Four factors are required for 3’-end cleavage of pre-mRNAs

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We reported previously that authentic polyadenylation of pre-mRNAs in vitro requires at least two factors: a cleavage/specificity factor (CSF) and a fraction containing nonspecific poly(A) polymerase activity. To study the molecular mechanisms underlying 3’ cleavage of pre-mRNAs, we fractionated CSF further and show that it consists of four separable subunits. One of these, called specificity factor (SF; Mr ~290,000), is required for both specific cleavage and for specific polyadenylation and thus appears responsible for the specificity of the reaction. Although SF has not been purified to homogeneity, several lines of evidence suggest that it may not contain an essential RNA component. Two other factors, designated cleavage factors I (CFI; Mr ~130,000) and II (CFII; Mr ~110,000), are sufficient to reconstitute accurate cleavage when mixed with SF. A fourth factor, termed cleavage stimulation factor (CstF; Mr ~200,000), enhances cleavage efficiency significantly when added to a mixture of the three other factors. CFI, CFII, and CstF do not contain RNA components, nor do they affect specific polyadenylation in the absence of cleavage. Although these four factors are necessary and sufficient to reconstitute efficient cleavage of one pre-RNA tested, poly(A) polymerase is also required to cleave several others. A model suggesting how these factors interact with the pre-mRNA and with each other is discussed.

[Key Words: Cleavage/specificity factor, poly(A), pre-RNA]

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Polyadenylation of eukaryotic pre-mRNAs is one of the most important steps in maturation of RNA polymerase II transcripts. This process plays an important role in gene expression, as mutations that block 3’-end formation prevent accumulation of mature mRNA (for reviews, see Birnstiel et al. 1985; Manley 1988). In addition, polyadenylation can play a role in the regulation of gene expression, because selection of alternative poly(A) sites in a single gene can lead to the synthesis of different mRNAs (for review, see Leff et al. 1986).

HeLa cell nuclear extracts are able to accurately process exogenously added pre-RNA molecules that contain the signals required for 3’-end formation [Moore and Sharp 1985]. Using such extracts, it has been shown that the two steps of the 3’-end processing reaction, i.e., endonucleolytic cleavage and polyadenylation, can be uncoupled and assayed separately [Moore and Sharp 1985; Moore et al. 1986; Zarkower et al. 1986; Sheets et al. 1987]. In the absence of divalent cation, pre-RNAs are accurately cleaved but not polyadenylated, generating upstream and downstream cleavage products. In the presence of Mg++, pre-RNAs can be polyadenylated at the 3’ ends of either the pre-RNAs, themselves, or upstream cleavage products [Manley 1983; Moore and Sharp 1985; Zarkower et al. 1986]. Both reactions absolutely require the conserved AAUAAA signal sequence [Proudfoot and Brownlee 1976], and the cleavage reaction is also influenced by sequences that lie just downstream of the cleavage site [for review, see Manley 1988]. Several studies have indicated that large complexes form on pre-mRNAs, which presumably contain the factors that catalyze the 3’-end formation reaction [Humphrey et al. 1987; Skolnik-David et al. 1987; Zarkower and Wickens 1987; Zhang and Cole 1987; McLauchlan et al. 1988; Moore et al. 1988b; Stefano and Adams 1988]. Based on UV cross-linking experiments, it has been proposed that two proteins (Mr 64,000–68,000 and 155,000) interact specifically with the sequences surrounding the poly(A) signal AAUAAA [Moore et al. 1988a; Wilusz and Shenk 1988].

Recently, several groups have begun fractionating HeLa cell nuclear extracts to identify the factors involved in the cleavage and polyadenylation reactions [for review, see Humphrey and Proudfoot 1988]. We reported previously that a cleavage specificity factor (CSF) that efficiently cleaves SV40 late pre-RNA at its poly(A) addition site can be separated chromatographically from a poly(A) polymerase [PAP; Takagaki et al. 1988]. Although this PAP activity functions only nonspecifically by itself; addition of CSF causes it to function in a poly(A) signal [AAUAAA]-dependent manner. On the other hand, the PAP activity is also required to cleave all other pre-RNAs tested so far. Using different fractionation methods, it has been shown that multiple factors are required for both cleavage and polyadenylation reactions [Gilmartin et al. 1988; McDevitt et al. 1988]. Christofori and Keller [1988] have recently demon-

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strated that three factors are required for cleavage of both SV40 late and Ad2 L3 pre-RNAs. Although two of these factors—cleavage polyadenylation factor and PAP—are also required for polyadenylation, a third factor—cleavage factor—is necessary only for the cleavage reaction.

Analogous to other pre-mRNA processing reactions, e.g., splicing (for review, see Green 1986; Padgett et al. 1986; Maniatis and Reed 1987) and histone mRNA 3'-end formation (for review, see Birnstiel et al. 1985; Mowry and Steitz 1988), the involvement of small nuclear ribonucleoproteins (snRNPs) in 3'-end formation of polyadenylated mRNAs has been suggested (Moore and Sharp 1984, 1985; Hashimoto and Steitz 1986; Sperry and Berget 1986; Christofori and Keller 1988; Gilmartin et al. 1988). However, it is unlikely that a major species of snRNA (e.g., U1, U2, U4, U5, and U6) is required for this process, because degradation of these snRNAs does not affect 3'-end processing reactions (Berget and Robberson 1986; Ryner and Manley 1987). Recently, Christofori and Keller (1988) reported that a fraction of U11 snRNP (Kramer 1987; Montzka and Steitz 1988) cofractionated with a factor required for both cleavage and polyadenylation reactions. The activity of this factor, however, was not strictly correlated with the amount of U11 snRNA. In addition, in contrast to snRNAs involved in splicing and 3'-end processing of histone pre-RNAs, the sequence of U11 snRNA displays no complementarity to sequences known to be required for polyadenylation (e.g. AAUAAA; Montzka and Steitz 1988). The question of snRNP involvement in pre-mRNA 3'-end processing thus remains an enigma.

To elucidate the molecular mechanisms underlying 3'-end processing of pre-mRNA, it is essential to identify and characterize all the factors that are involved in this process. In this study we have isolated from CSF four different factors that are necessary and sufficient to reconstitute accurate and efficient cleavage of SV40 late pre-RNA and characterized some of their functions and physical properties.

**Results**

**CSF can be fractionated into three factors**

We showed previously that a CSF that can efficiently cleave SV40 late pre-RNA at its poly(A) addition site can be separated from a nonspecific PAP. In addition, CSF is also required, along with PAP, to catalyze AAUAAA-dependent polyadenylation (Takagaki et al. 1988). To study CSF further, we subjected the factor to additional fractionation steps, as indicated in Figure 1.

As a first step, CSF obtained by Superose 6 chromatography (Materials and methods; Takagaki et al. 1988) was applied to a Mono Q anion exchange column. Fractions were assayed by incubating aliquots with a 32P-labeled 233-nucleotide SV40 late pre-RNA, either in the presence of Mg2+ and crude, Superose 6 PAP to assay specific polyadenylation, or in the absence of Mg2+ and other fractions to assay cleavage. Reaction products were analyzed by denaturing polyacrylamide gel electrophoresis, and the results of the experiment in which PAP was added are shown in Figure 2. A single, strong peak of specific polyadenylation activity was detected in fractions 19–21 (Fig. 2B). In contrast, when each of the

![Figure 1](https://example.com/f1.png)

**Figure 1.** Fractionation of factors required for in vitro 3'-end processing of pre-mRNAs. The fractionation methods used to separate and characterize the factors involved in 3'-end processing of pre-mRNAs are shown schematically. (Bottom) Sedimentation coefficients obtained by glycerol density gradient centrifugation, estimated molecular mass, and density (d) for SF.
Mono Q fractions was assayed alone, in either the presence or absence of Mg\(^{2+}\), neither polyadenylation nor cleavage was detected [results not shown]. An explanation for these results is that the activity detected [fractions 19–21] is a factor that interacts specifically with the AAUAAA sequence in the pre-RNA and leads to specific polyadenylation in the presence of PAP. However, additional factors, originally present in the CSF fraction but separated from the specificity-enhancing factor by Mono Q chromatography, are also required for cleavage.

To test this hypothesis, we performed mixing experiments using fraction 20 and each of the Mono Q fractions. As shown in Figure 3A, accurate cleavage activity was detected when fraction 20 was mixed with fractions eluted at lower salt concentrations [fractions 11–13]. However, recovery of cleavage activity was <10% of the total activity in the CSF-containing fractions that had been loaded on the Mono Q column. Therefore, we performed a second mixing experiment in which each Mono Q fraction was added to a mixture of fractions 12 [low salt] and 20 [high salt]. As summarized in Figure 3B, the flowthrough fraction [FT] was found to enhance cleavage activity significantly [cf. lanes 8 and 14]. An experiment in which the low salt and flowthrough fractions were mixed with each Mono Q fraction [Fig. 3C] revealed that the high salt activities required for cleavage and polyadenylation cofractionated precisely. In addition, neither the flowthrough fraction nor fraction 12 [low salt] had any effects on the specific polyadenylation reaction reconstituted by mixing fraction 20 [high salt] and PAP [data not shown]. It is unlikely that any additional factors are involved in specific cleavage of SV40 late pre-RNA, because the cleavage activity recovered after mixing the three fractions [flowthrough, low salt, and high salt fractions] was relatively high (>40% of the total activity present in CSF-containing fractions) and because an additional mixing experiment in which each Mono Q fraction was added to a mixture of these three fractions did not reveal any enhancement of the cleavage activity [data not shown].

These results strongly suggest that the three fractions separated from each other by Mono Q chromatography are necessary and sufficient to reconstitute efficient cleavage of SV40 late pre-RNA and that only one of these factors, which eluted at high salt, is required for specific polyadenylation. Based on these functional characteristics, the three factors recovered from the Mono Q column in high salt, low salt, and flowthrough fractions are designated specificity factor (SF), cleavage factor (CF), and cleavage stimulation factor (CstF), respectively.

Cleavage factors can function on adenovirus pre-RNA only in the presence of PAP.

We showed previously that both CSF and the PAP-containing fractions are required to reconstitute cleavage activity for three pre-RNAs other than SV40 late pre-
RNA (e.g., adenovirus L3 pre-RNA; Takagaki et al. 1988). We have recently fractionated PAP activities from HeLa cells and found that two chromatographically distinct forms of nonspecific poly(A) polymerase, called PAP type I and type II, can be separated from each other and purified extensively. Both of these enzymes were shown to be capable of functioning with CSF to bring about cleavage of the Ad2 L3 pre-RNA (Ryner et al. 1989b).

To test the generality of the functions of the three factors required to reconstitute cleavage of SV40 late pre-RNA, we mixed SF, CF, CstF, and PAP type I in all possible combinations in processing reaction mixtures that contained a 280-nucleotide Ad2 L3 pre-RNA as a substrate. Figure 4 shows that in contrast to the results obtained with the SV40 late pre-RNA (Fig. 3B), PAP was indispensable for cleavage of Ad2 L3 pre-RNA (cf. lanes 8 versus 11 and lanes 14 versus 15). It is noteworthy that the PAP did not detectably affect cleavage of the SV40 late pre-RNA [see Fig. 3B]. In addition, analogous to the results shown in Figure 3B, CstF significantly enhanced cleavage of Ad2 L3 pre-RNA [cf. lanes 11 and 15]. These results indicate that the three factors originally detected with the SV40 late pre-RNA are also sufficient to cleave Ad2 L3 pre-RNA when mixed with PAP and strongly suggest that the basic mechanisms of the cleavage reactions for both SV40 late and Ad2 L3 pre-RNAs are quite similar, except for the requirement for PAP.

Most snRNAs can be completely separated from the factors required for cleavage

To investigate the possible involvement of snRNPs in pre-mRNA 3'-end processing, we examined the distribution of snRNAs in the above Mono Q fractions by labeling endogenous RNAs at their 3'-ends and fractionating them on a 10% polyacrylamide–7 M urea gel (see Materials and methods). As shown in Figure 5, no snRNAs were detected in fractions containing CstF (flowthrough) or CF (fractions 11–13). Although a small fraction of U5 and U6 snRNAs was detected in fractions eluted at 800 mM (NH4)2SO4 (fractions 22 and 23), another snRNA, which was identified as U11 snRNA by Northern blotting, using a 5'-end-labeled complementary oligonucleotide (101; Montzka and Steitz 1988) as a probe [data not shown], was enriched in fractions 15 and 16, between CF and SF. Less than 10% of the total U11 snRNA found in CSF-containing fractions was present in fractions with SF activity [19–21].

Figure 3. Fractionation of CSF into three factors. (A) To reconstitute specific cleavage, mixing experiments were carried out in 7.5-μl standard reaction mixtures that contained 1.5 ng of pG3SVL-A pre-RNA, 1.5 μl of fraction 20, and 1.5 μl of each Mono Q fraction but no divalent cation. [CSF] CSF (2.0 μl) was used as a positive control; [−] 1.5 μl of fraction 20 only was used as a negative control; [FT] flowthrough fraction from Mono Q column; [M] DNA size markers; [Pre] unprocessed pre-RNA. [Right] The upstream and downstream cleavage products are indicated by solid and open arrows, respectively. (B) The indicated fractions obtained by Mono Q chromatography, as well as separately purified PAP [type I, see text], were mixed in all possible combinations. The mixing experiments were carried out in 10 μl standard reaction mixtures that contained 2.0 ng of pG3SVL-A pre-RNA, 0.5 μl of flowthrough fraction [FT], 2.0 μl of fraction 12 [low salt], 1.0 μl of fraction 20 [high salt], and/or 0.5 μl of PAP but no divalent cation. Four microliters of crude [NH4]2SO4 fraction [Am], 3 μl of CSF alone [CSF], or 3 μl of CSF plus 1 μl of PAP obtained by Superose 6 chromatography [C + A] were also used as controls. [C] One microliter of each Mono Q fraction was incubated with a mixture of 1 μl each of the Mono Q flowthrough fraction and fraction 12 in 7.5-μl standard reaction mixtures which also contained 1.5 ng of pG3SVL-A pre-RNA but no divalent cation. [CSF] CSF (2.0 μl) was used as a positive control; [−] flowthrough plus fraction 20 only, as a negative control.
3' End cleavage of pre-mRNAs

JL^.,X4^5^JL,8_9^10.1M213J4J5

Low Salt
High Salt

404-
309-
238-
180-

Figure 4. Reconstitution of cleavage activity for Ad2 L3 pre-RNA. Three fractions obtained by Mono Q chromatography (FT, low salt, and high salt fractions), as well as partially purified PAP (type I), were mixed and incubated under the conditions indicated in Fig. 3B, except that 2 ng of pG4L3-A (Ad2 L3) pre-RNA was used instead of pG3SVL-A pre-RNA. (Bottom) The structure of Ad2 L3 pre-RNA transcribed from pG4 L3-A DNA by SP6 RNA polymerase is shown schematically.

Characterization of the specificity factor

To characterize SF in more detail, the factor was further purified by phenyl Superose chromatography followed by CsCl buoyant density centrifugation. Figure 6, B and C, shows the results of mixing experiments, which indicate that phenyl Superose fractions 28–34 were able to reconstitute both specific cleavage and specific polyadenylation activities. When we examined the distribution of snRNAs in phenyl Superose fractions [Fig. 6D], U5 and U6 snRNAs were eluted over a broad range of (NH4)2SO4 concentrations. On the other hand, U11 snRNA was enriched in low salt fractions (26–32), which overlapped with SF-containing fractions [28–34]. However, although fraction 26, which contained significant U11 snRNA, showed no SF activity, fraction 34, with very little U11 snRNA, exhibited the same level of activity as fractions 30 and 32. The presence of U11 snRNA was thus not strictly correlated with the processing activities.

SF-containing fractions [30–34] were pooled and subjected to CsCl buoyant density centrifugation in the presence of 15 mm MgCl2 and 5 mm DTT to stabilize snRNPs during centrifugation [Reveillaud et al. 1984; Lelay-Taha et al. 1986]. As shown in Figure 7, B and C, both cleavage and polyadenylation activities were again detected in exactly the same fractions [11–13], at a density of 1.27–1.30 g/ml. These fractions also contained the majority of free proteins (Fig. 7A), indicating that little purification was achieved by this step. Figure 7D demonstrates the distribution of snRNAs. As expected, the major species of snRNAs (U1, U5, and U6) were detected in high-density fractions [6–9, 1.32–1.42 g/ml density]. Although some U11 snRNA was also present in these fractions, it was enriched in fractions 11 and 12, overlapping with SF activity. However, analogous to the results obtained by phenyl Superose chromatography [Fig. 6D], the presence of U11 snRNA and SF activity was not strictly correlated, because fraction 13, which contained virtually no snRNA, exhibited the same level of activity as fractions 11 and 12.

To address further the possible involvement of U11 snRNP in 3'-end processing, we performed immunodepletion experiments [Materials and methods; Kramer et al. 1984], using a monoclonal anti-Sm antibody [Y12, Lerner et al. 1981] conjugated to protein A-Sepharose [PAS]. These antibodies have been shown to bind U11 snRNP efficiently [Kramer 1987; Montzka and Steitz 1988]. SF-containing fractions obtained by CsCl buoyant density centrifugation were passed through a small anti-Sm antibody–PAS column, and the flowthrough and bound fractions were analyzed. As shown in Figure 8A, the anti-Sm flowthrough fraction contained only 10–15% of the total U11 snRNA (lane Sm, FT). In contrast, when a nonspecific monoclonal antibody of the same class [anti-p53 antibody, IgG2a] was used as a control, ~70% of the U11 snRNA recovered was found in...
Figure 6. Purification of SF by phenyl Superose chromatography. (A) The elution profile of proteins from the phenyl Superose column was monitored by UV absorbance at 280 nm. The concentration of (NH₄)₂SO₄ in the elution buffer is shown by the dotted line, and the elution position of SF is indicated by a bracket at top. After phenyl Superose chromatography, flowthrough fractions (4–6) and adjacent fractions starting from fraction 9 were pooled, concentrated, and dialyzed, as described in Materials and methods. Each pool is represented by an even-numbered fraction. (B) Specific polyadenylation activity was assayed in 7.5-μl standard reaction mixtures that contained 1.5 ng of pG3SLV-A pre-RNA, 1 mM MgCl₂, 1 μl of PAP obtained by Superose 6 chromatography, plus 2 μl of CSF, SF (Q high), or each phenyl Superose fraction. (FT) flowthrough fraction from the phenyl Superose column, (M) DNA size markers, (Pre) unprocessed pre-RNA. (C) Specific cleavage activity was assayed in 7.5-μl standard reaction mixtures [lacking divalent cations] that contained 1.5 ng of pG3SVL-A pre-RNA, 1 μl each of CF, and CstF-containing fractions, plus 1 μl of either SF (Q high) or each phenyl Superose fraction. (−) no phenyl Superose fraction, (FT) flowthrough fraction, (M) DNA size markers, (Pre) unprocessed pre-RNA. (D) RNAs isolated from CSF-containing fractions obtained by Superose 6 chromatography, from pooled SF-containing fractions from Mono Q chromatography (fractions 19–21, Q high), or from each of the phenyl Superose fractions were labeled at their 3' ends and fractionated on 10% polyacrylamide-7 M urea gel, as described in Materials and methods. (Right) snRNAs are identified. (M) DNA size markers.

the flowthrough fraction [lane p53, FT]. When SF activity was titrated, by measuring the ability of the flowthrough fractions to reconstitute both specific cleavage and specific polyadenylation with SV40 late pre-RNA [Fig. 8B,C], approximately equal activities were recovered in both anti-Sm and anti-p53 depleted fractions. The two titration curves, which were almost identical, suggest that SF was not present in excess, thereby excluding the possibility that saturating amounts of U11 snRNA were present in the anti-Sm antibody-depleted fraction.

To estimate the molecular weight of SF, SF-containing fractions (30–34) obtained by phenyl Superose chromatography were subjected to glycerol density gradient centrifugation. Both cleavage and polyadenylation activities were detected in exactly the same fractions with a sedimentation coefficient of 12.5S [data not shown]. Based on these results, the molecular weight of SF was estimated to be 290,000.

Cleavage factor consists of two subunits

To purify the cleavage factor further, CF-containing fractions obtained by Mono Q chromatography [11–13]...
Figure 7. CsCl buoyant density centrifugation of SF-containing fractions obtained by phenyl Superose chromatography. (A) After centrifugation, 15 fractions were collected and the protein concentration [solid line] and density [dotted line] of each fraction were measured. The positions of SF-containing fractions [SF] and snRNAs [U5, U6] are indicated by brackets at top. (B) Specific polyadenylation activity was assayed in 7.5-μl standard reaction mixtures that contained 1.5 ng of pG3SVL-A pre-RNA, 1 mM MgCl2, 1 μl of PAP obtained by Superose 6 chromatography, and 2 μl of either CSF (CSF), the SF-containing fraction obtained by phenyl Superose chromatography [Phe], or each of the CsCl density gradient fractions. (M) DNA size markers, (Pre) unprocessed pre-RNA. (C) Specific cleavage activity was assayed in 7.5-μl standard reaction mixtures that contained 1.5 ng of pG3SVL-A pre-RNA, 2 mM EDTA, 1 μl each of CF [fraction 12] and CstF [flowthrough]-containing fractions obtained by Mono Q chromatography, plus 1 μl of either the SF-containing fraction obtained by phenyl Superose chromatography or each of the CsCl gradient fractions. Two microliters of CSF was used as a positive control, and no added CsCl fraction [−] as a negative control. (D) RNAs isolated from CSF-containing fractions, pooled SF-containing fractions obtained by Mono Q [Q high] or phenyl Superose [Phe] chromatography, and each CsCl density gradient fraction were labeled at their 3′ ends and fractionated on 10% polyacrylamide-7 M urea gel, as described in Materials and methods. [Right] snRNAs are identified. [M] DNA size markers.

were applied to a Mono S cation exchange column. When proteins were eluted by a steep concentration gradient of [NH4]2SO4 [50–380 mM in 20 ml], a low level of activity was detected in fractions eluted at ~250 mM salt [data not shown]. However, when a more shallow gradient [50–380 mM in 25 ml] was used, no activity was recovered [data not shown]. Based on these results, we suspected that CF might have been separated into multiple fractions during Mono S chromatography. To test this hypothesis, we mixed separate fractions, eluted at higher [fraction 26] or lower [fraction 20] than 250 mM with each Mono S fraction in reaction mixtures that also contained SF and CstF obtained by Mono Q chromatography. As shown in Figure 9, B and C, fractions 18–20 and fractions 26–30 were able to complement high [fraction 26] and low [fraction 20] salt fractions, respectively, to recover efficient cleavage activity. Based on these results, the two complementary factors eluted at low and high salt concentrations were designated cleavage factors I [CFI] and II [CFII], respectively.

To purify CFI and CFII further and to estimate the molecular weights of these factors, we subjected CFI and CFII to glycerol density gradient centrifugation. Single peaks of activities were detected for both CFI and CFII.
Figure 8. Immunodepletion of SF-containing fractions obtained by CsCl buoyant density centrifugation. (A) Samples (200 μl) of the SF-containing fractions from the CsCl gradient were applied to anti-Sm or anti-p53 antibody-PAS columns as described in Materials and methods, and RNA was extracted from 60 μl of each flowthrough (FT) or the equivalent amount of each antibody-PAS conjugate (PAS). Isolated RNA was then labeled at its 3' end and fractionated on 10% polyacrylamide-7 M urea gel, as described in Fig. 5. (Right) The position of U11 snRNA. (M) DNA size markers. (B) Specific polyadenylation activity was assayed in 7.5-μl standard reaction mixtures that contained 1.5 ng of pG3SVL-A pre-RNA, 1 mM MgCl₂, 1 μl of PAP obtained by Superose 6 chromatography, and 2 μl of either CSF or undiluted (1/1) or 2-, 5-, or 10-fold diluted (1/2, 1/5, or 1/10, respectively) flowthrough fractions from the anti-Sm (Sm) or anti-p53 (p53) antibody-PAS columns. (C) Specific cleavage activity was assayed in 7.5-μl standard reaction mixtures that contained 1.5 ng of pG3SVL-A pre-RNA, 2 mM EDTA, 1 μl each of CF- and CstF-containing fractions obtained by Mono Q chromatography, and 1 μl of antibody-PAS column flowthrough fractions, as indicated in B. Two microliters of CSF was used as a positive control; [-] no flowthrough fraction, as a negative control. (M) DNA size markers; (Pre) unprocessed pre-RNA.

(data not shown). Based on the sedimentation coefficients of 6.5S and 5.85, the molecular weights of CFI and CFI were estimated to be 130,000 and 110,000, respectively.

CstF is distinct from PAP

To purify CstF further, the Mono Q flowthrough fraction was applied to a heparin–agarose column. As shown in Figure 10, A and B, CstF was eluted from the column as a single peak at ~200 mM (NH₄)₂SO₄. Recently, Christofori and Keller [1988, 1989] suggested that PAP is required to cleave not only Ad2 L3 but also SV40 late pre-RNAs. Although the mixing experiments presented above (Figs. 3B and 4) suggest that the effects of PAP and CstF on the cleavage reaction are different, to exclude the possibility that CstF is identical to PAP, nonspecific PAP activity in the CstF fraction was measured by the incorporation of radioactive ATP into bulk RNA in the presence of Mn²⁺ [Nevins and Joklik 1977; Takagaki et al. 1988]. No activity was detected in the heparin–agarose fractions that contained CstF [data not shown], providing additional evidence that CstF is not a PAP.

To estimate the molecular weight of CstF, heparin–agarose-purified CstF was subjected to glycerol density gradient centrifugation [data not shown]. A single peak of activity was detected, and based on a sedimentation coefficient of 9S, the molecular weight of CstF was estimated to be 200,000.

SF, CFI, CFI, and CstF are all required to reconstitute efficient cleavage

The above results describe the isolation and characterization of four separate factors from CSF. An important question is whether these factors are necessary and sufficient to catalyze 3'-end cleavage of pre-mRNAs. Thus, we have analyzed the effects of different combinations of these factors on processing of SV40 late and Ad2 L3 pre-RNAs. As sources of the factors we used phenyl superose [SF], Mono S [CFI and CFI], and heparin–agarose [CstF] fractions (see Fig. 1).

As described above [Fig. 3B], SF and CF were required to reconstitute accurate cleavage of SV40 late pre-RNA. As shown in Figure 11A, cleavage activity was detected only after mixing both CFI and CFI, as well as SF [lane 4], and efficient cleavage reaction was reconstituted by adding CstF to a mixture of these three factors [lane 8]. Note that the relative efficiency of the cleavage reaction was much lower in the absence of CstF than when CF was analyzed before fractionation on Mono S (see Fig.
3B). On the other hand, as shown in Figure 4, PAP, SF, and CF were required to cleave Ad2 L3 pre-RNA [cf. lanes 8 and 11]. When CFI and CFII were used instead of CF, both of these factors were required to reconstitute cleavage activity [lane 4]. This activity was again dramatically enhanced (~10-fold) by CstF [lane 8]. The relatively low activity reconstituted by SF, CFI, and CFII [and PAP], as compared to that by SF and CF [and PAP], may reflect a deleterious effect caused by the physical separation of CFI and CFII by Mono S chromatography. However, it appears that CstF is able to facilitate a functional interaction between CFI and CFII. These results show that the four factors obtained by fractionation of CSF are sufficient to catalyze efficient cleavage of SV40 late pre-RNA and that PAP is also required to cleave Ad2 L3 pre-RNA.

Discussion

In this study we have fractionated a CSF into four distinct factors and characterized their structural and functional properties. One of these factors, SF, is required for both specific cleavage and specific polyadenylation reactions, both of which require the poly(A) signal AAUAAA [Manley 1983; Manley et al. 1985; Zarkower et al. 1986; Skolnik-David et al. 1987]. As SF can complement PAP that has only a nonspecific catalytic function, to impart specificity, it is most likely that SF recognizes the polyadenylation signal and interacts in some fashion with PAP. However, in contrast to many eukaryotic transcription factors, which can specifically bind to promoter sequence with high affinity [for review, see Kadonaga et al. 1986], we have not detected specific binding of SF to an appropriate pre-RNA (Y. Takagaki and J.L. Manley, unpubl.), suggesting that the mechanism of poly(A) signal recognition by SF may be quite different from that of transcription factors. For example, transient interaction of SF with the signal sequence may be sufficient for the catalytic subunits [PAP and cleavage factors] to initiate their functions.

Three other factors, namely CFI, CFII, and CstF, appear to function together to reconstitute cleavage activity. The fact that CFI and CFII can accurately cleave SV40 late pre-RNA, when mixed with SF, suggests that these two factors play major roles in the cleavage reaction. On the other hand, although CstF, by itself, does not show any activity when mixed with SF, it strongly enhances the cleavage reaction. These results suggest that CstF may play a supportive role, e.g., by optimizing the interactions of the other factors with RNA substrate or with each other. In contrast to PAP, nonspecific activities could not be detected for CF [or CFI and CFII] and CstF, either alone or in combination.

Interestingly, the cleavage activity reconstituted by the four factors comprising CSF is specific for the SV40 late poly(A) site. Recently, we have analyzed the effects of cis-acting elements lying downstream of AAUAAA sequence on the efficiencies of in vitro cleavage reactions using partially purified fractions CSF and PAP [Ryner et al. 1989a]. These results indicated that an 11-nucleotide sequence encompassing the cleavage site itself is responsible for the selective cleavage of SV40 late pre-RNA by CSF. However, a longer RNA sequence extending up to over 20 nucleotides downstream of the poly(A) site is required for efficient cleavage of Ad2 L3 pre-RNA, which requires both CSF and PAP.

By combining these observations with the results obtained in this study, we can speculate on the modes of
interaction between trans-acting factors and cis-acting elements [Fig. 12]. The fact that only a short stretch of RNA surrounding the poly[A] site is sufficient for cleavage of the SV40 late pre-RNA suggests a possible interaction between this sequence and a catalytic subunit composed of CFI, CFII, and CstF. In the case of pre-RNAs other than SV40 late [e.g., Ad2 L3], PAP can interact with sequences farther downstream, perhaps to alter the structure of the RNA substrate around the poly[A] site to make it accessible for cleavage catalyzed by the other factors. Because PAP must interact with SF to initiate specific polyadenylation and must locate the 3' end of the substrate RNA to which it adds poly[A], PAP most likely contacts RNA sequences between the poly(A) signal AAUAAA and 3' end of the pre-RNA, supporting this hypothesis.

Figure 11. Reconstitution of pre-mRNA 3'-end cleavage activity. CstF-, CFI-, CFII- and SF-containing fractions were obtained by heparin–agarose, Mono S, Mono S, and phenyl Superose chromatography, respectively (see Fig. 1). PAP (type I) is the same preparation as in Figs. 3B and 4. (A) To assay specific cleavage activity with SV40 late pre-RNA, 1.0 μl of CstF, CFI, CFII, and SF was mixed in the indicated combinations at top. Standard reaction mixtures of 10 μl contained 2.0 ng of pG3SVL-A pre-RNA and 1 mM EDTA. Three microliters of CSF (CSF) was used as a positive control. [M] DNA size markers; [Pre] unprocessed pre-RNA. [B] To assay cleavage activity with Ad2 L3 pre-RNA, 0.5 μl of PAP and CstF and 1.0 μl of CFI, CFII, and SF were mixed in the indicated combinations. Reaction mixtures [10 μl] contained 2.0 ng of pG4L3-A pre-RNA and 1 mM EDTA. Four microliters of (NH₄)₂SO₄ fraction (20–40% saturation; see Fig. 1) [Am], 3 μl of CSF alone or 3 μl of CSF plus 1 μl of PAP obtained by Superose 6 chromatography [C + A] were used as controls.
Previously, we estimated the molecular weight of CSF to be ~360,000 (Takagaki et al. 1988). However, there was somewhat of a discrepancy between the results obtained by gel filtration ($M_r > 670,000$) and glycerol density gradient centrifugation (~10S, $M_r 220,000$). We suggested previously that this may indicate that CSF is asymmetrically shaped. The results presented here, however, offer an alternative explanation. Specifically, we believe that the larger estimate, obtained by gel filtration, probably represents the native size of CSF, because, assuming that one molecule each of the four factors described here (SF, CFI, CFII, and CstF) is present in the complex, the total molecular mass would be ~730,000 daltons. This is close not only to the size estimated previously but also to that of a heparin-resistant specific complex (25S) formed during incubation of crude nuclear extract with a pre-RNA substrate (Stefano and Adams 1988). The size estimated by glycerol density gradient centrifugation corresponds to that expected of a partially dissociated complex composed of SF ($M_r 290,000$), CFI + CFII ($M_r 240,000$), and CstF ($M_r 200,000$), perhaps due to the long-lasting gravitational force ($2.0 \times 10^5 \times g$ for 12 hr). The peak of activity detected (~10S) was located between those of SF (12.5S) and CstF (9S), where the hypothetical dissociated components would overlap. In addition, the fact that these three components can be separated from each other by Mono Q chromatography supports the existence of a larger complex.

Recently, Christofori and Keller (1988) fractionated HeLa cell nuclear extracts into three factors that are necessary for pre-mRNA 3'-end processing reactions. One of these factors, called cleavage polyadenylation factor (CPF), is required for both cleavage and polyadenylation reactions and is probably identical to our SF, as both have similar functional properties and molecular weights ($M_r 200,000$–$290,000$, 10–12.5S). Another factor, CF, was required only for the cleavage reaction. This factor is probably identical to our CF (CFI and CFII). CF was also fractionated by Christofori and Keller (1988) on Mono S, but it did not separate into two subunits, perhaps because the column was step-eluted. Therefore, a size estimate obtained by glycerol density gradient centrifugation (5S) could represent those of dissociated CFI and CFII, which we have shown to have similar sedimentation coefficients (5.8–6.5S).

Although Christofori and Keller reported that PAP is required to reconstitute cleavage activity for both SV40 late and Ad2 L3 pre-RNAs (Christofori and Keller 1988, 1989), we have demonstrated that besides SF and CF (or CFI + CFII), only CstF is required for efficient cleavage of SV40 late pre-RNA. Although CSF was clearly separated from PAP by gel filtration chromatography (Takagaki et al. 1988), the chromatographic behavior of CstF, which was isolated from CSF, is quite similar to that of PAP (Christofori and Keller 1989; Ryner et al. 1989b). Therefore, it is possible that the PAP purified by Christofori and Keller contains CstF, offering an explanation for the apparent requirement of PAP observed by these authors.

Several groups have suggested that snRNPs are involved in 3'-end processing reactions in crude nuclear extracts (Moore and Sharp 1984, 1985; Hashimoto and Steitz 1986; Sperry and Berget 1986) or with partially purified fractions (Christofori and Keller 1988; Gilmartin et al. 1988). Christofori and Keller demonstrated that a fraction of U11 snRNP cofractionates with CPF. However, the presence of U11 snRNA is not strictly correlated with CPF/SF activity. Indeed, >90% of the U11 snRNA in CSF can be separated from SF by Mono Q chromatography. In addition, the content of U11 snRNA was not strictly correlated with SF activity during two additional steps of purification. Surprisingly, the buoyant density of U11 snRNP measured in our experiments is low (1.28), which is similar to free proteins, and considerably lower than the density of U11 snRNP (1.45) measured by Christofori and Keller (1988). At present, we cannot explain this discrepancy, partly because little is known about the protein composition and physical properties of U11 snRNP. The protein composition and/or higher order structures of U11 snRNP may have been altered during fractionation. For example, it is possible

Figure 12. A model for the interaction between factors involved in pre-mRNA 3'-end cleavage and polyadenylation. Functional characteristics of four factors isolated from CSF and PAP are reflected in this model. Three of these factors (SF, CFI, and CFII) are necessary and sufficient to catalyze cleavage of SV40 late pre-RNA. To cleave other pre-RNAs (e.g., Ad2 L3), PAP is also required. CstF enhances the efficiency of the cleavage reaction. Only SF and PAP are required to catalyze AAUAAA-dependent polyadenylation. Cap structure ($m^7$GpppG), polyadenylation signal sequence (AAUAAA), and polyadenylation site (arrowhead) are indicated on the pre-mRNA sequence.
that U11 snRNP can form a complex with other species of snRNPs [Montzka and Steitz 1988], thereby raising its apparent density.

Several lines of evidence argue against the direct involvement of U11 snRNP in 3'-end processing of pre-mRNAs. First, there is no sequence complementarity between U11 snRNA and substrate RNA [Montzka and Steitz 1988]. Second, the presence of U11 snRNA is not strictly correlated with SF activity. This was most apparent in the Mono Q chromatography step. Whereas >90% of the total U11 snRNA found in SF-containing fractions was clearly isolated from SF, a high level of processing activity (40–50%) was recovered after Mono Q chromatography. Furthermore, nearly 90% of the parent in the Mono Q chromatography step. Whereas findings are possible (e.g., the form of Ul 1 snRNP active rate from SF. Although other explanations for these contained virtually no detectable Ul 1 snRNA. Finally, the vast majority of U11 snRNA could be separated from SF activity. By our sequential purification methods, we estimate that only ~1% of the total U11 snRNA found was in the starting [NH₄]₂SO₄ fraction of nuclear extract co-fractionated with SF. Furthermore, nearly 90% of the U11 snRNA found in SF-containing fractions obtained by CsCl gradient centrifugation was removed by monoclonal anti-Sm antibodies without affecting processing activity. Therefore, well over 99% of the U11 snRNA present in our starting fraction could be physically separated from SF. Although other explanations for these findings are possible (e.g., the form of U11 snRNP active in 3'-end formation lacks an exposed Sm epitope), we believe the simplest is that U11 snRNP is not required for pre-mRNA 3'-end formation. Because none of the other factors contain detectable RNA components and because U11 snRNA is the only detectable RNA found associated with SF, the possibility therefore exists that the pre-mRNA cleavage and polyadenylation reaction is catalyzed solely by protein factors.

Materials and methods

Plasmid constructions and in vitro transcription

Plasmids pG3SVL-A and pG4L3-A, which contain SV40 late and Ad2 L3 polyadenylation sites, respectively, were described previously [Takagaki et al. 1988]. These plasmid DNAs were digested with DraI and used for in vitro transcription reactions to prepare capped, ³²P-labeled pre-RNAs [Konarska et al. 1984; Melton et al. 1984].

RNA processing and analysis

Pre-RNAs were incubated with protein fractions in 7.5- or 10-µl standard reaction mixtures at 30°C for 1.5 hr [Moore and Sharp 1985; Takagaki et al. 1988]. The reaction system (10 µl) contained 4 µl of protein fractions, 8 mM Tris-HCl [pH 7.9], or HEPES—NaOH [pH 7.9], 8% [vol/vol] glycerol, 20 mM [NH₄]₂SO₄ or 40 mM NaCl, 0.08 mM EDTA, 0.2 mM DTT, 0.2 mM PMSF, 1 mM ATP, 20 mM creatine phosphate, 2.5% [wt/vol] polyvinyl alcohol, 2.0 µg prerNA, 0.4 µg Escherichia coli RNA, and 1 mM MgCl₂, no divalent cation, 1 mM or 2 mM EDTA, as indicated in the figure legends. After incubation, reaction products were isolated and fractionated on 5% polyacrylamide—8.3 M urea sequencing gel [Maxam and Gilbert 1980], as described previously [Ryner and Manley 1987, Takagaki et al. 1988].

Analysis of snRNAs

Sixty microliters of fractions dialyzed against buffer A or C [see below] containing 100 mM NaCl were digested with proteinase K [Boehringer-Mannheim], as described previously [Ryner and Manley 1987]. After digestion, RNAs were extracted with phenol/chloroform and precipitated with ethanol in the presence of 30 µg of glycogen and 0.3 M NaCl. RNAs were labeled at their 3'-ends with [5'-³²P]pCp (INEN) and T4 RNA ligase (New England Biolabs), as described [England et al. 1980]. Labeled RNAs were fractionated on 10% polyacrylamide—7 M urea sequencing gels [Maxam and Gilbert 1980].

Fractionation of factors involved in pre-mRNA 3'-end cleavage reactions

Buffer A contained 20 mM Tris-HCl [pH 7.9], 20% [vol/vol] glycerol, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF. Buffer B was identical to buffer A but contained only 10% [vol/vol] glycerol. Buffer C was the same as buffer A but contained 20 mM HEPES–NaOH [pH 7.9]. Buffer D was identical to buffer C but contained no glycerol.

HeLa cell nuclear extracts [Dignam et al. 1983] and [NH₄]₂SO₄ fraction [20–40% saturation] [Takagaki et al. 1988] were prepared as described previously. All procedures were carried out at 0–4°C. Fractions were quick-frozen on dry ice and stored at ~80°C. Protein concentrations were determined by the method of Bradford [1976].

Ten milliliters of [NH₄]₂SO₄ fraction [20–40% saturation] (~20 mg/ml protein), prepared from ~30 liters of HeLa cells, was passed through a UNIFLO filter (pore size 0.45 µm, Schleicher & Schuell), and the filtrate was loaded on a 2.6 × 65 cm FPLC Superose 6 column [Pharmacia] equilibrated with buffer B containing 200 mM [NH₄]₂SO₄ at a flow rate of 0.65 ml/min. Proteins were eluted at the same flow rate, and 7-ml fractions were collected. Adjacent fractions were pooled and proteins were recovered by precipitation with solid [NH₄]₂SO₄ (65% saturation). Protein pellets were resuspended in 1.5 ml of buffer containing 50 mM [NH₄]₂SO₄ and dialyzed against the same buffer.

CSF-containing fractions obtained from two Superose 6 columns were pooled and centrifuged in a microfuge for 3 min to remove insoluble material, and the supernatant [9 ml, 45 mg protein] was filtered through a UNIFLO filter and loaded on a 1 × 10 cm FPLC Mono Q column [Pharmacia] equilibrated with buffer A containing 50 mM [NH₄]₂SO₄ at a flow rate of 1.0 ml/min. After washing with two column volumes of the same buffer, proteins were eluted by a linear gradient of [NH₄]₂SO₄ [from 50 to 240 mM in 100 ml], followed by step elution at 800 mM [NH₄]₂SO₄ at a flow rate of 1.5 ml/min. Fractions of 6 ml were collected, concentrated to 1.5 ml using Centriloc CF-25 [Amicon], and dialyzed against buffer C containing 50 mM [NH₄]₂SO₄.

Proteins in SF-containing fractions [19–21] obtained from a single Mono Q column [5 mg of proteins] were precipitated with solid [NH₄]₂SO₄ (65% saturation) and resuspended in 2 ml of buffer D containing 1 M [NH₄]₂SO₄. After spinning in microfuge for 3 min, the supernatant was loaded on a 0.5 × 5-cm FPLC phenyl Superose column [Pharmacia] equilibrated with buffer D containing 1 M [NH₄]₂SO₄ at a flow rate of 0.25 ml/min. After washing with five column volumes of the same buffer, proteins were eluted by a linear gradient of [NH₄]₂SO₄.
perose chromatographies (2.5 mg of protein), MgCl₂, and DTT respectively, to stabilize snRNPs during centrifugation (Reveillaud et al. 1984; Lelay-Taha et al. 1986). Solid CsCl was then added to a final concentration of 40% (wt/wt). An equal volume of buffer containing 15 mM MgCl₂, 5 mM DTT, 50 mM (NH₄)₂SO₄, and 40% (wt/wt) CsCl was overlaid, and the sample was centrifuged at 35,000 rpm for 36 hr at 4°C in an SW 50.1 rotor. After centrifugation, 15 fractions were collected from the bottom of each tube. A small aliquot (60 µl) was saved to measure the density and protein concentration of each fraction, and the remainder was dialyzed against four changes of 500 ml of buffer C containing 50 mM (NH₄)₂SO₄ for a total of 9 hr.

Proteins in CF-containing fractions (11–13) obtained from two Mono Q preparations (4 ml, 7 mg of proteins) were applied to a 0.5 x 5-cm PFLC Mono S column [Pharmacia] equilibrated with buffer C containing 50 mM (NH₄)₂SO₄ at a flow rate of 0.25 ml/min. After washing with five column volumes of the same buffer, proteins were eluted by a linear gradient of [NH₄]₂SO₄ [from 50 to 380 mM in 25 ml], followed by a step elution at 800 mM [NH₄]₂SO₄ at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected, and adjacent fractions were pooled, concentrated to 0.5 ml by Centricon, and dialyzed against buffer C containing 50 mM (NH₄)₂SO₄.

Flowthrough fractions obtained by Mono Q chromatography [4 ml, 5.5 mg of proteins] were applied to a 1 x 4-cm heparin–agarose column [type I, Sigma] equilibrated with buffer C containing 50 mM (NH₄)₂SO₄, at a flow rate of 0.2 ml/min. After washing with three column volumes of the same buffer, proteins were eluted by a linear gradient of [NH₄]₂SO₄ [from 50 to 380 mM in 25 ml], followed by a step elution at 800 mM [NH₄]₂SO₄ at a flow rate of 0.2 ml/min. Fractions of 1 ml were collected, and adjacent fractions were pooled and dialyzed against buffer C containing 50 mM (NH₄)₂SO₄.

Glycerol density gradient centrifugation was carried out as described previously (Takagaki et al. 1988), except that active fractions were pooled and concentrated approximately fivefold by Centricon prior to centrifugation. The samples were centrifuged at 47,000 rpm for 14 or 18 hr at 4°C in an SW 50.1 rotor.

**Immunodepletion experiments**

Ninety micrograms of monoclonal anti-Sm [lgG2a, Lerner et al. 1981] or anti-p53 [lgG2a, generously provided by E. Wang] antibody was conjugated to 200 µl of packed protein A–Sepharose [PAS], as described previously (De Robertis et al. 1982). After washing with IPP buffer [10 mM Tris-HCl [pH 7.9], 500 mM NaCl, 0.1% NP-40] and equilibrating with buffer C containing 50 mM (NH₄)₂SO₄, the antibody–PAS conjugate was packed in a 1-ml pipette tip. Immunodepletion experiments were carried out at 4°C, according to Kramer et al. (1984) with modifications. Two hundred microliters of pooled SF-containing fractions obtained by CsCl buoyant density centrifugation was loaded on each column. The flowthrough fractions were reloaded three more times over 30 min. The buffer remaining in the column was completely recovered by centrifugation in a clinical tabletop centrifuge for 30 sec. Pooled flowthrough fractions (170 µl) was dialyzed against buffer C containing 100 mM NaCl for 5 hr. Sixty microliters of each dialyzed flowthrough fraction and equivalent amount of antibody–PAS conjugate were digested with proteinase K, and RNA was isolated and labeled at its 3′-end, as described above. To titrate SF activity recovered in the flowthrough fractions, dialyzed samples were diluted 2-, 5-, or 10-fold and added to processing reactions as indicated in the figure legends.

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