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Potential role of poly(A) polymerase in the assembly of polyadenylation-specific RNP complexes

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ABSTRACT

To elucidate the mechanism by which poly(A) polymerase functions in the 3'-end processing of pre-mRNAs, polyadenylation-specific RNP complexes were isolated by sedimentation in sucrose density gradients and the fractions were analyzed for the presence of the enzyme. At early stages of the reaction, the RNP complexes were resolved into distinct peaks which sedimented at ~18S and 25S. When reactions were carried out under conditions which support cleavage or polyadenylation, the pre-mRNA was specifically assembled into the larger 25S RNP complexes. Polyclonal antibodies raised against the enzyme purified from a rat hepatoma, which have been shown to inhibit cleavage and polyadenylation (Terns, M., and Jacob, S. T., Mol. Cell. Biol. 9:1435 - 1444, 1989) also prevented assembly of the 25S polyadenylation-specific RNP complexes. Furthermore, formation of these complexes required the presence of a chromatographic fraction containing poly(A) polymerase. UV cross-linking analysis indicated that the purified enzyme could be readily cross-linked to pre-mRNA but in an apparent sequence-independent manner. Reconstitution studies with the fractionated components showed that formation of the 25S RNP complex required the poly(A) polymerase fraction. Although the enzyme has not been directly localized to the specific complexes, the data presented in this report supports the role of poly(A) polymerase as an essential component of polyadenylation-specific complexes which functions both as a structural and enzymatic constituent.

INTRODUCTION

The 3'-end formation of eukaryotic pre-mRNA is a complex series of reactions involving recognition of sequences that are essential for cleavage at the proper poly(A) site, and subsequent polymerization of approximately 250 adenosine residues to the newly created termini (reviewed in 1−5). Our understanding of these critical post-transcriptional processing events has been greatly facilitated through the use of cell-free polyadenylation systems (6) which in most respects accurately represent the process of polyadenylation in intact cells. The determinants required for correct processing include the highly conserved AAUAAA hexamer element found 10−30 nucleotides upstream of a given poly(A) site (7−9) as well as GU or U rich sequences present downstream of the cleavage site (10−13). As observed *in vivo*, cleavage and polyadenylation reactions are tightly coupled in vitro under normal assay conditions.

Several investigators have demonstrated that pre-mRNA cleavage and polyadenylation reactions are performed by large, multicomponent ribonucleoprotein complexes. The discovery of these polyadenylation-specific RNP complexes resulted from an analysis of the complexes formed between radiolabeled polyadenylation substrates and specific proteins during polyadenylation reactions in HeLa extracts. Detection of these specific 3' end processing complexes has been accomplished by separation of these complexes after electrophoresis through native polyacrylamide gels (14−19) and by sedimentation through glycerol or sucrose density gradients (20, 21) as well as by RNase H protection studies (22).

Several lines of evidence support the hypothesis that the 3'-end cleavage and polyadenylation are due to formation of active polyadenylation-specific complexes. For example, assembly of these complexes is observed only with pre-mRNA substrates which contain the essential poly(A) sequences and only under reaction conditions which favor accurate *in vitro* cleavage and/or polyadenylation. Single point mutations within the AAUAAA hexamer which prevent both cleavage and polyadenylation, also inhibit active complex formation (15−18, 21) and deletions in the downstream element greatly reduce active complex formation (13, 15, 18). The kinetic profile of assembly and disassembly of the complex indicates that it is an essential reaction intermediate. Further support that these complexes are true functional intermediates in the polyadenylation process is derived from studies which directly analyzed the RNA in the complexes at different intervals during the reaction. These studies showed that polyadenylated products are first observed in these complexes (16, 20). A detailed characterization of these complexes has further revealed that the components in the extract specifically associate with both the upstream AAUAAA and downstream GU rich elements of the pre-mRNA (16, 21). UV cross-linking studies

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have shown AAUAAA-dependent binding components associated with the complexes (23, 24). Finally, antibodies directed against poly(A) polymerase have been shown to inhibit cleavage and polyadenylation as well as specifically affect the proper assembly of the complexes (19).

Significant progress has been accomplished in identification of the components responsible for pre-mRNA recognition, cleavage, and polyadenylation using a variety of approaches. One such approach which continues to yield important information regarding the factors involved in this reaction, is the biochemical fractionation of active extracts followed by identification of the individual functional components by complementation assays. These studies showed that a complete reconstitution of the cleavage and polyadenylation activities requires the concerted actions of a poly(A) polymerase, endonuclease, and additional factors which impart specificity and efficiency to these processes (25−31).

The essential role of the previously characterized poly(A) polymerase in both cleavage and polyadenylation was first demonstrated using antibodies directed against poly(A) polymerase purified from a rat hepatoma (19). This study concluded that tight coupling of cleavage and polyadenylation reactions is probably a reflection of the critical interaction between poly(A) polymerase and endonuclease in the polyadenylation-specific RNP complex. The direct involvement of such a classical poly(A) polymerase in specific polyadenylation was subsequently confirmed (32). Direct evidence that the poly(A) polymerase is a common factor required for both cleavage and polyadenylation has also surfaced from several biochemical fractionation studies (25−31). Cleavage in these fractionated systems requires mixing a fraction containing poly(A) polymerase with a fraction containing the endonuclease activity. Since poly(A) polymerase represents a key factor in both the cleavage and polyadenylation of pre-mRNA, the present study was undertaken to determine whether this enzyme is present in the polyadenylation-specific RNP complexes and is required for the formation of functional complexes.

MATERIALS AND METHODS

Materials

SP6 RNA polymerase, DNase I, and RNasin (Promega) [a^{32}P]UTP (NEN), 7meGpppG (Pharmacia), and ultra pure sucrose (Schwarz/Mann Biotech).

Preparation of Pre mRNA substrate

Precursor mRNA was generated which contained the adenovirus-2 L3 poly(A) site. The plasmid pSP64L3 used in these studies was a generous gift of Claire Moore (6). For the UV cross-linking studies, pSPL3MD (wild-type) and pSPL3MD-14 (point mutant) kindly provided by James Stefano, were employed. The point mutation (T to C) was introduced into the AATAAA hexamer element found 20 nucleotides upstream of the poly(A) site by site directed mutagenesis (21). 5′-capped, ^{32}P-labeled transcripts were produced by SP6-directed transcription of Dra-I digested template DNAs and purified as described (19, 33).

Preparation of HeLa cell nuclear extract and its fractionation

Nuclear extracts were prepared essentially as described (34) with the following modifications: PMSF was omitted from all buffers, the crude nuclear pellet was resuspended in 0.7 ml buffer C /ml packed cell volumes (instead of 3 ml/10^9 cells), and the final dialysate was centrifuged for 5 minutes at 5,000 rpm in a microfuge.

The 20−40% ammonium sulfate fraction of crude HeLa extract was prepared as described (30). This fraction was then dialyzed in HG_{20}MEAS (20 mM Hepes PH , 20% glycerol, 3 mM $MgCl_2$, 0.2 mM EDTA, and 50 mM ammonium sulfate) and loaded onto a DEAE-sepharose column equilibrated with the same buffer. The flow-through and fractions eluted with 600 mM salt was precipitated with $(NH_4)_2SO_4$ (60%), resuspended in a minimal volume of buffer D (20 mM Hepes PH 7.9, 20% glycerol, .1M KCl, and, 2 mM EDTA), dialyzed against the same buffer, and stored at −80°C until use. Gel Shift analysis of the fractions was performed essentially as previously reported (19, 34). The individual and combined fractions were incubated under polyadenylation conditions for 10 minutes, treated with heparin (4 mg/ml) on ice for ten minutes, and resolved by electrophoresis in 4% non-denaturing gels.

Purification of hepatoma poly(A) polymerase and production of anti-poly(A) polymerase antibodies

Nuclear Poly(A)Polymerase from the transplanted Morris hepatoma 3924A was purified essentially to homogeneity using the previously established purification scheme (36). Polyclonal antibodies against the purified enzyme were raised in rabbits as described previously (36,37) with the exception that the antigen was cut from the gel prior to immunization (19). IgG was purified by DEAE affi-gel blue chromatography (Bio-Rad Laboratories) of the sera followed by ammonium sulfate precipitation. Preimmune IgG used in controls was also prepared in this manner using the serum obtained from the rabbit prior to immunization.

Sedimentation analysis of polyadenylation reactions

The conditions for the sedimentation analysis of the polyadenylation reactions were similar to those described previously (21). Linear 4.8 ml sucrose gradients (5−20% w/v)were prepared in a buffer which was very similar in composition to the polyadenylation reaction buffer (8 mM Hepes PH 7.9, 40 mM KCl, 1mM $MgCl_2$, 0.1 mM EDTA, and freshly added 0.2 mM DTT and 1mM ATP). Standard polyadenylation reactions (scaled up to 100 μl) containing $1.0-1.5\times10^6$ cpm of ^{32}P labeled substrate RNA, 44% HeLa nuclear extract, 1mM ATP, 20 mM creatine phosphate, 0.5 mM $MgCl_2$, 1% polyvinyl alcohol, 0.25 mM dithiothreitol, and 40ng/μl of t-RNA were incubated at 30°C for various lengths of time as indicated in legends. Heparin, at a final concentration of 4.0 mg/ml, was generally added to the reactions and the samples were further incubated for ten minutes on ice to suppress non-specific RNA:protein interactions. When appropriate, 5 μl aliquots of the reaction were removed prior to sedimentation which served as unsedimented control samples. The remaining samples were then diluted to 200 μl with the same buffer used for making gradients and layered on top of a 5−20% (w/v) sucrose gradient.

For sedimentation analysis of the purified hepatoma poly(A) polymerase, the enzyme was first dialyzed against the gradient buffer and concentrated using Amicon concentration filters. Approximately 20 μg of the enzyme was loaded onto the gradients and centrifuged in a Beckman L8−80M ultracentrifuge with a SW55Ti rotor (Beckman) at 50,000 rpm for 180 minutes at 4°C. Approximately twenty, 250 μl fractions were collected using an ISCO model 185 density gradient fractionator. To estimate the sedimentation coefficients of the complexes as well as poly(A)

polymerase, 1.0 A_{260} unit of a mixture of 5,18, and 28S ribosomal RNAs was sedimented in parallel gradients. The position of the RNA standards was deduced following optical density readings of the individual fractions at A_{260}.

The ^{32}P profile of the gradients was obtained by counting a 25 μl aliquot of each of the individual fractions. Poly(A) polymerase activity was monitored across the gradients essentially as described by Rose and Jacob (37). To analyze the RNA/protein complexes distributed throughout the gradients, 75 μl samples of the fractions were subjected to electrophoresis under non denaturing conditions in polyacrylamide gels. The reaction products were analyzed after first deproteinizing entire gradient fractions by incubation with proteinase K (200 μg/ml) in the presence of 0.2% SDS for 30 minutes. RNA was then extracted with phenol and precipitated with ethanol in the presence of 10 μg of carrier yeast transfer RNA. The pelleted RNA was electrophoresed under denaturing (8M urea) conditions in polyacrylamide gels.

UV induced RNA/protein cross linking assay

The ultraviolet light-induced RNA/protein cross-linking was performed essentially as previously described (23, 24). Polyadenylation reactions (25 μl) containing approximately 1×10^5 cpm of ^{32}P-labeled substrate RNA were incubated in the presence of either 44% crude HeLa nuclear extract or 1.0 μg of purified hepatoma poly(A) polymerase for 10 minutes at 30°C. In a few experiments, yeast transfer RNA (200 μg/ml) was added to the samples directly after incubation to serve as a non-specific competitor RNA. Cross-linking was induced by irradiation of the samples on ice with ultraviolet light at 254 nm for ten minutes. The ultraviolet light source (UV products model UVG-54) was held 4.5 cm above the samples during the exposure. Pipeting the samples onto the caps of microfuge tubes was found to be a very convenient and reliable means to ensure uniform exposure of the samples to light (38). Samples were then digested with RNase A (1mg/ml) for 15 minutes at 37°C to remove unbound RNA. After digestion, the RNA-protein adducts were denatured by heating in the presence of an equal volume of gel loading buffer (0.125 M Tris-hydrochloride [pH 6.8], 2% SDS, 2% β-mercaptoethanol, 20% glycerol, and bromphenol blue) for 5 minutes at 90°C. The samples were centrifuged for 30 seconds in a microfuge and the solubilized mixture was electrophoresed through a 10% SDS-containing polyacrylamide gel. The gels were fixed in 50% ethanol: 10% acetic acid for an hour, soaked in 10% glycerol for 15 minutes and subsequently dried under vacuum. The proteins labeled by the covalent transfer of ^{32}P ribonucleotides were visualized after autoradiography of the gel.

RESULTS

Isolation of polyadenylation-specific RNA/protein complexes

Proper cleavage and polyadenylation of pre-mRNA are determined largely by the ability of processing factors to associate specifically with pre-mRNA to form large multicomponent 3′ end processing complexes. The RNA/protein (RNP) complexes which assemble during in vitro polyadenylation were characterized by sucrose gradient sedimentation analysis. For this purpose, ^{32}P-labeled adenovirus-2 L3 pre-mRNA was incubated for various periods in HeLa nuclear extract capable of in vitro cleavage and polyadenylation (Fig. 1). The reaction mixtures were then treated with heparin, centrifuged through 5−20% linear sucrose gradients, and fractionated (see Methods). The

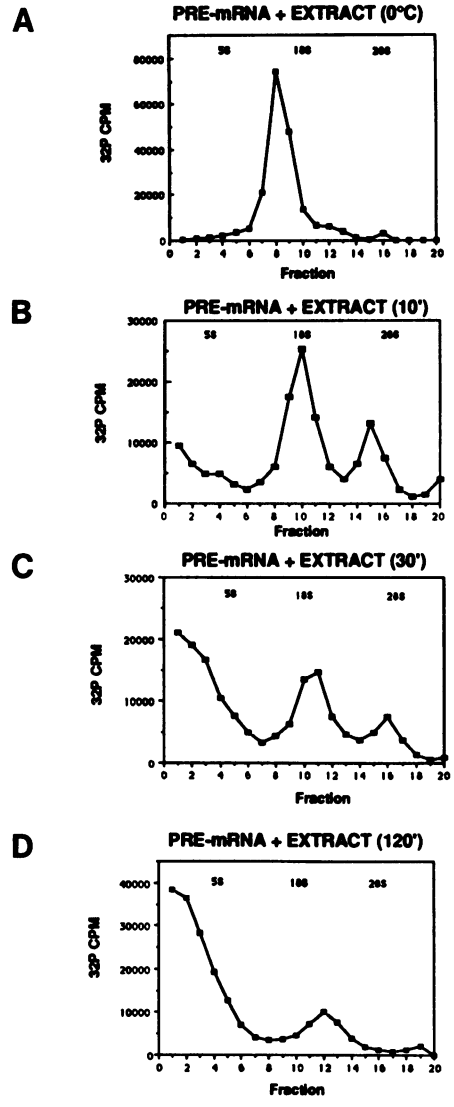

Figure 1. A kinetic profile of RNP complexes associated with polyadenylation. ^{32}P-labeled adenovirus L3 pre-mRNA was incubated in HeLa nuclear extract on ice (A) or at 30°C (B,C,D) for the indicated lengths of time (10, 30, and 120 min., respectively). The samples were treated with heparin and centrifuged through 5−20% linear sucrose density gradients. The direction of sedimentation is shown from left (top) to right (bottom of the gradient). The positions of 5S, 18S, and 28S ribosomal RNA standards are indicated. The profile of radioactivity across the gradients was determined by counting equal aliquots (30μl) of the individual fractions.

^{32}P profiles across the gradients were obtained by counting an equal aliquot of the individual gradient fractions. The untreated pre-mRNA sedimented to the first few fractions of the gradient (data not shown). In contrast, incubation of the pre-mRNA with extract resulted in an increase in the sedimentation of the RNA, as observed by a shift in the position of the ^{32}P peak(s).

When the pre-mRNA was incubated with extract under conditions which do not support polyadenylation (on ice; Fig. 1A), RNA assembled into RNP complexes which sedimented to approximately a 15 S position. A different profile of complex formation was observed when RNA was incubated with extract under an optimal reaction temperature (Fig. 1B,C, and D). At early stages of the reaction and prior to the accumulation of significant levels of cleaved and polyadenylated RNA, the RNP complexes were resolved as two distinct peaks which sedimented

at ~18 S and 25 S (Fig 1B). Formation of the faster sedimenting 25 S RNP complex was dependent on the sequences within the RNA. Thus, the slower sedimenting 18 S complex was formed with all RNA substrates tested whereas detection of the 25 S RNP complex was not observed with similarly sized RNA transcripts lacking poly(A) signals (pSP64-Pvu-II) or with substrate RNA containing a functionally disruptive point mutation in the critical upstream hexamer element (AAUAAA to AACAAA; data not shown). Both the 18 S and polyadenylation-specific 25 S complexes were observed at a stage in the reaction when both cleavage and polyadenylation were proceeding (Fig. 1C). Reactions analyzed at late stages, when the majority of the pre-mRNA was converted into poly(A)$^+$ product RNA, revealed a single ~20 S peak (Fig. 1D). The radioactivity present in the first few fractions of each of the gradients represents fragmented pre-mRNA which accumulates with increasing incubation time.

Next, the profile of both RNA and RNP complexes distributed across the gradient was examined using samples incubated for 20 minutes under polyadenylation reaction conditions (Fig. 2A). RNA present in the odd numbered fractions of this gradient was purified and analyzed by electrophoresis on denaturing polyacrylamide/urea gels and autoradiographed (Fig. 2B). It can be observed that the polyadenylated RNA co-sedimented with the 18 and 25 S RNP complexes. It was not detected in the top first few fractions of the gradient as would be anticipated if the poly (A)$^+$ RNA had not assembled into RNP complexes. When samples were analyzed in this manner after polyadenylation was allowed to proceed to near completion (120 minutes; Fig. 1D) poly(A)$^+$ RNA was localized predominantly to a 20 S position with less detectable levels trailing from the 25 to 20 S positions (data not shown). The profile of the RNP complexes throughout the gradient was monitored after directly analyzing an aliquot of the even numbered gradient fractions on non-denaturing gels (Fig. 2C). A comparison of the fractionated complexes with the unsedimented control (lane c) indicate that the 18 S and 25 S complexes isolated by sucrose density gradient fractionation appear identical in size to the preactive and active RNP complexes which we, and others, have previously characterized after direct electrophoresis of polyadenylation reactions on nondenaturing gel systems. It is evident from the experiments presented in Figures 1 and 2 that sedimentation analysis of polyadenylation reactions can provide the means to isolate and investigate the polyadenylation-specific 25 S complexes.

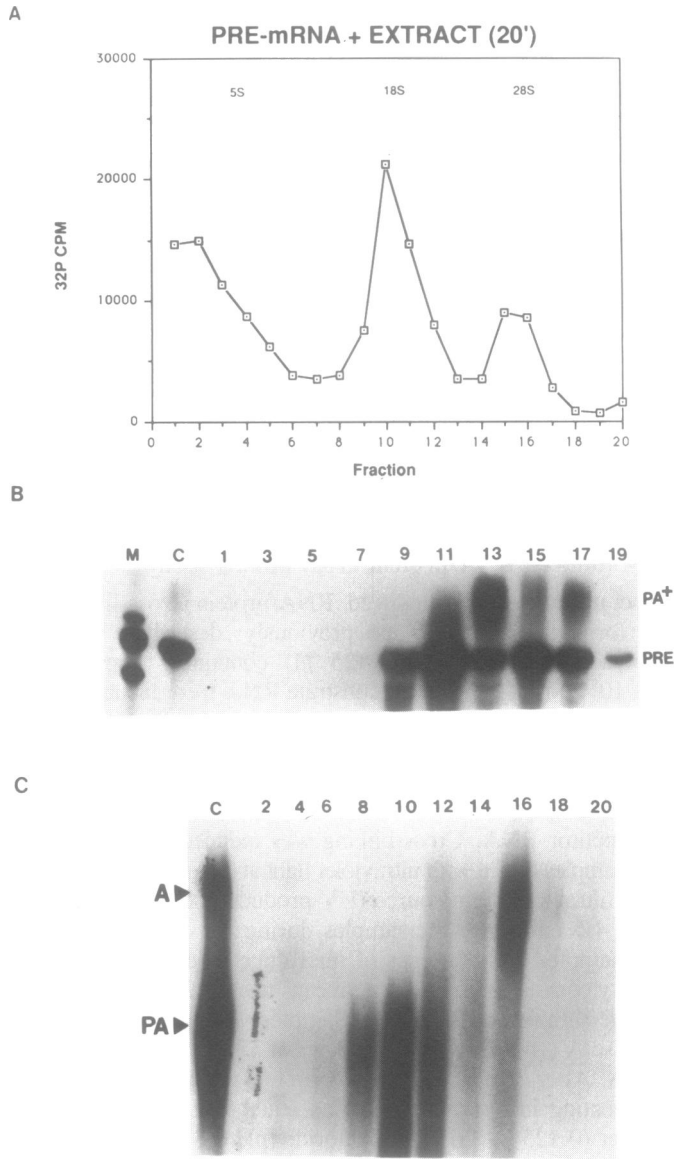

Figure 2. Distribution of RNA and RNP reaction products across the gradients. The sample from a 20 minute polyadenylation reaction was fractionated by sucrose gradient centrifugation (A). RNA present in the odd numbered fractions of this gradient was purified and analyzed on denaturing polyacrylamide/8 M urea gels and autoradiographed (B). The positions of both the polyadenylated (PA$^+$) and pre-mRNA (PRE) are indicated. ^{32}P-labeled DNA size markers are designated M and the 310 (upper), 271/281 (middle), and 194 (lower) fragments from a HindIII digestion of phage ϕX174 are displayed. The RNP complexes present in the even numbered gradient fractions were analyzed after a portion of the fractions was resolved by electrophoresis on non-denaturing gels followed by autoradiography (C). In both panels B and C, the lane designated c represents analysis of an unsedimented control sample. Positions of both faster migrating RNP (PA='preactive') as well as the slower migrating RNP (A='active') are indicated by arrows in panel C.

Anti-poly(A) polymerase antibodies which inhibit polyadenylation also prevent formation of 25 S polyadenylation-specific RNP complexes

We have previously reported that antibodies directed against purified poly(A) polymerase can specifically block the formation of cleaved and polyadenylated RNA (19). To investigate further the role of the polymerase in this reaction, the effect of direct addition of purified anti-poly(A) polymerase IgG on complex formation was explored (Fig. 3). When polyadenylation reactions were carried out for 20 minutes in the presence of HeLa nuclear extracts pretreated with preimmune control IgG (Fig. 3A), RNP complex formation (18/25S) as well as RNA formation were unaffected. In contrast, when the same reaction was carried out in the presence of an equal concentration of anti-poly(A) polymerase IgG (Fig. 3B) formation of the active 25S complex but not the preactive 18S complex was prevented. Furthermore, this treatment led to an increase in the amount of 18S complex relative to that formed in the presence of preimmune control IgG. In addition, inhibition of polyadenylated RNA formation was also observed, as only precursor RNA could be isolated from the gradient.

Location of poly(A) polymerase in the gradient fractions

To address whether the polymerase is indeed a component of the 18 S and/or 25 S RNP complexes the individual gradient fractions were assayed for the presence of poly(A) polymerase

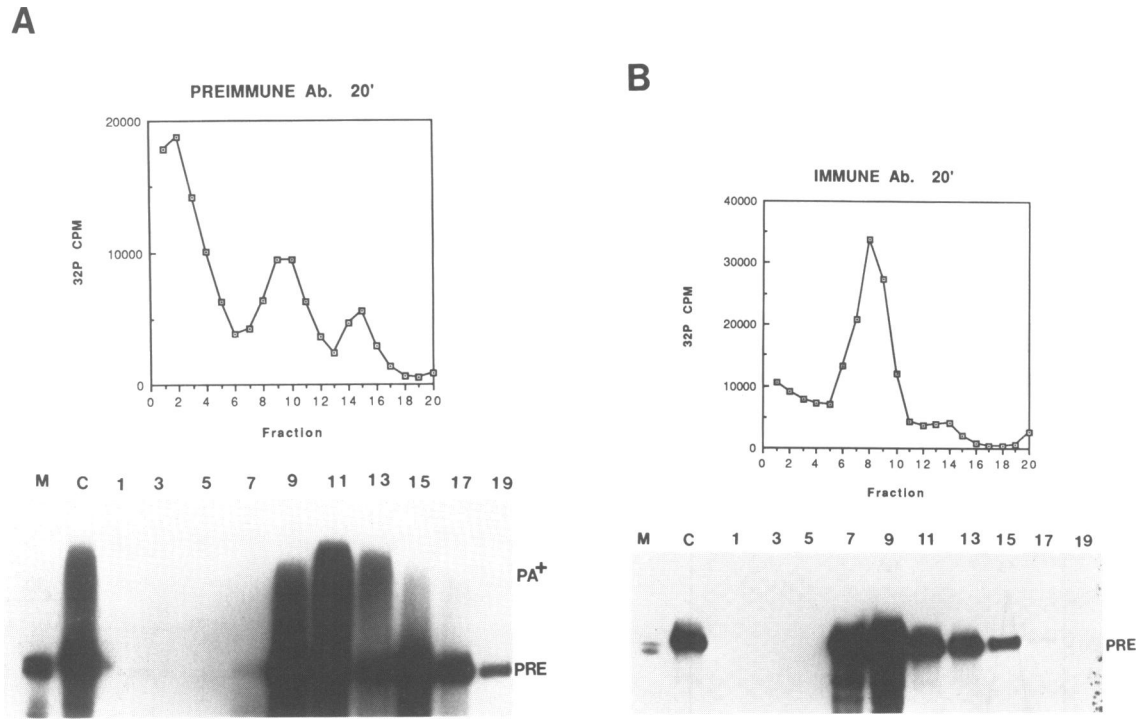

Figure 3. Effect of anti-poly(A) polymerase antibodies on gradient profiles. Polyadenylation reactions were carried out for 20 minutes in the presence of HeLa nuclear extracts pretreated with preimmune control IgG (Panel A) and an equal concentration (312 μg/44 μl nuclear extract) of anti-poly(A) polymerase IgG (Panel B). The samples were fractionated on sucrose gradients and the reaction products were analyzed as described in figure 2. RNA was isolated from the odd numbered fractions corresponding to the gradients shown directly above and analyzed. C represents an unsedimented control reaction sample. M corresponds to the marker lane exhibiting the ^{32}P-labeled 271/281 fragments from a HindIII digestion of phage ϕX174 DNA.

activity (Fig. 4). The poly(A) polymerase activity was assayed by a filter binding technique which monitors the incorporation of ^{3}H-AMP from ATP utilizing homopolymer poly(A) as the primer for the enzyme (36). Sedimentation of either purified hepatoma poly(A) polymerase (top panel) or crude HeLa nuclear extract (bottom panel) in the absence of exogenous pre-mRNA was performed. Consistent with our earlier observations (36), the polymerase activity of the purified poly(A) polymerase was localized to fractions sedimenting near the top of the gradient at approximately a 4–5 S region. The poly(A) polymerase activity of sedimented HeLa crude nuclear extract exhibited a peak at 4–5S as well as an additional highly reproducible peak at approximately a 12–15S position.

A shift of the poly(A) polymerase activity profile to the positions of the 18 S/25 S RNP complexes has not yet been observed when the substrate RNA was first incubated with the HeLa nuclear extract under a variety of experimental conditions and subsequently subjected to sedimentation analysis (data not shown). Similarly, immunoblot analysis of the fractions did not detect the enzyme in the complex (data not shown). It can be concluded from these studies that poly(A) polymerase is not a component of the complexes isolated by gradient fractionation.

Ultra-violet light induced cross-linking analysis of purified poly(A) polymerase

The next approach was to ascertain whether the purified poly(A) polymerase binds directly to pre-mRNA and if so, to determine whether the enzyme association is dependent on sequences or structures within the pre-mRNA. For this purpose, UV cross-linking was used, which has recently been applied to define protein components which specifically associate with polyadenylation substrates *in vitro* (23, 24).

The purified enzyme was incubated with the ^{32}P-labeled L3 pre-mRNA for ten minutes. The reaction mixture was then irradiated (254 nm), digested with RNase A, denatured, and resolved on SDS PAGE followed by autoradiography of cross-linked proteins (See Methods). As judged by the acquisition of the radiolabel, purified poly(A) polymerase readily cross-linked to this substrate (Fig. 5). This cross-linking was dependent on the use of UV light and was not observed when the reaction was first treated with proteinase K. Furthermore, a control protein (bovine serum albumin) did not become cross-linked under these conditions when used over a wide concentration range (data not shown). The interaction of the enzyme with adenovirus L3 pre-mRNA was sensitive to greater than 100 mM KCl in the reaction (Fig. 5A) and t-RNA was an effective competitor of this association (Fig. 5B). Furthermore, the pure enzyme was cross-linked with apparent equal efficiencies to wildtype pre-mRNA and functionally disruptive mutant derivatives of this substrate (data not shown). These substrates included a hexamer point mutant (AAUAAA to AACAAA), a downstream mutant lacking nucleotides beyond + 10, as well as bacterial RNA sequences which entirely lack poly(A) signals.

In an attempt to stabilize, by covalently cross-linking, the closely associated RNA binding components in crude nuclear extracts and to determine if the unpurified poly(A) polymerase directly binds to pre-mRNA, the cross-linking analysis was carried out using crude HeLa nuclear extract at 4°C (lanes 1–5) and 30°C (lanes 6–10) (Fig. 6). Both a wild-type substrate (AAUAAA) and one containing a hexamer point mutation (AA-

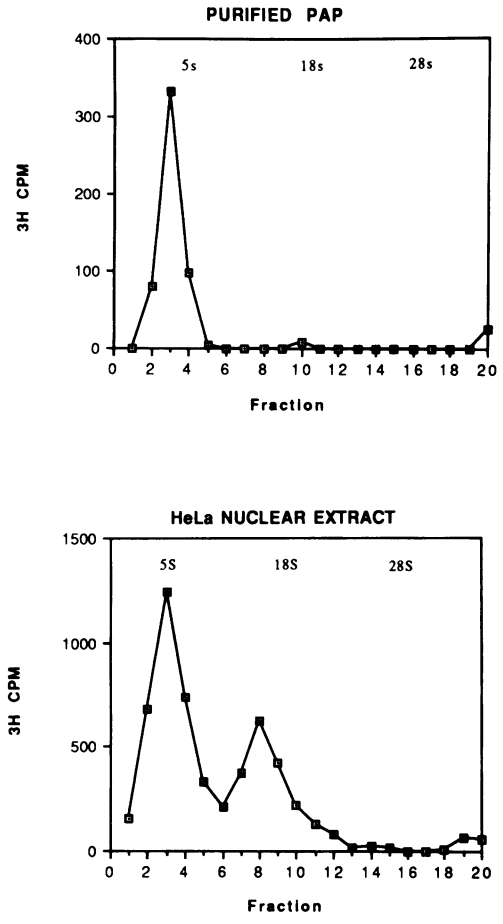

Figure 4. Analysis of the gradient fractions for the presence of poly(A) polymerase. Purified hepatoma poly(A) polymerase (20 μg; top panel) and crude HeLa nuclear extract (44 μl ; bottom panel) were fractionated on sucrose density gradients. The individual gradient fractions were assayed for the presence of poly(A) polymerase activity. Poly(A) polymerase activity was assayed by a filter binding technique which monitors the incorporation of ^{3}H-AMP from ATP utilizing homopolymer poly(A) as the primer for the enzyme as described in the Methods section. Positions of 5S, 18S, and 28S ribosomal RNA standards are indicated.

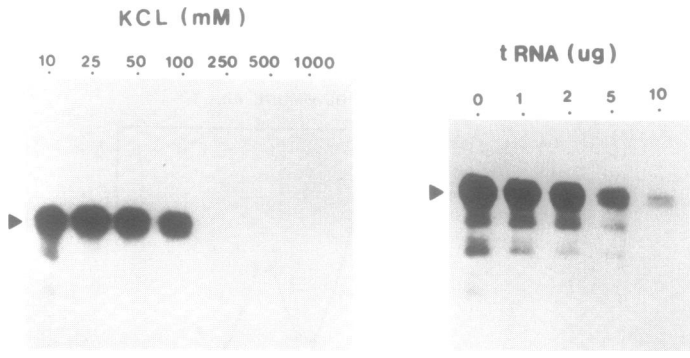

Figure 5. U.V. cross-linking analysis of purified poly(A) polymerase. Purified 48 kDa poly(A) polymerase was incubated with ^{32}P-labeled adenovirus L3 pre-mRNA under polyadenylation conditions in the presence of increasing concentrations of KCl (A) or yeast t-RNA (B). The solid arrow designates the position of the ^{32}P-labeled RNA/poly(A) polymerase adducts.

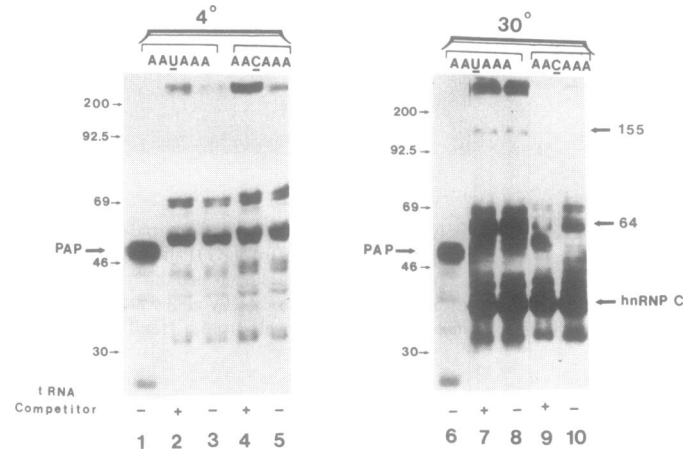

Figure 6. Comparison of the cross-linking pattern of purified poly(A) polymerase with that observed with active crude nuclear extracts. Cross-linking analysis was carried out with purified poly(A) polymerase and HeLa nuclear extract at 4°C (lanes 1–5) and 30°C (lanes 6–10). A wildtype substrate (AAUAAA) and one containing a point mutation (AACAAA) were employed. Cross-linking was performed both in the presence (+) and absence (–) of yeast transfer RNA (200 μg/ml) which was added to the samples directly after incubation and prior to irradiation. The previously reported major cross-linkable proteins (hnRNP C, 64 k Da, and 155 kDa) and the molecular weights of protein markers are indicated. Cross-linked poly(A) polymerase is denoted by a solid arrow.

CAAA) were employed. A distinct profile of cross-linked products was observed at the two temperatures employed. The use of nonspecific competitor tRNA (200 μg/ml) prior to UV irradiation appeared not to effect cross-linking of components under either reaction condition.

At 4°C, an identical pattern of cross-linked proteins was observed with both the wildtype and mutant substrate pre-mRNA. In contrast, incubation of the extract under more physiological temperatures (30°C) resulted in distinct array of cross-linked proteins which bound to each substrate. The results of this experiment are consistent with the findings of others (23, 24) who have previously identified the major crosslinkable proteins in crude extracts as being the hnRNP C1/C2 proteins, a 64–68 kDa, and a 155 kDa protein. For instance, while the hnRNP C proteins were bound to both wildtype and mutant substrate RNA, cross-linking of the 64 kDa protein was reduced or absent when the mutant substrate was used. Most strikingly, cross-linking of the 155 kDa protein was detected strictly on substrate RNA containing an intact hexamer element. Furthermore, the 155 kDa protein specifically cross-linked to wildtype pre-mRNA whether the substrate was radiolabeled with either ^{32}P-labeled UTP or ATP (data not shown).

The results of this analysis indicate that although the purified polymerase was cross-linked under the two assay conditions, a crosslinkable protein of the same molecular weight as the poly(A) polymerase was not observed in the crude extracts under either reaction condition. Apparently, poly(A) polymerase in crude extracts does not directly contact the pre-mRNA in a cross-linkable manner.

A poly(A) polymerase-containing fraction is needed to reconstitute polyadenylation-specific RNP complexes

The biochemical fractionation of active nuclear extract to isolate and characterize the components responsible for cleavage and polyadenylation is currently being pursued by a number of laboratories (25–31). A consensus has emerged from these

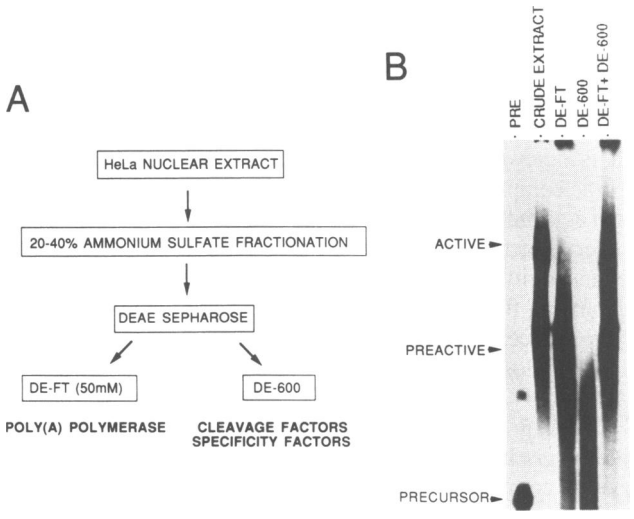

Figure 7. Gel shift analysis of the individual and combined column fractions. Chromatographic separation of poly(A) polymerase from additional factors participating in the cleavage and polyadenylation reaction was accomplished by the following fractionation scheme (A). The KCl concentrations (mM) of the fractions is indicated. Gel shift analysis was performed on the naked pre-mRNA (PRE), unfractionated extract (crude extract), and individual and combined chromatographic fractions (B). The position in the native gel where the naked pre-mRNA (PRE), non-specific RNP complex (preactive), and polyadenylation-specific RNP complex (active) migrated to is indicated by arrows on the left of the autoradiogram.

studies that multiple components are required for the entire reaction to proceed accurately and efficiently and that the poly(A) polymerase plays a pivotal role in both the cleavage and polyadenylation aspects of this reaction. Simple chromatographic separation of the poly(A) polymerase from the remaining important components of the reaction can be achieved by ion exchange chromatography. The polymerase is eluted entirely in the flow through fraction of a DEAE sepharose fractionation of ammonium sulfate fractionated extract whereas the other necessary reaction components can be eluted from this column at high salt concentrations (Fig. 7A). To address the issue as to which of these two fractions would bring about formation of the polyadenylation-specific RNP complexes, we assayed the individual fractions for there ability to retard the pre-mRNA in a native gel system. (Fig. 7B). The results of this analysis indicate that the individual fractions were not capable of shifting the RNA to the position of the gel to which the polyadenylation-specific complex migrated (compare the gel-shift pattern of the fractions with that of the unfractionated crude nuclear extract control sample). However, the combination of the polymerase-containing fraction with the high salt fraction which contain the cleavage/specificity factors, was necessary to reconstitute the active polyadenylation-specific complexes.

DISCUSSION

The principle objective of the present study was to investigate the role of poly(A) polymerase in the assembly of polyadenylation-specific RNP complexes. Incubation of pre-mRNA with HeLa nuclear extract resulted in the formation of both nonspecific (pre-active) and polyadenylation-specific (active) complexes. The nonspecific complexes which sedimented at approximately 15−18S, are probably due to interaction of general RNA-binding components, many of which may be of the hnRNP class (for review see 38). When incubated under conditions which support cleavage or polyadenylation, the pre-mRNA was specifically assembled into larger 25S RNP complexes. The increase in size of the complex is suggestive of the association of additional components with the pre-mRNA. Consistent with their functional role, the 25S complexes formed only on competent polyadenylation substrates. Substrates containing a mutated hexamer (AAUAAA to AACAAA) or transcripts which lacked poly(A) signals did not support the assembly of active complexes and were also not polyadenylated (data not shown). The 25S complexes remained relatively stable over the period of the reaction which corresponded to maximal polyadenylation but was converted to a unique 20S complex at later stages of the reaction when over 90% of the pre-mRNA had been converted to poly(A)$^+$ products. Polyadenylated RNA was first observed to co-sediment with the active 25S complexes which suggests that the RNA is originally processed by the components which comprise the 25S complexes.

Inhibition of the 25S complex formation by anti-poly(A) polymerase antibodies implies that the enzyme is a constituent of this processing complex. That the polyadenylation of pre-mRNA by poly(A) polymerase is dependent on the intact AAUAAA sequence following binding with a hexamer-binding specificity factor (32) would also support association of the enzyme with these complexes. The specificity of this reaction was demonstrated by the persistence of the 15S complex when the reaction was carried out in the presence of the antibodies. Further proof for the functional participation of the poly(A) polymerase in the 25S complex was provided by the inability of the extract to polyadenylate the pre-mRNA substrate in the presence of immune IgG. The inhibition of 25S complex formation by the anti poly(A) polymerase antibodies is in conflict with the report that stable complexes can be assembled with the mRNA precursor in the absence of poly(A) polymerase (28) as well as the inability to detect poly(A) polymerase in the complex by the conventional procedures (see the following paragraphs for discussion on this subject). It is conceivable that purification of the cleavage and specificity factors led to the loss of components required for interaction with poly(A) polymerase.

As stated above, the intriguing feature of the present studies was the failure to detect poly(A) polymerase in the 25S complexes by direct activity measurements or by immunoblot analysis (data not shown). Interestingly, the polymerase activity of the purified poly(A) polymerase did not co-sediment with the RNP complexes but instead was localized to fractions sedimenting near the top of the gradient at approximately a 4−5 S position. On the other hand, the activity of the sedimented HeLa crude nuclear extract exhibited a peak at 4−5S as well as an additional, highly reproducible, peak at approximately a 12−15S position (See figure 4). The sedimentation coefficients of the two peaks suggest molecular weights of approximately 50 kDa and 200−300 kDa for the poly(A) polymerase activities in the HeLa extract. A population of free form of the enzyme as well as a species of the polymerase complexed with additional components such as a specificity factor may exist in crude nuclear extracts and perhaps *in vivo*. This is consistent with the finding that pre-cleaved mRNAs can be polyadenylated in an AAUAAA-dependent manner by fractions containing only a poly(A) polymerase and specificity factor fraction (26, 31, 32). Alternatively, a pre-assembled poly(A) polymerase/endonuclease present in the extracts could account for this observation and may explain the tight coupling of the cleavage and polyadenylation reactions. The

nature of the additional poly(A) polymerase- containing entity in extracts must be further explored.

The failure to detect poly(A) polymerase in the isolated complexes conflicts with other data such as the requirement of the enzyme for both the cleavage and polyadenylation stages of the reaction (19, 26, 31) as well as the finding that anti-poly(A) polymerase antibodies prevent formation of the polyadenylation-specific complexes. The inability to detect poly(A) polymerase in the polyadenylation-specific complexes may relate to the properties of poly(A) polymerase or the conditions required to isolate the complexes. The reason why some factors survive the isolation procedure whereas poly(A) polymerase apparently does not, may be a reflection of the relative affinities these components have for either the RNA or proteins components within the complexes. As such, the interaction of poly(A) polymerase in the complex may not be sufficiently strong to withstand the centrifugal force during the complex isolation. Alternately, the reaction conditions could be contributing to the loss of the enzyme from the complexes. Heparin was used in these studies to dissociate the nonspecific complex components in order to obtain a more authentic profile of specific binding components. In the past (21), heparin-resistant, polyadenylation-specific complexes have been isolated which appear identical to those described in these studies. The heparin-resistant-complexes presumably retain components which bind to the AAUAAA element as well as critical downstream sequences (21). However, we failed to detect poly(A) polymerase in the complexes when they were treated with heparin over a wide range (0.1, −5 mg/ml) of concentrations or when heparin was omitted (data not shown). Similarly, the effects of various ionic strengths were tested. Low salt (40−50 mM KCL) favored detection of well resolved pre-active and active RNP complexes but poly(A) polymerase was not observed in these complexes. In the presence of higher salt (200 mM) the specific 25 S complexes were not detected on the gradients and interestingly only the 4−5 S peak of polyadenylation activity was observed. This suggests that the higher density poly(A) polymerase activity dissociates in the presence of high salt and does not, therefore, represent aggregation of multiple poly(A) polymerases through hydrophobic interactions. Increasing the concentration of substrate RNA did not result in the detection of poly(A) polymerase in the complexes. The use of precleaved pre-mRNA (3′ terminus located 10 nucleotides downstream of poly(A) site), which would presumably be a perfect candidate for the polyadenylation reaction in the absence of cleavage, also did not lead to association of the enzyme with RNA. The combined use of precleaved substrate RNA and cordycepin triphosphate (3′dATP) has been reported to stabilize polyadenylation-specific complexes (22, 1), probably due to tight binding of poly(A) polymerase to substrate RNA. This combination also failed to demonstrate association of poly(A) polymerase with the substrate.

The absence of poly(A) polymerase in the complexes may be due to its transient rather than stable association with the complex. However, since the enzyme is required for the cleavage reaction, it would be expected to occupy a position in the complex prior to the cleavage step as well as during the later events of poly(A) addition. We have examined the complexes over an extensive time course which represented many stages of the reaction and yet could not detect the enzyme in the RNP complexes. Recently, it has been demonstrated that dynamic changes in the components of the polyadenylation-specific complexes take place during cleavage and polyadenylation (24, 40). These findings are consistent with the concept that specific factors must first interact with pre-mRNA to form a polyadenylation-specific complex and that the events associated with processing lead to dissociation of the components. The strength of interaction between poly(A) polymerase and the complex may well be affected by any conformational changes occurring during these reaction events.

It is noteworthy that another group found polyadenylation-specific complexes isolated from glycerol gradients were inactive when assayed for cleavage or polyadenylation (20). The absence of every functional component in the complex might account for the inactivity of the complex. The most straight-forward explanation for the inability of the isolated complex to function in the processing reaction is that poly(A) polymerase was selectively lost from the complexes. In an attempt to reconstitute functionally-active complexes, fractions containing the two peaks of poly(A) polymerase activity (4−5 and 12−15 S) were mixed with the gradient fractions containing the 25 S polyadenylation-specific complexes. These attempts proved unsuccessful, as specific polyadenylation activity was not restored following reconstitution.

The absence of a key functional component in an RNP complex has also been reported in another RNA processing system. For example, despite the availability of data implicating the involvement of U1 snRNP in splicing activity as well as spliceosome formation (41−45), initial approaches failed to detect U1-snRNP in spliceosomes. Gradient-purified, avidin-selected spliceosomes formed on biotinylated pre-mRNA were found to contain expected quantities of U2, U4, U5, and U6 snRNA, but not U1 snRNA (46). Also, U1 snRNA was not observed in spliceosomes isolated on native polyacrylamide gels following Northern blot analysis (35, 47). These data were unexpected and occurred in the face of the accumulated evidence indicating that U1 snRNP was a critical factor for splicing and spliceosome formation. Subsequently, it was observed that U1 snRNP enters spliceosomes at early stages and is present throughout splicing steps but that its association with the complex is less stable than other components and reaction conditions contributed to the isolation of U1-deficient spliceosomes. (48, 49). The inability to detect poly(A) polymerase in polyadenylation-specific RNP complexes is reminiscent of the initial approach toward demonstrating the binding of U1 snRNP in splicing-specific (spliceosome) RNP complexes. Current procedures may not be ideal to capture the polyadenylating enzyme in a stable form as part of the RNP complex.

The results of the UV cross-linking studies indicate that purified poly(A) polymerase is capable of making direct contact with RNA but the enzyme does not appear to recognize the conserved features at poly(A) regions (figure 6). These results differ from those of two recent studies which found that partially purified HeLa extract fractions containing poly(A) polymerase did not become cross-linked to radiolabeled pre-mRNA (28, 50). The basis of the discrepancy is unknown but may relate to the degree of purity and the quantity of the poly(A) polymerase used in the cross-linking assays. The cross-linking of poly(A) polymerase to RNA is consistent with the ability of the enzyme to polyadenylate a wide array of nonspecific RNA primers. The observation that the enzyme cross-linked with equal efficiencies to a number of transcripts, was prevented from binding by moderate ionic strength, and was competed by t-RNA implies that the enzyme exhibits a general RNA binding activity. However, the polymerase does not appear to bind directly to pre-mRNA (in a cross-linkable manner) in crude nuclear extracts. The crosslinking method is a technique which detects a very specific subset of molecules, namely, those which directly bind

to RNA and are in contact with the radiolabeled residues. It is therefore possible that poly(A) polymerase occupies a position in the polyadenylation-specific complex associated through protein/protein interactions and as such the interaction would not be detectable by the cross-linking method.

A strong evidence for the role of poly(A) polymerase in the RNP complex formation has emerged from studies involving biochemical fractionation of components and reconstitution of these components into a polyadenylation-specific complex (See Fig. 7). For these studies, the extract was fractionated on a DEAE-sepharose column. The column flow-through fraction (DE-FT) contained poly(A) polymerase and no cross contamination of the poly(A) polymerase was observed in the high salt fraction (DE-600) as confirmed by poly(A) polymerase assay measurements and immunoblot analysis (data not shown). Reconstitution of cleavage and specific polyadenylation required mixing of these two fractions as the individual fractions alone were unable to support either of these activities (30). Incubation of radiolabeled pre-mRNA with either fraction alone did not yield RNP complexes which resembled those formed with the unfractionated extract (Fig. 7). In contrast, the combination of the flow-through fraction and high salt fraction restored ability to assemble the larger, polyadenylation-specific RNP complex. We postulate that the component in the flow-through fraction which contributes to complex formation is the poly(A) polymerase itself. This statement is primarily based upon the observations (26, 27, 51) that poly(A) polymerase is the only component of this fraction which is indispensable for reconstituting the cleavage and polyadenylation activities. The data presented here suggest that poly(A) polymerase is a component of polyadenylation-specific complexes and that the association of the enzyme with these structures is less stable than that of other components and is therefore not detectable by the variety of approaches used in this study. Poly(A) polymerase may be a member of the complex which is associated through protein:protein interactions.

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