The upstream sequence element of the C2 complement poly(A) signal activates mRNA 3' end formation by two distinct mechanisms

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The poly(A) signal of the C2 complement gene is unusual in that it possesses an upstream sequence element (USE) required for full activity in vivo. We describe here in vitro experiments demonstrating that this USE enhances both the cleavage and poly(A) addition reactions. We also show that the C2 USE can be cross-linked efficiently to a 55-kD protein that we identify as the polypyrimidine tract-binding protein (PTB), implicated previously in modulation of pre-mRNA splicing. Mutation of the PTB-binding site significantly reduces the efficiency of the C2 poly(A) site both in vivo and in vitro. Furthermore, addition of PTB to reconstituted processing reactions enhances cleavage at the C2 poly(A) site, indicating that PTB has a direct role in recognition of this signal. The C2 USE, however, also increases the affinity of general polyadenylation factors independently for the C2 poly(A) signal as detected by enhanced binding of cleavage-stimulation factor (CstF). Strikingly, this leads to a novel CstF-dependant enhancement of the poly(A) synthesis phase of the reaction. These studies both emphasize the interconnection between splicing and polyadenylation and indicate an unexpected flexibility in the organization of mammalian poly(A) sites.

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Polyadenylation of eukaryotic mRNAs involves the endonucleolytic cleavage of the pre-mRNA followed by the addition of a poly(A) tail to the upstream cleavage product. The signals that dictate the precise site of polyadenylation and determine the efficiency of the process have been studied extensively. These RNA signals are recognized by protein factors that act cooperatively to promote cleavage and poly(A) addition (for recent review, see Colgan and Manley 1997).

The sequence AAUAAA, present 15–30 nucleotides upstream of the poly(A) site of nearly all higher eukaryotic mRNAs (Proudfoot and Brownlee 1976; Wickens 1990), has been shown to have a central role in selection of a poly(A) signal and is recognized by the protein CPSF (cleavage polyadenylation specificity factor). CPSF is essential for both processes and is composed of four polypeptides, one of 160 kD, which interacts directly with the AAUAAA hexamer (Keller et al. 1991; Murthy and Manley 1995), a 100-kD (Jenny et al. 1994), and a 73-kD subunit (Jenny et al. 1996). A 30-kD polypeptide is also part of CPSF (Bienroth et al. 1991; Barabino et al. 1997), although it is not always essential for activity in vitro (Murthy and Manley 1992). A GU- or U-rich sequence is positioned downstream of the cleavage site and has been shown to enhance the efficiency of polyadenylation (Gil and Proudfoot 1984, 1987; McDevitt et al. 1984, 1986; McLauchlan et al. 1985; Chou et al. 1994). This element is known to bind CstF (cleavage stimulation factor; Weiss et al. 1991; MacDonald et al. 1994; Takagaki and Manley 1997), which in turn stabilizes the binding of CPSF to the RNA and greatly increases the efficiency of the 3’ cleavage reaction (Gilmartin and Nevins 1991; Murthy and Manley 1992). CstF has three subunits of 77, 64, and 50 kD (Takagaki et al. 1990). The 64-kD subunit (CstF-64) has a ribonucleoprotein (RNP)-type RNA-binding domain (Takagaki et al. 1992) and is responsible for the binding of CstF to pre-mRNA (Wilusz and Schenk 1988; Takagaki et al. 1990; Gilmartin and Nevins 1991; Takagaki and Manley 1997). The 77-kD...
subunit binds to the 160-kD subunit of CPSF (Murthy and Manley 1995), and also bridges the 64-kD and 50-kD subunits of CstF (Takagaki and Manley 1994). The nuclease responsible for the cleavage of the precursor RNA has not been characterized yet, although it is known that CFIIm and CFIm (cleavage factor I and II, mammalian) promote this reaction (Takagaki et al. 1989; Ruegsegger et al. 1996, 1998). Following cleavage, a tail of 200–300 adenylate residues is added to the 3' end of the pre-mRNA by poly(A) polymerase (PAP; Ryner et al. 1989; Raabe et al. 1991; Wahlle et al. 1991). Furthermore, PAP binds to the 160-kD subunit of CPSF and so further stabilizes the CPSF-RNA complex (Murthy and Manley 1992, 1995). Perhaps reflecting this, PAP is also required for cleavage of all poly(A) signals analyzed to date with the exception of SV40 late poly(A) signal (e.g., Takagaki et al. 1988).

USES (upstream sequence elements) were first identified in viral poly(A) signals where they participate in the selection of poly(A) sites in these complex transcription units—ground squirrel hepatitis virus (GSHV) (Russnak and Ganem 1990; Russnak 1991), adenovirus L1 (DeZazzo and Imperiale 1989), L3 (Prescott and Fack-Pedersen 1992, 1994), L4 (Sittler et al. 1994), SV40 late (Carswell and Alwine 1989), and HIV-1 (Brown et al. 1995; DeZazzo et al. 1991; Valsamakis et al. 1991; Cherryington and Ganem 1992). In the case of HIV-1, the RNA structure defined by transactivation response (TAR) element has been proposed to be necessary to bring the USE closer to the AAUAAA motif and promote cleavage/polyadenylation (Gilmartin et al. 1992). In two different cases, the binding of a protein to a USE results in the activation of polyadenylation. The USE of the HIV-1 poly(A) signal directly contacts CPSF (Gilmartin et al. 1995), whereas the USE of the SV40 late poly(A) signal interacts with the U1 small nuclear RNP (snRNP) protein A (Lutz and Alwine 1994), which stabilizes CPSF binding (Lutz et al. 1996).

Recently, we have identified USEs in two cellular genes, one encoding the complement factor C2 (Moreira et al. 1995) and the other inamin B2 (Brackenridge et al. 1998). We showed that polyadenylation of transcripts from both genes is activated by their respective USEs in vivo. Extensive mutagenesis of the C2 USE demonstrated that a 53-nucleotide sequence immediately upstream of the AAUAAA is required for full activity. As in the case of the viral and lamin B2 USEs, this element is U-rich (42%). Furthermore, the C2 USE is highly conserved between different mammals (Moreira et al. 1995).

Here we present in vitro data showing that the presence of the C2 USE enhances both cleavage and poly(A) addition reactions. Furthermore, a 55-kD protein specifically UV crosslinks to the USE and we identify this protein as the previously characterized polypyrimidine tract-binding protein (PTB). A direct role for PTB in the function of the USE is suggested strongly by experiments demonstrating that mutation of the PTB-binding site significantly reduces the efficiency of the C2 poly(A) signal in vivo and cleavage in vitro. Furthermore, we show that recombinant PTB can directly activate C2 RNA cleavage. PTB was originally isolated as an activity that binds to the polypyrimidine tract present near the 3' splice site of introns (Gil et al. 1991; Patton et al. 1991). It has subsequently been shown to be capable of affecting alternative splice site selection (Lin and Patton 1995; Singh et al. 1995). PTB has also been found associated with an intronic element capable of activating polyadenylation (Lou et al. 1996). Finally, we show that the C2 USE is required for efficient UV crosslinking of CstF-64 to the poly(A) signal, a function attributed previously only to downstream elements. We present additional data that in this context CstF can enhance the second step of the reaction, a previously undocumented activity of this factor. These data therefore indicate that the C2 USE mediates its effect by interaction with both PTB and components of the basal polyadenylation apparatus.

**Results**

The C2 USE enhances both cleavage and poly(A) addition in vitro

Previous data obtained by analysis of RNA isolated from transfected HeLa cells demonstrated that the USE of the C2 poly(A) signal activates 3' end-formation (Moreira et al. 1995). To investigate in vitro how the C2 USE functions, we made several pGEM-7 C2 poly(A) signal constructs to allow production of synthetic RNAs. Figure 1 represents schematically these synthetic RNAs, which are divided into those that contain entire poly(A) sites (Fig. 1A) or that end with a restriction site positioned near the site of polyadenylation (Fig. 1B). The wild-type C2 poly(A) signal (wt) is the 152-nucleotide Styl fragment shown previously to be required to promote efficient polyadenylation in vivo (Moreira et al. 1995). This DNA fragment contains 45 bp downstream of the cleavage site and 84 bp upstream of the AAUAAA. The USE is contained within 53 nucleotides immediately 5' of the AAUAAA. mt has a mutation in the AAUAAA (AAAGAAA). Sp has a spacer fragment of 150 nucleotides (isolated from the Escherichia coli lacZ gene) in place of the USE sequence, whereas Rev has the USE in reverse orientation. Although the rabbit C2 poly(A) signal is closely homologous to the human poly(A) signal, the sequence downstream of the cleavage site diverges, as it possesses a GU-rich element, in contrast to the human sequence (Moreira et al. 1995). To create a GU-rich downstream element (DSE) in the wild-type construct, 13 nucleotides downstream of the cleavage site were replaced by the same region from the rabbit sequence (Ra). RevRa has the upstream element inverted in this background. A construct that only contains the upstream element was also made (USE). The RNAs corresponding to these constructs were synthesised in vitro by T7 RNA polymerase in the presence of a radioactive nucleotide, and incubated with either nuclear extracts or partially purified protein fractions to test for mRNA 3' end formation activity. The sizes of the RNAs and expected cleavage products are shown underneath the wild-type construct.

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**Mechanisms of the C2 complement poly(A) signal USE**
In Figure 2A, cleavage reactions were performed in the presence of a partially purified 3' processing fraction called CSF (containing CPSF, CstF, CFIm, and CFIIm; Takagaki et al. 1988), with or without PAP, using the C2 substrates described above. EDTA was employed to block polyadenylation. As shown in lanes 2–7, SV40 late and adenovirus L3 poly(A) signals were effectively processed producing the expected upstream (open arrow) and downstream (closed arrow) cleavage products. Unlike other poly(A) signals, the SV40 late pre-mRNA does not require PAP to be cleaved (Ryner et al. 1989) (Fig. 2A, lanes 3, 4). Figure 2A, lanes 8–13, shows similar experiments with C2 substrates. As with the L3 poly(A) signal, the wild-type C2 pre-mRNA was only cleaved at detectable levels in the presence of PAP (Fig. 2A, lane 10). Ra, which contains both the USE and a GU-rich sequence downstream of the AAUAAA, is cleaved more efficiently than wild type (Fig. 2A, lane 13). In particular, the downstream cleavage product of $\sim 75$ nucleotides could be detected.

In Figure 2B, cleavage reactions were performed in the presence of a partially purified 3' processing fraction called CSF (containing CPSF, CstF, CFIm, and CFIIm; Takagaki et al. 1988), with or without PAP, using the C2 substrates described above. EDTA was employed to block polyadenylation. As shown in lanes 2–7, SV40 late and adenovirus L3 poly(A) signals were effectively processed producing the expected upstream (open arrow) and downstream (closed arrow) cleavage products. Unlike other poly(A) signals, the SV40 late pre-mRNA does not require PAP to be cleaved (Ryner et al. 1989) (Fig. 2A, lanes 3, 4). Figure 2A, lanes 8–13, shows similar experiments with C2 substrates. As with the L3 poly(A) signal, the wild-type C2 pre-mRNA was only cleaved at detectable levels in the presence of PAP (Fig. 2A, lane 10). Ra, which contains both the USE and a GU-rich sequence downstream of the AAUAAA, is cleaved more efficiently than wild type (Fig. 2A, lane 13). In particular, the downstream cleavage product of $\sim 75$ nucleotides could be detected. Figure 2B shows cleavage reactions carried out with the various mutated C2 substrates using unfractionated nuclear extracts. 3' Deoxy-ATP was employed to inhibit poly(A) addition in these experiments. As above, both wild-type and Ra substrates showed cleavage activity over the 60-min time course (Ra gave fourfold more cleavage product than wildtype). Inversion of the USE (Rev and RevRa), however, or its replacement with spacer sequence (Sp) both reduced C2 cleavage at least fivefold.

Polyadenylation using the pre-cleaved substrates with nuclear extract was also assayed (Fig. 2C). As observed for cleavage, poly(A) synthesis was also greatly enhanced by the presence of the intact USE in the precursor RNA. Therefore, over a 60-min time course, pre-wild type generated significant polyadenylated product, whereas pre-DUSE gave very little polyadenylation, at a similar low level to pre-mt, which has the mutated poly(A) sequence AAGAAA. Taken together, these results suggest that the C2 USE significantly enhances both cleavage and poly(A) addition of C2 pre-mRNA in vitro.

PTB cross-links to the USE

To investigate the mechanism of USE-enhanced polyadenylation, we first set out to determine whether pro-
teins that specifically interact with this element could be identified. Protein–RNA interactions can be detected by UV-cross-linking assays where label transfer from a $^{32}$P–RNA to the protein is measured. RNA precursors double-labeled with C and U were therefore incubated with nuclear extract or protein fractions to allow cleavage/polyadenylation complexes to form. Following irradiation with UV light, the reaction mixtures were treated with RNase A and the labeled proteins analyzed by SDS-gel electrophoresis. Labeling with A and G gave identical results (data not shown). As shown in Figure 3A, using different C2 RNAs incubated with nuclear extract, a protein of ~55-kD cross-linked to USE-containing pre-mRNAs (lanes 1, 3, 5, 6). Furthermore, Figure 3A, lanes 2 and 4, indicates that when the USE is positioned in the reverse orientation, cross-linking to the 55-kD protein is reduced greatly. The different number of labeled residues present in the sequence of Rev in comparison with wild type (29 and 51 nucleotides, respectively), does not account for the difference observed in the intensity of the bands. Ra and RevRa show similar results to wild type and Rev suggesting that the GU-rich DSE present in these RNAs does not affect 55-kD protein binding (Fig. 3A, cf. lanes 1 and 2 with 3 and 4). USE RNA alone (which corresponds to the 84-nucleotide sequence upstream of the AAUAAA) also cross-linked efficiently to the 55-kD protein, indicating that this sequence alone is sufficient for binding (Fig 3A, lane 5). Finally, cross-linking of the 55-kD protein was not affected by a point mutation in the AAUAAA sequence [AAGAAA mutant (mt) lane 6 of Fig. 3A]. This suggests that the interaction of 55-kD protein with the RNA does not depend on the simultaneous binding of CPSF to the AAUAAA, as CPSF binding requires an intact AAUAAA sequence (e.g., Bardwell et al. 1991).

Adenovirus L3 produced only a faint 55-kD protein band on cross-linking (Fig. 3A, lane 7). Although UV-cross-linking assays are nonquantitative, it is interesting to note that a 64-kD protein cross-links efficiently to the L3 RNA and less strongly to the C2 RNAs. It has been shown previously that CstF-64 has a high affinity for the L3 poly(A) signal because of its GU-rich downstream sequence (MacDonald et al. 1994), suggesting that it may be this band. Consistent with this, the 64-kD protein also bound more strongly to the rabbit C2 RNAs, which contain a GU-rich DSE (Fig. 3A, cf. lanes 3 and 4 with 1 and 2). Low levels of cross-linking of a 64-kD protein can be seen in lane 5, where the USE transcript was used. This may suggest a direct and specific interaction of USE and CstF-64, although weak nonspecific binding of CstF

Figure 2. The C2 USE activates both in vitro cleavage and polyadenylation. (A) In vitro cleavage reactions (see Materials and Methods), containing radiolabeled SV40 (lanes 2–4), L3 (lanes 5–7), wild type (lanes 8–10), and Ra (lanes 11–13) RNAs and CSF (Takagaki et al. 1988). Cleavage was assayed in the presence (+) or absence (−) of PAP and the cleavage products were analysed on denaturing polyacrylamide gels. Substrate RNAs are indicated by solid arrows, upstream cleavage products by open arrows and downstream cleavage products by dots. Molecular weight markers are denoted by M (lane 1) and input pre-mRNAs by P. (B) Time course of cleavage reactions using wild type, Rev, Sp, Ra, and RevRa RNAs. As before, substrate RNAs are indicated by solid arrows and upstream cleavage products by open arrows. Time in minutes is indicated above each lane. The dependence of the C2 cleavage reaction on the USE sequence is evident. (C) Time course of polyadenylation reactions using pre-wild type, pre-mt, and pre-ΔUSE RNAs over a 60-min period (see Materials and Methods). The appearance of the poly(A) product is evident in pre-wild type (lanes 2–5) and drastically reduced in pre-mt (lanes 6–9) and pre-ΔUSE (lanes 10–13).
The results indicate that a protein of the nuclear extract used in Figure 3A. Taken together, the concentration of the 55-kD protein in this fraction than in (Fig. 3B, lane 7) is likely to be caused by the higher concentration of the 55-kD protein to L3 in this experiment (Fig. 3B, lane 7) is likely to be caused by the higher concentration of the 55-kD protein in this fraction than in the nuclear extract used in Figure 3A. Taken together, these results indicate that a protein of ∼55 kD can be cross-linked to the C2 USE and this process appears to be independent of the AAUAAA and downstream sequences.

PTB is an ∼57-kD protein capable of binding to the polypyrimidine tract present near the 3' splice site of certain introns (Gil et al. 1991; Patton et al. 1991). Because the C2 USE is pyrimidine-rich and PTB has a similar size to the UV-cross-linking 55-kD protein, we tested whether the 55-kD protein is recognized by PTB-specific antibodies. Immunoprecipitations of the proteins UV-cross-linked to the wild-type C2 RNA were performed, using a polyclonal antibody against PTB. As shown in Figure 3C, the 55-kD protein cross-linked to the wild-type C2 RNA (Fig. 3C, lane 3) was immunoprecipitated with the anti-PTB antibody (lane 4). No band was detected when preimmune serum was used (Fig. 3C, lane 5). Figure 3C, lane 6, corresponds to immunoprecipitation using a monoclonal anti-CstF-64 antibody, and lane 7 to immunoprecipitation with an isotype-matched control antibody, OX1. The absence of any immunoprecipitated proteins in these lanes underlines the specificity of the PTB interaction. The 37-kD protein that also cross-links to the wild-type RNA (Fig. 3C, lane 3) is likely to be heterogeneous nuclear RNP (hnRNP) C, which is known to bind U-rich sequences nonspecifically, as present in this RNA (Wilusz et al. 1990). This has not been tested directly.

To confirm that PTB interacts with the C2 RNA, recombinant PTB was used in the UV-cross-linking assay. As shown in lanes 1 and 2 of Figure 3C, a protein band similar to the one detected with the 55-kD protein fraction is observed when both a smaller or larger amount of recombinant PTB is used. The PTB band detected in lane 4 of Figure 3C is slightly retarded because of excess IgG light chain.

Specific mutation of the PTB-binding site inactivates the C2 poly(A) signal

Two studies have defined consensus RNA sequences that bind PTB based on the selection of random sequence by PTB (Singh et al. 1995; Perez et al. 1997). Although neither sequence precisely matches any part of the C2 USE, a central U-rich region has significant homology to these PTB-binding sequences and has been show to directly bind PTB in vitro (data not shown). A specific mutation was therefore generated in this putative PTB-binding site as shown in Figure 4A. Figure 4B shows UV cross-linking of proteins in nuclear extract to the C2 wild-type and ∆PTB RNAs (lanes 1,2). It should be noted that the extract employed in these experiments was prepared differently to that used in Figure 3, and approximately equal cross-linking of CstF-64 and PTB is now seen with the wild-type RNA. The identity of the two major cross-linking species seen in this extract was confirmed by immunoprecipitation using αCstF (Fig. 4B, lane 4) and αPTB (lane 5) antibodies. Strikingly, the ∆PTB mutation results in greatly reduced cross-linking of PTB, even though the signal for CstF-64 is undiminished. This loss of cross-linking signal for the ∆PTB
Initially we investigated the effect of this \( \Delta PTB \) mutation on the efficiency of the C2 poly(A) signal, using an in vivo poly(A) site competition assay as described previously (Moreira et al. 1995). As shown in Figure 5A, the C2 wild-type and \( \Delta PTB \) poly(A) signals were placed downstream of the \( \alpha 2 \)-globin gene in competition with its own poly(A) signal. Following transfection into HeLa cells, the ratio of \( \alpha 2 \) to C2 poly(A) signal usage was measured by S1 nuclease analysis. As can be seen, the wild-type C2 poly(A) signal is used with approximately the same frequency as the \( \alpha 2 \) globin signal; in marked contrast, the \( \Delta PTB \) mutation reduces use of the C2 poly(A) signal \(~10\) fold. This suggests that PTB binding to the C2 USE has a critical role in the activity of the C2 poly(A) signal.

Similar experiments were carried out in vitro to measure the effect of the \( \Delta PTB \) mutation on either cleavage or poly(A) addition of RNA containing the C2 poly(A) signal. As shown in Figure 5B, the \( \Delta PTB \) mutation had a significant inhibitory effect (threefold) on the efficiency of wild-type RNA cleavage. In contrast, as shown in Figure 5C, \( \Delta PTB \) had no significant effect on pre-wild-type RNA poly(A) addition. We conclude from these in vitro experiments that PTB binding to the USE has an activatory effect on cleavage but not polyadenylation. It is possible that the greater effect (10-fold as compared with 3-fold) of this mutation in vivo may result from the ability of PTB to still bind weakly the C2 USE in vitro. Such binding may be excluded in the more tightly regulated in vivo situation.

Recombinant PTB activates cleavage of the C2 poly(A) site

To obtain direct evidence that PTB activates cleavage of the C2 poly(A) site, we tested the effect of adding recombinant PTB or the 55-kD-enriched fraction to in vitro 3' processing reactions. Because PTB is already present in unfraccionated nuclear extract and to a lesser but significant degree in the CSF fraction used in Figure 2A, we used more highly purified cleavage/polyadenylation fractions. These fractions are less efficient at 3' processing than nuclear extracts, so cleavage reactions were carried out comparing the more efficient C2 Rα RNA substrate with the L3 control. Figure 6 shows the effect of increasing amounts of PTB on cleavage reactions with L3 and Rα substrates, using a mixture of more extensively purified factors (CPSF, CFIm and IIIm, CstF, and PAP). As controls for these experiments, both nuclear extract and CSF were employed and in the case of CSF, the effect of adding the 55-kD fraction was also tested. As shown in Figure 6A, lanes 3–6, it is apparent that PTB has no stimulatory effect on the formation of L3 cleavage products. At the highest concentration of PTB (200 ng, Fig 6A, lane 6), L3 cleavage is inhibited twofold. In contrast, although Rα cleavage is very inefficient with these purified fractions, a low level cleavage product is detectable in Figure 6A, lane 11 which is enhanced twofold with 67 ng (lane 13) and sixfold with 200 ng (lane 14) of added PTB (based on the average of three independent experiments). Increased accumulation of the downstream cleavage product in response to added PTB is also apparent from longer exposures of this gel (data not...
Figure 5. Mutation of PTB-binding site in the C2 USE sequence inhibits in vivo mRNA 3'-end formation and in vitro 3' cleavage. (A) In vivo poly(A) site competition analysis of C2 wild-type and ΔPTB poly(A) signals placed downstream of the human α2-globin gene in competition with its poly(A) signal. The diagram below shows the 3' end of the α2-globin/C2 poly(A) site construct with the positions of the poly(A) sites and S1 probe signals indicated (Moreira et al. 1995). The S1 nuclease mapping experiment is shown above. Bands corresponding to usage of either the C2 or α2 poly(A) sites are indicated alongside DNA markers. The ΔPTB mutation results in ~10-fold inhibition of the C2 poly(A) site (based on PhosphorImager quantitative analysis). (B) Time course of cleavage reactions comparing wild-type and ΔPTB pre-mRNAs. As before, substrate RNAs are indicated by solid arrows, and upstream cleavage products by open arrows. Time in minutes is indicated above each lane. The ΔPTB mutation reduces cleavage activity threefold based on PhosphorImager quantitative analysis. (C) Time course of polyadenylation reactions using pre-wild-type and pre-ΔPTB RNAs over a 60-min period (see Materials and Methods). The appearance of the poly(A) product is evident for both RNAs indicating that the ΔPTB mutation has only a negligible effect on polyadenylation of C2 RNA.

shown). The fact that high amounts of added PTB actually inhibits cleavage of the L3 template, but activates C2, is strong evidence that the binding of PTB to the C2 USE directly activates cleavage of this RNA substrate. Figure 6, A and B (shorter exposure of the indicated lanes in Fig. 6A), also shows the effect of adding 55-kD fraction to the CSF+PAP-mediated cleavage reactions of L3 and Ra. The 55-kD fraction caused a threefold activation of Ra cleavage (Fig 6, lanes 15, 16), but had no significant effect on L3 processing (Fig. 6, lanes 7,8). This argues against the stimulatory effect of the 55-kD fraction being mediated simply by the presence of general cleavage and polyadenylation factors and confirms the importance of PTB for processing at the C2 poly(A) signal. Note that the downstream products of the cleavage reactions (Fig. 6A, •) were correspondingly increased in the Ra cleavage reaction.

We conclude from these experiments and from the analysis of the ΔPTB mutation shown in Figures 4 and 5, that PTB has a direct role in enhancing the cleavage reaction of the C2 pre-mRNA.
to RNAs with the USE reversed (Fig. 7, lanes 3 and 5). No CstF cross-linking was detected in the absence of CPSF, consistent with the known cooperative effects of these two proteins (data not shown). Because wild-type RNA lacks GU-rich downstream sequence (normally required for CstF binding), whereas pre-wild-type has no downstream sequence, it is likely that CstF-64 interacts with the USE sequence. USE alone retains some binding capacity (Fig. 7, lane 2).

The above results raise the possibility that the USE-dependent binding of CstF-64 may be responsible for the PTB-independent effect of the USE on the poly(A) addition step of the reaction. Although CstF has not been observed previously to influence this step, we tested whether increasing concentrations of CstF could enhance poly(A) addition catalyzed by CPSF plus PAP, using as substrates pre-cleaved wild-type C2 RNA (pre-wild-type) and a deletion mutant lacking the USE (pre-ΔUSE). In the absence of CstF, both substrates were polyadenylated very poorly (Fig. 7B, lanes 2,8). Increasing concentrations of CstF stimulated polyadenylation of both the pre-ΔUSE (Fig. 7, lanes 3–6) and pre-wild-type (lanes 9–12) RNAs, most likely by stabilizing the binding of CPSF and PAP to the RNAs. The presence of the USE, however, results in greater stimulation consistent with CstF binding to this sequence. Stable binding of CstF to the pre-wild-type RNA greatly stimulates poly(A) addition, whereas little or no stable interaction of CstF with the pre-ΔUSE RNA diminishes the effect.

We note that poly(A) length shortens at the highest CstF concentration, which also suggests an effect on poly(A) length control. Taken together, the results presented in Figure 7 indicate that CstF is capable of both interacting with upstream sequences and enhancing the second step of the polyadenylation reaction.

**Discussion**

In this study we show that the USE of the C2 poly(A) signal activates both cleavage and poly(A) addition in vitro (Fig. 2), which agrees well with our previous in vivo data (Moreira et al. 1995). Using both crude extracts and a size-selected fraction, we demonstrate that a protein of ∼55 kD cross-links to this element (Figs. 3 and 4) and further show by immunoprecipitation and UV-cross-linking that it is PTB (Fig. 3). Several lines of evidence implicate PTB in activation of the C2 poly(A) site. First, we identify the binding site for PTB on the USE sequence and then show that mutation of this sequence blocks PTB binding (Fig. 4). Second, this same mutation has a dramatic 10-fold inhibitory effect on the in vivo efficiency of the C2 poly(A) signal. We have previously mutated this region of the C2 poly(A) signal (Moreira et al. 1995) but observed only small, two- to threefold inhibitory effects. Significantly, in this earlier study we either mutated only a part of the PTB-binding site or in one case mutated the whole binding site by replacing Us for Cs and vice versa. In each of these mutations, PTB-binding is still not excluded. We also show in these present studies that the ΔPTB mutation inhibits C2 cleavage,
A general theme of these different examples of elements that affect the efficiency of nearby poly(A) signals is that there is an overlap between factors associated with poly(A) site selection and splice site selection. PTB was originally isolated as a factor that interacts with the pyrimidine tract adjacent to the pre-mRNA branch site (Gil et al. 1991; Patton et al. 1991). The essential splicing factor U2AF, not PTB, however, was shown to function in recognition of the branch site by interacting with the pyrimidine tract and recruiting U2 snRNP (Zamore and Green 1991). In some cases of alternative splicing, competition between PTB and U2AF may occur such that PTB binds to the pyrimidine tract of an acceptor site, blocking U2AF binding and subsequent splicing. For example, in the case of α-tropomyosin, exons 2 and 3 behave in a mutually exclusive fashion that in part is associated with the binding of PTB to the polypyrimidine tract of the exon 3 acceptor site, which represses the selection of this 3' splice site (Gooding et al. 1994; Singh et al. 1995). Splice donor sites are also known to regulate the use of poly(A) signals, as it has been demonstrated in the HIV-1 provirus that the major splice donor site inhibits the 5' long-term repeat (LTR) poly(A) site situated 200 nucleotides upstream (Ashe et al. 1995, 1997), whereas in the case of bovine papillomavirus, a donor site upstream of the late poly(A) site represses its activity (Furth et al. 1994; Gunderson et al. 1998). In both of these examples of donor site poly(A) site inhibition, it is likely that U1 snRNA-binding to the donor site targets snRNA-binding proteins close to the poly(A) site and so blocks polyadenylation by direct protein–protein interactions. Further examples of such regulation of poly(A) site selection are reviewed by Proudfoot (1996).

We have shown in these experiments that the C2 USE not only activates 3' end cleavage but also has a significant activating effect on poly(A) addition. Although PTB activates the cleavage reaction, it does not affect this second step of the reaction. We therefore reasoned that the USE may have a secondary role of enhancing the affinity of general polyadenylation factors for the C2 poly(A) signal. Consistent with this notion, we found that the USE enhances CPSF-dependent binding of CstF to the C2 poly(A) site, as judged by UV-cross-linking of CstF-64. To our knowledge, this is the first demonstration that CPSF-CstF cooperative binding can be mediated by sequences in the pre-mRNA upstream of AAUAAA. Although CPSF-CstF interactions have been well documented (Wilusz et al. 1990; Weiss et al. 1991; Murthy and Manley 1992, 1995), they have always been shown or presumed to involve downstream sequences of the poly(A) signals. In our previous experiments, the downstream region could be deleted with only minimal effects (Ashfield et al. 1991), whereas the USE was essential (Moreira et al. 1995). We show here that binding of CstF to the USE is functionally significant, by demonstrating that CstF can enhance poly(A) addition in a USE-dependent manner. This is not only the first demonstration that CstF can function in response to upstream sequences, but also the first indication that CstF can enhance the second step of the reaction. As its name

Figure 7. CstF activates USE-dependent polyadenylation of C2 precleaved substrate. (A) UV cross-linking of purified CPSF and CstF to various [32P]-labeled C2 RNAs. The cross-linked CstF-64 protein is evident, especially in lanes 1 (wt) and 4 (pre-wt). (B) Polyadenylation reactions using pre-USE and pre-wild-type substrates with purified CPSF and PAP together with increasing amounts of purified CstF (0, 5, 15, 50, and 150 ng). Polyadenylated products are indicated. Lanes P correspond to the input RNAs.
Mechanisms of the C2 complement poly(A) signal USE

implies, CstF was initially identified as an activity that enhances the cleavage reaction (Takagaki et al. 1989; Gilmartin and Nevins 1991), and it has never been observed to affect poly(A) addition. It now seems likely that this reflects the nature of the precleaved RNA substrates used that lack the downstream sequences usually required for CstF function. When binding sites are present upstream, CstF will enhance poly(A) addition. Our data indicate that, as with many transcriptional regulators, CstF can function both upstream and downstream of its target (i.e., CPSF). Although we have not investigated the mechanism by which CstF activates poly(A) synthesis, it likely reflects stabilization of CPSF binding, thereby facilitating interaction between CPSF and PAP (Murthy and Manley 1992, 1995; Bienroth et al. 1993).

It is intriguing to compare the structure and function of the C2 poly(A) site with that emerging as a typical site in Saccharomyces cerevisiae. Although it has been difficult to define clear consensus sequence elements, Guo and Sherman (1996) suggested recently the existence of two sequences upstream of the cleavage site, a 3′ “positioning element” and a 5′ “efficiency element”, that appear to be the principal signals for 3′-end formation. Based on location, sequence and function, the positioning element may be analogous to AAUAAA. If so, then the efficiency element may be related to the GU-rich downstream element found in vertebrate genes, despite their different positions (Manley and Takagaki 1996). It is striking that this organization resembles that of the C2 gene, especially in humans where there appears to be no DSE (Moreira et al. 1995). It is not known which of the characterized yeast polyadenylation factors recognizes the positioning or efficiency elements. If the efficiency element is indeed analogous to the mammalian GU-rich sequence, however, then the yeast factor CFIy is a good candidate to bind it because two of its subunits, RNA14 and RNA15, are the apparent homologs of CstF-77 and CstF-64 (M invielle-Sebastia et al. 1994; Takagaki and Manley 1994). Interestingly, CFIy is required for both cleavage and poly(A) addition in yeast (Chen and Moore 1992), which had appeared to distinguish it from CstF. Our data demonstrating that CstF can participate in both steps of 3′-end formation removes this apparent difference between the two factors, and further emphasizes the similarities between the proteins required for polyadenylation in yeast and mammals. Finally, it is noteworthy that the distance between the 3′ end of the C2 gene and the 5′ end of the next gene is only 412 nucleotides (Wu et al. 1987) and a very similar gene arrangement exists for the other USE-containing gene Lamin B2 (Brackenridge et al. 1998). Such gene organization is more typical of yeast than mammals. It is intriguing to speculate that this explains the reliance on USEs both in yeast and in closely spaced mammalian genes.

Materials and methods

Constrasts used in the in vitro and UV cross-linking assays

The wild-type and mutant (AAUAAA → AAGAAA) full-length C2 poly(A) signal DNA fragments were isolated by Styl diges-

In vitro cleavage and poly(A) addition analysis

In vitro transcription All of the pGemC2 plasmids were linearized with BamHI, whereas SV40 was linearized with Dral and L3 with BamHI. One microgram of linearized plasmid was transcribed in the presence of 10µCi [α-32P]UTP (800 Ci/ mmole) and T7 (for pGemC2 plasmids) or T3 RNA polymerase (for SV40 and L3). When RNAs for UV-cross-linking experiments were synthesized, two different radionucleotides were used—12.5 µCi [α-32P]UTP and 12.5 µCi [α-32P]CTP. In the experiment shown in Figure 4B, [α-32P]ATP was used in place of CTP to allow efficient labeling of the ΔPTB A-rich mutant sequence.

In vitro RNA 3′-processing reactions A mix (6.5 µl) containing 40 µg/µl of E. coli tRNA, 1 mM of MgCl2 (for polyadenylation) and either 2 mM EDTA and 1 mM ATP or 1 mM 3′-deoxy-ATP (for cleavage), 20 mM creatine phosphate and 2.5% PVA was incubated with 1 µl of radiolabeled RNA substrate (50 fmole) and 5 µl of nuclear extract or purified protein fractions in buffer D (3 µl of CSF or CPSF, 1 µl of PAP and 1 µl of the 55-kD protein fraction were used). When a time course cleavage or polyade-
nlation reaction was performed the final volume of the reaction was 100 µl and aliquots were taken from the tube incubated at 30°C at the time required. Cleavage or poly(A) addition reactions were otherwise allowed to proceed at 30°C for 1.5 hr. The proteins in the reaction were digested with protease K, by addition of 112.5 µl of the mixture 20 mM Tris-HCl at pH 7.9, 100 mM NaCl, 10 mM EDTA, 1% SDS, and 0.33 mg/ml proteinase K and incubated at 30°C for 15 min. In time course experiments, 12.5 µl of the reaction were taken from 30°C at the time required, added to a tube with the proteinase K mixture and incubated for 15 min. The cleavage/poly(A) addition RNA products were separated by denaturing (8.3 M urea) polyacrylamide gel electrophoresis.

Nuclear extract and protein fractions Nuclear extract was prepared according to the method of Dignam et al. (1983) with minor modifications (Takagaki et al. 1988). Figures 2, B and C, 48, and 5, B and C, all employed nuclear extract made according to Wahle and Kelier (1993). CSF is a Supersose 6 gel filtration column chromatography protein fraction (Takagaki et al. 1988). CSF and PAP are Mono-S ion exchange column chromatography protein fractions (Ryner et al. 1989; Takagaki et al. 1990). CPSF and CFI+II are Mono-Q ion exchange column chromatography protein fractions (Takagaki et al. 1989). Partially purified 55-kD protein fraction was obtained from a Supersose 6 gel filtration column loaded with the 20%-40% ammonium sulfate fraction precipitated from the nuclear extract. Recombinant histidine-tagged PTB was a kind gift of C. Gooding and C.W.J. Smith, Cambridge University.

UV cross-linking of proteins to RNA

The protocol described by Moore et al. (1988) was used with some modifications. Cleavage reaction mixtures containing 20 fmoles of substrate RNA were incubated at 30°C for 10 min to allow the cleavage/polyadenylation complexes to form on the RNA. In the UV cross-linking competition experiments the labeled RNA substrate was incubated together with the competitor DNA oligonucleotide and the protein fraction. E. coli tRNA was added at final concentration of 0.2 mg/ml to dissociate weakly bound proteins. Proteins were cross-linked to the RNA by exposure to UV light at 254 nm for 10 min at 4°C. The UV light source (Mineralight, UVS-54, 220 V, 50 Hz, 0.12 A, Ultra Violet Products Inc., San Gabriel, CA) was supported 3 cm above the samples. The RNA was digested with 15 µg of RNAse A and incubated at 37°C for 30 min. An equal volume of 2× protein gel loading buffer was added to the samples and the proteins were denatured by incubation at 95°C for 5 min. Prenstained Sigma and Rainbow molecular weight markers were used. The proteins were separated on a 5% stacking, 10% resolving gel. The RNA was digested with 15 µg of RNase A, by incubation at 37°C for 30 min. An equal volume of 2× protein gel loading buffer was added to the samples and the proteins were denatured by incubation at 95°C for 5 min. Prenstained Sigma and Rainbow molecular weight markers were used. The proteins were separated on a 5% stacking, 10% resolving gel. After electrophoresis, the gel was incubated in 10% acetic acid, 2% glycerol for 30 min, washed with water twice for 5 min, and incubated in 1× saline-citric acid for 30 min, to intensify the signal. Autoradiography was performed at ~70°C with intensifying screens, after drying the gel.

Immunoprecipitation

Immunoprecipitation of UV cross-linked proteins was performed according to Takagaki et al. (1990), with minor modifications. For immunoprecipitation using polyclonal antibodies, after UV cross-linking, 10 µl of the RNase A-treated samples was added to 100 µl of protein A-Sepharose beads in IP-2 buffer (10% (vol/vol) i antibody (5 µl of anti-PTB serum or preimmune serum). For immunoprecipitations using the monoclonal antibodies anti-64 kD and OX1, rabbit anti-mouse immunoglobulin was mixed on a vertical wheel with protein A-Sepharose beads (40 µg of RAM/100 µl protein A-Sepharose, 1.5 hr, 4°C). The beads were washed three times, resuspended in 200 µl of hybridoma culture supernatants, and added to the UV cross-linked, RNase A-treated samples. The mixtures were rotated at 4°C for 16 hr. Antibody-antigen complexes formed and bound to protein A-Sepharose beads were resuspended in 20 µl of 1× protein loading buffer and separated on an SDS-polyacrylamide gel as described above.

Antibodies The rabbit polyclonal anti-PTB antibody was raised against an alglutathione-S-transferase (GST)-PTB fusion protein (Kaminski et al. 1995), and was a generous gift from R.J. Jackson (Cambridge University). Rabbit pre-immune serum was also obtained from R.J. Jackson. The anti-64 kD mouse mAb was prepared according to Takagaki et al. (1990). The mouse anti-rat CD45 mAb OX1 (Sunderland et al. 1979), used as a negative control for the immunoprecipitation with the anti-64 kD antibody, was a kind gift from Alexandre Carmo (University of Oxford).

In vivo analysis of wild-type vs. ΔPTB C2 poly(A) signals

The C2 Sty1 fragment was isolated from ΔPTB or wild-type pGEM plasmids and inserted into the 3′-flanking region PvuI site of α23 PspSved (see Moreira et al. 1995). These two plasmids (wild type and ΔPTB) were transfected transiently into HeLa cells and the cytoplasmic RNA isolated from these two transfections was subjected to S1 mapping, all described by Moreira et al. (1995).

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