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Purification of the Cleavage and Polyadenylation Factor Involved in the 3'-Processing of Messenger RNA Precursors*

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Polyadenylation of messenger RNA precursors requires the nucleotide sequence AAUAAA and two factors: poly(A) polymerase and a specificity factor termed cleavage and polyadenylation factor (CPF). We have purified CPF from calf thymus and from HeLa cells to near homogeneity. Four polypeptides with molecular masses of 160, 100, 73, and 30 kDa cofractionate with CPF activity. Glycerol gradient centrifugation and gel filtration indicate that these four proteins form one large complex with a sedimentation constant of 12 S, a Stokes radius near 100 Å, and a native molecular mass near 500 kDa. Purified CPF binds specifically to an RNA that contains the AAUAAA sequence. Mutation of the AAUAAA sequence inhibits CPF binding as well as polyadenylation. Purified CPF contains only trace amounts of RNA and does not react with antibodies against common epitopes of small nuclear ribonucleoprotein particles. Thus, contrary to previous indications, CPF does not appear to be a small nuclear ribonucleoprotein particle.

The 3' ends of eukaryotic messenger RNAs are formed by endonucleolytic cleavage of the primary transcript and the subsequent addition of approximately 200 adenosine residues to the upstream cleavage product. Two cis-acting sequence elements confer specificity to the 3'-processing reaction; 10–30 nucleotides upstream of the polyadenylation site lies the highly conserved sequence AAUAAA. Less conserved GU- or U-rich sequence elements are located downstream (for reviews, see Birnstiel *et al.* (1985), Manley (1988), Wickens (1990), and Proudfoot (1991). Single base substitutions or modifications in the AAUAAA sequence inhibit or completely abolish cleavage as well as polyadenylation. The incorporation of substrate RNA into the so-called 3'-processing complex is also strongly dependent on the intact AAUAAA motif (Humphrey *et al.* 1987; Zarkower and Wickens, 1987a, 1987b; Skolnik-David *et al.*, 1987; Zhang and Cole, 1987; Moore *et al.*, 1988; Stefano and Adams, 1988). Cleavage and polyadenylation are normally tightly coupled *in vivo* and *in vitro*. However, cleaved RNA lacking poly(A) tails accumulates *in vitro* in the presence of EDTA or a chain-terminating ATP analogue (Moore and Sharp, 1985). Polyadenylation can be examined independently of cleavage by use of a synthetic RNA substrate that terminates at or close to the natural cleavage and polyadenylation site.

At least four factors are involved in the complete 3'-processing reaction. One factor that has been purified to apparent homogeneity and characterized in detail is poly(A) polymerase (Wahle, 1991), a protein of 60 kDa that nonspecifically polyadenylates any RNA in the presence of manganese ions. In the presence of magnesium ions, the polymerase is barely active. Under these conditions, another factor, cleavage and polyadenylation factor (CPF),[1] endows the poly(A) polymerase with activity specific for AAUAAA-containing RNAs (Christofori and Keller, 1988, 1989; Takagaki *et al.*, 1988, 1989; McDevitt *et al.*, 1988; Wahle, 1991). CPF, which has also been called specificity factor (SF; Takagaki *et al.* (1989)) or polyadenylation factor 2 (PF2; Gilmartin and Nevins (1989)), directly interacts with the AAUAAA sequence (Gilmartin and Nevins, 1989; Bardwell *et al.* 1991).[2] Poly(A) polymerase and CPF are sufficient to polyadenylate an RNA substrate that includes the AAUAAA sequence close to its 3' end.

In addition to these two components, at least two more factors participate in the cleavage reaction (Christofori and Keller, 1988; Gilmartin *et al.*, 1988; Gilmartin and Nevins, 1989; Takagaki *et al.*, 1989; Takagaki *et al.*, 1990).

A U-type small nuclear ribonucleoprotein particle (U-snRNP) has been suspected to be involved in the 3'-processing reaction since other RNA-processing reactions such as RNA splicing and histone 3' end processing require the activity of different snRNPs (Lamond *et al.*, 1990; Birnstiel and Schaufele, 1988). In agreement with this expectation, anti-Sm antibodies directed against a common epitope of all U-type snRNPs have been shown to inhibit the 3'-processing reaction (Moore and Sharp, 1984; Berget and Robberson, 1986). In addition, an RNA fragment that contained the AAUAAA sequence was immunoprecipitated from processing reactions by snRNP-specific antibodies (Hashimoto and Steitz, 1986; Stefano and Adams, 1988). Furthermore, it has been reported that a 155-kDa protein that could be specifically cross-linked to the AAUAAA sequence could be immunoprecipitated with the same antibodies (Moore *et al.*, 1988). During the fractionation of 3'-processing factors, a copurification of the U11 snRNP (Krämer, 1987) with CPF activity was observed (Christofori and Keller, 1988). However, no direct proof for the involvement of the U11 snRNP in the 3'-processing reaction was found.

Here, we report the purification of CPF to near homogeneity from calf thymus and from HeLa cell nuclear extract. CPF was found to be a complex of four polypeptides, and it

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[1] The abbreviations used are: CPF, cleavage and polyadenylation factor; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein; m_3G, 2,2,7-trimethylguanosine; FPLC, fast protein liquid chromatography.

[2] W. Keller, S., Bienroth, K. Lang, and G. Christofori, manuscript in preparation.

contained only trace amounts of U11 RNA. Proteins common to all U snRNPs were not detectable.

EXPERIMENTAL PROCEDURES

Materials—Poly(A) polymerase was purified as described (Wahle, 1991). SP6 RNA polymerase, T7 RNA polymerase, and proteinase K were from Boehringer Mannheim. RNAguard was from Pharmacia LKB Biotechnology Inc., bovine serum albumin (RNase-free) from Biofinex (CH-1724 Praroman), and bovine serum albumin (fraction V) from Boehringer Mannheim. Monoclonal antibodies directed against the common 2,2,7-trimethylguanosine cap structure of snRNPs and coupled to Sepharose 4B (anti-m_3G affinity column) (Bochnig *et al.*, 1987) were a gift from R. Lührmann, University of Marburg, FRG. Spermine agarose was from Sigma. Blue Sepharose was made from Sepharose CL-6B (Pharmacia) and reactive blue (Basilen Blue E-3G, Sigma no. B-5520) according to Böhme *et al.* (1972). 1 g of reactive blue was used for 100 ml (packed volume) of Sepharose CL-6B. All other resins were from Pharmacia. Polyvinyl alcohol (molecular mass, 40 kDa), polyvinylpyrrolidone (molecular mass, 40 kDa), and herring sperm DNA (type XIV) were from Sigma, ammonium sulfate from Bethesda Research Laboratories, Nonidet P-40 from Fluka, dithiothreitol from Calbiochem, dextran sulfate and Ficoll-400 from Pharmacia, phenylmethylsulfonyl fluoride from Boehringer Mannheim, and leupeptin and pepstatin from Bachem AG (CH-4416 Bubendorf). Labeled nucleoside triphosphates were from Amersham Corp., and nonlabeled nucleoside triphosphates were from Pharmacia.

In Vitro Synthesis of Pre-mRNA—The RNAs used as substrates for the specific polyadenylation reaction contained the adenovirus-2 L3 polyadenylation site. The precleaved substrates (L3pre, L3preΔ), terminating one nucleotide upstream of the natural polyadenylation site, have been described (Christofori and Keller, 1988). L3pre contained an intact AAUAAA recognition signal, whereas L3preΔ has a U to G point mutation in this sequence. The *in vitro* transcription by SP6 RNA polymerase from a truncated plasmid DNA included the addition of a GpppG cap to the RNA and the incorporation of [α-^{32}P]UTP (Frendewey and Keller, 1985; Krämer and Keller, 1985). The UTP concentration was 0.1 mM.

Unlabeled competitor RNA was synthesized essentially as described above with the following modifications. The reaction volume was increased to 100 μl, and incubation was performed for 90 min. The concentration of template DNA was 0.02 μg/μl, the UTP concentration was 0.5 mM, and [α-^{32}P]UTP was omitted from the reaction. After purification by electrophoresis on a 6% denaturing polyacrylamide gel, the RNA was visualized by UV shadowing (254 nm), excised from the gel, and eluted overnight at 37 °C in 0.75 M ammonium acetate, 10 mM magnesium acetate, 1% (v/v) phenol, 0.1% (w/v) SDS, and 0.1 mM EDTA. The eluted RNA was purified by extraction with phenol and chloroform:isoamyl alcohol (24:1) and ethanol-precipitated. The RNA pellet was resuspended in 300 μl of H_2O and quantified spectrophotometrically.

In Vitro Synthesis of U11 Sense and Antisense RNA—Synthetic U11 RNA was derived from phage M13 mp18 DNA that contained, cloned in its *Hin*dIII site, a fragment with the T7 promoter upstream of the human U11 snRNA gene (CS 23) (Suter-Crazzolara and Keller, 1991). For run-off transcription, the vector was linearized with *Dra*I. The transcript contained all 134 nucleotides of U11 RNA and 3 guanosine residues at its 5′ end (Montzka and Steitz, 1988; Suter-Crazzolara and Keller, 1991). Unlabeled RNA was synthesized in a total volume of 100 μl, containing 40 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 10 mM dithiothreitol, 0.5 mM each of ATP, GTP, CTP, and UTP, 2 μg of template DNA, 120 units of RNAguard, and 20 units of T7 RNA polymerase. After incubation for 60 min at 37 °C, the transcripts were purified as described above.

Radiolabeled U11 antisense RNA was synthesized by T7 RNA polymerase from a Bluescript KS$^+$ vector that contained the human U11 gene in an inverted orientation downstream of the T7 promoter (*Fis* I) and which was linearized with *Pst*I. *Fis*I was made by cloning a *Sac*I-*Hin*dIII fragment containing the U11 sequence into *Sac*I- and *Hin*dIII-cut Bluescript KS$^+$. The transcript contained, at its 5′ end, 11 nucleotides of Bluescript sequence followed by 25 nucleotides of M13 mp18 sequence and 127 nucleotides of sequence complementary to U11 RNA and, at its 3′ end, 5 nucleotides of M13 mp18 sequence. Transcriptions were performed in 20 μl containing 40 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 10 mM dithiothreitol, 0.5 mM each of ATP, CTP, and GTP, 0.02 mM UTP, 50 μCi of [α-^{32}P]UTP (800 Ci/mmol), 30 units of RNAguard, 10 units of T7 RNA polymerase, and 1 μg of template DNA for 30 min at 37 °C. The RNA was purified as described (Frendewey and Keller, 1985).

Specific Polyadenylation—The polyadenylation reaction was as described (Christofori and Keller, 1988), with the following modifications. 0.01% Nonidet P-40 and 0.2 mg/ml RNase-free bovine serum albumin were included. 60 fmol of RNA ($1–5 \times 10^4$ cpm) and 8–10 units of purified poly(A) polymerase were used per reaction. All reactions were incubated for 30 min at 30 °C and stopped by the addition of proteinase K mix (20 μg of proteinase K and 5 μg of tRNA in 180 μl of 100 mM Tris-HCl, pH 7.5, 20 mM EDTA, 2% *N*-lauroylsarcosine) and incubation for 30 min at 30 °C. The RNA was precipitated by the addition of 100 μl of 7.5 M ammonium acetate and 750 μl of ethanol. After one wash with 70% ethanol, the RNA was analyzed on a 12% denaturing polyacrylamide gel (Sambrook *et al.*, 1989).

For quantitation of CPF activity, elongated and nonelongated substrate RNA was excised from the gel. Gel pieces were placed in a scintillation mixture (Instagel, Packard Instrument Co.) and counted in a scintillation counter. One unit of CPF is defined as the amount that leads to the polyadenylation of 50% of the substrate RNA.

Gel Retardation Assay—The standard reaction used for the gel retardation assay was essentially the same as the 3′-processing reaction, with the following modifications. Substrate RNA was increased to 100 fmol ($2–8 \times 10^4$ cpm) per reaction and the ATP concentration was raised to 1.6 mM. After 10 min at 30 °C, the reaction mixtures were loaded directly onto a 4% nondenaturing polyacrylamide gel (Frendewey *et al.*, 1987). Bromphenol blue and xylene cyanol were applied to neighboring lanes, and electrophoresis was carried out at room temperature at 20 mA until the xylene cyanol had migrated 10.5 cm into the gel. Complexes were visualized by autoradiography.

Purification of Cleavage and Polyadenylation Factor from Calf Thymus—Buffer A contained 10% (v/v) glycerol, 3 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.0, 0.2 mM EDTA, 0.5 mM dithiothreitol. Buffer B was the same as buffer A but contained 20% glycerol. The pH of the Tris buffer was adjusted as a 1 M stock solution at room temperature. Salt concentrations were as indicated. All columns were run at or close to 0 °C and 1 column volume/h, unless indicated otherwise. Protein concentrations were determined according to Bradford (1976) with BSA (fraction V) as a standard.

Extract was prepared from 1 kg of calf thymus as described (Wahle, 1991), except that the tissue was homogenized in buffer A containing 100 mM KCl and 1 mM EDTA.

The crude extract was applied to a DEAE-Sepharose column of 45 × 10 cm, equilibrated in buffer A plus 100 mM KCl, with 10 mg of protein loaded per ml of bed volume. The column was washed with 1 volume of buffer A containing 100 mM KCl and eluted with a gradient (3 column volumes) from 100 to 300 mM KCl. CPF activity eluted from 175 to 250 mM KCl. The pooled fractions were adjusted to 45% saturation with solid ammonium sulfate and stirred overnight on ice. After centrifugation (60 min, 8000 rpm ($10,800 \times g_{max}$) Sorvall GS3 rotor), the pellet was resuspended in 80 ml of buffer A, dialyzed for 8 h against 4 × 2 liters or overnight against 10 liter of buffer A plus 100 mM KCl, and clarified by centrifugation for 15 min at 15,000 rpm ($27,000 \times g_{max}$) in a Sorvall SS34 rotor. The fraction was diluted to the conductivity of buffer A containing 100 mM KCl.

A blue Sepharose column (20 × 10 cm) was equilibrated with buffer A containing 100 mM KCl. The dialyzed precipitate was applied with 2 mg of protein/ml of column bed volume. The column was washed with 1 volume of buffer A plus 100 mM KCl and developed with a gradient from 0.1 to 1 M KCl in buffer A (5 column volumes). The active fractions (eluted at 600–900 mM KCl) were pooled and dialyzed for 6 h against 3 × 2 liters of buffer B and then for 2 h against 6 liters of buffer B plus 100 mM KCl.

The blue Sepharose pool was applied to a heparin-Sepharose column (15 × 3.5 cm) equilibrated with buffer B plus 100 mM KCl. Loading was done overnight with 2 mg of protein/ml of column bed volume and followed by washing with 2 column volumes of buffer B plus 100 mM KCl. CPF was eluted with a 5-column volume gradient from 100 to 300 mM KCl and was recovered between 180 and 240 mM KCl. After dialysis (6 h against 3 × 2 liters of buffer A plus 100 mM KCl) the solution was centrifuged for 30 min at 12,000 rpm ($23,400 \times g_{max}$) in a GSA rotor.

An 8-ml Mono Q FPLC column was equilibrated with buffer A plus 100 mM KCl, and the heparin-Sepharose pool was loaded. After washing with 2 column volumes, the protein was eluted with a 40-column volume gradient from 100 to 500 mM KCl in buffer A at 2 ml/min. To the pooled fractions (eluted at 270–300 mM KCl), Nonidet

P-40 was added to a final concentration of 0.02%. The inclusion of Nonidet P-40 in this and all following buffers resulted in increased stability and recovery of CPF activity. The Mono Q pool was dialyzed for 6 h against 3 × 1 liter of buffer B containing 100 mM KCl.

A spermine-agarose column (13 × 1 cm) was equilibrated with buffer B plus 100 mM KCl, lacking EDTA. After loading (1 mg of protein/ml of column bed volume) the column was washed with 2 column volumes of the same buffer and developed with a gradient (8 column volumes) from 100 to 320 mM KCl in buffer B minus EDTA and finally washed with 1 column volume of 320 mM KCl. CPF activity was found between 220 and 300 mM KCl, pooled, and dialyzed against buffer B containing 50 mM KCl (4.5 h, 3 × 0.5 liter).

A poly(A)-Sepharose column (13 × 1 cm) was equilibrated with buffer B plus 50 mM KCl. The spermine-agarose pool was applied to the column with 0.25 mg of protein/ml of column bed volume, and the column was washed with 1 column volume of buffer B plus 50 mM KCl and 2 column volumes of buffer B plus 100 mM KCl. CPF was eluted with a 1 M KCl step in buffer B.

More recently, we have omitted the spermine-agarose column and eluted the poly(A)-Sepharose column with a 0.5 M KCl step. The yield with this purification was 2-fold higher, and the purity was as high as that of the preparation described in detail above.

Purification of CPF from HeLa Cells—Nuclear extracts from 500 g of HeLa cells were prepared according to Dignam *et al.* (1983). The same purification steps as described above were performed with the following modifications. The DEAE-Sepharose pool was loaded directly onto a blue-Sepharose column without prior ammonium sulfate precipitation. The heparin-Sepharose fraction was applied to a 1-ml Mono Q FPLC column. Finally, the spermine-agarose column was omitted and the Mono Q pool was directly applied to the poly(A)-Sepharose column. During all fractionation steps, HeLa CPF behaved comparable with calf thymus CPF.

Glycerol Gradient Centrifugation—1 ml of a poly(A)-Sepharose pool of calf thymus CPF was dialyzed for 4 h against 2 × 0.5 liter of buffer A plus 100 mM KCl, and 300 μl of the dialysate was layered on top of a 15–40% glycerol gradient (13 ml, 14 × 95-mm tubes) in 50 mM Tris-HCl, pH 7.0, 100 mM KCl, 3 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol. Sedimentation was performed for 34 h at 4 °C and 39,000 rpm (273,000 × g_{max}) in a Kontron TST 41.14 rotor. The gradient was unloaded from the top in 300-μl fractions (40 fractions/tube), and CPF was identified by its activity.

CsCl Buoyant Density Centrifugation—4 ml of a calf thymus CPF Mono Q pool (0.4 mg/ml protein) in buffer A plus 100 mM KCl were adjusted to a final density of 1.45 g/cm^3 with solid CsCl. Centrifugation was performed for 43 h at 4 °C at 52,000 rpm (200,000 × g_{max}) in a Kontron TFT 80.4 rotor. 150-μl fractions were collected from the top of the gradient and dialyzed overnight in a microdialysis system (Bethesda Research Laboratories) against buffer B plus 100 mM KCl. In the same run, CsCl gradients were formed with bovine serum albumin (0.5 mg/ml) or a Mono Q fraction containing a random assortment of protein (0.3 mg/ml) but no snRNPs. Proteins were quantified by their UV absorbance.

Affinity Chromatography of CPF on an Anti-m_3G Column—Partially purified CPF was applied to an affinity column that carried monoclonal antibodies against the trimethylguanosine cap structure of snRNPs (see above). A 100-μl affinity column was made in a 200-μl pipette tip and equilibrated with TMN 300 (20 mM Tris, pH 7.9, 10 mM $MgCl_2$, 300 mM NH_4Cl, 0.5 mM dithiothreitol, 10% glycerol). 20 μl (1600 units) of a concentrated spermine-agarose fraction (concentrated 20-fold on a 1-ml Mono Q column) was applied. The column was washed with 100 μl of TMN 300, and the flow-through was reloaded several times for 1 h, total. Unbound material was eluted with 500 μl of TMN 300. Bound material was eluted with 500 μl of TMN 100 (as TMN 300 but 100 mM NH_4Cl), including 10 mM 7-methylguanosine (Bochnig *et al.*, 1987).

Northern Blotting—20 × SSC and 50 × Denhardt's solution were according to Sambrook *et al.* (1989). Blotting buffer consisted of 100 mM Tris acetate, 50 mM sodium acetate, 5 mM EDTA, pH 7.8, and hybridization buffer was 50% formamide, 5 × SSC, 5 × Denhardt's solution, 1% SDS, 2.5% dextran sulfate, 50 mM sodium phosphate, pH 6.5, 0.1 mg/ml herring sperm DNA.

For quantitation of U11 RNA in various CPF fractions, appropriate amounts of each fraction were treated with proteinase K, ethanol-precipitated as described above, and applied to an 8% polyacrylamide-urea gel (Sambrook *et al.*, 1989). As a standard, known quantities (1–20 fmol) of *in vitro* synthesized U11 RNA were also applied. After the run, the gel was soaked for 2 × 15 min in blotting buffer, and the RNA was transferred to a GeneScreen membrane (Du Pont-New England Nuclear) for 1 h at 150 mA in a semidry blotting apparatus in blotting buffer. The membrane was UV-irradiated for 75 s with 2000 microwatts/cm^2 and baked for 1 h at 80 °C. After 2 h of prehybridization at 42 °C in hybridization buffer, U11 antisense RNA (3–5 × 10^5 cpm/ml of hybridization buffer) was added, and hybridization was allowed for 16 h at 42 °C. Washing was performed for 2 × 30 min in 1 × SSC, 0.1% SDS and for 2 × 30 min in 0.5 × SSC and 0.1% SDS at room temperature. After exposure to film, radioactive bands were excised from the membrane and counted in a scintillation counter. The amount of U11 in CPF fractions was determined by comparison to the calibration curve generated with synthetic U11 RNA.

SDS-Polyacrylamide Gel Electrophoresis—Appropriate amounts of CPF fractions were precipitated by the addition of 1 volume of 50% trichloroacetic acid. After 30 min on ice and 20 min of centrifugation (4 °C, 12,000 rpm, 48,000 × g_{max}), the pellets were washed two times with acetone (cooled to −20 °C), dried, and resuspended in loading buffer (3% SDS (w/v), 10% glycerol (v/v), 63 mM Tris-HCl, pH 6.8, and 5% 2-mercaptoethanol (v/v)). The samples were heated for 10 min at 90 °C and separated on a 9% SDS-polyacrylamide gel according to Laemmli (1970). The gels were stained with either Coomassie Brilliant Blue or with silver.

RESULTS

Purification of CPF from Calf Thymus—CPF was purified from extracts of calf thymus. The factor was assayed by its ability to complement purified poly(A) polymerase in the polyadenylation of radiolabeled substrate RNA that contained the adenovirus L3 polyadenylation site. The reaction, which was dependent on the AAUAAA signal (see above), was analyzed by gel electrophoresis. For quantitation of CPF activity, elongated and nonelongated RNA was cut from the gel and counted in a scintillation counter. CPF activity could be observed in the crude calf thymus extract but could not be quantitated due to the high nuclease content. In the first two purification steps, the quantitation of CPF activity was therefore unreliable.

Purification of CPF was about 500-fold with a yield of 6% relative to the DEAE pool (Table I). We estimate that the total purification was at least 2000-fold. In the last two purification steps, the addition of 0.02% Nonidet P-40 stabilized CPF activity. In a preparation that was performed without Nonidet P-40, the recovery of activity was 10 times lower.

On the spermine-agarose column, four polypeptides with molecular weights of 160,000, 100,000, 73,000, and 30,000 were the predominant protein species and co-fractionated with CPF activity (Fig. 1). Quantitation by scanning densitometry showed that the amount of the four polypeptides relative to each other remained constant through the peak fractions. The pooled spermine agarose fractions were applied to a poly(A)-Sepharose column. In this purification step, the set of the four proteins again copurified with CPF activity in a salt gradient, whereas most of the remaining polypeptides did not bind to the column (data not shown). A better recovery of CPF was obtained with a single high salt elution step. Fig. 2 shows the proteins present in the pooled poly(A)-Sepharose eluate (*lane 3*). In addition to some minor contaminants, the 160-, 100-, 73-, and 30-kDa polypeptides were present in molar ratios of 1:1.3:1.5:1.6, as determined by scanning of the stained SDS gel. Since the staining intensity with Coomassie Brilliant Blue varies for different proteins, this result is not inconsistent with an equimolar ratio of all four polypeptides.

Another preparation of CPF was carried out in the absence of Mg^{2+} and in the presence of the protease inhibitors EDTA (1 mM), phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (0.4 μg/ml), and pepstatin (0.7 μg/ml). This purification resulted in essentially the same ratio of the four polypeptides

TABLE I

Purification of CPF from calf thymus

The purification from 1 kg of calf thymus was carried out as described under "Experimental Procedures." CPF activity could be observed but not quantitated in the crude extract. The purification factor and recovery are relative to the DEAE pool. Protein concentrations were determined according to Bradford (1976) with BSA as a standard; only the protein concentration in the poly(A)-Sepharose pool was estimated by comparison with BSA standards on the same polyacrylamide gel.

Fraction	Protein	Activity	Specific activity	Purification	Recovery
	mg	*units* × 10^{-3}	*units* × 10^{-3}/*mg*	*-fold*	*%*
Extract	37,000				
DEAE-Sepharose	4,300	1,230	0.29	1.0	100.0
$AmSO_4$ precipitate	1,950	1,200	0.62	2.1	97.6
Blue-Sepharose	231	575	2.5	8.6	46.7
Heparin-Sepharose	25	350	14.0	48.3	28.5
Mono Q	10	280	28.0	96.6	22.8
Spermine-agarose	2.6	104	40.0	138.0	8.5
Poly(A)-Sepharose	~0.5	74	~148	~510	6.0

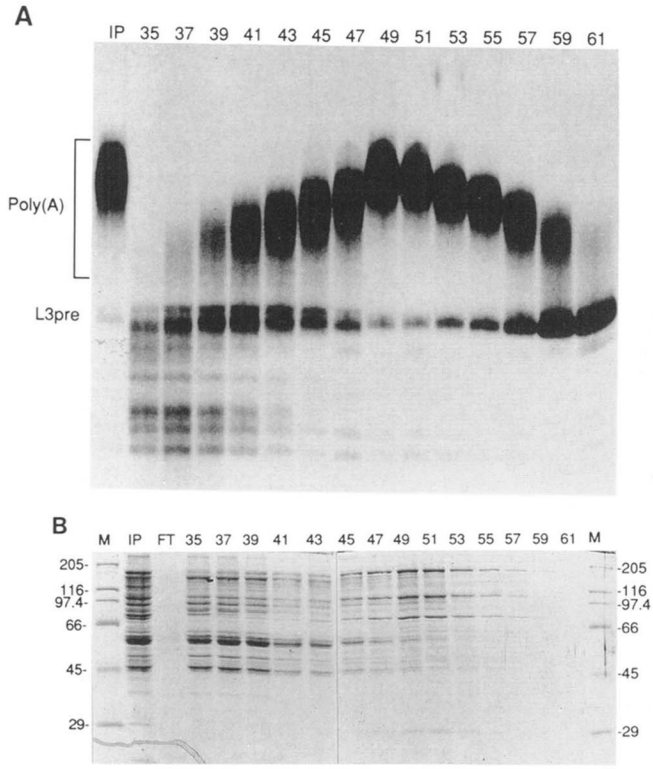

FIG. 1. **Chromatography of CPF on spermine-agarose.** *A*, A Mono Q pool of calf thymus CPF was run on spermine-agarose, as described under "Experimental Procedures." CPF activity was measured with 0.5 μl of each fraction in a 12.5 μl reaction (see "Experimental Procedures"). *Fraction numbers* are indicated at the *top*. *B*, SDS-polyacrylamide gel electrophoresis of spermine-agarose fractions, 20-μl aliquots of the fractions indicated were analyzed on two 9% gels and visualized by staining with Coomassie Brilliant Blue. The molecular masses of marker proteins in kilodaltons are indicated on the *left*. *M*, marker proteins; *IP*, input; *FT*, flow-through.

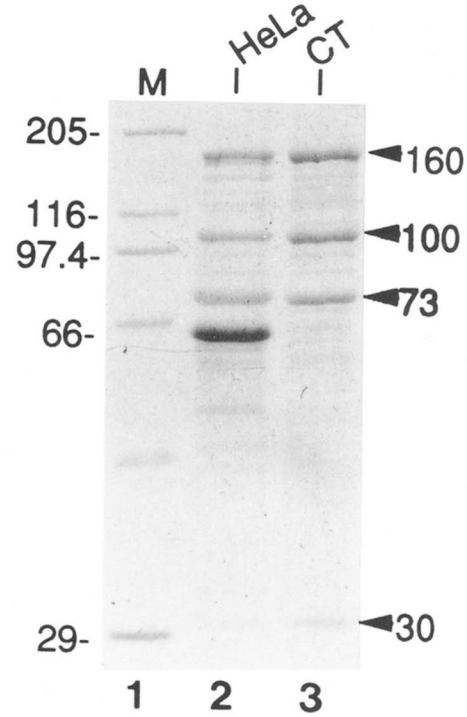

FIG. 2. **SDS-gel electrophoresis of calf thymus (*CT*) and HeLa cell CPF.** Aliquots of the poly(A)-Sepharose pools of calf thymus CPF (*lane 3*; see Table I) and HeLa cell CPF (*lane 2*; Table II) were analyzed on a 9% polyacrylamide gel as described under "Experimental Procedures." The same protein pattern was observed when the samples were applied to SDS-gel electrophoresis without prior precipitation with trichloroacetic acid and heat treatment. The four predominant polypeptides in these purified fractions are indicated on the *right* of the figure. The molecular mass of marker proteins in kilodaltons is indicated on the *left* of the figure. The gel was stained with Coomassie Brilliant Blue and the concentrations of the four polypeptides were estimated by densitometry of the gel and comparison with BSA standards run on the same gel. *M*, marker proteins.

to each other. In addition, a protein of 140 kDa copurified with CPF (see below).

Purification of CPF from HeLa Cell Nuclear Extract—CPF was also purified from HeLa cell nuclear extract with the same assay and essentially the same purification steps that were used to purify the factor from calf thymus. After five steps, CPF was purified about 540-fold with a yield of 1.9% relative to the nuclear extract (Table II). The specific activity was similar to that of calf thymus CPF. Fig. 2 shows the proteins present in the poly(A)-Sepharose pool (*lane 2*). The four polypeptides of 160, 100, 73, and 30 kDa again eluted with CPF activity in relative molar concentrations of 1:1.1:1.9:1.7. An additional protein with a molecular weight of 65,000 was present in this fraction. Obviously, this protein was present in much larger quantities than any of the four CPF subunits. We assume that the 65-kDa protein was a contamination that was not removed due to the omission of two purification steps in the CPF preparation from HeLa cells (see "Experimental Procedures").

Comparison of the most purified CPF from calf thymus and HeLa cell nuclear extracts showed that proteins of similar

TABLE II

Purification of CPF from HeLa nuclear extract

CPF was purified from 500 g of HeLa cells, as described under "Experimental Procedures." The purification and recovery are relative to the nuclear extract. Protein concentrations were determined as described for the calf thymus purification.

Fraction	Protein	Activity	Specific activity	Purification	Recovery
	mg	*units* $\times 10^{-3}$	*units* $\times 10^{-3}$/*mg*	*-fold*	*%*
Extract	860	365	0.42	1	100
DEAE-Sepharose	250	88.2	0.35	0.8	24
Blue-Sepharose	18.4	43.7	2.4	5.7	12
Heparin-Sepharose	1.6	10.0	6.3	15.0	2.7
Mono Q	0.25	6.1	24.4	58.1	1.7
Poly(A)-Sepharose	~0.03	7.0	~230	~548	1.9

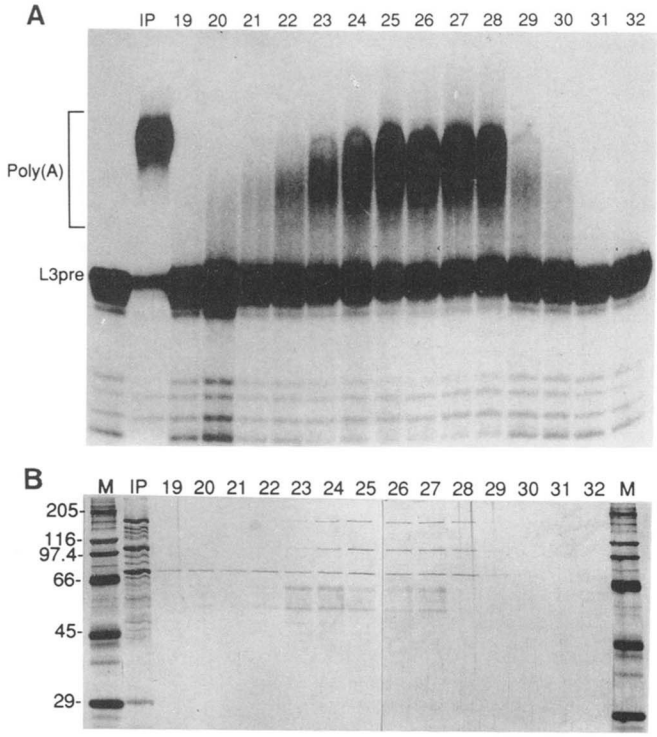

FIG. 3. **Sedimentation of CPF in a glycerol gradient.** Glycerol gradient centrifugation was performed as described under "Experimental Procedures." *A*, in the activity assay, 0.5 μl of the loaded sample (*IP*) and 2 μl of each gradient fraction were used. The gradient fractions are indicated at the *top*. *B*, SDS gel electrophoresis of the glycerol gradient fractions. 15 μl of the input material (*IP*) and 25 μl of each gradient fraction were precipitated with trichloroacetic acid and analyzed on two 9% SDS polyacrylamide gels. The molecular masses of the marker proteins (*M*) in kilodaltons are indicated on the *left*.

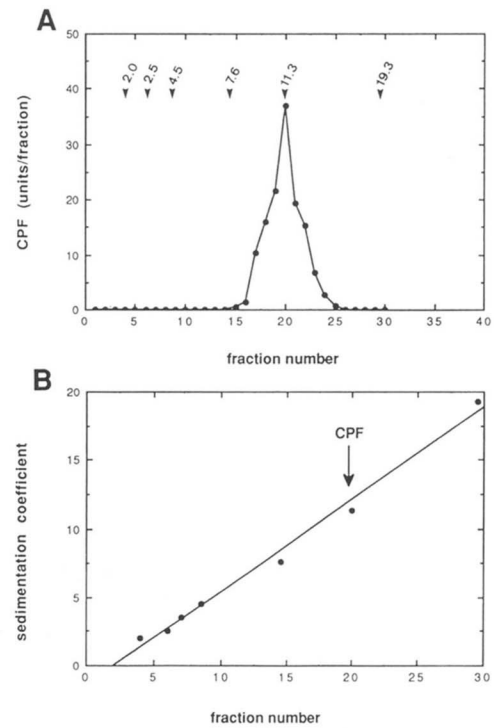

FIG. 4. **Sedimentation Constant of CPF.** The sedimentation constant of CPF was determined with a poly(A)-Sepharose fraction that had been purified in the absence of detergent (see text). Sedimentation through a glycerol gradient was performed as described under "Experimental Procedures." The following proteins were applied to parallel gradients in pairwise combinations: RNase A (2 S), chymotrypsinogen (2.5 S), ovalbumin (3.5 S), BSA (4.5 S), aldolase (7.35 S), yeast alcohol dehydrogenase (7.6 S), catalase (11.3 S), and thyroglobulin (19.3 S). The marker proteins were located by their UV absorbance and CPF by activity assays. *A*, sedimentation profile of CPF. The positions of some of the marker proteins are indicated with their sedimentation constants. *B*, data of all proteins were replotted. The position of CPF is indicated by the *arrow*.

molecular weights were purified from either source and that the ratio of the four polypeptides to each other was comparable in both preparations (Fig. 2). For the following experiments, we used a poly(A)-Sepharose fraction purified from calf thymus extract if not indicated otherwise.

CPF Is a Complex of Four Polypeptides—Upon centrifugation through glycerol gradients, all four polypeptides cosedimented with CPF activity in a single peak (Fig. 3). Only the 73-kDa protein was partially separated from CPF activity.

The protein content of the complex formed between CPF and the substrate RNA was consistent with the sedimentation data. CPF was run on a native polyacrylamide gel after incubation with or without radiolabeled L3pre RNA. The complex formed with RNA was located by autoradiography. Elution of the proteins out of this complex into a second dimension SDS-polyacrylamide gel resulted in the pattern of the four proteins (data not shown). The same protein pattern was observed in the second dimension analysis of the corresponding gel region in the absence of substrate RNA. Most likely, the short RNA molecule did not significantly influence the migration behavior of the protein complex to which it was bound.

For determination of the sedimentation coefficient of CPF, another glycerol gradient centrifugation was performed with CPF that had been purified in the absence of detergent. In addition, this preparation had been performed in the presence of protease inhibitors and in the absence of $MgCl_2$ (see above). The poly(A)-Sepharose fraction of this preparation was about 3-fold less pure than the corresponding standard preparation. Comparison to marker proteins resulted in a sedimentation coefficient of 11.5 S (Fig. 4).

The same fraction was analyzed by gel filtration chromatography on a Superose 6 column. On this column, CPF behaved slightly heterogeneously (Fig. 5). The Stokes radius as determined by comparison to standard proteins (Siegel and Monty, 1966) was near 100 Å. From the sedimentation coefficient, the Stokes radius and an assumed partial specific volume of 0.73 mg/ml, a native molecular mass of approximately 500 kDa was calculated (Siegel and Monty, 1966). The sum of the molecular weights of the four polypeptides determined by gel electrophoresis is 360,000. In the glycerol gradient, as well as in the gel filtration of Fig. 5, an additional protein with a molecular mass of 140 kDa comigrated with CPF, which was not observed in other preparations. The presence of this protein may explain the discrepancy between the native molecular weight and the sum of the denatured molecular weights of the standard CPF preparation.

For the detection of disulfide bonds involved in the interaction of the four polypeptides, an aliquot of CPF was precipitated with trichloroacetic acid and resuspended in gel-loading buffer lacking 2-mercaptoethanol. Half of this sample then received 5% 2-mercaptoethanol, and both aliquots were heat-denatured and analyzed on an SDS gel. The 30-kDa polypeptide was visible only in the sample boiled in the presence of the reducing agent (Fig. 6). This suggests that the 30-kDa polypeptide may be bound via disulfide bonds to one of the other CPF subunits, although none of them changed in size under nonreducing conditions.

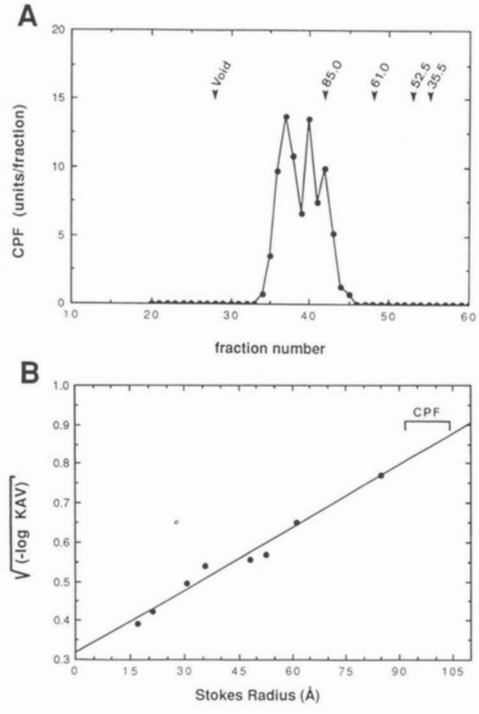

FIG. 5. **Determination of the Stokes radius by gel filtration.** The detergent-free fraction was concentrated to approximately 0.3 mg/ml in a Centricon 30 device (Amicon) and 200 μl of the concentrate were applied to a Superose 6 FPLC column (1 × 30 cm, Pharmacia) that was equilibrated in buffer A plus 100 mM KCl. The column was developed with the same buffer, and fraction of 300 μl were collected. Marker proteins and their Stokes radii were cytochrome *c* (17.0 Å), chymotrypsinogen (20.9 Å), ovalbumin (30.5 Å), BSA (35.5 Å), aldolase (48.1 Å), catalase (52.2 Å), ferritin (61.0 Å), and thyroglobulin (85 Å). The void volume of the column was determined with blue dextran. *A*, elution profile of CPF. The void volume and the positions of some marker proteins together with their radii are indicated with *arrowheads*. *B*, determination of the Stokes radius of CPF according to Siegel and Monty (1966). The position of CPF is indicated.

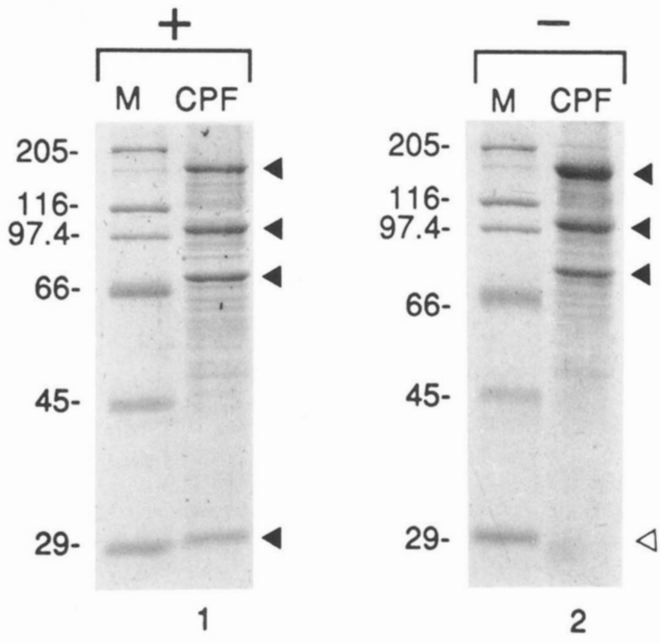

FIG. 6. **SDS gel electrophoresis of CPF under reducing and nonreducing conditions.** A poly(A)-Sepharose fraction (~500 units of CPF activity) was precipitated with trichloroacetic acid and resuspended in loading buffer lacking 2-mercaptoethanol. Prior to loading, one-half of the sample was heat-denatured in the presence (*lane 1*), and the other half in the absence, of 5% 2-mercaptoethanol (*lane 2*). The loaded samples were separated by several lanes to avoid diffusion of the reducing agent through the gel. The marker proteins were also loaded in the presence or absence of 5% 2-mercaptoethanol. The molecular masses of the marker proteins in kilodaltons are indicated. *Arrowheads* mark the positions of the 160-, 100-, 73-, and 30-kDa polypeptides. *M*, marker proteins.

CPF Is Not a snRNP—Previous experiments (Christofori and Keller, 1988) had indicated that the U11 snRNP might play a role in CPF activity. Partially purified CPF was analyzed by CsCl buoyant density centrifugation as described under "Experimental Procedures." CPF activity was found in fractions 1–8 at a density of 1.32–1.40 g/cm^3 (Fig. 7*A*), with the peak of activity in fraction 1 (1.32 mg/cm^3). Total protein showed a very similar distribution (Fig. 7*B*). U11 RNA had a broad distribution from fraction 1 to fraction 14 with peaks in fraction 2 (1.34 mg/cm^3) and fraction 8 (1.40 mg/cm^3) (Fig. 7*C*). snRNPs generally show a heterogenous distribution in Mono Q FPLC chromatography.[3] Specifically, U11 snRNP particles eluting at low salt concentrations from Mono Q appeared as more slowly migrating complexes in a native polyacrylamide gel, compared with the U11 snRNPs eluted at higher salt concentrations (data not shown). Therefore, CsCl density centrifugation was repeated with a particular Mono Q fraction that had been eluted at a higher salt concentration and contained U11 snRNP, as well as CPF activity. In this gradient, CPF again banded at the density of a protein (1.31 mg/cm^3), whereas U11 snRNA was mainly found at a snRNP-specific density (1.40–1.44 mg/cm^3) (data not shown). These results thus clearly show that CPF has the density of a protein in CsCl buoyant density gradients, whereas the U11 snRNP has a heterogeneous density distribution. The majority of U11 does not cofractionate with CPF in this type of separation.

The removal of U11 RNA in the course of CPF purification was quantitated by northern blot analysis (Table III). A 1,700-fold reduction of U11 snRNA relative to CPF was obtained. The most efficient separation of U11 RNA from CPF was

[3] S. Bienroth, A., Krämer, and W. Keller, unpublished observation.

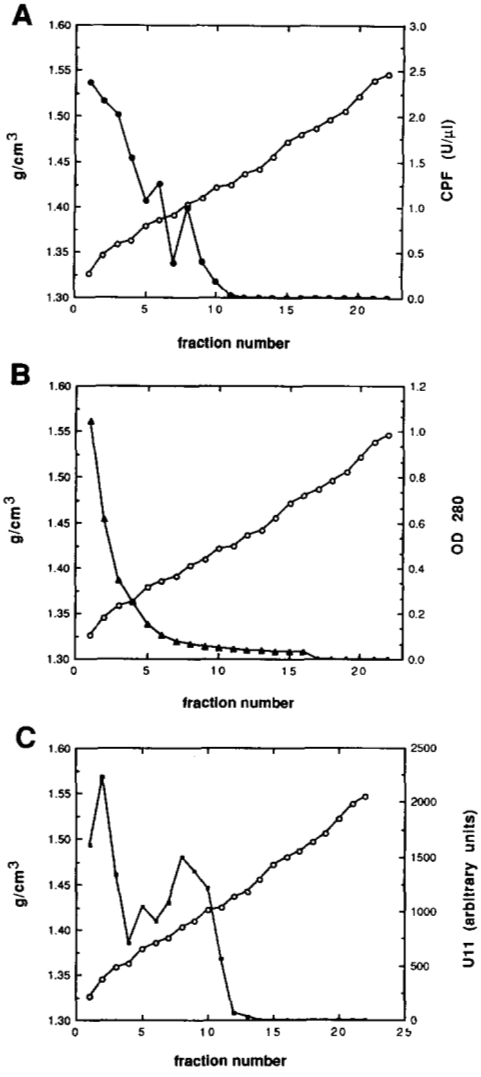

FIG. 7. **CsCl buoyant density centrifugation of CPF.** CsCl gradients were performed as described under "Experimental Procedures." The CsCl gradients were analyzed for the distribution of CPF activity, proteins, and U11 snRNA. *A*, CPF activity is indicated by *filled circles*. *Open circles* show the density of the fractions as determined by weighing of 100-µl aliquots. *B*, centrifugation of a protein mixture (Mono Q fraction; see "Experimental Procedures"). The UV absorbance is indicated by *triangles*, and the density, by *open circles*. The same protein distribution was obtained in a gradient that contained pure BSA (not shown). *C*, distribution of U11 RNA in the gradient. U11 RNA was measured by Northern blotting (see "Experimental Procedures") and scanning densitometry. *Boxes* indicate U11 RNA, and *open circles*, the density of the fractions.

TABLE III

Separation of U11 RNA from CPF

The concentration of U11 RNA was determined by Northern blot analysis in the various steps of the calf thymus CPF purification (see "Experimental Procedures").

Fraction	U11 RNA	U11 RNA/CPF activity
	pmoles	*pmoles/units* × 10^{-3}
Extract	33,600	
DEAE-Sepharose	10,420	8.5
$AmSO_4$ pellet	11,890	9.9
Blue-Sepharose	1,260	2.2
Heparin-Sepharose	153	0.44
Mono Q	45.5	0.16
Spermine-agarose	26.4	0.25
Poly(A)-Sepharose	0.375	0.005

TABLE IV

Affinity chromatography of CPF with monoclonal antibodies directed against the trimethylguanosine cap of snRNAs

An aliquot of a spermine-agarose fraction of calf thymus CPF was loaded on an anti-m_3G affinity column as described under "Experimental Procedures."

Fraction	CPF activity	Recovery of CPF	U11 RNA	Recovery of U11 RNA
	units	%	*fmol*	%
Input	1600		8000	
Unbound	880	55	660	8.3
Bound	3.9	0.2	4000	50

observed upon poly(A)-Sepharose chromatography, where 98% of U11 RNA did not bind. The poly(A)-Sepharose fraction was also tested for the presence of other snRNAs by 3′ end-labeling with pCp and RNA ligase (Krämer, 1987). In addition to U11 RNA, comparable amounts of U1, U2, and U5 RNA were found (data not shown).

CPF was analyzed by Western blotting with antibodies against common snRNP proteins (Lührmann, 1988). The monoclonal antibody Y12 (Lerner *et al.*, 1981), directed against the Sm epitope of snRNP proteins, and the monoclonal antibody H57 against the BB′ proteins[4] did not detect any of the common snRNP proteins (data not shown). Since the sample contained small amounts of U RNAs (see above), snRNP proteins were probably present in concentrations too low to be detectable by this method. However, the results rule out the presence of substantial quantities of an unknown snRNP whose RNA escaped detection of 3′ end-labeling.

A spermine-agarose CPF fraction was applied to an affinity column that carried monoclonal antibodies directed against the trimethylguanosine cap structure of snRNAs (anti-m_3G affinity column). Recovery of CPF activity was 55% in the flow-through and 0.24% in the eluate. In contrast, most of the U11 RNA bound to the affinity matrix. Only 8% of the initial amount of U11 RNA did not bind (Table IV). Application of the poly(A)-Sepharose pool to the anti-m_3G affinity column resulted in a similar separation of CPF activity from U11 RNA (data not shown).

The poly(A)-Sepharose pool used in the experiment shown in Fig. 8 (see below) contained 5 fmol of U11 RNA/µl, as determined by quantitative Northern blot analysis. However, 1 µl of this CPF fraction bound 100 fmol of labeled substrate RNA in a gel shift assay. U11, therefore, cannot be responsible for binding of CPF to the substrate RNA. The concentration of the 160-kDa protein in the same fraction was estimated by comparison to a BSA standard on an SDS-polyacrylamide gel and amounted to ~60 fmol/µl. This protein is thus sufficiently abundant to account for RNA binding.

Based on these data, we conclude that U11 snRNP is not related to CPF.

Specificity of Purified CPF for the AAUAAA Sequence—An RNA substrate (L3pre) containing the AAUAAA signal was polyadenylated by poly(A) polymerase that was complemented with the poly(A)-Sepharose fraction of CPF. A single U to G conversion in the recognition signal (L3preΔ) abolished polyadenylation (not shown). Polyadenylation of labeled L3pre RNA was almost completely competed by a 5-fold excess of unlabeled L3pre RNA but was not affected at all by the addition of a 10-fold excess of unlabeled L3preΔ substrate (Fig. 8*A*).

CPF in the absence of any other factor binds to an RNA containing the AAUAAA signal (Gilmartin and Nevins, 1989).[2] Incubation of CPF with L3pre and analysis of the

[4] R. Lührmann, personal communication.

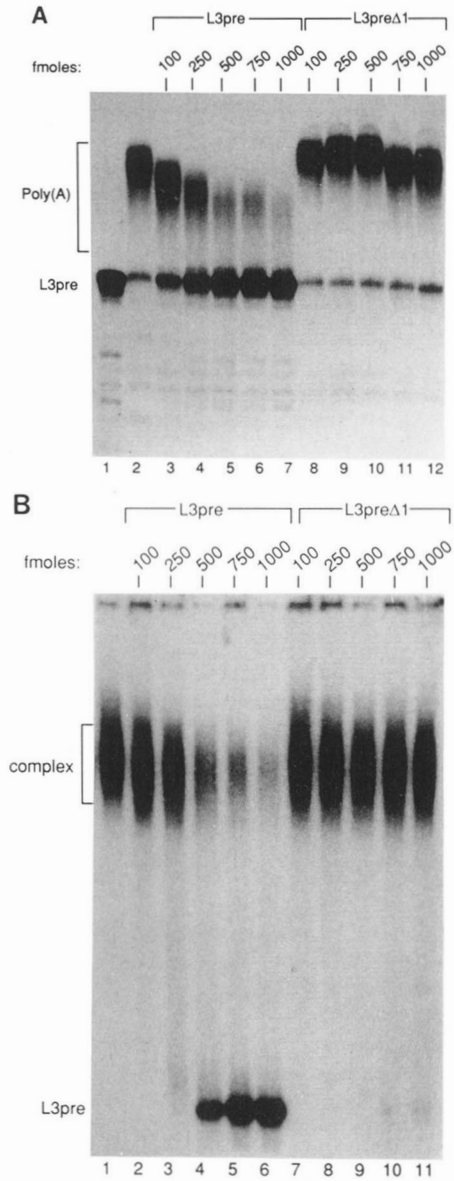

FIG. 8. **CPF-dependent polyadenylation and binding of CPF to the substrate RNA are dependent on the AAUAAA signal.** *A*, CPF (9 units of a poly(A)-Sepharose pool) was incubated under polyadenylation conditions with 100 fmol of labeled L3pre RNA and different concentrations of nonlabeled competitor RNA. The reactions were analyzed on a denaturing 12% polyacrylamide gel. *Lane 1*, minus CPF; *lane 2*, minus competitor; *lanes 3–7*, 100–1000 fmol of L3pre; *lanes 8–12*, 100–1000 fmol of L3preΔ. The amount of competitor RNA in each reaction is indicated at the *top* of the *panel*. *B*, CPF (9 units) was incubated under complex formation conditions with 100 fmol of labeled L3pre in a 6.25-μl reaction in the presence of wild-type and mutant competitor RNA. *Lane 1*, minus competitor; *lanes 2–6*, 100–1000 fmol of L3pre; *lanes 7–11*, 100–1000 fmol of L3preΔ. Competitor RNA concentrations are indicated at the *top* of the *panel*.

reaction on a native polyacrylamide gel resulted in the appearance of a specific, slowly migrating band. This gel shift activity coincided with the polyadenylation activity in all purification steps examined. No binding was observed when the mutant substrate was used (not shown). The specific binding was competed by an unlabeled wild-type RNA but not by a 10-fold excess of the mutant (Fig. 8*B*).

These experiments demonstrate that the binding of purified CPF is specific for the AAUAAA signal. Furthermore, CPF alone is sufficient for specific binding to the substrate RNA.

DISCUSSION

We have described the purification of CPF as a complex of four polypeptides of 160, 100, 73, and 30 kDa. Purification from calf thymus homogenate and from HeLa cell nuclear extract gave essentially the same protein pattern. The four proteins were present in approximately equimolar amounts, and their ratios and molecular weights did not change in response to the presence or absence of protease inhibitors during the purification. Sedimentation, gel filtration, and native gel electrophoresis demonstrated the formation of a tight complex between the four proteins. The native molecular weight of the complex was roughly consistent with the sum of the denatured molecular weights of its components. Finally, the patterns of tryptic fragments of the four polypeptides showed no obvious similarities (data not shown). Taken together, these results suggest that all four polypeptides may be genuine subunits of CPF and that none of the polypeptides is a proteolytic degradation product of any of the others. A partial separation of the 73-kDa polypeptide from CPF in glycerol sedimentation indicates that this protein is less tightly held in the complex than the others. Whether it is dispensable for CPF activity remains to be shown.

In the sedimentation and gel filtration of CPF purified in the presence of protease inhibitors, an additional polypeptide of 140 kDa comigrated with the activity. However, all of the smaller polypeptides were also present in the same amount as in other preparations. Therefore, it appears that the smaller polypeptides are not degradation products of the 140-kDa protein. The latter is thus not an essential subunit of CPF.

The involvement of snRNPs in mRNA 3′ end formation has been suggested by several lines of evidence (see above). Specifically, the observation that the AAUAAA sequence could be immunoprecipitated from 3′-processing reactions by anti-Sm antibodies and by anti-trimethylguanosine antibodies (Hashimoto and Steitz, 1986; Stefano and Adams, 1988; Moore *et al.*, 1988) indicated the association of a snRNP with the AAUAAA sequence. Furthermore, in a partial purification of CPF, the U11 snRNP appeared associated with CPF activity (Christofori and Keller, 1988). However, in the purification reported here a 1,700-fold reduction of the ratio of U11 RNA to CPF was observed. By Western blot analysis we also did not detect any snRNP-specific proteins in purified CPF fractions. CPF did not bind to antibodies directed against the trimethylguanosine cap common to most U-type snRNAs. In addition, CPF had the density of a protein in CsCl buoyant density centrifugation. An earlier report that CPF has the density of a snRNP (Christofori and Keller, 1988) could not be confirmed. Finally, the amount of U11 RNA (or any other RNA) in purified CPF was not sufficient to account for the amount of substrate RNA bound. In contrast, the CPF polypeptide subunits were present in the appropriate quantities. Thus we conclude, in agreement with other reports (Takagaki *et al.*, 1989; Bardwell *et al.*, 1991), that CPF is not a snRNP. We have observed that partially purified CPF fractions could be immunoprecipitated after UV cross-linking to L3pre RNA with anti-Sm and anti-BB′ antibody. Thus it is possible that CPF has a propensity to bind snRNPs specifically or nonspecifically. Since none of the other processing factors has so far shown any indication of containing an RNA component, 3′ end formation of polyadenylated mRNA may be the only major RNA-processing event that proceeds without the involvement of a snRNP. However, it remains possible that a snRNP is involved in the cleavage reaction.

CPF and poly(A) polymerase, both purified to near homogeneity, were sufficient to reconstitute AAUAAA-dependent polyadenylation. This confirms earlier results obtained with

partially purified components (see above). It has been demonstrated before (Gilmartin and Nevins, 1989; Bardwell *et al.*, 1991),[2] and confirmed in this work, that CPF binds to AAUAAA. UV cross-linking studies identified two proteins of 160 and 30 kDa as AAUAAA-specific RNA-binding components (Moore *et al.*, 1988; Gilmartin and Nevins, 1991).[2] These two polypeptides are very likely to be the largest and smallest subunits of CPF. In a partially purified CPF fraction, a 170-kDa protein that specifically cross-linked to an AAUAAA-containing RNA was recognized by human SLE serum of the Sm serotype (Gilmartin and Nevins, 1991). However, in a Western blot analysis with monoclonal antibodies directed against the Sm epitope, we have not found a reaction with any of the polypeptides in CPF. Possibly, the systemic lupus erythematosus serum contained additional antibodies. The same systemic lupus erythematosus serum resulted in the coimmunoprecipitation of five polypeptides of 170, 130, 100, 74, and 42 kDa (Gilmartin and Nevins, 1991). By size, three of these polypeptides might correspond to the purified CPF subunits. Resolution of the four subunits and reconstitution of CPF are required to establish the roles of each of the subunits in the recognition of AAUAAA and the interaction with other processing factors.

The recognition of the AAUAAA sequence by CPF implies that this factor already acts in the first step of the 3′ end-processing reaction, the endonucleolytic cleavage. Previous studies showed that partially purified CPF is required for this cleavage reaction in the presence of additional cleavage factors (Christofori and Keller, 1988; Gilmartin *et al.*, 1988; Gilmartin and Nevins, 1989; Takagaki *et al.*, 1989; Takagaki *et al.*, 1990). We have purified CPF based on its ability to reconstitute specific polyadenylation. However, additional experiments have shown that this CPF preparation also supports cleavage.[5]

It is intriguing that CPF is an oligomeric complex with a high molecular weight. Poly(A) polymerase in contrast is a simple enzyme consisting of a single polypeptide (Wahle, 1991). This difference in complexity probably reflects the fact that during 3′ processing CPF has to interact with the RNA substrate but also with poly(A) polymerase and the cleavage factors via protein-protein contacts. The elucidation of these multiple interactions will be the subject of future studies.

Acknowledgments—We thank Dr. Reinhard Lührmann for the anti-m_3G affinity column and Drs. Lührmann and Joan Steitz for antibodies. We are most grateful to Drs. Angela Krämer, Paul Jenö, and Ariel Lustig for helpful discussions. We thank Dr. Gerhard Christofori for his advice to one of us (S. B.) at the beginning of this work.

[5] K. Beyer, E. Wahle, and W. Keller, unpublished observation.

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