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METHOD

Isolation and characterization of polyadenylation complexes assembled in vitro

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ABSTRACT

We developed a two-step purification of mammalian polyadenylation complexes assembled in vitro. Biotinylated pre-mRNAs containing viral or immunoglobulin poly(A) sites were incubated with nuclear extracts prepared from mouse myeloma cells under conditions permissive for in vitro cleavage and polyadenylation and the mixture was fractionated by gel filtration; complexes containing biotinylated pre-mRNA and bound proteins were affinity purified on avidin-agarose resin. Western analysis of known components of the polyadenylation complex demonstrated copurification of polyadenylation factors with poly(A) site-containing RNA but not with control RNA substrates containing either no polyadenylation signals or a point mutation of the AAUAAA polyadenylation signal. Polyadenylation complexes that were assembled on exogenous RNA eluted from the Sephacryl column in fractions consistent with their size range extending from 2 to 4 × 10^6 M_r. Complexes endogenous to the extract were of approximately the same apparent size, but more heterogeneous in distribution. This method can be used to study polyadenylation/cleavage complexes that may form upon a number of different RNA sequences, an important step towards defining which factors might differentially associate with specific RNAs.

Keywords: pre-mRNA 3′-end processing; plasma cells

INTRODUCTION

Over the past decade *trans*-acting factors necessary and sufficient for accurate in vitro cleavage and polyadenylation of pre-mRNA have been have been identified, primarily from HeLa cells and calf thymus; many have been cloned and characterized as reviewed (Wahle & Keller, 1992; Keller, 1995; Zhao et al., 1999). Although functional interactions of polyadenylation factors with splicing (Lutz et al., 1996; Gunderson et al., 1997, 1998) or transcription factors (Dantonel et al., 1997) have been described, the purification of intact polyadenylation complexes containing these factors and a systematic study of their assembly has been lacking.

The four-subunit (160, 100, 73 and 30 kDa) cleavage-polyadenylation specificity factor (CPSF) is required for both cleavage and polyadenylation (Bienroth et al., 1991; Gilmartin & Nevins, 1991; Murthy & Manley, 1992). The 160-kDa subunit of CPSF (CPSF-160) recognizes the AAUAAA element in RNA (Gilmartin & Nevins, 1991; Keller et al., 1991; Jenny et al., 1994). CPSF-160 also facilitates the interaction of CPSF and cleavage stimulation factor (CstF) (Murthy & Manley, 1995) that is required to form the stable RNA–protein complex for correct recognition of the cleavage and polyadenylation site. The 77-kDa subunit of CstF bridges the 64- and 50-kDa subunits while interacting directly with CPSF by protein–protein interactions (Takagaki & Manley, 2000). The 64-kDa subunit of CstF (CstF-64) contains an RNA-binding domain of the RNP-type (Takagaki et al., 1992) that interacts directly with the downstream GU-rich element of transcripts containing poly(A) signals

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Abbreviations: CPSF: cleavage and polyadenylation specificity factor; CstF-64: 64-kDa subunit of cleavage stimulation factor CstF; Ig: immunoglobulin; M_r: relative molecular mass/size; mu-mb, IgM membrane-specific poly(A) site.

(Wilusz et al., 1990; Takagaki et al., 1992; MacDonald et al., 1994; Beyer et al., 1997; Takagaki & Manley, 1997). The strength of CstF-64 interaction with the pre-mRNA is an indicator of the strength of the poly(A) site (MacDonald et al., 1994; Chen et al., 1995). The amount of CstF-64 is regulated with the transition from Go to S (Martincic et al., 1998) and alternative forms of CstF-64 have been seen in calf thymus (Beyer et al., 1997) and in mouse spermatocytes (Wallace et al., 1999).

Also required for polyadenylation and cleavage are cleavage factors I_m and II_m (CF I_m and II_m), poly(A) polymerase, and poly(A)-binding protein II. Cleavage factor I_m has been purified to near homogeneity from HeLa cell nuclear extracts and consists of heterodimers of 25- and 59-, 68-, or 72-kDa subunits (Rüegsegger et al., 1996, 1998). Poly(A) polymerase is required for both cleavage and polyadenylation and, in conjunction with CPSF, specifically initiates polyadenylation of AAUAAA-containing transcripts (Takagaki et al., 1988; Christofori & Keller, 1989). Poly(A) polymerase is differentially phosphorylated during the cell cycle and several isoforms have been reported (Zhao et al., 1999). Poly(A)-binding protein II attaches to the growing poly(A) tail and acts as a stimulatory factor for elongation (Wahle, 1991).

We have developed a method to purify polyadenylation complexes assembled on exogenously added poly(A) sites that allowed us to analyze the size and protein composition of the assemblage. We used a two-step procedure, employing gel filtration and affinity purification, to analyze polyadenylation complexes assembled in vitro on RNAs containing viral and immunoglobulin sites by using nuclear extracts prepared from cells of the fully differentiated B-lineage, namely myeloma cells. This method, adapted from the HeLa spliceosome purification scheme (Reed, 1990), provides a means of efficiently separating in vitro-assembled RNA–protein complexes from endogenous complexes of similar size, and allows analysis of their protein content both by western blots and silver staining. This method can be applied to a variety of cell types and pre-mRNAs and may be a valuable tool in investigation of systems in which poly(A) site choice is regulated and/or different isoforms of the basal factors are expressed.

RESULTS

Mouse myeloma cell nuclear extracts are competent for pre-mRNA processing

The mouse myeloma cell line AxJ produces primarily secretory-specific immunoglobulin heavy chain mRNA and protein (Milcarek et al., 1996). Nuclear extracts were prepared from the myeloma cell line using a variation of the Dignam extraction (see Materials and methods). We previously reported that myeloma extracts prepared by this method were competent for in vitro cleavage and polyadenylation activity. Several pre-mRNA substrates used in the experiments reported here are shown in Figure 1A. Data shown in Figure 1B demonstrate the efficient cleavage of pre-mRNAs containing the SV40 late poly(A) site (SVL). A construct that contains only the sequence downstream of the AAUAAA (SVL-dsc) is not cleaved, demonstrating that the myeloma nuclear extracts require upstream sequences in the RNA substrate, including AAUAAA, for in vitro cleavage. Shown in Figure 1C is a time course for cleavage with an RNA containing the immunoglobulin mu membrane-specific (mu-mb) poly(A) site. The size of the cleavage product is that predicted from the sequence; accurately cleaved RNA is detectable within 10 min. A nearly identical construct, differing only by the presence of a mutation in the AAUAAA converting it to the inefficient signal AAGAAA, is incapable of being cleaved (Fig. 1D), again demonstrating the accuracy and specificity of in vitro cleavage in these extracts. The polyadenylation of mu-mb RNA, although quite efficient, takes longer than the cleavage reaction to achieve maximal activity, as demonstrated by the time course shown in Figure 1E. Figure 1F shows the immunoprecipitation of CstF-64 crosslinked to RNAs shown in Figure 1A that are used in subsequent experiments; the results demonstrate that CstF-64 binding to the RNA is dependent upon the AAUAAA polyadenylation signal in these extracts. We have not characterized the higher M_r protein species seen with mu-mb and G_4SV pre-mRNAs. As measured by UV crosslinking, binding of CstF-64 to poly(A) site-containing RNAs plateaus within 5 min and remains stable for at least 20 min (data not shown). Table 1 summarizes the time courses of the RNA processing activities we observed in these extracts. Cleavage and polyadenylation activities are maximal within 15–20 min and 1–2 h, respectively. Extracts prepared by this method are also competent for splicing of viral substrates (not shown).

Optimization of conditions for in vitro polyadenylation complex formation and elution from avidin agarose

For efficient in vitro assembly of polyadenylation complexes on biotinylated RNA substrates we modified our previously described conditions (Edwalds-Gilbert & Milcarek, 1995). Specifically, 15–25 pmol of mu-mb pre-mRNA (containing ~1 biotinylated U per molecule and [^{32}P] tracer) were incubated for 20 min at 30 °C in a mixture containing 3–6.25 mg nuclear extract, 40 μg/mL yeast tRNA, 1 mM ATP, 0.7 mM $MgCl_2$, 20% glycerol (v/v), 80 U/mL RNasin, 2 mM DTT, and 230 mM potassium glutamate in 20 mM HEPES, pH 7.9, in a volume of ~1 mL. Electrophoretic mobility shift assays using mu-mb RNA demonstrate that very large ATP-

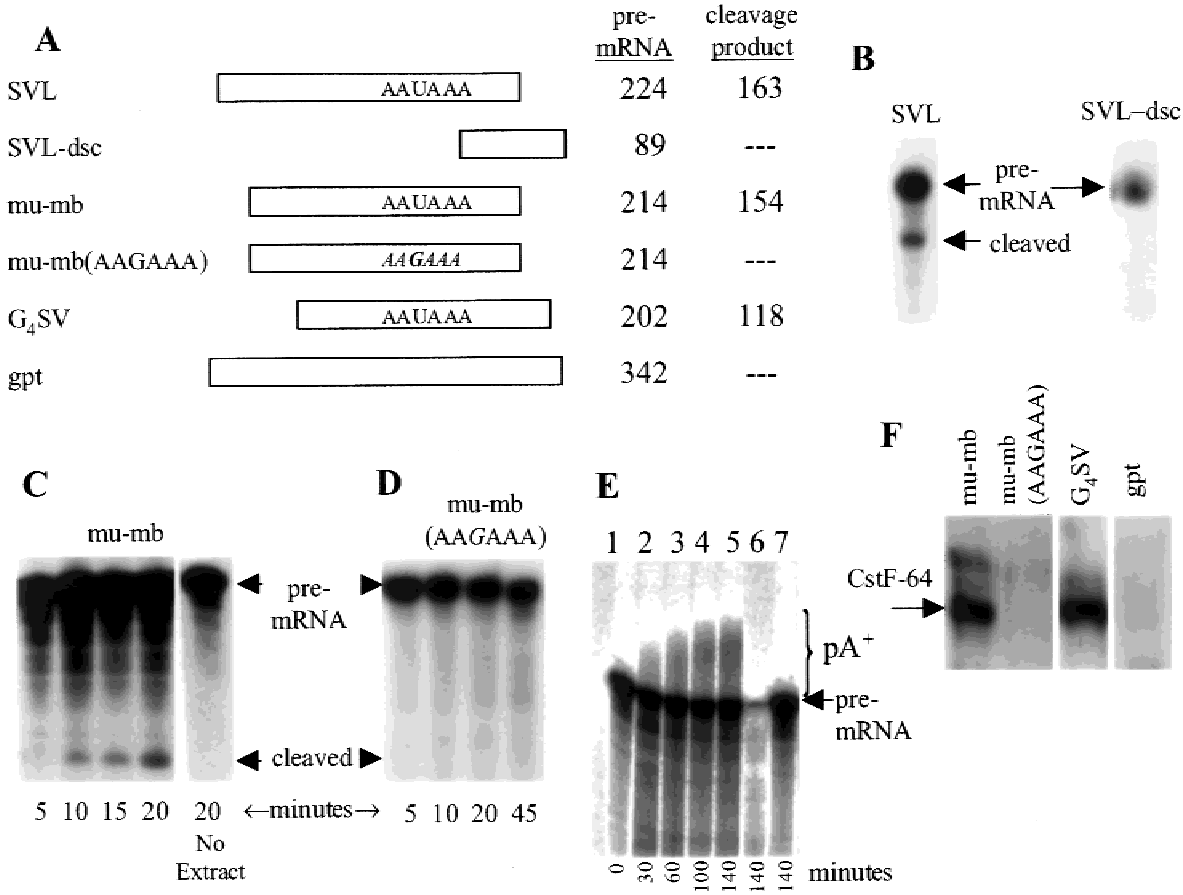

FIGURE 1. Mouse myeloma cell nuclear extracts are competent for in vitro pre-mRNA processing. **A**: Substrates used for in vitro studies. Sizes of the pre-mRNAs and cleavage products are indicated on the right and the approximate location of the AAUAAA polyadenylation signal is noted. The poly(A) sites included in these substrates are as follows: SVL: SV40 late; SVL-dsc: the region downstream of the poly(A) addition site of SVL; mu-mb: mouse IgM membrane; mu-mb AAGAAA: a substitution of one nucleotide in the mu-mb poly(A) signal; G_4SV: chimeric IgG2b/SV40 early; gpt: a portion of the *E. coli* xanthine-guanine phosphoribosyl transferase gene. **B**–**F**: Autoradiograms of dried gels. In **B**–**E**, [^{32}P]-labeled RNA was electrophoresed on 5% acrylamide:8 M urea. **B**: The indicated RNAs were incubated with myeloma nuclear extract for 20 min under cleavage conditions (see Materials and methods). **C**: mu-mb pre-mRNA was incubated for the indicated times under cleavage conditions. **D**: The point mutant, mu-mb (AAGAAA) pre-mRNA, was incubated for the indicated times under cleavage conditions. **E**: mu-mb pre-mRNA was incubated for the indicated times under polyadenylation conditions as described in Materials and methods and electrophoresed in lanes 1 to 5. In lane 6 the reaction was carried out without added ATP and in lane 7 nuclear extract was omitted. **F**: The indicated [^{32}P]-labeled RNAs were incubated with myeloma nuclear extract, UV irradiated, and immunoprecipitated as described in Materials and methods. Following RNase digestion, proteins were separated by SDS-10% PAGE.

dependent complexes form under these conditions and remain grossly intact well beyond a 20 min incubation period (data not shown).

Before fractionating these complexes of biotinylated pre-RNA and protein by size exclusion chromatography, we determined their stability during the washing and elution conditions that we would employ with avidin agarose. Assembly reactions were diluted in 1× PCB-1 column buffer and incubated with avidin resin at 4 °C for various periods; maximum binding occurred in 18 h, as judged by the binding of [^{32}P] tracer. The resin was subjected to various washing and elution conditions to minimize nonspecific, biotin-independent binding without compromising the binding and elution of the

TABLE 1. Kinetics of in vitro RNA processing assays[a].

Time	CstF-64 UV crosslinking	Complex formation[b]	Cleavage	Polyadenylation
0	–	–	–	–
5 min	0	+	+/–	–
10 min	0	++	++	+/–
20 min	+++	+++	+++	++
90 min	0	++	+++	+++

[a]Summary of various RNA processing assays using the mu-mb pre-mRNA substrate in AxJ nuclear extract as described in Materials and methods. Maximal activity in each category was assigned a value of "+++" and other values were compared to the maximum.

[b]Complex formation was evaluated on the basis of an electrophoretic mobility shift assay of the specific complex formed by the mu-mb pre-mRNA in AxJ myeloma nuclear extracts (data not shown).

specific complexes. Washing with buffer containing 150 mM NaCl reduced nonspecific tracer binding as compared to 50 mM NaCl. However, washing with 300 mM NaCl did not demonstrate additional improvement (data not shown); thus, all subsequent washes of the resin were with 150 mM NaCl in 20 mM Tris, pH 7.6. Blocking the affinity resin with either BSA or yeast tRNA prior to use did not diminish nonspecific binding of myeloma nuclear proteins (data not shown).

After washing and RNase A treatment of the resin-bound complexes, various elution conditions were tested (Fig. 2). Western analysis of the eluted protein reveals that RNase treatment alone is not sufficient to release CstF-64, CstF-77, and CPSF-100 from the biotinylated RNA (Fig. 2, lanes 1–4, +); these proteins are eluted most abundantly from the biotinylated RNA–resin when an SDS-containing buffer is employed (Fig. 2, lane 5+). These data suggest that protein–protein interactions hold the polyadenylation complexes together tightly and protect the RNA tethering the protein to the resin from digestion. Based upon these results, all subsequent protein elution from the resin was performed with a buffer containing 20 mM Tris, pH 7.6, 2% SDS and 20 mM DTT. Also demonstrated in Figure 2A is a low level of binding of some polyadenylation factors to the affinity resin (see lane 6, RNA synthesized without biotin.) Although elution of polyadenylation factors from the resin required detergent-containing buffers, analysis by silver staining revealed some proteins eluted by each of the buffers tested (Fig. 2B). Some of these proteins appeared to be specific to the in vitro-assembled complex [see especially in the 1 M NaCl elution (Fig. 2B, lane 4+)], suggesting the purification of distinct, more loosely associated proteins along with the basal factors. The 100-kDa subunit of CPSF appears in Figure 2A, lanes 5 and 6, in the minus biotin fractions, indicating that some of its adherence to the avidin resin is nonspecific. However there is always more CPSF-100 in the biotinylated RNA fractions, indicating that specific interactions with the RNA are also allowing to bind to the column.

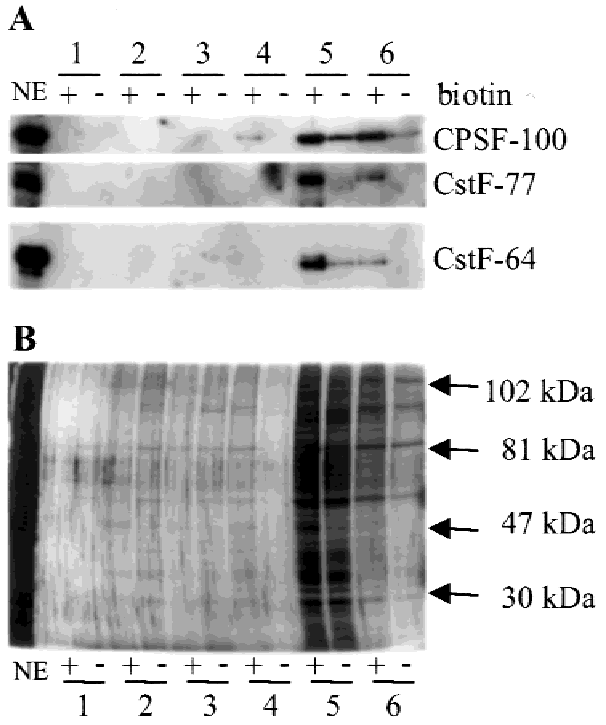

FIGURE 2. Elution of proteins bound to biotinylated RNA from avidin affinity resin. Mu-mb pre-mRNAs with (+) and without (−) biotin were incubated with myeloma nuclear extract under complex-assembly conditions and incubated 12 h with avidin agarose resin. The resin was washed and incubated with RNase A as described in Materials and methods. Sequential elutions of the RNase-treated resin were conducted using the indicated buffers (lanes 1–5). Elutions performed at 4 °C for 10 min with 20 mM Tris, pH 7.6 (lane 1); Tris plus 300 mM NaCl (lane 2); Tris plus 600 mM NaCl (lane 3); Tris plus 1 M NaCl (lane 4); Tris, 2% SDS, and 20 mM DTT (lane 5). After the final elution, the resin was boiled in 1.5× SDS-sample buffer to release any remaining proteins (lane 6). The samples were analyzed by SDS-10% PAGE and western immunoblot with the indicated antibodies (**A**) or silver staining (**B**). The migration of molecular weight markers is indicated in **B**. NE: 2 μg of unfractionated nuclear extract.

Gel filtration analysis of polyadenylation factors and RNA in myeloma nuclear extract

We fractionated nuclear extract prepared from myeloma cells over a 1.5 × 50 cm Sephacryl S-500HR column, collected fractions of approximately 1.25 mL, and analyzed the total protein from alternate fractions via separation by SDS-10% PAGE and western immunoblot with the indicated antibodies (Fig. 3A). Shown are the experimentally determined void volume, V_0 (~40 ± 5% of bed volume), total included volume, V_{tot} (100% of bed volume), and elution volume of a molecular weight standard, thyroglobulin (M_r = 669,000; ~80% of bed volume). Polyadenylation factors elute across the entire fractionation range of the column, indicating that they are present in heterogeneous endogenous complexes. However, coincident elution peaks of all four polyadenylation factors assayed occurred at approximately 60–70% of the total bed volume, at an apparent molecular weight range of ~2–4 × 10^6 M_r, suggesting that this is the relative molecular size of endogenous polyadenylation complexes. An almost identical profile of the total protein was seen both in the presence and absence of exogenously added biotinylated RNAs (data not shown).

Interestingly, several molecular weight forms of CstF-64, CstF-77, and CPSF-30 were seen. The ~70-kDa form of CstF-64 in myeloma cells was preferentially associated with complexes eluting apparently larger than thyroglobulin, whereas the 64-kDa form was seen in the fractions eluting after thyroglobulin. Two forms of CPSF-30 were observed, with the ~28-kDa species eluting with the other polyadenylation factors, whereas the 30-kDa form was seen primarily in the fractions

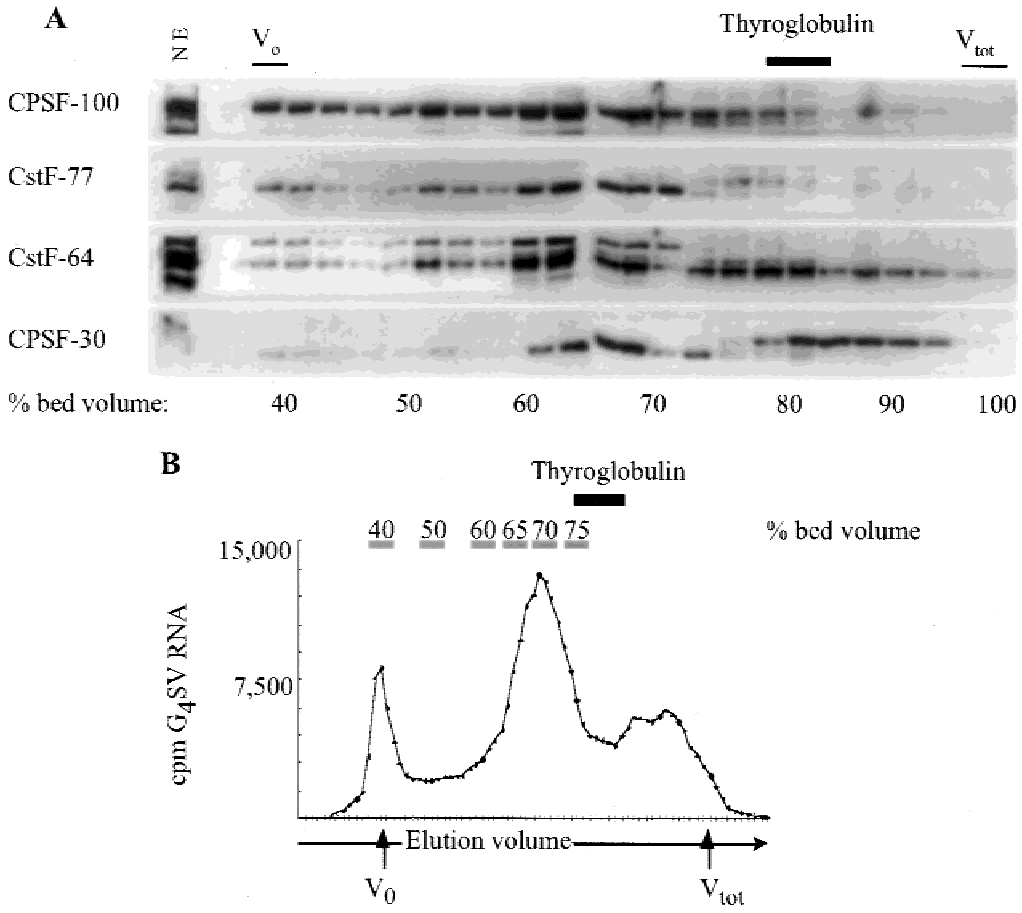

FIGURE 3. Gel filtration profiles. **A**: Fractionation of endogenous myeloma nuclear extract protein. Nuclear extract was incubated at 30 °C for 20 min under complex-assembly conditions and fractionated by gel filtration on Sephacryl S-500 HR (see Materials and methods). Protein (5 μg) from alternate fractions was precipitated, electrophoresed on SDS-10% PAGE, immunoblotted, and probed with the indicated antibodies. V_0: void volume; V_{tot}: total included volume; M_r: relative molecular size. The total elution volume of the column was set at 100% bed volume; the void volume of the column was 40% of the bed volume and the intervening fractions labeled as a percentage of the total bed volume. The black bar indicates the fractions in which thyroglobulin eluted from a parallel column. Nuclear Extract (NE) lane contains 2 μg of unfractionated myeloma nuclear extract. **B**: Polyadenylation complexes were formed on biotinylated and [^{32}P]-labeled G_4SV pre-mRNA and fractionated by gel filtration. The radioactivity in 1/10 of each fraction was determined as described in Materials and methods. Gray bars show the origin of fractions that were pooled for subsequent affinity purification and analysis in Figures 4 and 5.

eluting with or after thyroglobulin. CPSF-100 is distributed throughout the column fractions from 40 through 80% of the elution volume, indicating that the protein either interacts with a large number of other components or is nonspecifically adherent.

Figure 3B illustrates the elution profile of biotinylated, [^{32}P]-labeled G_4SV pre-mRNA incubated with a nuclear extract under "assembly" conditions; this is a typical column profile for exogenously added RNA in these extracts. G_4SV pre-mRNA is a chimera of a portion of the SV40 early poly(A) site and Ig sequences and produces a very efficient polyadenylation site when used in reporter constructs in myeloma cells; this same site was assayed in vivo in construct pSM2 (Matis et al., 1996). Generally, a large fraction of the input RNA elutes between 60 and 75% of the total included volume. The later peak of radioactivity in Figure 3B, at >80% bed volume, contains approximately 20% of the total input counts and consists of free nucleotides, very short RNAs, including 3′-cleavage products, incomplete transcripts, and degradation products from nucleases. The small gray bars in this figure show the individual fractions that were pooled for subsequent analyses.

Comparison of protein profiles with individual steps versus two-step purification

To assess the efficacy of each of the purification steps we fractionated the proteins obtained from each step of the procedure on SDS-PAGE and analyzed the complexity of the profiles by silver staining the gel. The profile of the unfractionated nuclear extract is shown in Figure 4, lane 1, and the material that does not bind to the single-step avidin agarose affinity matrix is shown

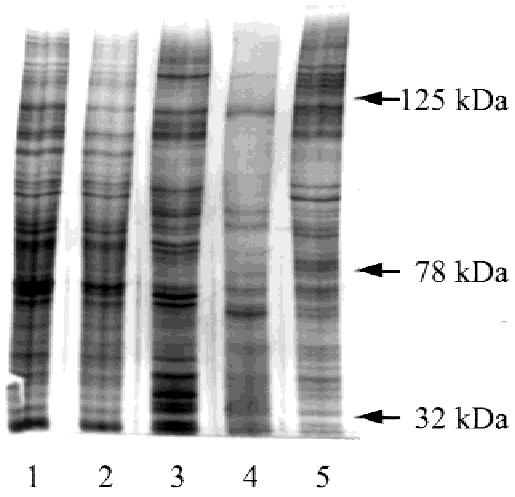

FIGURE 4. Profile of proteins purified by the affinity versus size fractionation plus affinity steps. Proteins were size fractionated on SDS-7.5% PAGE and stained with silver as described in Material and methods. Lane 1: Proteins from a total myeloma nuclear extract (0.5 μg). Lanes 2 and 3: Pre-mRNA with biotin and [^{32}P]-label (G_4SV) was incubated in 0.3 mL of the standard complex formation reaction and then mixed with avidin agarose resin overnight at 4 °C. The resin was washed, treated with RNase A, and the proteins eluted with 2% SDS, 20 mM DTT, 20 mM Tris-HCl (pH 7.6). The material not bound to the resin was analyzed in lane 2 (0.5 μg protein). In lane 3, 8% of the material bound and then eluted from the affinity resin was analyzed. Lanes 4 and 5: Biotinylated and [^{32}P]-labeled G_4SV pre-mRNA was incubated in 1 mL of the standard complex formation reaction, incubated, and run on Sephacryl. Fractions eluting from 60 to 65% of the bed volume were pooled and subjected to avidin affinity purification. In lane 4, 5% of the affinity purified and eluted fraction was analyzed. Lane 5: 0.5 μg of the material not bound to the avidin agarose column was analyzed. The migration of size markers, run in another lane, are indicated by arrows.

in lane 2. As shown in Figure 4, lane 3, employing just the affinity step with biotinylated G_4SV RNA and avidin-agarose produced a mixture of proteins almost as complex as that seen in the unfractionated nuclear extract. A different, but equally complex, set of proteins was found in the pooled fractions eluting at 60–70% of the bed volume of the Sephacryl S-500 column (Fig. 4, lane 5). We analyzed the protein profile seen in the two-step procedure, that is, Sephacryl column followed by affinity purification, in Figure 4, lane 4 and saw a much simplified pattern relative to the other lanes. The apparent molecular weights of the major bands in Figure 4, lane 4 correspond to some of the previously described cleavage and polyadenylation factors, especially in the range of 50 to 77 kDa. Additional bands in the range of 80 to 130 kDa are also observed; the identity of these has not been determined. These may represent novel factors associated with the polyadenylation complex.

The material in Figure 4, lane 3, represents the yield from approximately half as much starting proteins as was used to produce the material in lane 4. Although the protein yield in the two-step procedure is considerably reduced relative to affinity purification alone, we conclude that employing both steps results in a significant purification of some proteins associated with the input RNA over and above either step alone.

Identification of known cleavage-polyadenylation factors following size fractionation and affinity purification

We wanted to assess the fractionation of the known polyadenylation/cleavage factors with the two-step purification procedure. Complexes were assembled on a biotinylated, [32P]-containing RNA substrate with the G_4SV poly(A) site and downstream sequences; when the complexes were fractionated by gel filtration chromatography, the profile on western blots of total protein fractions was identical to that shown in Figure 3A (data not shown). This is not surprising, as only a fraction of the proteins in the extract were assembled on the biotinylated RNA (less than 20%) as determined by protein assays and silver staining. The individual column fractions indicated by the small grey bars in Figure 3B were pooled and incubated with avidin agarose; the eluted proteins were separated by SDS-8% PAGE, and analyzed by western immunoblot using the indicated antibodies (Fig. 5). We found the bulk of the affinity-purified CstF-64, CstF-77, CPSF-160, and CPSF-100 proteins in fractions resolved at 60–70% of the bed volume of the Sephacryl column (Fig. 5A). This observation suggests that the in vitro assembled complexes resemble the endogenous complexes in size and composition. The affinity purified proteins included those that bind RNA directly (e.g., CstF-64) as well as indirectly (e.g., CstF-77). The in vitro assembled complexes are also stable to rechromatography (data not shown).

When we used a biotinylated RNA substrate lacking any poly(A) signals (gpt in Fig. 1A) in a parallel complex formation and two-step purification, we saw no cleavage/polyadenylation-specific proteins eluting from the avidin agarose (Fig. 5B). This indicates specificity for poly(A) signals in the complex assembly reaction.

Mutation of the AAUAAA poly(A) signal disrupts polyadenylation complex formation

We next analyzed complexes formed on an RNA containing the immunoglobulin mu membrane poly(A) site (Fig. 5C); the complexes formed on the mu-mb pre-mRNA are as large or larger than those seen with G_4SV RNA and the endogenous complexes. When we used a substrate in which the poly(A) signal was mutated to the inactive AAGAAA (mu-mb AAGAAA in Fig. 1A), stable complexes containing the cleavage/polyadenylation factors could not be detected on the point mutant RNA (Fig. 5D). This result indicates that formation of these in

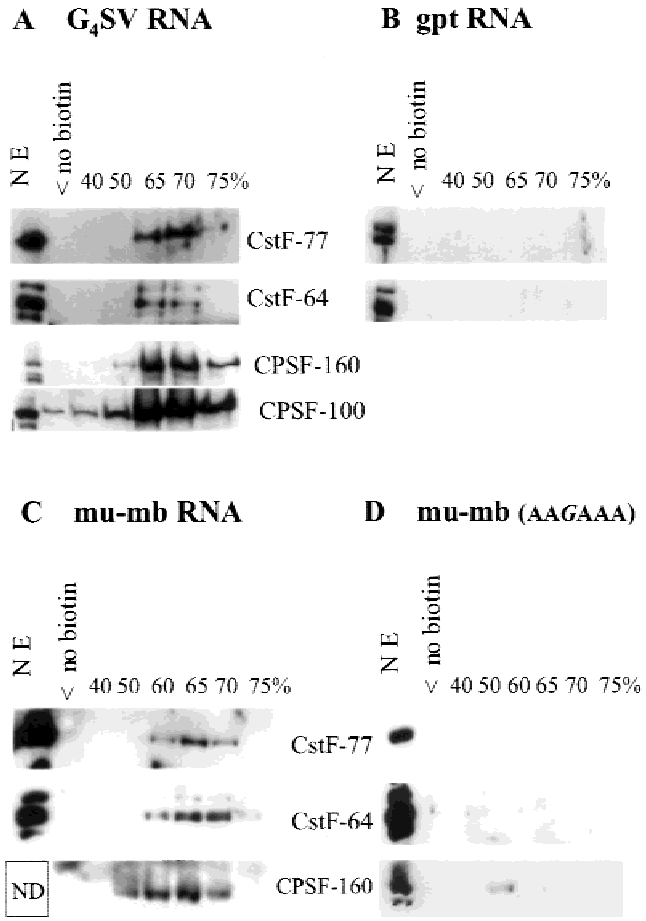

FIGURE 5. Western analysis of affinity-purified proteins of complexes fractionated on Sephacryl columns. Polyadenylation complexes were formed on the indicated biotinylated, [^{32}P]-labeled pre-mRNAs, fractionated on Sephacryl S-500 HR, and affinity purified on avidin agarose. The proteins eluted from avidin agarose after RNase A treatment were immunoblotted and probed with the indicated antibodies, as described in Materials and methods. The RNA profiles of all the pre-mRNAs were similar; the location of the fractions pooled for analyses are shown in Figure 3B. Lanes labeled NE (Nuclear Extract) contain 2 μg of unfractionated myeloma nuclear extract. The "no-biotin" lane is a control for background binding to the affinity resin; proteins in the unfractionated nuclear extract were mixed with RNA lacking biotin, incubated under an assembly conditions, avidin agarose was added, and then RNase A and SDS elution was conducted. The "%" values refer to the percent bed volume of the fraction analyzed (see Fig. 3B for profile). **A**: G_4SV pre-mRNA associated proteins. **B**: gpt RNA associated proteins. **C**: mu-mb pre-mRNA associated proteins. ND: not done on this sample. **D**: mu-mb(AA*G*AAA) RNA associated proteins.

vitro-assembled polyadenylation complexes is highly dependent on the classic poly(A) signal, AAUAAA.

DISCUSSION

We have adapted the column chromatography plus affinity selection technique to the purification of proteins bound to substrates containing cleavage/polyadenylation signals. Both steps in the purification are necessary to achieve maximal enrichment of a small subset of proteins. The association of the known factors with the input polyadenylation substrate is dependent on the presence of the *cis*-acting sequences for polyadenylation and cleavage including AAUAAA in the pre-mRNA. The purification of in vitro-assembled spliceosomes has been invaluable in the analysis of their assembly and composition (Reed, 1990; Bennett et al., 1992). The modifications of this method for the purification of in vitro assembled polyadenylation complexes provide us with a valuable tool for further analysis of the 3′-end formation pathway.

Analysis of the gel filtration pattern of polyadenylation factors from myeloma nuclear extract demonstrated that they are associated with endogenous complexes of widely varying sizes (Fig. 3A). This may be the result of the association of polyadenylation factors with a number of different cellular factors, including components of the transcription machinery (Dantonel et al., 1997; McCracken et al., 1997). In addition, the potential diversity of the polyadenylation complexes themselves, especially because of the CF I_m heterodimers, makes it likely that they would be of different sizes. Although polyadenylation factors eluted across the entire fractionation range of the column, we found that several basal polyadenylation factors coeluted at a peak at approximately 60–70% of bed volume. This observation suggests that these fractions contain the bulk of the endogenous polyadenylation factors associated in a large complex. We were surprised at the remarkable stability of both the endogenous complexes and those formed on exogenously added RNA. The complexes were stable to rechromatography over a period of hours, indicating the strength of the protein–RNA and protein–protein interactions, and suggesting that there is little dynamic exchange of components once the complex is formed.

The minimal size of a polyadenylation complex can be estimated to be 7–8 $\times$ 10^5 Da based upon the molecular weights of the basal polypeptide components. The fractionation range of the sizing columns used for our studies extends to globular proteins of approximately 8 million daltons. Plasma cell polyadenylation complexes formed in vitro and purified by our two-step method elute at a relative molecular size larger than thyroglobulin, indicating that they are likely within the range of 2 to 4 $\times$ 10^6 Da (Figs. 3 and 5). Using 60S and 40S ribosomal subunits as size markers, previous sedimentation analyses estimated the size of in vitro assembled polyadenylation-specific complexes to be 25S (3.3 $\times$ 10^5 Da) (Stefano & Adams, 1988) or 50S (2 $\times$ 10^6 Da) (Moore et al., 1988). Complexes in those studies were not analyzed for protein content; the affinity-based purification method we have developed permits the rapid and simple isolation of in vitro-assembled complexes on a large enough scale with sufficient purity to examine the protein components of the complexes.

This two-step technique can be used to study the composition of complexes that form upon a virtually unlimited number of RNA sequences. It will allow the characterization of which basal factors and/or isoforms of factors as well as novel proteins are found in specific RNA–protein assemblies. More importantly, we believe that our success with mouse myeloma nuclear extracts indicates that this technique can be adapted to study the complexes that form in nuclear extracts prepared from a variety of other cell types in which the choice of polyadenylation site is regulated. There is an ever growing number of complex transcription units with multiple poly(A) sites whose regulation could take place at the level of poly(A) site choice (Edwalds-Gilbert et al., 1997).

MATERIALS AND METHODS

Cell culture and nuclear extract preparation

The AxJ mouse myeloma cell line produces secreted γ2a immunoglobulin, with mRNA encoding the heavy-chain secreted form exceeding the membrane form by 36:1; (Milcarek et al., 1996). The continuous cell line was grown at the National Cell Culture Center (Cellex Biosciences, Inc., Minneapolis, Minnesota) in "slosh" culture in Iscove's modified Dulbecco medium containing 5% horse serum, as described previously (Edwalds-Gilbert & Milcarek, 1995; Milcarek et al., 1996). Nuclear extracts were prepared from myeloma cells using the Dignam protocol (Dignam et al., 1983) with the following modifications: nuclei were extracted in buffer containing 300 mM NaCl and additional protease inhibitors: 100 μg/mL Tosyl-L-phenylalanine chloromethyl ketone, and 1 μg/mL each aprotinin, leupeptin, pepstatin A, and trypsin-chymotrypsin inhibitor. The nuclear extracts were dialyzed against modified Dignam dialysis buffer (myeloma dialysis buffer D) containing 230 mM potassium glutamate instead of 100 mM KCl, 1 mM $MgCl_2$, and protease inhibitors as above. Protein concentrations were determined by a Bradford assay (BioRad; Hercules, California) and were typically between 4–6 mg/mL. Extracts prepared by this method are competent for in vitro cleavage, polyadenylation, and splicing.

Plasmid constructs and in vitro transcription of RNA

The SVL and SVL-dsc constructs were generously provided by Dr. Jeffrey Wilusz (Wilusz & Shenk, 1990). The pG_4SV insert in pGEM4 is a chimera between the weak immunoglobulin γ2b secretory poly(A) site and the SV40 early poly(A) site; a 31-nt fragment containing the cleavage site and downstream consensus sequence of the Ig γ2b secretory poly(A) site, located 17 nt downstream of the AAUAAA poly(A) signal, was replaced by the SV40 early cleavage site and downstream consensus making it a strong, unregulated poly(A) site in transfection studies (Matis et al., 1996). The mu-mb construct includes the AAUAAA polyadenylation signal, cleavage site, and downstream GU-rich region of the Ig mu membrane poly(A) site cloned into pGEM4. Mu-mb(AAGAAA) is a point mutation of the Mu-mb construct that changes the AAUAAA polyadenylation signal to AAGAAA. The gpt construct consists of the *Hin*dIII-*Kpn*I fragment of the 5′ end of the *Escherichia coli* xanthine-guanine phosphoribosyl transferase gene (GenBank accession M10382) cloned into pGEM3 and lacks recognizable poly(A) sites and signals as well as splice sites.

Plasmids were linearized and transcribed with SP6 RNA polymerase (Promega Corp., Madison, Wisconsin) in 20-100 μL reactions containing 500 μM cap analog (m7G(5′)ppp(5′)G), 100 μM each UTP and GTP, 500 μM each ATP and CTP, 1 U/μL of RNasin ribonuclease inhibitor (Promega), and 8–40 μCi α-^{32}P-UTP (New England Nuclear; Boston, Massachusetts). For biotinylated transcripts, 30 μM biotin-11-UTP (ENZO Diagnostics, Inc., supplied through Sigma Chemical Co., St. Louis, Missouri) was included; at this concentration 1–3 molecules of biotinylated nucleotide is expected per transcript (Grabowski & Sharp, 1986). Transcriptions were carried out at 37 °C for 60–90 min, template digested by DNase I at 37 °C for 15 min, and the transcribed RNA phenol-chloroform extracted and ethanol precipitated as previously described (Edwalds-Gilbert & Milcarek, 1995). Full-length transcripts were gel purified; >80% of this naked biotin-tagged RNA bound to the avidin resin. Typically, from 50 to 400 pmol of RNA ~200 nt in length, based on the size of the reaction, were synthesized in these reactions with specific activities in the range of 50 to 100 cpm/fmol RNA molecule.

Gel filtration chromatography and affinity purification of polyadenylation complexes

Polyadenylation complexes were assembled on ice. The 1-mL reactions typically contained 20 pmol of [^{32}P] and biotin-labeled substrate RNA, ~3 mg nuclear extract, 40 μg/mL yeast tRNA, 1 mM ATP, 10 mM creatine phosphate, 0.7 mM $MgCl_2$, 2 mM DTT, 80 U/mL RNasin ribonuclease inhibitor, and 20% glycerol (v/v) in 20 mM HEPES, pH 7.9. The reactions were incubated at 30 °C for 20 min. The mixture was loaded over a 1.5 × 50 cm (85–90 cc bed volume) Sephacryl S-500HR (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey) gel filtration column at 4 °C and separated using a 1× PCB-1 buffer system (150 mM KCl, 0.1% Triton X-100, and 0.5 mM each DTT and phenylmethylsulfonyl fluoride in 20 mM Tris, pH 7.6). Prior to use, the columns were blocked with nuclear extract to minimize nonspecific interaction of complex-associated proteins with the matrix. Columns were run at a flow rate of 0.35 mL · min^{-1} and 1-mL fractions were collected until at least one bed volume was eluted from the column.

Cerenkov counts (Matthews, 1968) were obtained for 100 μL of each fraction to determine the elution profile of the RNA from the column. Peak fractions were chosen to pool for subsequent purification and a small portion of each pool was removed for western analysis of total protein. The remaining samples were incubated with 15–20 μL/mL Avidin D Agarose (Vector Laboratories Inc., Burlingame, California) with gentle rocking at 4 °C for at least 6 h. The affinity resin was washed at 4 °C with gentle rocking once for 15 min and three times for 10 min with 150 mM NaCl in 20 mM Tris, pH 7.6, and once for 10 min with 50 mM NaCl in 20 mM Tris, pH 7.6. Cerenkov counts were determined for the washed resin to calculate the fraction of input RNA associated with the resin. Between 25 and 60% of the bio-

tinylated RNA–protein complex in the pooled fractions bound to the avidin resin, versus less than 1% binding of non-biotinylated RNA.

The RNA–protein complexes were released from the affinity resin by incubation with 0.6 μg RNaseA at 37 °C for 20 min followed by elution with 250 μL of elution buffer (2% SDS, 20 mM DTT in 20 mM Tris, pH 7.6) with gentle rocking at 4 °C for 10 min. The resin was recounted to determine the efficiency of the RNase/elution step; generally, 85–95% of bound RNA was released. The eluents were heated at 65–70 °C for 5–10 min, 40 μg yeast tRNA or glycogen carrier added, and 4 vol ice-cold acetone added to precipitate the proteins. The samples were incubated on ice for 10–20 min and pelleted in a 4 °C microfuge for 20 min. The air-dried pellets were resuspended in 1.5× SDS sample buffer for subsequent analysis by SDS-PAGE and western blotting.

Starting with approximately 20 pmol of substrate RNA and assuming about 20 proteins of average molecular weight of 70 kDa per RNA molecule, we calculate that we could theoretically isolate about 28 μg protein in the two-step purification. In practice, the yields are diminished to less than 25% of that by the elution of ~50% of the RNA in the complex at 60–75% bed volume on the column and the ~50% binding efficiency on the avidin agarose.

Silver staining

SDS-PAGE gels were silver stained for analysis of the protein profiles (Poehling & Neuhoff, 1981). Briefly, gels were soaked in 50% methanol plus 10% acetic acid for fixation of the proteins and washed three times with 15% methanol; proteins were crosslinked with 5% glutaraldehyde and the gels were washed eight times with four washes each of alternating 15% methanol then deionized water. In the hood, a freshly prepared solution of 1.6 g of $AgNO_3$ in 8 mL water (117 mM $AgNO_3$) was added all at once to 160 mL of 20 mN NaOH with stirring. About 2 mL of concentrated NH_4OH was added drop-wise until the solution cleared and then the mixture was adjusted to 200 mL final volume. Gels were incubated in the silver solution for 10 min then washed with water and developed with 0.005% citric acid/0.0185% formaldehyde for 3–4 min with shaking; gels were rinsed quickly in water and development stopped in 5% acetic acid.

Western analysis and antibodies

Protein samples were separated on SDS-PAGE, electroblotted to polyvinylidene difluoride membrane (PolyScreen® PVDF Transfer Membrane; NEN™ Life Science Products, Inc., Boston, Massachusetts) and probed with the indicated antibodies. Rabbit anti-peptide antibodies against CstF-64 and CstF-77 were previously described (Martincic et al., 1998). Rabbit polyclonal antibodies against peptides of CPSF-160 were raised to the COOH-terminal 27 amino acids of the human sequence by coupling the peptide to KLH-carrier proteins. Rabbit polyclonal antibodies against CPSF-100 and CPSF-30 were generously provided by Drs. Silvia Barabino, Ursula Rüegsegger, and Walter Keller. Peroxidase-coupled secondary antibodies were purchased from Boehringer Mannheim and detected by chemiluminescence.

In vitro cleavage and polyadenylation assays

Cleavage assays were performed as previously reported (Moore & Sharp, 1985; Edwalds-Gilbert & Milcarek, 1995) and contained 10–30 μg nuclear extract, 5 mM creatine phosphate, 0.5 mM 3′-dATP, 2.5% polyvinyl alcohol, 4–5 U RNasin RNase inhibitor, and 3–5 × 10^4 cpm of high specific activity [^{32}P]-labeled RNA substrate in a 20-μL volume. In "No Extract" controls, nuclear extract volume was replaced with myeloma dialysis buffer D. Reactions were incubated at 30 °C for the times indicated then digested with 2.5 mg/mL proteinase K at 37 °C for 15 min. The reactions were phenol-chloroform extracted and the RNA precipitated and electrophoresed on 5% polyacrylamide-8 M urea gels. Polyadenylation assays differed only in that the chain terminating 3′-dATP was omitted and 0.8 mM ATP and 0.1 mg/mL yeast tRNA were included.

UV crosslinking and immunoprecipitation

UV crosslinking reactions were preformed as previously described (Edwalds-Gilbert & Milcarek, 1995) with high specific activity [^{32}P]-labeled substrate RNA (at least 2 × 10^5 cpm), 5 μg AxJ nuclear extract, 1 mM ATP, 0.7 mM $MgCl_2$, and 40 μg/mL yeast tRNA brought up to a final volume of 25 μL with nuclear extract dialysis buffer. Reaction mixtures were incubated for 5 min at 30 °C, placed on ice, and irradiated with 1.8 J/cm^2 ultraviolet light. CstF-64 was immunoprecipitated with anti-CstF-64 antibody (3A7) and the precipitate was incubated with 1.25 μg RNase A for 15 min at 37 °C, the proteins boiled for 5 min in 1× SDS sample buffer (0.1 M DTT, 2% SDS, 80 mM Tris, pH 6.8, 10% glycerol, 0.2% bromophenol blue) and separated by SDS-10% PAGE.

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