. Histone pre-mRNA 3′ end formation is known to be dependent on the presence of minor, small nuclear ribonucleoprotein particles (snRNPs) both *in vivo* and *in vitro* (Strub *et al.*, 1984; Gick *et al.*, 1986; Strub and Birnstiel, 1986; Mowry and Steitz, 1987).

During pre-mRNA splicing, the five major nucleoplasmic snRNPs, U1, U2, U4, U5 and U6, are essential for both the assembly of multicomponent splicing complexes and for the removal of introns from the pre-mRNA (for reviews see Green, 1986; Padgett *et al.*, 1986; Maniatis and Reed, 1987; Sharp, 1987). The involvement of snRNPs in pre-mRNA cleavage and polyadenylation has been suggested (Moore and Sharp, 1984, 1985; Sperry and Berget, 1986). Furthermore, a minor snRNP is thought to bind to the AAUAAA sequence upstream of the polyadenylation site during the 3′ processing reaction *in vitro* as determined by immunoprecipitation with snRNP-specific antibodies (Hashimoto and Steitz, 1986). However, this snRNP has not yet been identified. By analogy to the splicing reaction, it is conceivable that interaction of components of the HeLa cell nuclear extract with a pre-mRNA substrate requires the assembly of a specific complex prior to cleavage and polyadenylation.

The results presented here demonstrate that cleavage and polyadenylation of pre-mRNA *in vitro* is preceded by the assembly of specific complexes. This requires an intact AAUAAA sequence on the pre-mRNA. Formation of the complexes involves the binding of components in the nuclear extract to a specific region of the pre-mRNA and leads to the protection from RNase T1 digestion of a sequence that includes the AAUAAA signal and the GU-rich sequence downstream of the poly(A) site.

Results

A series of model pre-mRNA substrates, illustrated in Figure 1, were used to study the early events in 3′ end formation of pre-mRNA. Purified precursor RNA was incubated in HeLa cell nuclear extract (Dignam *et al.*, 1983) under polyadenylation conditions (see Materials and methods). To analyze complex formation, an aliquot was taken from the reaction mixture, incubated with heparin and then directly electrophoresed on a 4% native

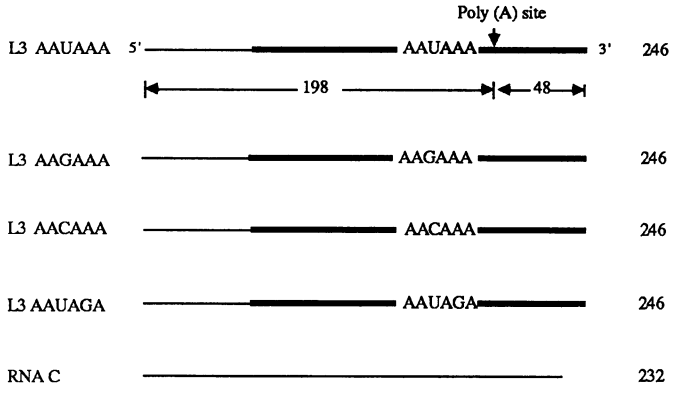

Fig. 1. Schematic representation of the pre-mRNAs used in this study. An *AluI* fragment containing the adenovirus polyadenylation site (indicated by a thick line) was inserted into pSP64 (indicated by a thin line) to give pSP6 L3. Plasmids pSP6 L3 AAGAAA, pSP6 L3 AACAAA and pSP6 L3 AAUAGA were generated by site directed mutagenesis within the AAUAAA sequence (see Materials and methods for details). Plasmid pSP6 L3 and mutants thereof were truncated with restriction endonuclease, *DraI*, resulting in run off transcripts of 246 nt in length. RNA C is a pre-mRNA produced from pSP64 cleaved with *PvuII* resulting in run off transcripts of 232 nt. The thin lines and the numbers below RNA L3 AAUAAA indicate the length of the upstream and the downstream fragments produced by cleavage at the poly(A) site. Numbers at the 3′ end of the RNAs give the total length in nucleotides. Poly(A) site, polyadenylation site.

polyacrylamide gel. Cleavage and polyadenylation were analyzed by electrophoresis of RNA species extracted from the reaction mixture on denaturing polyacrylamide gels. The pre-mRNA was correctly cleaved prior to polyadenylation (data not shown). Accurate cleavage without polyadenylation was assayed by substituting 2′,3′-dideoxyadenosinetriphosphate (ddATP) for ATP in the standard reaction. Incorporation of ddATP at the cleaved 3′ end prevents further polyadenylation (Moore and Sharp, 1985) and yields a 'cleaved' RNA of 199 nucleotides (nt). Under both cleavage and polyadenylation conditions, the 48 nt downstream fragment is rapidly degraded.

Incubation of L3 pre-mRNA with nuclear extract results in complex formation

Multiple factors within HeLa cell nuclear extract have been shown to interact with intron-containing pre-mRNAs *in vitro*, resulting in a stepwise assembly of splicing complexes followed by intron excision (Frendewey and Keller, 1985; Grabowski *et al.*, 1985). We investigated the possibility that 3′ end formation of pre-mRNA is also accompanied by the assembly of specific RNA complexes *in vitro*. Pre-mRNA was incubated for various times to determine whether it is assembled into specific complexes prior to the appearance of reaction products. Initial observations showed that complexes formed immediately upon incubation of pre-mRNA substrate with the nuclear extract (data not shown). To determine whether the assembly of such complexes takes place in discrete steps, the nuclear extract was pre-incubated for 30 min at 30°C to delay the appearance of specific complexes. As has been shown earlier (Ruskin and Green, 1985), pre-incubation of nuclear extract in the absence of creatine phosphate leads to depletion of the endogenous ATP pool. Unfortunately, the pre-incubation also results in low efficiency of the cleavage and polyadenylation reaction. Incubation of L3 pre-mRNA with nuclear extract results in the rapid formation of distinct complexes. In the presence of ddATP, where cleavage takes place but polyadenylation is prevented, two major complexes, termed H and S, and two minor complexes are formed (Figure 2, panel A). When ATP is included in the reactions, cleavage and subsequent polyadenylation occur. Under these conditions we observe a similar pattern of complex formation for the first 20 min of the time course (Figure 2, panel B). However, at later times an additional complex, termed P, appears; this complex migrates between complexes S and H. Formation of the H, S and two minor complexes takes place within the first minutes of incubation, and precedes the appearance of cleaved and polyadenylated RNA (see Figure 2, panels C and D, respectively).

Complex H assembles immediately upon incubation with pre-incubated nuclear extract and is the fastest migrating complex upon gel electrophoresis. It is the only complex that assembles on RNA C, a non-specific RNA that lacks polyadenylation signals and is of comparable size with RNA L3 (cf. Figure 3, panel A, lanes 2 and 10). As determined by sucrose gradients, complex H was found to have a sedimentation value of ~22S (data not shown). We conclude that complex H corresponds to heterogeneous complexes described previously and therefore represents non-specific binding of components to the pre-mRNA (Frendewey and Keller, 1985; Konarska and Sharp, 1986).

The major complex observed is designated S in Figure 2, panels A and B; it actually consists of two complexes with similar electrophoretic mobilities. The S complexes migrate between splicing complexes A and B (Konarska and Sharp, 1986; cf. Figure 3, panel A, lanes 2 and 4). In sucrose gradients they sediment at ~40S (data not shown). The low electrophoretic mobility and the high S value indicates that the S complexes represent large RNPs which may contain several components. When the polyadenylation reaction is incubated with the ATP analog, AMPCPP, or with 1 mM EDTA, polyadenylation is inhibited; however, cleavage of the L3 precursor still occurs (Moore and Sharp, 1985). Formation of the two S complexes was also found to occur under these conditions (data not shown). In the time course presented in Figure 2, both complexes assemble after a short delay of ~1 min. This delay was only observed with pre-incubated nuclear extract (see above). In the presence of ddATP, the S complexes are present throughout the entire time course. In contrast, in the presence of ATP, the S complexes gradually decrease in amount at later times.

The third major complex is detected after 30 min incubation in the presence of ATP (Figure 2B; complex P). The P complex does not occur under cleavage conditions (cf. Figure 2, panels A and B) and its appearance coincides with the production of polyadenylated RNA (cf. Figure 2, panels B and D). Therefore, complex P may be an RNP-containing polyadenylated RNA released from the specific complexes. The electrophoretic mobility of the P complex is dependent on the heparin concentration added to the reaction. An optimal separation pattern is achieved with a final heparin concentration of 5 mg/ml. This concentration does not prevent specific complex formation when added to the processing reaction, demonstrating that the P complex does not result from dissociation of the S complexes (data not shown).

The two minor complexes (open and filled arrowheads in Figure 2) form immediately upon incubation of pre-mRNA in nuclear extract, but behave differently over time. The upper one (open arrowhead) disappears within the first few minutes of the time course, whereas the lower complex (filled arrowhead) is visible for a longer time. Both minor complexes can only be detected with pre-mRNA containing an intact AAUAAA sequence, and are therefore considered to be specific complexes. The two minor complexes may represent intermediates in the formation of the specific complexes; however, they have not been further characterized.

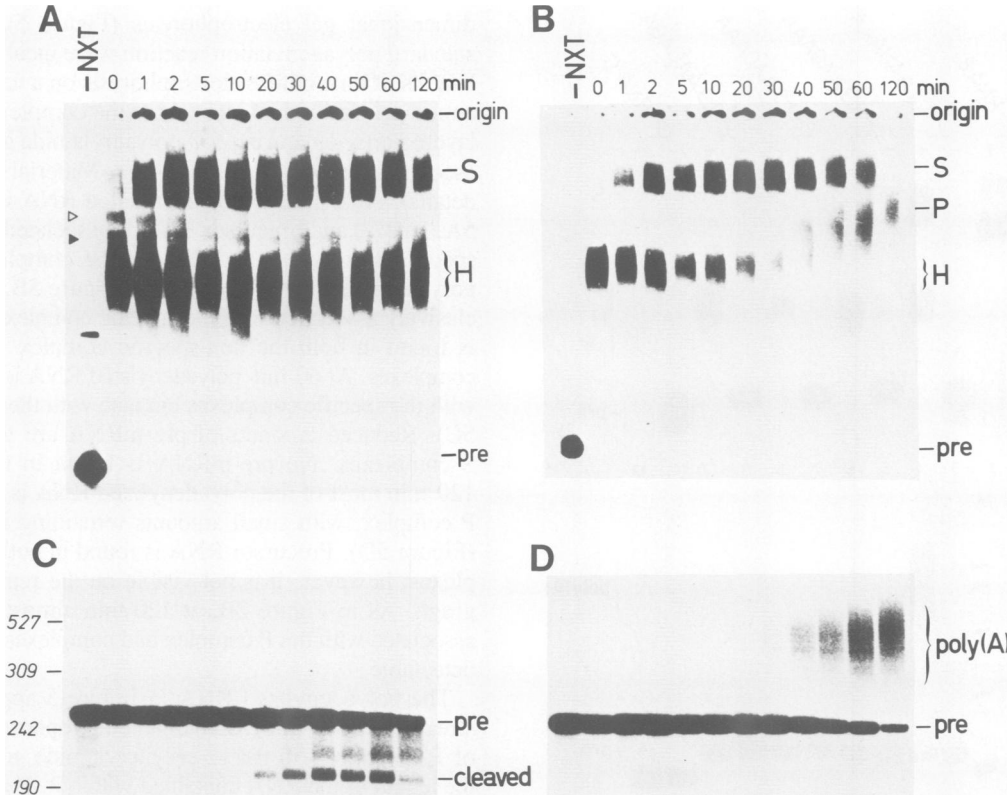

Fig. 2. Formation of complexes precedes the appearance of reaction products under cleavage and polyadenylation conditions. (**A**) Time course of complex formation under cleavage conditions. ^{32}P-Labeled L3 pre-mRNA was incubated in a HeLa cell nuclear extract in the presence of ddATP for the times indicated at the top of each lane (see Materials and methods). The first lane was incubated without nuclear extract (−NXT) as indicated. Aliquots (5 μl) were removed from the reaction mixture and incubated with 1 μl heparin (25 μg/μl) for 10 min on ice before electrophoresis on a native 4% polyacrylamide gel. The origin of the gel is indicated. The positions of the precursor RNA (pre) and the H and S complexes (see text) are indicated. The two minor complexes (see text) are indicated by open and filled arrowheads, respectively. (**B**) Time course of complex formation under polyadenylation conditions. ^{32}P-Labeled L3 pre-mRNA was incubated in a HeLa cell nuclear extract in the presence of ATP for the times indicated at the top of each lane (see Materials and methods). The first lane was incubated without nuclear extract (−NXT) as indicated. Aliquots from the polyadenylation reaction were removed and treated as described above. Symbols are as above. (**C**) Time course of 3′ processing under cleavage conditions. ^{32}P-Labeled RNA was extracted from the cleavage reactions shown in (A) and analyzed on a denaturing 6% polyacrylamide gel. Unprocessed pre-mRNA and 'cleaved' precursor are indicated (pre and cleaved, respectively). DNA size markers are indicated at the left side of the panel (^{32}P-labeled DNA fragments from a *Hpa*II digestion of pBR322). The numbers are given in nucleotides. (**D**) Time course of 3′ processing under polyadenylation conditions. ^{32}P-Labeled RNA was extracted from the polyadenylation reactions shown in (B) and separated by electrophoresis on a denaturing 6% polyacrylamide gel. Unprocessed pre-mRNA and polyadenylated RNA are indicated [pre and poly(A), respectively].

Specific complex formation is dependent on an intact AAUAAA sequence

Point mutations within the AAUAAA sequence reduce cleavage at the poly(A) site by as much as 50-fold (Montell *et al.*, 1983; Wickens and Stephenson, 1984). In addition, polyadenylation of synthetic RNAs ending at the poly(A) site also have strict requirements for this canonical sequence (Manley, 1983; Manley *et al.*, 1985; Moore *et al.*, 1986; Zarkower *et al.*, 1986). It was recently demonstrated that during the cleavage and polyadenylation reaction binding of oligodeoxynucleotides complementary to this sequence was prevented, suggesting that a complex was formed at this site (Zarkower and Wickens, 1987). To determine directly whether specific complex formation also requires an intact AAUAAA signal, three single base substitution mutations within the AAUAAA sequence of the L3 precursor were made (see Figure 1) and their effect on complex formation and polyadenylation analyzed. Figure 3A shows that incubation of the mutant pre-mRNAs, L3 AAGAAA, L3 AACAAA and L3 AAUAGA, under polyadenylation conditions results in the formation of the non-specific complex H but the S complexes do not form (cf. Figure 3A, lanes 4, 6, 8 and 10). In addition, no cleaved or polyadenylated RNAs could be detected with these mutant pre-mRNAs (see Figure 3B, lanes 6, 8 and 10). Therefore, the formation of S complexes, like cleavage and polyadenylation of the pre-mRNA, is dependent on the presence of an intact AAUAAA sequence within the mRNA precursor.

Competition experiments further demonstrated the requirement of an intact AAUAAA sequence for the formation of the specific complexes. Preincubation of nuclear extract with increasing concentrations of unlabeled L3 pre-mRNA prior to the addition of radioactively labeled L3 mRNA-precursor led to the reduction (at 50-, 250- and 500-fold molar excess of competitor) and eventual disappearance (at 1000-fold molar excess of competitor) of the S complexes (Figure 4A, lanes 2−6). Polyadenylation activity, associated with the presence of the S complexes, was also found to be similarly reduced (Figure 4B, lanes 2−6). Addition of increasing amounts of mutant pre-mRNAs, L3 AAGAAA, L3 AACAAA, L3 AAUAGA, or RNA C did not result in the inhibition of S complex formation (Figure 4A, lanes 7−22); it also did not inhibit the polyadenylation reaction (Figure

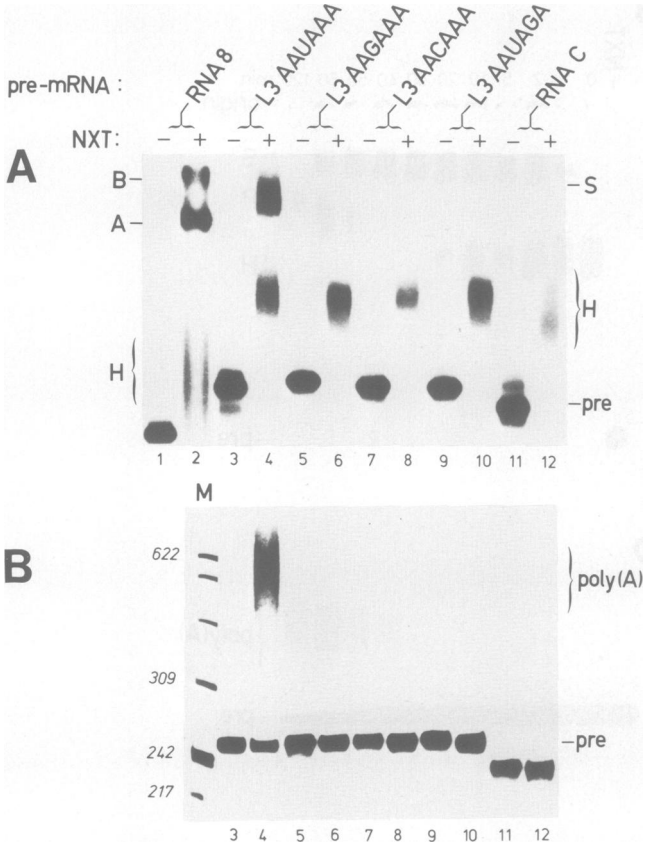

Fig. 3. Formation of the S complexes is dependent on an intact AAUAAA sequence within the pre-mRNA. **(A)** Analysis of complex formation with different RNA substrates. ^{32}P-Labeled pre-mRNAs were incubated for 90 min with nuclear extract under polyadenylation conditions. RNA 8, a splicing precursor, was incubated with and without HeLa cell nuclear extract (**lanes 2** and **1**, respectively) under conditions described previously (Frendewey and Keller, 1985). The splicing complexes are indicated according to the nomenclature described by Frendewey *et al.* (1987). Polyadenylation reactions containing the pre-mRNAs indicated at the top of each pair of lanes (see Figure 1) were incubated with (**lanes 4, 6, 8, 10** and **12**) and without (**lanes 3, 5, 7, 9** and **11**) nuclear extract. Aliquots were removed from the reaction mix and incubated with 1 μl (1 μg/μl) heparin for 10 min at room temperature before electrophoresis on a 4% native polyacrylamide gel. The positions of the precursor RNA (pre), the H, and the S complexes are indicated. **(B)** Analysis of polyadenylation with different RNA substrates. ^{32}P-Labeled RNA was extracted from the polyadenylation reactions shown in (A) (**lanes 3–12**) and analyzed by electrophoresis on a denaturing 6% polyacrylamide gel. Unprocessed pre-mRNA and polyadenylated RNA are indicated [pre and poly(A), respectively]. **Lane M**, DNA size markers as described in Figure 2.

4B, lanes 7–22). These results demonstrate the importance of the AAUAAA sequence in the formation of S complexes. The competition experiments also suggest that the GU-rich downstream sequences alone are unable to compete for S complex formation. Furthermore, assembly of the S complexes appears to be required for the polyadenylation reaction because processing of the pre-mRNA substrates does not occur in their absence. Therefore, we propose that the S complexes are specifically required for cleavage and polyadenylation of pre-mRNA.

Cleaved and polyadenylated pre-mRNA is found within the specific complexes prior to its release

If the formation of the specific complexes is required for cleavage and polyadenylation, then intermediates or products of the reaction should be associated with these complexes. Therefore, we analyzed the RNA content of the major complexes by two-dimensional gel electrophoresis (Figure 5). Aliquots from a standard polyadenylation reaction were incubated for increasing lengths of time before electrophoresis on a native 4% polyacrylamide gel. Individual lanes from the complex gel were excised, layered on a denaturing 6% polyacrylamide gel and electrophoresed in a second dimension (see Materials and methods for details). At 1 min no polyadenylated RNA is observed (Figure 5A, lane C) and precursor RNA is associated with both the non-specific complex H and the specific complexes S. At 30 min polyadenylated RNA is detected (Figure 5B, lane C) and is exclusively associated with the specific complexes. Precursor RNA is found in both the non-specific complex H and the specific complexes. At 60 min polyadenylated RNA is associated not only with the specific complexes but also with the P complex (Figure 5C). Reduced amounts of pre-mRNA are found in the H and S complexes. No pre-mRNA is found in the P complex. At 120 min most of the polyadenylated RNA is associated with the P complex, with small amounts remaining in the S complexes (Figure 5D). Precursor RNA is found in both the H and S complexes; however, it is not visible on the reproduced autoradiograph. As in Figure 2B, at 120 min almost all of the RNA is associated with the P complex and complexes H and S are barely detectable.

The polyadenylated RNA in Figure 5 appears as a diagonal streak after two-dimensional gel electrophoresis. The migration of P complexes in native polyacrylamide gels is dependent on the length of the RNA contained within it. Because of the length-heterogeneity of the poly(A) tail, P complexes of different electrophoretic mobility are formed. This, in combination with the length-dependent separation of the RNAs by electrophoresis on a denaturing gel, results in the formation of the diagonal streak.

Because complex P migrates faster than the S complexes (Figures 2B and 5) and because second dimensional analysis shows that it contains only polyadenylated RNA, we propose that the P complex contains polyadenylated RNA released from the specific complexes. The P complex may contain poly(A)-binding proteins in addition to the non-specific binding proteins normally associated with RNA in nuclear extracts. Since no P complex can be formed in the presence of ddATP, we determined whether the 'cleaved' RNA is released from the specific complexes as well. Pre-mRNA was incubated under cleavage conditions and analyzed by two-dimensional gel electrophoresis. After 120 min precursor RNA and 'cleaved' RNA migrate in the region of the H and S complexes (Figure 6), indicating that some of the 'cleaved' precursor is released from the specific complexes. At earlier time points the 'cleaved' RNA is associated with the S complexes (results not shown), indicating that cleavage without subsequent polyadenylation also takes place in the S complexes.

The S complexes protect a region containing the AAUAAA signal and the GU-rich downstream sequences from RNase T1 digestion

Components from HeLa cell nuclear extracts interact with the L3 pre-mRNA, forming specific complexes. To locate the binding site of these components on the L3 pre-mRNA, RNase T1 protection analysis was performed. Aliquots taken from a polyadenylation reaction containing [^{32}P]UTP L3 pre-mRNA were digested with different amounts of RNase T1. Two T1-resistant bands comparable in mobility with the S complexes were detected on a native 4% polyacrylamide gel (Figure 7A, lane 3, U and L). No protected complex comparable in electrophoretic mobility with either the H complex or the P complex was detected.

To determine the size of the protected RNA fragments the RNA

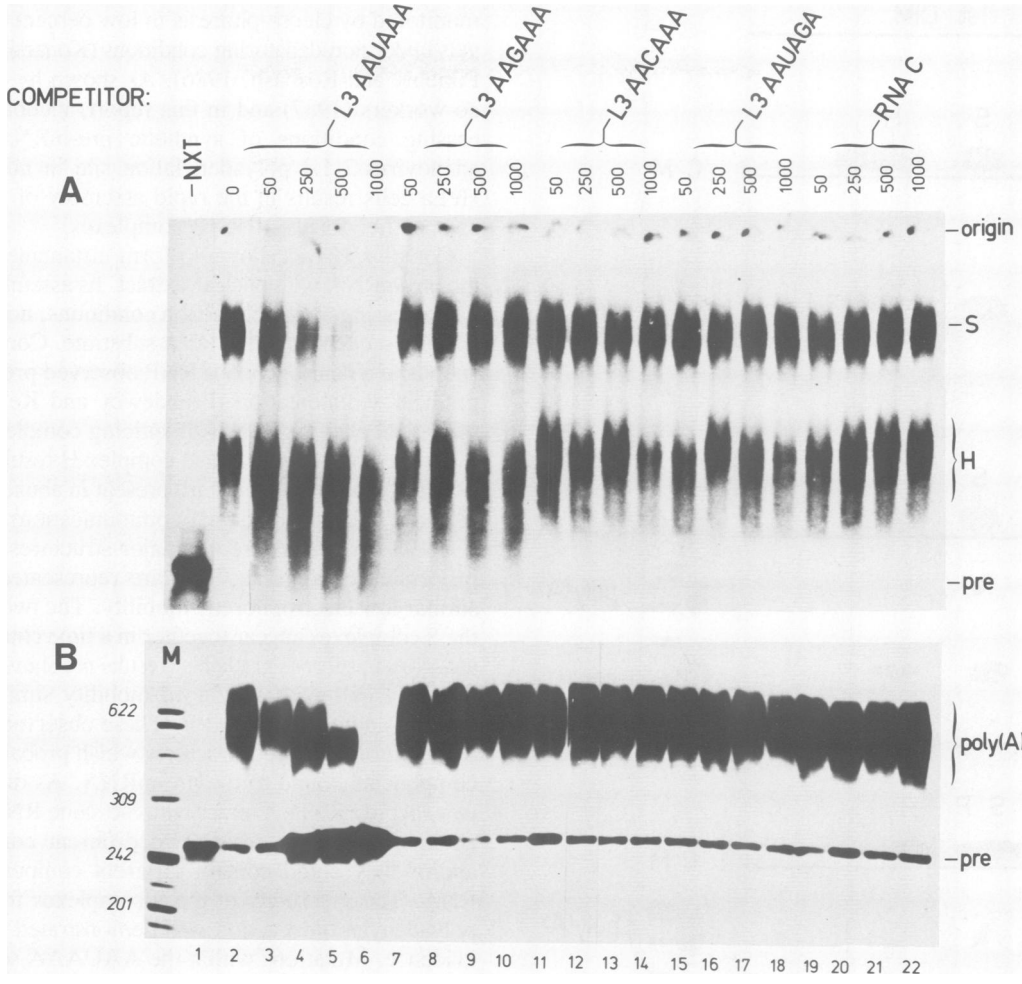

Fig. 4. Competition for specific complex formation and polyadenylation by L3 and mutant L3 pre-mRNAs. **(A)** Analysis of the assembly of the S complexes in the presence of different competitor RNAs. ^{32}P-Labeled L3 pre-mRNA was processed in polyadenylation reactions pre-incubated with various amounts of unlabeled competitor RNA (see Materials and methods and below). Aliquots (5 μl) were removed from the reaction, incubated with 1 μl (1 μg/μl) heparin for 10 min at room temperature and analyzed by electrophoresis on a native 4% polyacrylamide gel. **Lane 1**, pre-mRNA incubated in the absence of nuclear extract (−NXT). **Lanes 2−6**, reactions pre-incubated with 0-, 50-, 250-, 500- and 1000-fold molar excess of unlabeled L3 AAUAAA, respectively. **Lanes 7−10**, reactions pre-incubated with 50-, 250-, 500- and 1000-fold molar excess of unlabeled L3 AAGAAA, respectively. **Lanes 11−14**, reactions pre-incubated with 50-, 250-, 500- and 1000-fold molar excess of unlabeled L3 AACAAA, respectively. **Lanes 15−18**, reactions pre-incubated with 50-, 250-, 500- and 1000-fold molar excess of unlabeled L3 AAUAGA, respectively. **Lanes 19−22**, reactions pre-incubated with 50-, 250-, 500- and 1000-fold molar excess of unlabeled RNA C, respectively. Symbols are as in Figure 2. **(B)** Analysis of polyadenylation activity in the presence of different competitor RNAs. RNA was extracted from the reactions shown in (A) and analyzed by electrophoresis on a denaturing 6% polyacrylamide gel. **Lane M**, DNA size markers. Symbols are as in Figure 2.

was released from the complexes by proteinase K treatment and separated by electrophoresis on a 12% polyacrylamide denaturing gel. Figure 7B shows that both the upper (U) and the lower (L) RNase T1 resistant complexes contain an abundant RNA species of 67 nt. These bands were eluted from the gel and digested to completion with RNase T1 to locate the protected RNA on the L3 precursor. The resulting RNA fragments were separated by electrophoresis on a denaturing 20% polyacrylamide gel (Figure 7C, lanes 2 and 3). Fragments of 21, 15, 10, 6 and 1 nt in length were obtained from both the U and the L RNAs, indicating that both the upper and the lower T1-resistant complexes protect the same RNA sequences. The labeled mononucleotide is not detectable on the reproduced autoradiographs. Since the RNase T1 fragments of 21, 15 and 10 nt are unique, and therefore diagnostic for the L3 pre-mRNA, a RNase T1 protection map was compiled (Figure 8). The sequences of the fragments were further confirmed by complete digestion of the oligonucleotides with RNase T2 and RNase A, followed by analysis of the resulting labeled nucleotides (data not shown). The precise borders of the protected region were further analyzed by repeating the procedure with precursor RNAs labeled with [^{32}P]ATP and [^{32}P]CTP (data not shown). The RNase T1 protection map illustrated in Figure 8 shows that the 67 nt protected sequence extends from 34 nt upstream of the poly(A) site, including the AAUAAA signal, to 33 nt downstream of the poly(A) site, containing the GU-rich sequences.

Discussion

The results reported above lead us to conclude that the 3′ processing of pre-mRNA *in vitro* is determined by the interaction of components present in the nuclear extract with RNA sequences located in the vicinity of the poly(A) site. As first observed with pre-mRNA splicing substrates, the association of specific components in the nuclear extract with radiolabeled RNAs leads to the formation of large RNP complexes which can be conveniently

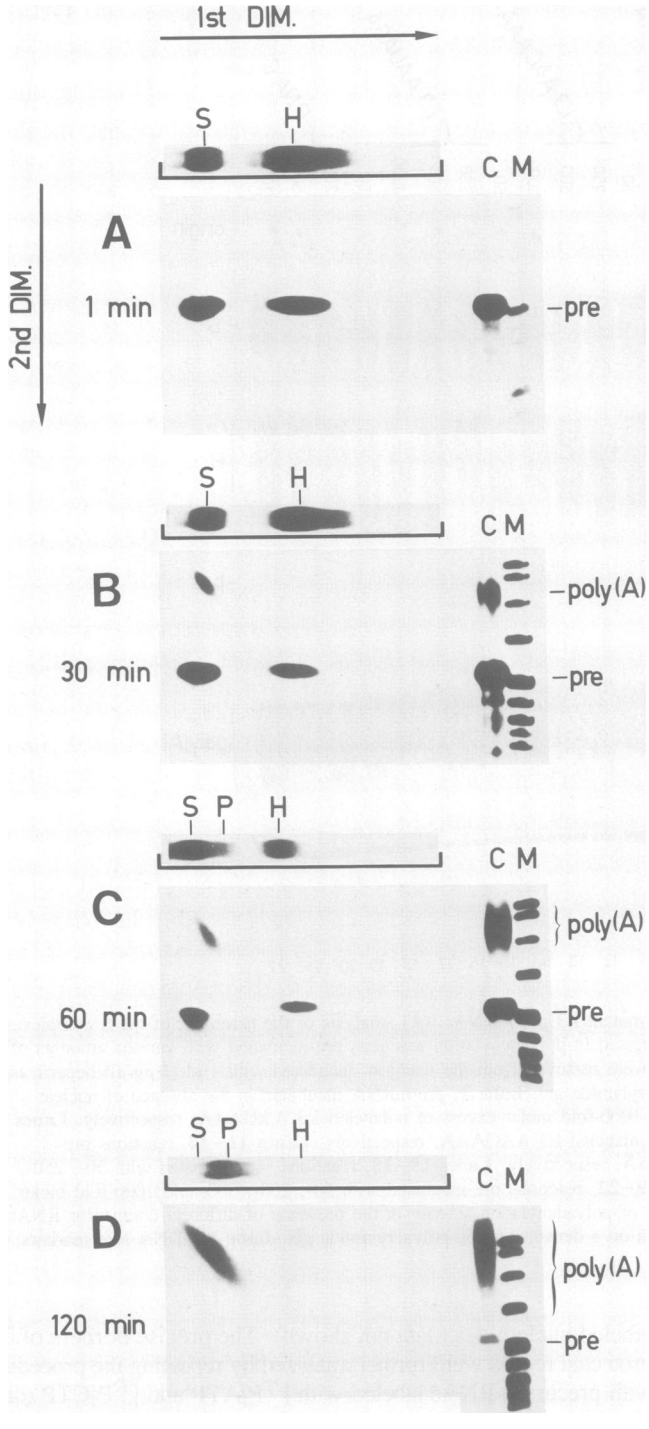

Fig. 5. Analysis of the RNA content of the various complexes by two-dimensional gel electrophoresis. First dimension: ^{32}P-labeled L3 pre-mRNA was incubated for various times in HeLa cell nuclear extract. Aliquots from these polyadenylation reactions were electrophoresed on a native gel and visualized by autoradiography. The autoradiograph of the first dimensional gel (indicated by a horizontal bracket) is pictured above that of the second dimension. The positions of complexes H, P and S are indicated. Second dimension: lanes from the native gel were excised and treated as described in Materials and methods. The gel strip was layered horizontally on a denaturing 6% polyacrylamide gel and the RNA was separated by electrophoresis. **Lane C**, RNA extracted from each time point of the polyadenylation reaction was co-electrophoresed on the denaturing gel. **Lane M**, DNA size markers as in Figure 2. Unprocessed pre-mRNA and polyadenylated RNA are indicated [pre and poly(A), respectively]. Arrows above the figure indicate the direction of the first and second dimensions (indicated by 1st DIM and 2nd DIM, respectively). Incubation times: **(A)** 1 min; **(B)** 30 min; **(C)** 60 min; **(D)** 120 min.

monitored by electrophoresis in low percentage polyacrylamide gels under non-denaturing conditions (Konarska and Sharp, 1986; Pikielny and Rosbash, 1986). As shown by Skolnik-David and co-workers (1987) and in this report, incubation under 3′ processing conditions of synthetic pre-mRNAs containing the adenovirus-2 L3 polyadenylation site in nuclear extract from HeLa cells results in the rapid assembly of unspecific, as well as specific, forms of RNP complexes.

Complex H was shown to form instantaneously upon mixing the pre-mRNA with nuclear extract. Its assembly does not require 3′ processing-specific reaction conditions, nor does it depend on specific sequences in the RNA substrate. Complex H thus corresponds to a heterogeneous RNP observed previously by sucrose gradient sedimentation (Frendewey and Keller, 1985) and by native gel electrophoresis of splicing complexes (Konarska and Sharp, 1986). It is likely that complex H results from the binding of hnRNP-proteins which are present in abundance in the nuclear extract (U.Utans, personal communication).

The S complexes are the major structures observed under 3′ processing conditions. They are represented by two bands of similar low electrophoretic mobility. The two RNPs comprising the S complexes appear together in a time course. They sediment at ~40S in sucrose gradients (results not shown) and they migrate in native gels with a reduced mobility similar to the splicing-specific complexes A and B. These observations imply that the S complexes are large structures which probably contain multiple components bound to the pre-mRNA. As discussed below, the two specific RNPs interact with the same RNA sequences. They could therefore represent two different conformational states and/or they could contain different components bound to the RNA. The specificity of the S complexes for the cleavage and polyadenylation reaction was demonstrated by several lines of evidence. Mutations within the AAUAAA sequence previously shown to be important for the cleavage (Montell *et al.*, 1983; Wickens and Stephenson, 1984) and the polyadenylation reaction (Manley, 1983; Manley *et al.*, 1985; Zarkower *et al.*, 1986) completely prevent formation of the S complexes.

Competition experiments show that addition of increasing amounts of unlabeled L3 mRNA precursor to the polyadenylation reaction results in a reduction and the eventual loss of specific complex formation and polyadenylation activity. This result strongly suggests that the presence of the specific complex is required for correct 3′ processing, since cleavage and polyadenylation of pre-mRNA does not occur in its absence. Addition of

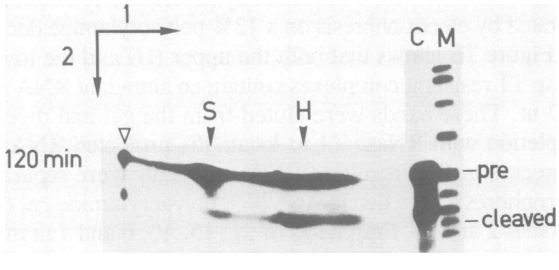

Fig. 6. Release of 'cleaved' RNA from the S complexes. ^{32}P-Labeled pre-mRNA was incubated for 120 min with HeLa cell nuclear extract in the presence of ddATP. An aliquot of the reaction mixture was analyzed by two-dimensional gel electrophoresis as described in the legend to Figure 5. Arrows above the figure indicate the directions of the first and second dimensions. The autoradiograph of the first dimension is not shown but corresponds to the 120 min time point of Figure 2A. The positions of complexes H and S are indicated on the second dimensional gel. The open arrowhead indicates the origin of the first dimensional gel. Unprocessed pre-mRNA and 'cleaved' RNA are indicated (pre and cleaved, respectively).

unlabeled mutant precursor containing base substitutions within the AAUAAA sequence does not compete with the formation of the S complexes. This indicates that the AAUAAA signal plays a key role in the assembly of specific complexes. All other sequence elements on the L3 precursor, including the GU-rich downstream sequences, do not compete for processing activity or associated specific complex formation.

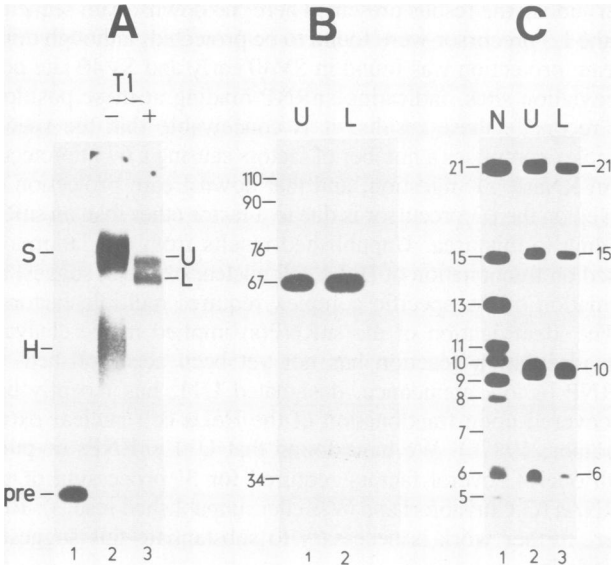

Fig. 7. RNase T1 protection analysis of complex formation on L3 mRNA precursor. **(A)** RNase T1 treatment of 3′ processing complexes. ^{32}P-Labeled L3 pre-mRNA was incubated in nuclear extract and subjected to RNase T1 digestion. T1-resistant complexes were separated by electrophoresis on a native 4% polyacrylamide gel (see Materials and methods). **Lane 1**, RNA incubated without nuclear extract. **Lane 2**, RNA incubated with nuclear extract. **Lane 3**, RNA incubated with nuclear extract and subsequently digested with RNase T1. The upper (U) and lower (L) protected complexes are indicated. Other symbols are as in Figure 2. **(B)** Isolation of the RNA from the upper and lower RNase T1-resistant complexes. The upper (U) and lower (L) complexes from (A) were excised, treated with proteinase K, and the RNA was electrophoresed on a denaturing 12% polyacrylamide gel (see Materials and methods). The sizes of the protected RNA fragments were determined by comparison with DNA size markers (indicated on the left side of the panel; described in Figure 2A). **Lane 1**, upper complex (U); **lane 2**, lower complex (L). **(C)** Analysis of the 67 nt protected RNA sequence. The 67 nt RNA fragments from (B) were eluted from the gel, digested with RNase T1 and electrophoresed on a 20% denaturing polyacrylamide gel (see Materials and methods). **Lane 1**, RNA fragments resulting from RNase T1 digestion of naked (N) L3 pre-mRNA. The sizes of the RNA fragments were ascertained by comparison with DNA size markers. **Lane 2**, RNA fragments from the 67 nt RNA contained in the upper (U) T1-resistant complex. **Lane 3**, RNA fragments from the 67 nt RNA contained in the lower (L) T1-resistant complex. The sizes of the RNA fragments are given in nucleotides. Because the samples were precipitated with ethanol, the smaller fragments are missing on this autoradiograph. Although there are six fragments of 7 nt length, they are always faint on the original autoradiographs, and are not visible on the reproduction.

Two minor complexes also assemble immediately upon incubation of pre-mRNA in the HeLa cell nuclear extract, but their stability during the time course is different. The larger complex disappears before the detection of the first reaction product, whereas the smaller decreases more gradually and is still present after the first reaction products are observed. Both of these minor complexes contain unprocessed pre-mRNA. They form only on pre-mRNA containing an intact AAUAAA sequence and are therefore considered to be specific complexes. However, since no precursor−product relationship could be established, the possible role of these complexes as intermediates in a stepwise assembly of the specific complexes remains to be investigated.

RNase T1 digestion of the polyadenylation reaction mixture converts the two S complexes into the slightly faster migrating forms, U and L. The other observed complexes (H, P and the two minor specific complexes) are degraded by the activity of the nuclease. The T1-resistant complexes U and L are derived from the two S complexes: they are detectable throughout a time course and do not form on pre-mRNA containing a mutation in the AAUAAA sequence. Both complexes, U and L, protect an identical RNA fragment of 67 nt in length. This region contains the AAUAAA signal upstream of the polyadenylation site and the GU-rich sequence downstream of the cleavage site, known to be required for correct 3′ end formation (reviewed in Birnstiel *et al.*, 1985; Proudfoot and Whitelaw, 1987).

The RNA content of the S complexes was analyzed by electrophoresis on denaturing gels. Increasing amounts of correctly cleaved and polyadenylated RNA accumulate within the specific complexes during the time course. After the RNA has been polyadenylated it is released from the specific complexes, S, and migrates between complexes S and H upon native gel electrophoresis. The appearance of this complex P is strongly correlated with the appearance of polyadenylated RNA. Complex P is not detected under cleavage conditions in the presence of ddATP. The assembly of complex P probably results from the association of poly(A) binding proteins with the polyadenylated RNA in addition to non-specific components normally bound to the RNA. 'Cleaved' but not polyadenylated RNA produced in the presence of ddATP migrates in the region of the H and S complexes. Because the incorporation of ddATP into the cleaved intermediate is a result of the polyadenylation reaction and the 'cleaved' RNA cannot be further processed, it appears to be treated as a final reaction product and is released from the S complexes. The correctly cleaved intermediate resulting from incubation in the presence of ATP is never released prior to polyadenylation. In the presence of ddATP we do not observe a decrease of the S

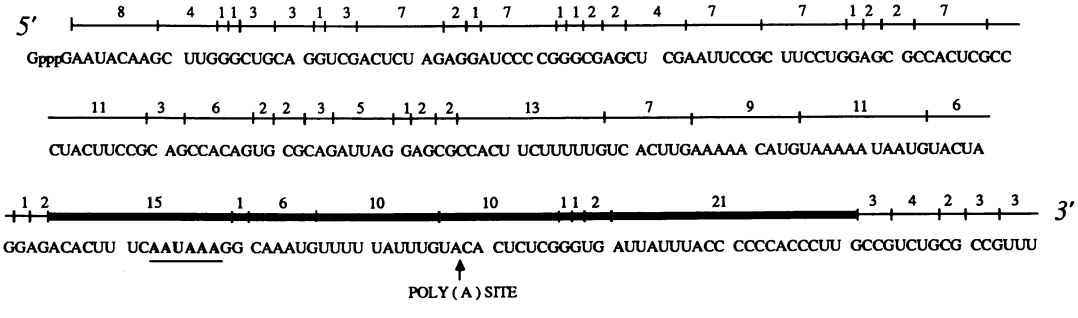

Fig. 8. Location of the RNase T1 protected region on L3 pre-mRNA. The nucleotide sequence of the entire L3 pre-mRNA is schematically represented. Numbers above the sequence indicate the RNA fragments produced by RNase T1 digestion of naked pre-mRNA. The thick line indicates sequences protected from RNase T1 digestion by assembly of the specific complexes. The AAUAAA sequence is underlined and the poly(A) site is indicated.

complexes at later time points. Therefore, the S complexes seem to be stabilized by a lower turnover of the substrate. In contrast, in the presence of ATP the turnover of the substrate is higher and results in the disappearance of the H and S complexes and the accumulation of complex P during the time course. In this context it is interesting to note parallels between 3′ processing and splicing of pre-mRNA. In both cases the reaction takes place in large complexes and the final products are released from these structures (Konarska and Sharp, 1986, 1987; Sholnik-David *et al.*, 1987; Frendewey *et al.*, 1987).

We conclude that the S complexes represent a rapid interaction of specific factors in a HeLa cell nuclear extract with the AAUAAA signal of the L3 pre-mRNA protecting a 67 nt stretch of the precursor. These complexes are associated with cleavage and polyadenylation activity, and their formation is thought to represent a necessary step in 3′ processing. However, whether the formation of the specific complexes is a prerequisite for cleavage and for polyadenylation or just for one of these reactions is not known. The importance of specific complex formation for the cleavage reaction has been directly demonstrated by incubation of the L3 precursor with AMPCPP or EDTA. Under these conditions, pre-mRNA cleavage (but not polyadenylation) is observed and S complexes are formed (results not shown). The role of the S complexes in the polyadenylation reaction *per se* has not been established. However, it is unlikely that the complexes are required for cleavage and not for polyadenylation since cleaved precursor is always found to be polyadenylated (Montell *et al.*, 1983; Wickens and Stephenson, 1984). In addition, both processes require the AAUAAA sequence and are not separable under standard conditions. Furthermore, since polyadenylated RNA is initially associated with the S complexes we suggest that both cleavage and polyadenylation take place within these structures.

Zarkower and Wickens (1987) have recently proposed that a complex forms at the AAUAAA sequence on the pre-mRNA and is required for cleavage and polyadenylation. Evidence for these conclusions was based on experiments in which complex formation was assayed for by accessibility of a complementary oligodeoxynucleotide to bind to precursor RNA, as detected by RNase H digestion. Their results also indicate that mutations within the AAUAAA sequence prevent complex formation. Interestingly, this study demonstrated that pre-cleaved RNAs could form complexes associated with the AAUAAA sequence. However, substitution of the downstream sequences with unspecific RNA prevented specific complex formation (D.Zarkower and M.Wickens, personal communication). The competition experiments presented here suggest that downstream sequences do not play an important role in the initial step of specific complex formation. However, the RNase T1 protection experiments clearly show a protected region encompassing the GU-rich sequences up to 33 nt downstream of the poly(A) site. This implies that these sequences are involved in the 3′ processing reaction, as previously shown by a variety of mutational and deletional analyses (reviewed by Birnstiel *et al.*, 1985; Proudfoot and Whitelaw, 1987).

The involvement of snRNPs in the cleavage and polyadenylation reaction has been suggested by inhibition of the *in vitro* polyadenylation reaction with antibodies known to interact with snRNPs (Moore and Sharp, 1985; Sperry and Berget, 1986; reviewed by Birnstiel and Schaufele, 1987). Furthermore, coupled RNase T1 digestion and immunoprecipitation with antibodies directed against either the Sm-epitopes or the trimethylguanosine-cap structure of U-type snRNAs revealed protected RNA fragments containing the AAUAAA signal (Hashimoto and Steitz, 1986). In the latter study the major sequence protected from RNase T1 digestion shares the same 5′ protection border on the L3 mRNA as demonstrated here, 34 nt upstream of the poly(A) site. However, unlike the results presented here, no downstream sequences on the L3 precursor were found to be protected, although downstream protection was found in SV40 early and SV40 late polyadenylation sites, indicating snRNP binding at these positions. To reconcile these results, it is conceivable that the specific complex comprises a number of factors causing a 67 nt protection from RNase T1 digestion, and that downstream protection observed on the L3 precursor is due to a factor other than an snRNP binding to this area. Unpublished results from our laboratory, based on fractionation of HeLa cell nuclear extracts, suggest that formation of the specific complex requires multiple factors.

The identification of the snRNP(s) implied in the cleavage/polyadenylation reaction has not yet been accomplished. An snRNP of low abundance, designated U11, has recently been discovered upon fractionation of the HeLa cell nuclear extract (Krämer, 1987b). We have found that U11 snRNPs co-purify with one of several factors required for 3′ processing of pre-mRNAs (G.Christofori and W.Keller, unpublished results). However, further work is necessary to substantiate this suggestive correlation.

Five snRNPs in addition to several protein factors are involved in pre-mRNA splicing. Here, the assembly proceeds in several steps and leads to the formation of the active spliceosome. Upon the generation of the spliceosome the first intermediate and products of the splicing reaction can be immediately observed. Therefore, the lag-phase of *in vitro* splicing correlates with the time required to assemble spliceosomes (reviewed by Green, 1986; Padgett *et al.*, 1986; Maniatis and Reed, 1987; Sharp, 1987). In contrast to splicing, the formation of the specific 3′ processing complexes occurs very rapidly *in vitro*, with a short lag-period only detectable upon pre-incubation of the nuclear extract (which presumably causes an exhaustion of residual ATP). There is, however, a considerable delay between the formation of specific complexes and the emergence of the first reaction product. The cause of this delay is not known. It is our hope that isolation of the components involved in 3′ processing by biochemical fractionation will help to elucidate the mechanism of this reaction.

Materials and methods

Materials

The following materials are listed with their suppliers: restriction endonuclease, Bethesda Research Laboratories, New England Biolabs and Boehringer Mannheim; SP6 RNA polymerase, Boehringer Mannheim; pSP64 and RNasin, Promega Biotec; [α^{32}P]nucleoside triphosphates (400 Ci/mmol), Amersham Buchler; GpppG, P-L Biochemicals; heparin, Sigma; glycogen, Renner Dannstadt.

Construction of SP6 templates

To obtain plasmid pSP6 L3 an *Alu*I fragment containing the L3 polyadenylation site of adenovirus-2 was isolated from cloned *Eco*RI B-fragment of the viral DNA (a gift from Dr W.Doerfler, Köln). Following *Eco*RI-linker ligation, the *Alu*I fragment was inserted into the *Eco*RI site of pSP64. Site directed oligodeoxynucleotide mutagenesis was performed as described (Kramer *et al.*, 1984) to obtain DNA-templates with single nucleotide substitution mutations within the AAUAAA sequence (L3 AAGAAA; L3 AACAAA and L3 AAUAGA). The mutations were confirmed by dideoxynucleotide sequencing (Sanger *et al.*, 1977).

In vitro synthesis of pre-mRNAs

In vitro transcription of truncated SP6 templates (as indicated in Figure 1) was performed as described by Melton *et al.* (1984) with a cap-priming protocol (Contreras *et al.*, 1982; Georgiev and Birnstiel, 1985) as described by Krämer and Keller (1985). The transcripts were purified and stored as described by Frendewey & Keller (1985). The pre-mRNAs used as substrates for the *in vitro* polyadenylation reactions are displayed schematically in Figure 1.

In vitro pre-mRNA processing reactions

The standard polyadenylation reaction contained, in a total volume of 25 μl, 0.8 mM ATP, 20 mM creatine phosphate, 0.5 mM $MgCl_2$, 10% glycerol, 10 mM Hepes–KOH (pH 7.9), 50 mM KCl, 0.05 mM EDTA, 0.25 mM DTT, 1% polyvinylalcohol, 0.1 mg/ml tRNA, 10 μl HeLa cell nuclear extract (Dignam *et al.*, 1983) and $1-5 \times 10^4$ Cerenkov c.p.m. of [^{32}P]pre-mRNA substrate (0.08–0.4 nM final concentration). For the standard pre-mRNA cleavage reaction identical conditions to the polyadenylation reaction were used except that 0.8 mM ATP is replaced by 0.8 mM ddATP. Reactions were incubated at 30°C and were either stopped by quick freezing in a dry ice/ethanol bath (for native gel electrophoresis) or by addition of 60 μl of proteinase K mixture [2% (w/v) sarcosyl, 100 mM Tris–HCl, pH 7.5, 20 mM EDTA, 400 μg/ml proteinase K] followed by incubation for 10 min at 30°C. The RNA was phenol/chloroform extracted and an equal volume of 7.5 M ammonium acetate was added to the aqueous phase before ethanol precipitation.

Polyacrylamide gel electrophoresis

Extracted RNAs were separated by electrophoresis on denaturing polyacrylamide gels as previously described (Krämer *et al.*, 1984) and were visualized by autoradiography.

Gel electrophoresis of 3' processing complexes

A 5 μl aliquot ($0.2-1.0 \times 10^4$ c.p.m.) of the processing reaction was incubated with different concentrations of heparin as indicated in the figure legends for 10 min at room temperature or on ice. Following electrophoresis on a 4% polyacrylamide gel (acrylamide:bisacrylamide = 80:1, in 25 mM Tris base, 25 mM boric acid and 1 mM EDTA, pH 8 [TBE]) complexes were visualized by autoradiography.

Second dimensional analysis of 3' processing complexes

Lanes from a native gel were excised, soaked in 0.1% SDS in TBE buffer for 10 min at room temperature, and then incubated for 30 min at 30°C in TBE buffer containing 0.1% SDS and proteinase K (0.5 mg/ml). The gel strip was layered horizontally on a denaturing 6% polyacrylamide gel to separate the RNA species contained within the particular complexes.

Competition experiments

Unlabeled competitor RNA was synthesized *in vitro* in a total volume of 100 μl, containing 50 μM GTP, 2.5 mM ATP, 2.5 mM CTP, 2.5 mM UTP, 10 mM DTT, 1.5 units/μl RNasin, 25 μM GpppG, 0.1 mg/ml SP6 DNA-template, 200 mM Tris–HCl, pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine and 30 units SP6 polymerase. The reaction was incubated for 90 min at 40°C. After phenol/chloroform extraction the RNA was ethanol precipitated in the presence of 1 μg glycogen. Full-length transcripts were eluted from a polyacrylamide gel (Frendewey and Keller, 1985) and the RNA yield was quantified spectrophotometrically. Polyadenylation reactions were preincubated with cold competitor RNA for 25 min at 30°C prior to addition of labeled RNA precursor. The reaction was then further incubated for 150 min at 30°C.

RNase T1 protection analysis

For the RNAse T1 protection studies high sp. act. ^{32}P-labeled pre-mRNA was synthesized as described previously (Krämer, 1987a). A standard 25 μl polyadenylation reaction containing 10×10^6 c.p.m. of [^{32}P]L3 pre-mRNA was incubated for 20 min at 30°C. A 5 μl aliquot was removed and incubated with 0.02 units of RNase T1 at room temperature for 10 min. After 10 m incubation with heparin (0.2 mg/ml) at room temperature, the sample was electrophoresed on a native 4% polyacrylamide gel. Complexes were visualized by autoradiography, excised from the gel, and incubated with proteinase K as described above. The excised gel piece was layered onto a denaturing 12% polyacrylamide gel to separate protected RNA fragments. To determine the sequence of the protected region of the L3 pre-mRNA, the predominant T1-resistant fragment was eluted from the gel and digested to completion in 4 μl of 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, containing 2.5 mg/ml tRNA and 2 units of RNase T1. The reaction was lyophilized and the resulting oligonucleotides were separated by electrophoresis on a denaturing 20% polyacrylamide gel.

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