A multisubunit factor, CstF, is required for polyadenylation of mammalian pre-mRNAs

Yoshio Takagaki,¹ James L. Manley,¹ Clinton C. MacDonald,² Jeffrey Wilusz,³ and Thomas Shenk²

¹Department of Biological Sciences, Columbia University, New York, New York 10027 USA; ²Howard Hughes Medical Institute, Department of Biology, Princeton University, Princeton, New Jersey 08544 USA; ³Department of Microbiology and Molecular Genetics, UMDNJ–New Jersey Medical School, Newark, New Jersey 07103 USA

We have purified and characterized a factor required for accurate polyadenylation of mammalian pre-mRNAs in vitro. This factor, called cleavage-stimulation factor (CstF), is composed of three distinct polypeptide subunits of 77, 64, and 50 kD. Using monoclonal antibodies directed against the 64- and 50-kD subunits, we show that CstF is required for efficient cleavage of polyadenylation substrates. Furthermore, CstF present in unfractionated nuclear extracts interacts with pre-mRNAs containing the signal sequence AAUAAA, but not AAGAAA, in such a manner that the 64-kD subunit can be cross-linked to the RNA by UV light. This polypeptide is thus identical to the previously described 64-kD nuclear protein that binds to AAUAAA-containing RNAs. Finally, indirect immunofluorescence of fixed cells indicates that CstF is distributed diffusely throughout the nucleus in a granular pattern distinct from the “speckled” pattern displayed by factors involved in pre-mRNA splicing, but similar to that of heterogeneous nuclear ribonucleoproteins. A model is presented in which CstF binds specifically to nascent RNA polymerase II transcripts and, by interacting with other factors, results in a rapid initiation of 3'-end processing of pre-mRNAs.

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Nearly all mammalian mRNAs are post-transcriptionally modified by the addition of a 3' poly(A) tail. This occurs by a two-step reaction in which an RNA polymerase II primary transcript is first endonucleolytically cleaved at the site of polyadenylation, and a poly(A) stretch of 200–300 nucleotides is then added to the 3' end of the upstream cleavage product (Nevins and Darnell 1978; Manley et al. 1982; Moore et al. 1986; Sheets et al. 1987; for reviews, see Humphrey and Proudfoot 1988; Manley 1988). Although these two steps are tightly coupled in vivo, they can be uncoupled and studied separately in vitro (Manley 1983; Moore and Sharp 1985; Zarkower et al. 1986). Using such assays, it has been shown that the nearly ubiquitous AAUAAA consensus sequence located 10–30 nucleotides upstream of the polyadenylation site (Proudfoot and Brownlee 1976; Fitzgerald and Shenk 1981; Higgs et al. 1983) is necessary for both cleavage and polyadenylation (Manley et al. 1985; Zarkower et al. 1986; Skolnik-David et al. 1987) and that a complex set of *trans*-acting factors is required to catalyze accurate polyadenylation.

Biochemical fractionation of nuclear extracts has demonstrated that multiple factors are required for both cleavage and polyadenylation reactions. One of these factors, designated cleavage–polyadenylation or specificity factor (SF), with an estimated molecular mass of

200–290 kD, is required for both reactions (Christofori and Keller 1988; Gilmartin and Nevins 1989; Takagaki et al. 1989). Poly(A) polymerase (PAP; 40–60 kD), which, by itself, synthesizes poly(A) tracts onto any RNA primer, is the only other factor necessary for AAUAAA-dependent poly(A) addition (Christofori and Keller 1989; Gilmartin and Nevins 1989; Ryner et al. 1989; Bardwell et al. 1990). Three additional factors, designated cleavage factors I (CFI; 130 kD) and II (CFII; 110 kD) and cleavage-stimulation factor (CstF; 200 kD), together with SF, are necessary and sufficient to reconstitute efficient cleavage activity with an SV40 late pre-mRNA (Takagaki et al. 1989). To cleave other pre-mRNAs, however, PAP is also necessary (Christofori and Keller 1988, 1989; Takagaki et al. 1988, 1989; Gilmartin and Nevins 1989; Ryner et al. 1989; Terns and Jacob 1989; Bardwell et al. 1990). To date, none of the factors required for polyadenylation have been purified to homogeneity. Using crude nuclear extracts, however, it was found that 64-kD (Moore et al. 1988b; Wilusz and Shenk 1988) and 155-kD (Moore et al. 1988b) polypeptides can be specifically UV cross-linked to pre-mRNAs that contain the AAUAAA sequence. Recently, it was shown that specific cross-linking of the 64-kD protein can be reconstituted with partially purified fractions (Wilusz et al. 1990). Only SF and CstF fractions are nec-

essary, suggesting that the 64-kD protein is a component of one of these two factors.

Apart from the problem of mechanism, an important question is when and where in the mRNA biosynthetic pathway polyadenylation occurs. Early experiments examining 3'-end formation in the adenovirus late transcription unit suggested that polyadenylation could occur very rapidly following passage of RNA polymerase past the poly(A) addition site, perhaps while the RNA is still a nascent chain (Nevins and Darnell 1978; Chen-Kiang et al. 1982; Manley et al. 1982). In keeping with the idea that polyadenylation and transcription might be linked, it has been shown in some cases that 3'-end formation precedes splicing (Weber et al. 1980). However, this situation is not universal (Zeevi et al. 1981), and models that assume a later position for 3'-end processing in the mRNA biosynthetic pathway are in some ways attractive. For example, polyadenylation might be linked to transport of mRNA from nucleus to cytoplasm. Also, based on experiments showing that certain factors involved in pre-mRNA splicing appear to be localized in discrete "speckles" within the nucleus (Lerner et al. 1981; Nyman et al. 1986; Fu and Maniatis 1990), it has been suggested that splicing might occur only in specific parts of the nucleus (Fu and Maniatis 1990). Might 3' processing occur in similar (or identical) nuclear substructures?

To address these issues, we have purified one of the factors required for pre-mRNA 3'-end cleavage and studied its biochemical and cell biological characteristics using monoclonal antibodies. This factor, CstF, is composed of three distinct subunits, one of which can be specifically cross-linked to AAUAAA-containing RNAs. Indirect immunofluorescence microscopy reveals a diffuse distribution of CstF throughout the nucleus, suggesting that CstF may bind to nascent RNA polymerase II transcripts. The roles of CstF in the 3'-end cleavage of pre-mRNAs are discussed.

Results and Discussion

Purification and characterization of CstF

As mentioned above, RNA 3' cleavage and polyadenylation can be uncoupled *in vitro* and assayed separately. For example, a pre-mRNA can be cleaved, but not polyadenylated, by a multicomponent complex called cleavage/specificity factor (CSF; Fig. 1B, lane 3) (Takagaki et al. 1988). We reported previously that CSF can be fractionated into four distinct subunits (Takagaki et al. 1989). CstF activity can be assayed by adding appropriate fractions to a mixture of SF- and CFI + CFII-containing fractions (cf. Fig. 1B, lanes 4 with 5), using a ³²P-labeled SV40 late pre-mRNA substrate (for structure, see Fig. 1B, bottom).

We have purified CstF to near homogeneity by a combination of chromatography methods and glycerol density gradient centrifugation (see Materials and methods). In the last two steps of purification, Mono S chromatography (data not shown) and glycerol gradient centrifugation (Fig. 1), three polypeptides with estimated molec-

Figure 1. Cofractionation of three polypeptides with CstF activity. (A) SDS-polyacrylamide gel profile of glycerol gradient fractions. Fraction numbers and the positions of the glycerol gradient molecular weight markers (200 kD, β -amylase; 150 kD, alcohol dehydrogenase) are shown at top. Estimated molecular weights of the three polypeptides are indicated at left. Note that three protein bands are detected in the 64-kD region. (B) Assay of glycerol gradient fractions for CstF activity. One microliter of each of the glycerol gradient fractions (lanes 6–11), CstF-containing fraction obtained by Mono S chromatography (lane 5), or buffer C containing 50 mM $(\text{NH}_4)_2\text{SO}_4$ (lane 4) was added to reaction mixtures containing SF, CFI, and CFII (see Materials and methods), together with an SV40 late pre-mRNA. Three microliters of CSF (Takagaki et al. 1988) (lane 3) was also used as a positive control. The sizes of the *Hpa*II-digested pBR322 DNA fragments (lane 1) are indicated at left. The positions of the pre-mRNA (lane 2, Pre), and the upstream (solid arrow) and downstream (open arrow) cleavage products are also shown at right. The structure of the SV40 late pre-mRNA transcribed from pG3SVL-A DNA (Takagaki et al. 1988) by SP6 RNA polymerase is shown at bottom. The 5' cap structure ($m^7\text{GpppG}$), polyadenylation signal sequence (AAUAAA), polyadenylation site (arrowhead), and size of the pre-mRNA are indicated.

ular weights of 77, 64, and 50 kD (Fig. 1A, fractions 8–10, lanes 2–4) exactly correlated with CstF activity (Fig. 1B, lanes 7–9). We hypothesized that CstF is composed of these three polypeptides based on two lines of evidence. First, the total molecular mass of these three polypeptides, 191 kD, is close to the native size of CstF, estimated to be 200 kD by glycerol gradient centrifugation (Fig. 1A). Second, the molar ratio of these polypeptides estimated by staining with Coomassie blue (data not shown) and silver (Fig. 1A) is approximately 1 : 1 : 1. Note that there is heterogeneity in the 64-kD polypeptide. Usually two major protein bands (62 and 64 kD)

and one minor one (66 kD) are detected (Fig. 1A; see below).

Anti-CstF monoclonal antibodies

To investigate the biochemical properties of CstF in more detail, we raised monoclonal antibodies against the polypeptides that cofractionated with CstF activity by injecting CstF-containing fractions obtained by Mono S chromatography into mice (see Materials and methods). We successfully cloned four hybridomas that secreted antibodies against the 64-kD polypeptide and two specific for the 50-kD polypeptide. In Figure 2A, proteins in the CstF-containing fraction used to immunize mice (lane 1) were subjected to Western blot analysis with preimmune serum (lane 2), anti-64-kD mAb ($\alpha 64k$, lane 3), or anti-50-kD mAb ($\alpha 50k$, lane 4). Note that the three polypeptides in the 64-kD region that were detected by protein staining (Fig. 2A, lane 1; also see Fig. 1A) were all recognized by $\alpha 64k$ monoclonal antibody (mAb) (lane 3), suggesting that the observed heterogeneity is generated by post-translational modifications. The fact that each of the six monoclonal antibodies obtained from cloned hybridomas recognized only one of the three polypeptides (64 or 50 kD) suggests that these proteins are not closely related to one another in their sequence (data not shown). None of the monoclonal antibodies used in these studies cross-reacted with other polypeptides, even when crude nuclear ex-

tract was used as an antigen for Western blotting (data not shown).

To prove that the 64- and 50-kD proteins are components of CstF and not simply abundant contaminants of this activity, we performed immunodepletion experiments (Fig. 2B). For this purpose, the crude CstF-containing fraction obtained by Mono Q chromatography (see Materials and methods) was depleted with monoclonal antibodies conjugated to protein G–Sepharose (PGS), and the flowthrough fractions were mixed with SF, CFI, and CFII to assay CstF activity (see Materials and methods). When CstF-containing fractions were treated with PGS alone (lane 5) or with an anti-Sm mAb–PGS conjugate containing antibodies against the snRNP Sm epitope (Lerner et al. 1981) (αSm , lane 6), high levels of CstF activity were recovered in the flowthrough. In contrast, both $\alpha 64k$ mAb- and $\alpha 50k$ mAb-treated fractions (lanes 9 and 10) exhibited only very low cleavage activity, virtually identical to that detected in the absence of CstF (lane 4). Consistent with these results, only 5%, at most, of the total antigen present in the untreated Mono Q fraction was detected by Western blotting in $\alpha 64k$ mAb- and $\alpha 50k$ mAb-depleted samples (data not shown). Furthermore, cleavage activity was completely recovered when highly purified CstF was added back to the reaction mixtures (lanes 7 and 8), confirming that the inhibition of cleavage activity was caused by removal of CstF. These results indicate that the 64- and 50-kD polypeptides are both re-

Figure 2. Characterization of monoclonal antibodies raised against CstF. (A) Western blotting with $\alpha 64k$ and $\alpha 50k$ mAbs. CstF-containing fraction purified through Mono S chromatography was fractionated on a 10% SDS–polyacrylamide gel and electrotransferred to a nitrocellulose membrane. Transferred proteins were stained with India ink (lane 1) or probed with preimmune serum (lane 2), $\alpha 64k$ mAb (lane 3), or $\alpha 50k$ mAb (lane 4). The positions of the three polypeptides that cofractionated with CstF activity are indicated on the left side of the figure. Note that three bands can be seen in the 64-kD region in lanes 1 and 3. (B) Immunodepletion of CstF activity with monoclonal antibodies. The reaction mixtures were as described in Fig. 1B, except for the origin of CstF. A CstF-containing fraction obtained by Mono Q chromatography (Takagaki et al. 1989) was passed through a small column of protein G–Sepharose (PGS) alone (lane 5), or of αSm mAb (Y12; Lerner et al. 1981; lane 6), $\alpha 64k$ mAb (lanes 7 and 9), or $\alpha 50k$ mAb (lanes 8 and 10) – PGS conjugate, and in each case 1 μ l of the eluate was used. In lanes 7 and 8, reaction mixtures were supplemented with 1 μ l of CstF purified through Mono S chromatography in which ~90% of the total protein was accounted for by the 77-, 64-, and 50-kD polypeptides. An equivalent amount of untreated Mono Q fraction (lane 3) or 3 μ l of CSF alone (lane 2) was used as a positive control, or no Mono Q fraction was added (lane 4) as a negative control.

quired for CstF activity but are consistent with either of two possibilities. First, the 64- and 50-kD polypeptides may have fortuitously cofractionated, and each is independently required for CstF activity. Second, these two polypeptides, and presumably the 77-kD polypeptide, exist as a protein complex that functions in 3'-end formation.

CstF is composed of three subunits

To determine whether the 77-, 64-, and 50-kD polypeptides are indeed components of a multisubunit factor, we carried out immunoprecipitation experiments using 125 I-labeled CstF as an antigen (Fig. 3; see Materials and methods). Among the three polypeptides that cofractionated with CstF activity, the 77-kD protein was more efficiently labeled with 125 I-labeled Bolton–Hunter reagent than were the 64- and 50-kD polypeptides (lane 1). When native CstF was used as an antigen, both α 64k and α 50k mAbs not only precipitated their target polypeptides but also the other two polypeptides (lanes 3 and 4). In contrast, preimmune serum (lane 2) did not precipitate any of these polypeptides. The slight difference in the signal of these three polypeptides between input and immunoprecipitates was probably caused by partial dissociation of the polypeptides during the labeling reaction and/or immunoprecipitation (see Materials and methods; cf. lane 1 with lanes 3 and 4). Note that with both α 64k and α 50k mAbs, the target protein and the 77-kD polypeptide were precipitated more efficiently than the third polypeptide. This could reflect a direct

Figure 3. Immunoprecipitation of CstF with monoclonal antibodies. (Lane 1) CstF-containing fraction labeled with 125 I (input). Native 125 I-labeled CstF was immunoprecipitated with preimmune serum (lane 2), α 64k mAb (lane 3), or α 50k mAb (lane 4). The positions of the molecular weight markers and 77-, 64-, and 50-kD polypeptides are indicated at left and right, respectively.

Figure 4. Immunoprecipitation of UV cross-linked 64-kD polypeptide with monoclonal antibodies. HeLa cell nuclear extract was incubated with 32 P-labeled SV40 late pre-mRNA containing a wild-type (AAUAAA) or mutated (AAGAAA) polyadenylation signal, irradiated with UV light, and digested with RNase A. In lanes 2 and 3, total proteins were fractionated directly on a 10% SDS-polyacrylamide gel. In lanes 4–9, samples were immunoprecipitated with α 64k mAb (lanes 4 and 5), α 50k mAb (lanes 6 and 7), or an unrelated mAb against the adenovirus 5 E1B protein (α E1B mAb, lanes 8 and 9) immediately after RNase digestion. In lanes 10–15, RNase A-treated samples were boiled in the presence of 0.05% NP-40 for 5 min and immunoprecipitated with α 64k mAb (lanes 10 and 11), α 50k mAb (lanes 12 and 13), or α E1B mAb (lanes 14 and 15). The positions of the molecular weight markers (M, lane 1) and UV cross-linked 64-kD polypeptide (open arrow) are indicated at left and right respectively.

interaction between the 77-kD polypeptide and both 64- and 50-kD polypeptides, and the absence of contact between the 64- and 50-kD polypeptides. These results combined with the data demonstrating an equal molarity of the three polypeptides present in CstF-containing fractions (Fig. 1A) indicate that native CstF is composed of three subunits with estimated molecular weights of 77, 64, and 50 kD.

Specific UV cross-linking of a CstF subunit to pre-mRNA

We recently reported (Wilusz et al. 1990) that only SF- and CstF-containing fractions are necessary to reconstitute the AAUAAA-dependent UV cross-linking of a 64-kD polypeptide to pre-mRNA that was originally detected using crude nuclear extracts (Fig. 4, lanes 2 and 3; Moore et al. 1988b; Wilusz and Shenk 1988). To determine whether the UV cross-linked 64-kD polypeptide corresponds to the 64-kD subunit of CstF, we subjected nuclear proteins cross-linked with a pre-mRNA con-

taining either a wild-type (AAUAAA) or mutated (AAGAAA) sequence to immunoprecipitation with monoclonal antibodies (Fig. 4). When immunoprecipitation was carried out immediately following UV irradiation and RNase A digestion, both α 64k and α 50k mAbs, but not an unrelated control monoclonal antibody, efficiently precipitated a 32 P-labeled 64-kD polypeptide with the wild-type but not the mutant RNA precursor (lanes 4–9). These results are consistent with either of two possibilities. First, the 64-kD polypeptide is a subunit of CstF, and it can therefore be precipitated with either the α 64k or the α 50k mAb. Second, the cross-linkable 64-kD polypeptide is not present in CstF. However, it is immunoprecipitated with monoclonal antibodies against CstF because factors involved in polyadenylation form a high-molecular-weight complex (Humphrey et al. 1987; Skolnik-David et al. 1987; Zarkower and Wickens 1987; Zhang and Cole 1987; McLauchlan et al. 1988; Moore et al. 1988a; Stefano and Adams 1988; Gilmartin and Nevins 1989), and all of its components, including a distinct 64-kD protein, are precipitated with the anti-CstF antibodies. To differentiate between these two possibilities, samples were boiled prior to immunoprecipitation to dissociate any noncovalent complexes. Under these conditions, the α 64k mAb was still able to immunoprecipitate the cross-linked 64-kD polypeptide, but the α 50k mAb and control monoclonal antibody were not (lanes 10–15). These results indicate that the 64-kD subunit of CstF is the nuclear protein that can be UV cross-linked specifically to AAUAAA-containing pre-mRNAs.

Nuclear localization of CstF

Finally, we examined the subcellular localization of CstF by indirect immunofluorescence microscopy using α 64k and α 50k mAbs and compared the distribution of this factor with that of other proteins involved in nuclear RNA metabolism. Both the 64-kD (Fig. 5A) and the 50-kD (data not shown) polypeptides were detected throughout the nucleus (except for nucleoli) but not in the cytoplasm. At higher magnification, the 64-kD polypeptide was observed as fine granules that were distributed diffusely throughout the nucleus (data not shown). Proteins involved in pre-mRNA splicing show different patterns of distributions. For example, Sm antigen (Fig. 5B, Lerner et al. 1981) and small nuclear ribonucleoproteins (snRNPs) (Nyman et al. 1986) are detected not only in the nucleoplasm but also in large nuclear speckles. In addition, a protein factor required for spliceosome assembly was observed only in the speckles (Fu and Maniatis 1990). On the basis of these observations, Fu and Maniatis (1990) proposed that pre-mRNA splicing and/or spliceosome assembly are compartmentalized in the speckled regions. The Sm snRNPs detected in the nucleoplasm might reflect the initial step of splicing, that is, binding of certain snRNPs to nascent RNA polymerase II transcripts (Beyer and Osheim 1988). It is known that hnRNP proteins can bind and form protein complexes on nascent RNA transcripts (Dreyfuss et al. 1988). Furthermore, these proteins [e.g., 120 kD (Drey-

Figure 5. Nuclear localization of CstF. Indirect immunofluorescent staining of HeLa cells with α 64k mAb (A), α Sm mAb (Y12; Lerner et al. 1981; B), or α hnRNP A1 protein mAb (4B10; Pinol-Roma et al. 1989; C). Mouse monoclonal antibodies were probed with fluorescein-conjugated anti-mouse IgG secondary antibody.

fuss et al. 1984), A1 (Fig. 5C, Pinol-Roma et al. 1989), and C (data not shown; Choi and Dreyfuss 1984)] are detected only in the nucleoplasm, where they are distributed diffusely in a granular pattern very similar to that of the 64- and 50-kD polypeptides (cf. Fig. 5A with C).

On the basis of the above results, we suggest that CstF is distributed ubiquitously throughout the nucleus where it can interact with nascent RNA polymerase II transcripts to initiate 3'-end formation. This hypothesis

is consistent with the findings mentioned above indicating that polyadenylation can occur immediately after transcription. Additionally, in several instances it has been shown that a functional polyadenylation sequence is required for subsequent transcription termination (Whitelaw and Proudfoot 1986; Logan et al. 1987; Connelly and Manley 1988; Lanoix and Acheson 1988), consistent with the idea that 3'-end formation occurs on nascent RNA chains. Splicing might usually occur later than 3'-end processing if pre-mRNAs (probably with certain snRNPs already attached) need to be transported to the speckles before complete spliceosomes are formed. However, it appears that in some cases splicing can also occur on nascent RNA chains (e.g., Beyer and Osheim 1988).

The characteristic fine granular structure observed by immunofluorescent staining with α 64k or α 50k mAb might represent high-molecular-weight complexes formed by polyadenylation factors and pre-mRNAs. Another intriguing possibility is that the similar fine granules observed for both hnRNP proteins and CstF might reflect the coexistence or interaction of heterogeneous nuclear RNP (hnRNP) particles and the factors that are directly involved in 3'-end processing. This hypothesis is supported by the fact that at least one of the hnRNP proteins (C proteins) can interact with pre-mRNA sequences located downstream of the polyadenylation site, suggesting a possible involvement of this protein in the 3'-end processing events (Wilusz et al. 1988).

Roles of CstF in the 3'-end cleavage of pre-mRNAs

The results presented here, coupled with previous findings, provide new insights into how the multiple factors involved in pre-mRNA polyadenylation interact both with each other and with the RNA substrate (see Fig. 6). As mentioned above, specificity factor (SF) is the only one of these factors that is necessary both for cleavage and for accurate poly(A) addition. Because of this, and because the other factor required for poly(A) addition, PAP, lacks intrinsic specificity, it is most likely that SF directly recognizes the AAUAAA sequence. However, we have shown here that the only polypeptide that can be UV cross-linked specifically to pre-mRNAs containing an AAUAAA sequence is the 64-kD subunit of CstF. This factor is required only for the cleavage reaction (Takagaki et al. 1989) and thus probably does not, itself, contact AAUAAA directly. Several properties of SF and CstF, however, offer an explanation for this apparent paradox. First, SF- and CstF-containing fractions are necessary and sufficient to recover specific UV cross-linking of the 64-kD polypeptide (Wilusz et al. 1990), implying a direct interaction between SF and CstF. Second, the 64-kD protein in extensively purified CstF can be UV cross-linked to nonspecific RNA substrates, indicating that CstF by itself has affinity for RNA (Wilusz et al. 1990). Third, the binding of SF alone to AAUAAA-containing pre-mRNA is not strong enough to withstand high-salt concentration (Ta-

Figure 6. A model for the interaction between the factors involved in the 3'-end processing of pre-mRNAs. This model reflects functional characteristics of the three subunits of CstF, as well as other factors that are required for 3'-end cleavage and polyadenylation of pre-mRNAs (Takagaki et al. 1989). SF, CFI, and CFII, and CstF are necessary and sufficient for efficient cleavage of SV40 late pre-mRNA. One of the three subunits of CstF, the 64-kD polypeptide, can be specifically UV cross-linked to pre-mRNAs containing a functional polyadenylation signal. SF and PAP are necessary and sufficient for AAUAAA-dependent polyadenylation. The 5' cap structure (m⁷GpppG), polyadenylation signal sequence (AAUAAA), and polyadenylation site (arrowhead) are indicated on the pre-mRNA sequence.

kagaki et al. 1989; Y. Takagaki and J.L. Manley, unpubl.). Therefore, we suggest that SF, via direct protein-protein interactions, imparts specificity to the binding of CstF to pre-mRNA, which is mediated by the 64-kD subunit. At the same time, CstF stabilizes the interaction between SF and the AAUAAA sequence, setting the stage for subsequent cleavage and polyadenylation.

CstF is one of four separable factors required for efficient 3'-end cleavage of pre-mRNAs (Takagaki et al. 1989; see also Christofori and Keller 1988; Gilmartin and Nevins 1989). In addition to SF, these include CFI and CFII, which are presumably involved in the actual endonucleolytic cleavage. We imagine that CstF also binds to these two factors to stabilize their interactions with pre-mRNAs and to enhance their catalytic function. A model to explain these interactions suggests that CstF is located between SF and CFI and CFII, where it contacts the pre-mRNA as well as these three factors (see Fig. 6). This model raises the intriguing possibility that CstF may play an important role in determining the efficiency of the cleavage reaction.

Materials and methods

Purification of CstF

CstF, CFI, CFII, and SF were partially purified from nuclear extracts (Dignam et al. 1983) prepared from ~400 liters of HeLa cells as described previously (Takagaki et al. 1988, 1989). CstF-containing fractions obtained from four heparin-agarose columns (1 × 4 cm) were dialyzed against buffer C [20 mM HEPES-NaOH (pH 7.9), 20% (vol/vol) glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF] containing 10 mM (NH₄)₂SO₄ (6.2 mg of protein, 12 ml) and loaded on a 0.5 × 5-cm FPLC Mono S column equilibrated with the dialysis buffer at a flow rate of 0.25 ml/min. Proteins were eluted by a linear gradient of

$(\text{NH}_4)_2\text{SO}_4$ (from 10 to 100 mM in 25 ml) at a flow rate of 0.5 ml/min, and fractions were dialyzed against buffer C containing 10% (vol/vol) glycerol and 50 mM $(\text{NH}_4)_2\text{SO}_4$. CstF-containing fractions (0.6 mg of protein, 3 ml) were pooled, concentrated to 400 μl using a Centricon 30 (Amicon), and loaded onto a 4.3-ml glycerol gradient [12–30% (vol/vol)] made in buffer C containing 50 mM $(\text{NH}_4)_2\text{SO}_4$. After centrifugation at 47,000 rpm and 4°C for 14 hr in an SW50.1 rotor, fractions (~0.25 ml) were collected from the bottom of the tube. A mixture of the molecular weight markers was centrifuged in a parallel gradient.

Protein analysis

To analyze the protein profile, 50 μl of each glycerol gradient fraction was precipitated with 10% (wt/vol) TCA in the presence of 0.017% (wt/vol) sodium deoxycholate. Proteins were resuspended in 20 μl of loading buffer containing 2% SDS and 0.1 M DTT, fractionated on 10% SDS–polyacrylamide gel (Laemmli 1970), and stained with silver (Merrill et al. 1981).

RNA processing and analysis

CstF activity was assayed using capped SV40 late pre-mRNA transcribed from pG3SVL-A DNA (Takagaki et al. 1988) by SP6 RNA polymerase as a substrate (Takagaki et al. 1989). Reaction mixtures (7.5 μl) contained 1.5 ng of ^{32}P -labeled SV40 late pre-mRNA, 1 μl each of SF- and CF1 + CFII-containing fractions obtained by Mono Q chromatography, 1 μl of CstF-containing fraction, 8 mM HEPES–NaOH (pH 7.9), 6.7–9.3% (vol/vol) glycerol, 20 mM $(\text{NH}_4)_2\text{SO}_4$, 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, and 0.3 μg *E. coli* RNA. After incubation at 30 °C for 1.5 hr, reaction products were isolated and fractionated on a 5% polyacrylamide–8.3 M urea sequencing-type gel.

Preparation of hybridomas

CstF purified through Mono S chromatography (see above) was dialyzed against PBS, concentrated to ~0.5 mg protein/ml using a Centricon 30, and used as an antigen to immunize mice. First, female BALB/c mice were injected intraperitoneally with 100 μg of the antigen plus complete Freund's adjuvant. The mice were then boosted with 50 μg of the antigen plus incomplete Freund's adjuvant three times at intervals of 2 weeks prior to fusion of their spleen cells with SP2/0 myeloma cells to produce hybridomas (Harlow and Lane 1988). First, 650 pools of hybridomas were screened by dot blotting (Hawkes et al. 1982), using Mono S fraction as an antigen. Culture supernatants from positive hybridomas were then tested for reactivity to the three polypeptides (77, 64, and 50 kD) that cofractionated with CstF activity (Fig. 1) by strip Western blotting. 13, 20, and 67 hybridomas were positive for 77-, 64-, and 50-kD polypeptides, respectively. Four 64-kD and two 50-kD hybridomas were successfully cloned to homogeneity. Hybridoma cells were then grown in large scale in RPMI medium supplemented with 10% FBS, and culture supernatants were used for Western blotting (Fig. 2A), immunodepletion (Fig. 2B), immunoprecipitation (Figs. 3 and 4), or indirect immunofluorescence microscopy (Fig. 5).

Western blotting

For Western blotting, 5 μg of CstF-containing fraction purified through the Mono S step was loaded into a large well (0.1 $\times$ 1.5 cm) and fractionated on a 10% SDS–polyacrylamide gel

(Laemmli 1970). Fractionated proteins were then electroblotted (Towbin et al. 1979) onto nitrocellulose membrane BA 85 (Schleicher & Schuell) using a Transphor TE 22 (Hoefer Scientific Instruments) at 200 mA for 2 hr. Nitrocellulose membrane was cut into strips (0.2 $\times$ 7 cm) and stained with India ink (Pelikan) (Hancock and Tsang 1983) or probed with monoclonal antibodies using alkaline phosphatase-conjugated anti-mouse IgG (Sigma) as a secondary antibody.

Immunodepletion experiments

Immunodepletion experiments were carried out as described previously (Takagaki et al. 1989). As both $\alpha 64\text{k}$ and $\alpha 50\text{k}$ mAbs belong to IgG1 subclass, the monoclonal antibodies were conjugated with protein G–Sepharose (PGS, Pharmacia) by gently mixing 1 ml of hybridoma culture supernatant with 100 μl of swollen PGS at 4°C for 2 hr. mAb–PGS conjugates were washed with IPP buffer [10 mM Tris–HCl (pH 7.9), 500 mM NaCl, 0.1% (vol/vol) NP-40], equilibrated with buffer C containing 50 mM $(\text{NH}_4)_2\text{SO}_4$, and packed in 1-ml pipette tips. A 100- μl aliquot of the CstF-containing fraction obtained by Mono Q chromatography (Takagaki et al. 1989) was passed through each mAb–PGS column four times over 30 min, and sample remaining in the columns was completely recovered by centrifugation. Mono Q fractions thus treated were assayed for CstF activity in the reaction system described above, except that the total volume was increased to 8.5 μl .

Immunoprecipitation experiments

The CstF-containing fraction obtained by Mono S chromatography (0.5 mg/ml) was dialyzed against 0.1 M sodium borate (pH 8.5) overnight, and 20 μl of the protein solution was labeled with 250 μCi of ^{125}I -labeled Bolton–Hunter reagent (ICN) by allowing the mixture to stand on ice for 15 min (Bolton and Hunter 1973). Then 200 μl of stop solution [0.1 M sodium borate (pH 8.5), 0.5 M ethanolamine, 10% (vol/vol) glycerol, 0.1% (wt/vol) xylene cyanol] was added and the mixture was incubated at room temperature for 5 min. CstF labeled with ^{125}I was separated from free ^{125}I -labeled Bolton–Hunter reagent by gel filtration through a P-10 column (0.6 $\times$ 25 cm; Bio-Rad) equilibrated with PBS containing 0.25% (wt/vol) gelatin and washed with PBS. For immunoprecipitation, 0.1 μg of ^{125}I -labeled CstF was first mixed with 100 μl of hybridoma culture supernatant and 400 μl of IP-1 buffer [50 mM Tris–HCl (pH 7.9), 150 mM NaCl, 1% NP-40], and the mixture was allowed to stand on ice with occasional shaking. After 30 min, 0.5 μl of rabbit anti-mouse IgG antibody (Sigma) was added and the mixture was incubated on ice for another 30 min. Then 100 μl of 10% (vol/vol) suspension of protein A–Sepharose (PAS, Pharmacia) in IP-1 buffer was added and the mixture was shaken gently at 4°C for 1 hr. Antigen–antibody complexes bound to PAS were collected by brief centrifugation in a microfuge at 4°C and washed three times with 1 ml of ice-cold IP-1 buffer. Immunoprecipitated proteins were eluted from PAS by heating at 85°C for 10 min in 50 μl of loading buffer and fractionated on a 10% SDS–polyacrylamide gel (Laemmli 1970).

UV cross-linking experiments

HeLa cell nuclear extract was incubated with [^{32}P]UTP-labeled SV40 late pre-mRNA under polyadenylation conditions, irradiated with UV light, and digested with RNase A as described previously (Wilusz and Shenk 1988). For immunoprecipitation of UV cross-linked proteins, 12 μl of each RNase A-treated sample was diluted with 200 μl of IP-2 buffer [50 mM Tris–HCl

(pH 7.6), 50 mM NaCl, 0.05% (vol/vol) NP-40], precleared, and mixed with 100 μ l of hybridoma culture supernatant. The mixtures were allowed to stand on ice for 1 hr with occasional shaking. Antigen-antibody complexes were then conjugated to PAS via rabbit anti-mouse IgG antibody. The complexes were collected, washed with IP-2 buffer, and the bound proteins were eluted and fractionated as described above.

Immunofluorescence microscopy

HeLa cells were grown overnight on cover glasses (Fisher Scientific), fixed in 3.7% (wt/vol) formaldehyde-PBS for 20 min at room temperature, and washed with PBS for 10 min. The cells were then permeabilized in 1% (vol/vol) NP-40-PBS for 20 min at room temperature and washed twice with PBS for 5 min each. For indirect immunofluorescence microscopy, the cells were incubated with 10 μ l of hybridoma culture supernatant (α 64k and α Sm mAbs) or 10 μ l of ascites fluid diluted 1:1000 in PBS (α hnRNP A1 protein mAb) at 37°C for 45 min and washed six times in PBS. The mouse monoclonal antibodies were probed with fluorescein-labeled anti-mouse IgG secondary antibody (1:10 dilution in PBS; Cappel) at 37°C for 45 min, washed six times in PBS, and mounted.

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Y Takagaki, J L Manley, C C MacDonald, et al.

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