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Poly(A) Tail Length Is Controlled by the Nuclear Poly(A)-binding Protein Regulating the Interaction between Poly(A) Polymerase and the Cleavage and Polyadenylation Specificity Factor*[S]

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Poly(A) tails of mRNAs are synthesized in the cell nucleus with a defined length, ~250 nucleotides in mammalian cells. The same type of length control is seen in an *in vitro* polyadenylation system reconstituted from three proteins: poly(A) polymerase, cleavage and polyadenylation specificity factor (CPSF), and the nuclear poly(A)-binding protein (PABPN1). CPSF, binding the polyadenylation signal AAUAAA, and PABPN1, binding the growing poly(A) tail, cooperatively stimulate poly(A) polymerase such that a complete poly(A) tail is synthesized in one processive event, which terminates at a length of ~250 nucleotides. We report that PABPN1 is required to restrict CPSF binding to the AAUAAA sequence and to permit the stimulation of poly(A) polymerase by AAUAAA-bound CPSF to be maintained throughout the elongation reaction. The stimulation by CPSF is disrupted when the poly(A) tail has reached a length of ~250 nucleotides, and this terminates processive elongation. PABPN1 measures the length of the tail and is responsible for disrupting the CPSF-poly(A) polymerase interaction.

The poly(A) tails present at the 3′ end of almost all eukaryotic mRNAs have two major functions. The first function is in the control of mRNA decay; degradation of the poly(A) tail by a 3′ exonuclease (deadenylation) is the first step in both of the two main pathways of mRNA decay, and the completion of deadenylation triggers the second step, either cap hydrolysis or further 3′–5′ degradation. Because the rate of deadenylation is governed by sequence elements in the mRNA, it is specific for each mRNA species and serves as a major determinant of mRNA half-life (1–3). Obviously, a control of mRNA stability by the rate of deadenylation requires a defined poly(A) length as a starting point. The second function of the poly(A) tail is in the initiation of translation; the cytoplasmic poly(A)-binding protein associated with the poly(A) tail promotes the initiation of translation by an interaction with the initiation factor eIF4G and probably through additional mechanisms (4–7). In this process, poly(A) tail length can also be important. For example, gene regulation during oocyte maturation and early embryonic development of animals depends on translational regulation of maternal mRNAs, and changes in poly(A) tail lengths of specific mRNAs, determined both by deadenylation and by regulated cytoplasmic poly(A) extension, play a major role in this translational regulation. Long poly(A) tails favor translation, whereas a shortening of the tail promotes translational inactivation of the message (8, 9). Similar mechanisms seem to operate in neurons (10, 11) and possibly in other somatic cells (12).

Because the length of the poly(A) tail is important for its function, it is not surprising that poly(A) tails are generally synthesized with a defined length, which is species-specific, ~70–90 nucleotides in *Saccharomyces cerevisiae* (13, 14) and ~250 nucleotides in mammalian cells (15). Subtle differences between newly made poly(A) tails of different mRNAs have been described (13), and there is even a class of mRNAs that never receives more than an oligo(A) tail (16, 17). However, the heterogeneous length distribution seen in the steady-state mRNA population is the result of cytoplasmic shortening starting from a relatively well defined initial tail length; heterogeneity of tail length reflects age differences of the mRNA molecules. The oligo(A) tails present on inactive mRNAs in oocytes or embryos are also generated by shortening of full-length tails made in the cell nucleus (18).

The poly(A) tail is added during 3′ end processing of mRNA precursors in the cell nucleus (19–21). This reaction consists of two steps: an endonucleolytic cleavage followed by the addition of the poly(A) tail to the upstream cleavage product. Whereas a large protein machinery of some 20 or more polypeptides (22) is required for the cleavage reaction, subsequent polyadenylation has much simpler protein requirements. In the mammalian system, it can be reconstituted from three proteins: poly(A) polymerase, the enzyme catalyzing primer-dependent polymerization of AMP using ATP as a precursor (23–25); the cleavage and

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polyadenylation specificity factor (CPSF),[6] which binds the cleavage and polyadenylation signal AAUAAA (26, 27); and the nuclear poly(A)-binding protein (PABPN1), which binds the growing poly(A) tail (28, 29). Note that PABPN1 is distinct from the family of cytoplasmic poly(A)-binding proteins (30). Roles of poly(A) polymerase and CPSF in polyadenylation *in vivo* have been most clearly demonstrated by genetic analysis of the orthologues in *S. cerevisiae* (21, 31). PABPN1 has no functional orthologue in budding yeast (32); its function in polyadenylation has been confirmed in mammalian cells (33) and in *Drosophila* (34).

Whereas PABPN1 and poly(A) polymerase are monomeric proteins, CPSF is a hetero-oligomer, which has not yet been reconstituted from recombinant proteins (22, 26, 35–40). Poly(A) polymerase on its own is barely active because of a low affinity for its RNA substrate and thus acts distributively, *i.e.* it dissociates from the RNA after each polymerization step, and presumably often before it has incorporated any nucleotide; the enzyme also has no significant sequence specificity and will elongate any RNA with a free 3′ OH (24). Both CPSF and PABPN1 enhance the activity of the polymerase by recruiting the enzyme to its substrate through direct interactions (38, 41). Sequence specificity of poly(A) addition reflects the RNA binding specificities of the two stimulatory factors: CPSF recruits the polymerase to RNAs containing the AAUAAA sequence in the vicinity of their 3′ ends (24, 42, 43), and PABPN1 recruits the enzyme to substrate RNAs carrying a terminal oligo(A) tract (29). Each factor alone endows the polymerase with modest processivity, such that it can incorporate maybe two to five nucleotides before dissociating (44). RNAs containing both the AAUAAA sequence and an oligo(A) tail and thus resembling intermediates of the polyadenylation reaction support a cooperative or synergistic stimulation of poly(A) polymerase by both CPSF and PABPN1. Under these conditions, addition of the poly(A) tail occurs in a processive manner, *i.e.* without intermittent dissociation of the protein complex from its substrate RNA (29, 44).

Interestingly, the reconstituted polyadenylation reaction also shows proper length control, generating poly(A) tails of the same length as seen *in vivo*; tails grow to a relatively well defined length of 250–300 nucleotides in a rapid, processive reaction (29, 44). Length control is due to termination of this processive elongation; extension beyond 250 A residues is largely distributive and therefore slow (45). These kinetics of *in vitro* poly(A) tail synthesis are fully consistent with the *in vivo* kinetics derived from pulse-labeling studies (46). *In vitro*, poly(A) tail elongation rates beyond 250 A residues are similar when either CPSF or PABPN1 or both are present. In other words, substrates with long poly(A) tails no longer support the cooperative stimulation of poly(A) polymerase by both CPSF and PABPN1 that is the basis of processive elongation (45). The termination of processive elongation must be mediated by a change in the RNA-protein complex that remains to be defined. When RNAs carrying poly(A) tails of different lengths are used as substrates for polyadenylation, the tails are always elongated processively to 250 nucleotides, independently of the initial length, whereas extension of a tail of 250 or more nucleotides in length is slow and distributive from the start of the reaction. Thus, poly(A) tail length control is based on some kind of AMP residue counting or length measurement, not on a kinetic mechanism (45).

In this paper, we address the two problems outlined above: first, how does the polyadenylation complex change to terminate processive poly(A) tail elongation, and second, how is the length of the tail measured? We provide evidence that PABPN1 is the active component in the mechanism of length control. The protein promotes the interaction between CPSF and poly(A) polymerase when bound to a short poly(A) tail. PABPN1 no longer promotes or even actively disrupts this interaction when bound to a poly(A) tail of 250 nucleotides or longer and thereby terminates the cooperative, processive elongation reaction in a poly(A) tail length-dependent manner. Only poly(A) sequences are counted as part of the tail. Because this reflects the binding specificity of PABPN1 and because disruption of the CPSF-poly(A) polymerase interaction requires complete coverage of the poly(A) tail by this protein, PABPN1 is also the protein that measures the length of the tail.

EXPERIMENTAL PROCEDURES

Plasmids—The expression plasmid containing a partially synthetic PABPN1 gene has been described (47). The LALA double mutant was generated by introduction of the L119A mutation into the plasmid pGM-synPABPN1 L136A (41). The PABPN1 open reading frame was then subcloned with NdeI and BamHI into the expression vector pUK (47). For expression of poly(A) polymerase, the PAP open reading frame from pGM10-PAP82 (48) was subcloned into the pUK plasmid with NdeI and BamHI.

Plasmids encoding various polyadenylated derivatives of the L3pre RNA have been described (41). In addition, pSP64-L3preA$_{75}$ was generated by the same procedure. The mutagenesis of the polyadenylation signal (Δ variant: AA**G**AAA) of pSP64-L3preA$_{15}$ and derivatives (41) was done by PCR using Δ forward and reverse primers (supplemental Table S1) and Pwo DNA polymerase (Peqlab). The PCR fragment was digested with PstI and PvuII and subcloned into the PstI/PvuII opened plasmids.

RNA—A_{280} was obtained by size fractionation of commercial poly(A) and labeled as described (49). Poly(A) concentrations were determined by UV spectroscopy with ϵ (AMP) = 9800 $cm^{-1}\ M^{-1}$.

Other RNAs were synthesized by run-off transcription with SP6 RNA polymerase (Roche Applied Science) and [α-^{32}P]UTP (GE Healthcare or Hartmann Analytics). No 5′ cap was added. RNAs were gel-purified and quantified as described (45). Templates for run-off transcription were either plasmid DNA-digested with appropriate restriction enzymes or PCR fragments that were amplified with an SP6 promotor forward primer (supplemental Table S1) and a reverse primer that hybridized downstream to the plasmid. DNA templates were purified by phenol/chloroform extraction followed by ethanol precipitation.

[6] The abbreviations used are: CPSF, cleavage and polyadenylation specificity factor; PABPN1, poly(A)-binding protein nuclear 1; nt, nucleotide(s); PAP, poly(A) polymerase.

Polyadenylated RNAs L3preA$_{15}$, L3preA$_{45}$, L3preA$_{75}$, and L3preA$_{105}$ and the corresponding versions with a point mutation in AAUAAA were generated by transcription of suitable plasmids templates (41) as above. RNAs with poly(A) tails longer than 105 nucleotides were obtained by further elongation of L3preA$_{15}$ or similar RNAs as described (45). The RNA substrates L3pre-N$_{49}$A$_{15}$ and L3preA$_{15}$N$_{63}$A$_{15}$ were synthesized from PCR fragments generated as described above using the plasmids pSP64-L3pre and pSP64-L3preA$_{15}$ as templates and the SP6 promotor primer and the corresponding reverse primers (supplemental Table S1).

A first set of RNA processing substrates with no poly(A) tail and with increasing distance between the AAUAAA sequence and the 3′ end were generated by transcription from the plasmids pSP64-L3 or pSP64-L3preΔ linearized with RsaI close to the 3′ processing cleavage site (1 nt upstream of the cleavage site, 19 nt downstream of AAUAAA) or further downstream with DraI (48 nt downstream of the cleavage site), AflIII (101 nt), EcoRI (162 nt), and MbiI (205 nt) and from a PCR fragment (299 nt distance) generated by use of the SP6 promotor primer and a second oligonucleotide priming about 300 nt downstream of the RsaI site (L299 rev; supplemental Table S1). A second set of processing substrates with increasing distance between the AAUAAA sequence and the 3′ end was synthesized from PCR fragments generated from the templates pSP64-L3 and pSP64-L3preΔ, respectively, with the help of the SP6 promotor primer and different downstream primers (supplemental Table S1). The downstream primers were designed such that all RNAs carried the same additional sequence (-UGUA) at the 3′ end. When RNAdraw (54) predicted that the 3′ end of a particular RNA was included in a double-stranded region, the additional 3′ terminal sequence was extended to UGUUGUA to disrupt the double-stranded structure.

Proteins—Expression of His-tagged PABPN1 LALA was done as described (47) with the following modifications: M9 minimal medium was used, and induction was started at A_{600} = 1 by addition of 0.4 mM isopropyl β-D-thiogalactopyranoside at 25–30 °C for 3 h. The cells were harvested by centrifugation, resuspended in 50 mM sodium phosphate (pH 8.0), 300 mM KCl, 10% glycerol, and lysed with a Basic Z cell disruptor (Constant Systems Ltd.) at 2200 bar. Nickel-nitrilotriacetic acid chromatography was performed as described (47). Eluted proteins were dialyzed first against RS buffer (50 mM sodium phosphate, pH 6.5, 10% glycerol, 1 mM dithiothreitol, 1 mM EDTA) containing 1 M NaCl and then against RS buffer containing 300 mM salt. After dilution to a salt concentration below 150 mM, nucleic acids and contaminating proteins were removed by chromatography on a 1-ml Resource S column (GE Healthcare). Wild-type PABPN1 was expressed in a fermentor-grown culture and purified by a similar procedure. Because recombinant His-tagged PABPN1 is not always fully active, the amount required to saturate the poly(A) tails of substrate RNAs was determined by titrations in gel mobility shift assays. For some experiments, calf thymus PABPN1 was used (50).

CPSF was purified essentially as described (45). Full-length bovine poly(A) polymerase was also expressed in *Escherichia coli* Bl21 pUBS. Bacteria were grown in SOB medium to A_{600} = 1 and induced by 1 mM isopropyl β-D-thiogalactopyranoside. The cells were harvested after 16 h at 25–30 °C and disrupted as above. The His-tagged PAP was purified by nickel-nitrilotriacetic acid chromatography (Qiagen) followed by a Hitrap Heparin-Sepharose and a MonoQ column (GE Healthcare).

Concentrations of PABPN1 and poly(A) polymerase were determined from the absorption at 280 nm, and extinction coefficients were calculated by the Lasergene software package (DNAStar Inc.). The concentration of CPSF was estimated by titrations in gel mobility shift and polyadenylation assays, assuming that a 1:1 ratio to the substrate RNA was required to saturate the reaction.

Polyadenylation Assays—Polyadenylation assays were performed at 37 °C as described (45) except that 2% (w/v) polyethylene glycol 6000 was substituted for polyvinyl alcohol in the reaction buffer. The reaction volume was 20 or 25 μl or multiples thereof when several time points were taken from one reaction. RNA substrates, proteins, and incubation times are indicated in the figure legends. Amounts of RNA and protein always refer to the unit reaction volume of 20 or 25 μl. Molar quantities of RNA refer to polymers, not mononucleotides. The reaction products were resolved on 5 or 6% polyacrylamide gels containing 8.3 M urea, and radioactivity was detected by phosphorimaging. Polyadenylation assays often included a reaction containing a 10-fold amount of poly(A) polymerase to control for the ability of the RNA to be elongated and to gauge the stimulatory effects of the RNA binding proteins.

Quantitation of PAP Stimulation by CPSF—Polyadenylation reactions were performed with 80 fmol of radioactively labeled RNA, 80 fmol of PAP, and an appropriate amount of CPSF (approximately equimolar to the RNA) in the presence of 1.25 μg of competitor tRNA for 0, 2, and 10 min at 37 °C. To measure the activity of unstimulated PAP, control reactions with 800 fmol of PAP were performed in the absence of CPSF for 10 min at 37 °C. Polyadenylation products were analyzed as above and quantified with ImageQuant 5.0 (GE Healthcare). Efficiency of polyadenylation was determined as follows: for each lane, a line graph was integrated in Excel (Microsoft Corp.) to determine the total amount of radioactivity. Then the position in the gel was determined that divided the lane into two portions each containing the same amount of radioactivity. DNA size standards in neighboring lanes were used to determine the RNA length corresponding to this position. The elongation of an RNA is given by the difference between this position and that of the same RNA without incubation (t = 0 min). PAP stimulation was calculated as the ratio of stimulated elongation (in the presence of CPSF) over unstimulated elongation (in the absence of CPSF). Because extension by 1× poly(A) polymerase (80 fmol) was barely detectable, stimulation was normalized to 1/10 of the extension seen in the 10× poly(A) polymerase lane. Because the RNA intensities are not weighted by the length, this type of quantitation measures mostly the quantity of RNA that is elongated and puts less emphasis on the length of poly(A) that is added. This is desired because the former parameter more accurately reflects the AAUAAA-dependent stimulation by CPSF.

Electrophoretic Mobility Shift Assays— 80 fmol of RNA was incubated under reaction conditions in the absence of ATP. The reaction volume was 20 μl, and proteins were used as indi-

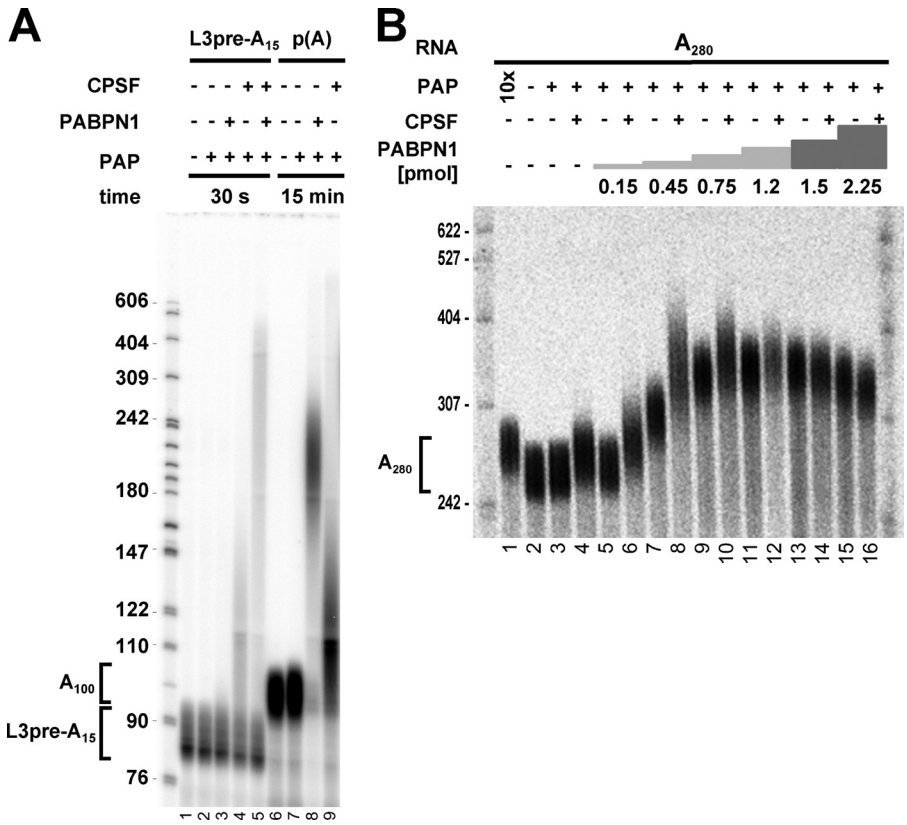

FIGURE 1. **Stimulation of poly(A) polymerase by CPSF bound to straight poly(A) is sensitive to PABPN1.** *A*, CPSF can stimulate poly(A) polymerase when bound to straight poly(A). 80 fmol of $L3preA_{15}$ or A_{100} was incubated in a 25-μl reaction with an approximately equimolar amount of CPSF, 20 fmol of PAP and 1.6 pmol of His-PABPN1 as indicated. After 2 min of preincubation at 37 °C, the reactions were started by the addition of ATP. The reactions were stopped after 30 s or 15 min as indicated, and RNAs were analyzed by gel electrophoresis. The size of DNA markers in nucleotides is indicated on the *left*. *B*, PABPN1 inhibits the stimulatory activity of CPSF on straight poly(A). 80 fmol of A_{280} was incubated in 20-μl reactions with an approximately equimolar amount of CPSF, 32 fmol of PAP, and increasing amounts of PABPN1 as indicated. 1.5 pmol of PABPN1 was sufficient for A_{280} coverage as judged by an independent gel mobility shift assay. The reactions were preincubated for 10 or 15 min at 37 °C and started by addition of ATP. They were stopped after 15 min and analyzed by gel electrophoresis. A control reaction containing a 10-fold amount of poly(A) polymerase was included as explained under "Experimental Procedures."

cated in the figure legend. After 30 min of incubation at room temperature, protein-RNA complexes were resolved by native gel electrophoresis as described (49).

RESULTS

PABPN1 Maintains the AAUAAA Dependence of CPSF Binding—With RNA substrates carrying no poly(A) tails, the stimulation of polyadenylation by CPSF is strongly dependent on the presence of the CPSF-binding site, AAUAAA. Because of the similarity of this sequence with poly(A) and because CPSF binds tightly to a poly(A) column (26), the possibility was tested that CPSF could stimulate polyadenylation also by binding to the growing poly(A) tail. Indeed, CPSF stimulated extension of size-fractionated poly(A) by poly(A) polymerase quite efficiently, although less so than PABPN1 (Fig. 1*A*; activity of the same proteins on the regular polyadenylation substrate is shown as a control in the *first five lanes*). When PABPN1 was titrated into these poly(A) elongation reactions, the rate of elongation increased, but the CPSF-dependent stimulation was reduced to zero at a concentration of PABPN1 sufficient to cover the poly(A) substrate completely (Fig. 1*B*, compare *lanes plus* and *minus CPSF*). Thus, CPSF can stimulate poly(A) polymerase when bound to naked poly(A), but it cannot bind the poly(A)-PABPN1 complex. The effect of CPSF was then examined with a "regular" polyadenylation substrate carrying an AAUAAA sequence; the L3pre RNA corresponds to the cleaved intermediate of a 3′ processing reaction at the L3 site of the adenovirus major late transcript. In L3preΔ, the polyadenylation signal is mutated to AAGAAA. Variants of these RNAs were used that carried different lengths of 3′ terminal poly(A) already at the start of the elongation reaction, thus representing intermediates in the elongation reaction. In the absence of PABPN1, CPSF stimulation of the extension of such substrates became increasingly insensitive to a point mutation in the AAUAAA sequence (L3preΔ substrate) with increasing poly(A) tail length; AAUAAA dependence was pronounced with an A_{15} tail (Fig. 2, compare *lanes 7–9* with *lanes 10–12*), weak with an A_{105} tail (*lanes 19–21 versus lanes 22–24*), and absent with an A_{190} tail (*lanes 31–33 versus lanes 34–36*). This confirms stimulation of poly(A) polymerase by CPSF bound to poly(A). The addition of PABPN1 restored AAUAAA dependence (Fig. 2; compare, for example, *lanes 25–30* with *lanes 31–36*). Therefore, in all subsequent experiments, a complete coverage of the poly(A) tail with PABPN1 was ascertained by titration experiments analyzed by gel mobility shifts. These controls guaranteed that any stimulation by CPSF was due to its occupancy of the AAUAAA site.

PABPN1 Facilitates the Stimulation of Poly(A) Polymerase by CPSF—We have previously reported that CPSF can stimulate poly(A) polymerase efficiently even on RNAs carrying long poly(A) tails (45). The results described in the preceding section now suggest that this may have been due to CPSF binding to the poly(A) tail. Therefore, RNAs without a poly(A) tail and with an increasing distance between the AAUAAA sequence and the 3′ end were tested for their ability to support the stimulation of poly(A) polymerase by CPSF. Two such series of RNA substrates were used. Because poly(A) polymerase is sensitive to the type of nucleotides at the 3′ end, the second series of RNAs was designed to have a uniform 3′ end sequence (-UGUA). Two corresponding series of RNAs carrying a point mutation in the AAUAAA sequence served as controls. With the two mutant series of substrates, CPSF stimulation was weak and essentially independent of the length of the RNA (Fig. 3; an example of raw

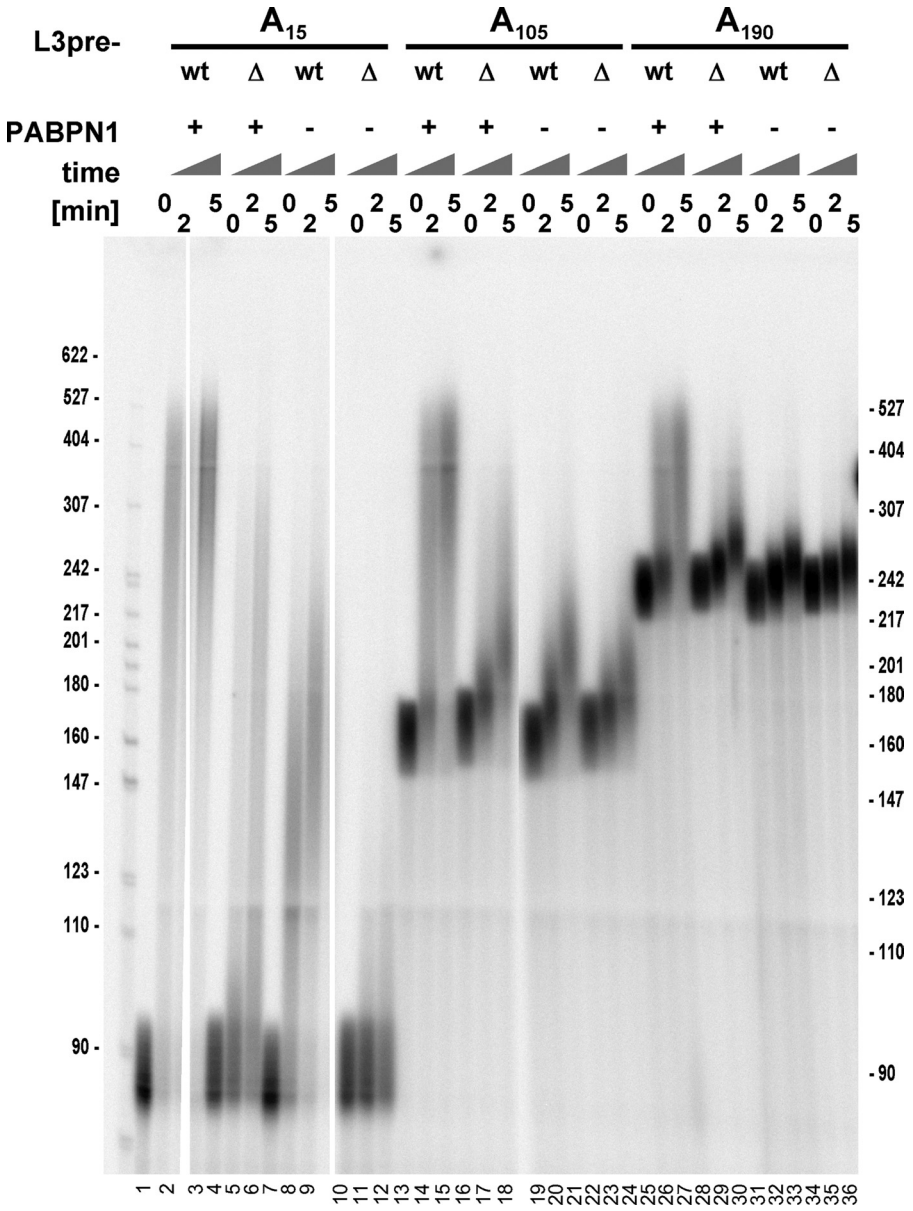

FIGURE 2. **PABPN1 maintains the AAUAA dependence of CPSF stimulation.** The reactions were set up containing 80 fmol of RNA/25 μl of unit volume, either wild type (*wt*) or with a point mutation in the polyadenylation signal (Δ) and with different poly(A) tail lengths, as indicated. The amounts of CPSF were approximately equimolar to the RNA, 20 fmol of poly(A) polymerase, and 1.6 pmol PABPN1 was used as indicated. The reaction mixtures were prewarmed for 3 min at 37 °C, the samples were withdrawn for the 0 min time points, and the reactions were started by the addition of ATP. Aliquots were taken at the time points indicated, and RNA was analyzed by gel electrophoresis. The sizes of DNA markers are indicated on both sides.

data is shown in supplemental Fig. S1). With the wild-type series of substrates, the stimulatory effect of CPSF was strong when the RNA ended at the normal cleavage site and got weaker with an increasing distance. The decrease in polyadenylation efficiency was more gradual in one series of RNAs and more precipitous in the other (Fig. 3 and supplemental Fig. S1). In contrast to this pronounced distance sensitivity of the CPSF effect, the rate of the processive elongation reaction in the presence of both CPSF and PABPN1 remains constant at ~25 nt/s all the way up to a tail length of 250 nucleotides (Fig. 3 in Ref. 45). From the efficiency of elongation we infer that, in the reaction lacking PABPN1, the interaction between CPSF and poly(A) polymerase is progressively weakened with an increasing distance of the two proteins on the RNA, whereas in the presence of PABPN1, the interaction is maintained over a distance of up to 250 nucleotides. In other words, PABPN1 does not merely passively permit CPSF to interact with poly(A) polymerase; rather, PABPN1 actively promotes this interaction, presumably by facilitating a folding back of the RNA. With increasing distance between the 3′ end undergoing elongation and the CPSF-binding site, the CPSF-poly(A) polymerase interaction becomes increasingly dependent on PABPN1.

Both CPSF and PABPN1 Remain Associated with the RNA Substrate after Termination of Processive Polyadenylation—How does the polyadenylation complex change to terminate processive elongation? Cooperative stimulation of poly(A) polymerase by CPSF and PABPN1 is essential for processive elongation, and the elongation beyond ~250 nucleotides proceeds at a rate characteristic for the stimulation by either factor alone (45). Thus, termination of the processive reaction might be caused by the dissociation or displacement of one of the two stimulatory proteins. For example, PABPN1 might displace CPSF from the AAUAAA sequence because of the similarity of the binding site. This hypothesis was tested by gel shift experiments. PABPN1 was used at a concentration sufficient to saturate the L3preA_{300} substrate (Fig. 4, *lane 3*). In the absence of PABPN1, 80% of the RNA were bound by CPSF (Fig. 4, *lanes 4* and *5*). When PABPN1 was added in addition, ~50% of the PABPN1-associated RNA bound CPSF simultaneously, as shown by the supershift (Fig. 4, *lanes 7* and *8*). Controls with the L3preΔA_{300} substrate showed that binding of CPSF was AAUAAA-independent (Fig. 4, compare *lanes 12* and *13* with *lanes 4* and *5*), confirming binding to the poly(A) tail as seen in the functional assays reported above (Figs. 1 and 2). Binding of CPSF to the poly(A) tail was prevented by its saturation with PABPN1 (Fig. 4, compare *lanes 9* and *10* with *lanes 7* and *8*). The experiment shows that RNAs with a long poly(A) tail that can no longer undergo processive elongation can nevertheless bind CPSF and PABPN1 simultaneously.

The binding of CPSF to RNAs carrying different poly(A) tail lengths was also examined by UV cross-linking experiments. Although cross-linking of the 30-kDa subunit of CPSF was very

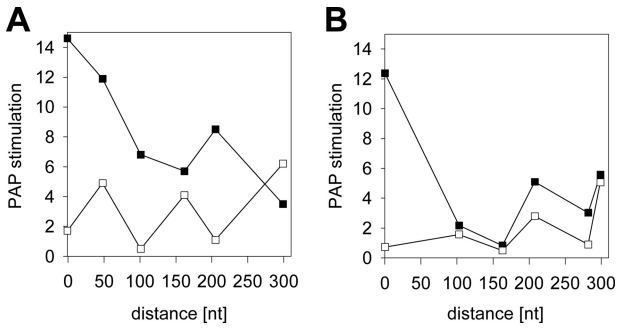

FIGURE 3. **CPSF stimulation is sensitive to the distance between AAUAAA and the 3′ end.** The stimulation of poly(A) polymerase by CPSF was measured with two sets of RNA substrates with an increasing distance between the CPSF-binding site AAUAAA and the 3′ end. In each case, an identical set of RNAs with a point mutation in AAUAAA was used as a control. Polyadenylation reactions contained, per unit volume of 25 μl, 80 fmol of RNA, and 80 fmol of PAP in the presence or absence of CPSF (amount approximately equimolar with RNA). After warming to 37 °C, aliquots for the 0-min time point were withdrawn, the reactions were started by the addition of ATP, and additional aliquots were withdrawn after 2 and 10 min. A control reaction containing a 10-fold higher amount of poly(A) polymerase (800 fmol) was incubated for 10 min in the absence of CPSF. PAP stimulation was analyzed as described under "Experimental Procedures" based on the 10-min time points. The plotted data were averaged from at least two experiments for each set of RNAs. Raw data for the first series of RNAs and the mutant controls are shown in supplemental Fig. S1. *A*, distance-dependent stimulation with the first set of RNA substrates having different 3′ end sequences. The *black squares* represent the data for RNAs with a wild-type AAUAAA sequence and show a decreasing elongation efficiency in proportion to an increasing distance between AAUAAA sequence and 3′ end. *Open squares* represent the control data obtained with the corresponding RNAs having a point mutation in the AAUAAA sequence. Elongation is less efficient than with the wild-type sequence and essentially independent of the length of the RNA. *B*, distance-dependent stimulation with the second set of RNA substrates having uniform 3′ end sequences (-UGUA). The data for the wild-type and mutant RNAs are shown as in *A*.

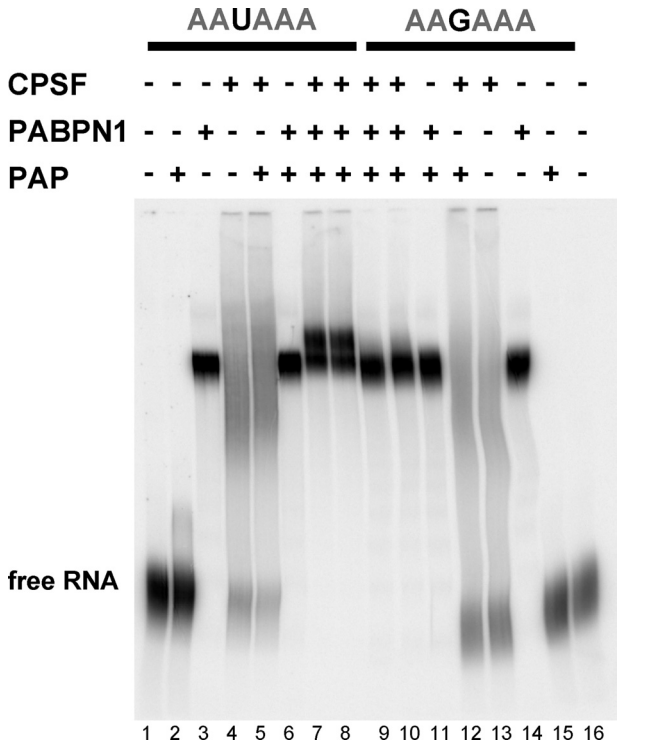

FIGURE 4. **CPSF and PABPN1 can bind simultaneously to an RNA with a long poly(A) tail.** 80 fmol of L3pre-A_{300} orL3preΔ-A_{300} was incubated in a 20-μl reaction volume in the absence of ATP with 100 fmol of PAP, 2400 fmol of calf thymus PABPN1 (saturating as judged by titration in an independent gel mobility shift assay) and CPSF (approximately equimolar with RNA) in the presence of 1.5 μg of competitor tRNA. After 30 min of incubation at room temperature, complexes were resolved on a native polyacrylamide/agarose composite gel. The reactions containing all three proteins were carried out twice (*lanes 7* and *8* and *lanes 12* and *13*, respectively).

inefficient under all conditions tested, there was only a slight decrease when RNAs with long poly(A) tails and saturating concentrations of PABPN1 were used (supplemental Fig. S2). From both the gel shift and the cross-linking experiments, we conclude that a dissociation or displacement of CPSF does not play a role in the termination of processive polyadenylation.

PABPN1 Disrupts the Stimulation of Poly(A) Polymerase by CPSF to Terminate Processive Polyadenylation—An alternative mechanism to terminate processive elongation might involve a disruption of the interaction between poly(A) polymerase and one of the two stimulatory factors without a displacement of either protein. It seems plausible that it is the interaction with CPSF that is lost; the CPSF stimulation gets progressively weaker with increasing distance between the CPSF-binding site and the RNA 3′ end (see above), presumably because the interaction between CPSF and the enzyme can be maintained only if the RNA folds back on itself. In contrast, the stimulation by PABPN1 is not affected by increasing poly(A) tail length (45); the activity of poly(A) polymerase continuously creates new binding sites for PABPN1, and PABPN1 must remain close to the polymerase (41). Thus, an attractive model for length control would be that there is a length-dependent conformational transition in the poly(A)-PABPN1 complex such that PABPN1 no longer facilitates or even actively disrupts the contact between CPSF and the polymerase. According to this model, the relatively slow elongation taking place after termination of processive elongation should then be due to the remaining stimulation of poly(A) polymerase by just PABPN1. That the rate of extension of long poly(A) tails in the presence of PABPN1 is insensitive to the presence or absence of CPSF (45) is consistent with this model but does not prove it. However, the model makes a simple but very specific prediction; if, on long tails, PABPN1 stimulates poly(A) polymerase in the normal fashion but disrupts the stimulation by CPSF, then a mutation in PABPN1 making this protein unable to stimulate poly(A) polymerase should uncover its inhibitory influence on the CPSF-poly(A) polymerase interaction. Moreover, this inhibitory activity should be visible only on long tails, whereas the mutant PABPN1 should still support the CPSF-poly(A) polymerase interaction on short tails.

Point mutations in the hydrophobic face of the coiled-coil domain of PABPN1, most strongly L136S or L136A, abolish the ability of the protein to stimulate poly(A) polymerase without affecting poly(A) binding (41). The corresponding mutation of the *Drosophila* orthologue of PABPN1, I61S, fails to rescue a null allele (34). For the purpose of testing the proposed model, we used the double mutant L119A/L136A (LALA for short). In control reactions with a substrate RNA carrying a short oligo(A) tail (L3preA_{15}), the mutant protein showed a barely detectable stimulation of poly(A) polymerase in the absence of CPSF over a time course of 5 min (Fig. 5, compare *lanes 17–20* with *lanes 1–4*). In the presence of both CPSF and PABPN1 LALA, the rate of elongation was very similar to that seen with

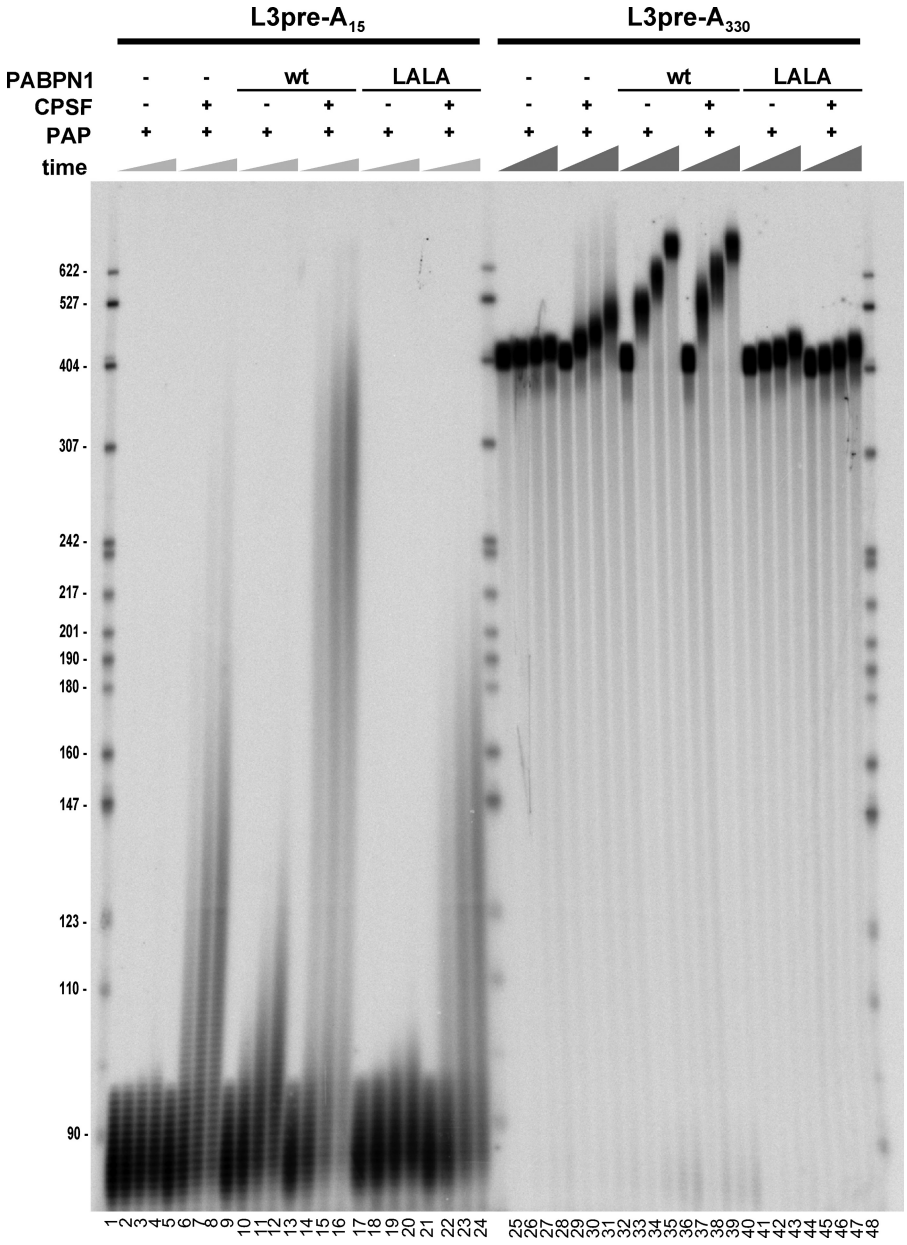

FIGURE 5. **PABPN1 inhibits poly(A) polymerase stimulation by CPSF on long tails.** Polyadenylation reactions were set up containing, per unit volume of 20 μl, 80 fmol of RNA, 32 fmol poly(A) polymerase and, where indicated, CPSF at a concentration approximately equimolar with RNA and/or 3.5 pmol of PABPN1 wild-type or LALA mutant. RNA, poly(A) polymerase, and PABPN1 were assembled on ice, and the mixture was prewarmed for 10 min at 37 °C. Next CPSF was added, followed by a second preincubation for 15 min at 37 °C. 20-μl aliquots were withdrawn for the 0-min time points, and then polyadenylation was started by the addition of ATP. Additional aliquots were withdrawn after 1, 3, and 5 min for L3pre-A_{15} (*small light gray wedges*, *lanes 1–24*) and after 15, 30, and 60 min for L3pre-A_{330} (*large dark gray wedges*, *lanes 25–48*). RNAs were resolved by polyacrylamide gel electrophoresis. The sizes of DNA markers in nucleotides are indicated on the *left*.

CPSF alone; in other words the mutant PABPN1 behaved as if it were absent (Fig. 5, compare *lanes 21–24* with *lanes 5–8*) (41). In contrast, a second control reaction with wild-type PABPN1 and CPSF showed the expected processive elongation (Fig. 5, compare *lanes 13–16* with *lanes 5–8* and *9–12*). With an RNA substrate carrying an A_{330} tail, 60-min time courses were used because of the low elongation rates caused by the lack of processivity. Both wild-type PABPN1 and CPSF stimulated extension individually, as expected (Fig. 5, compare *lanes 33–36* and *29–32* with *lanes 25–28*). Elongation in the presence of both was very similar to the reaction seen with PABPN1 alone, demonstrating the absence of a cooperative effect, also as expected (Fig. 5, *lanes 37–40*). PABPN1 LALA by itself was again unable to stimulate poly(A) extension to any significant extent (Fig. 5, *lanes 41–44*). Remarkably, however, when the mutant PABPN1 was used together with CPSF, no stimulation of polyadenylation was seen, *i.e.* the stimulation by CPSF alone was suppressed (Fig. 5, *lanes 45–48*), and the rate of elongation was very similar to that caused by poly(A) polymerase alone (*lanes 25–28*). These results provide very strong support for the model outlined above. On a long poly(A) tail, PABPN1 disrupts the interaction between CPSF and poly(A) polymerase. With wild-type protein, this is not evident because PABPN1 itself stimulates poly(A) tail extension. The inhibitory effect of PABPN1 on the CPSF-poly(A) polymerase interaction becomes visible when mutant PABPN1 is used that cannot stimulate polyadenylation itself. Disruption of the CPSF-poly(A) polymerase interaction is limited to long poly(A) tails; the interaction is normal on short tails.

Because this model is proposed to explain length control, the dependence of the switch in PABPN1 activity on the length of the poly(A) tail is a central feature. Therefore, additional poly(A) tail lengths were tested, all at a uniform elongation time of 15 min. In this experiment, PABPN1 LALA behaved again neutral as expected with an RNA carrying an A_{15} tail (Fig. 6, *lanes 1–8*). On two RNAs with A_{280} and A_{300} tails, mutant PABPN1 behaved as an inhibitor, just like on the RNA with the A_{330} tail discussed above (Fig. 6, *lanes 17-32*). On an RNA with an intermediate tail length of 110 nucleotides, PABPN1 LALA by itself was unable to stimulate, as expected. When it was used together with CPSF, the latter was still able to stimulate polyadenylation, albeit slightly less efficiently than on its own (Fig. 6, *lanes 9–16*). Thus, the inhibition of the CPSF-poly(A) polymerase interaction by PABPN1 occurs at a poly(A) tail length of more than 110 nucleotides and less than 280 nucleotides. This supports the idea that this activity of PABPN1 is at the heart of the length control mechanism.

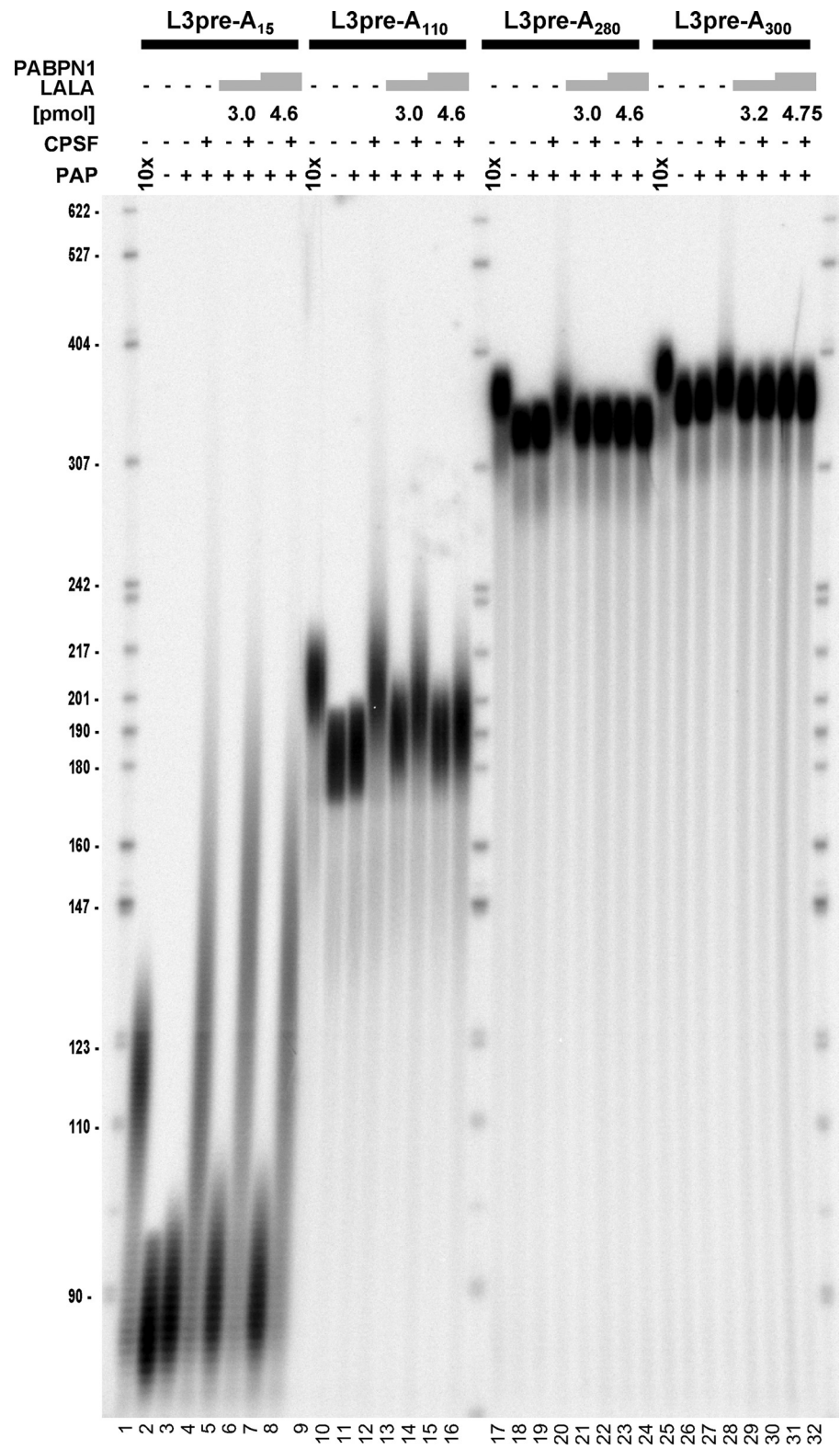

FIGURE 6. **Tail length dependence of the inhibitory effect of PABPN1.** The reactions of 20 μl were assembled containing, as indicated, 80 fmol of L3pre-based RNAs with various poly(A) tail lengths, 32 fmol of PAP, CPSF (approximately equimolar with RNA), and increasing amounts of PABPN1 LALA. For each RNA a control reaction containing a 10-fold amount of poly(A) polymerase was also carried out. After addition of RNA, poly(A) polymerase and PABPN1 the reaction mixtures were prewarmed for 10 min at 37 °C. After CPSF addition, prewarming was continued for 15 min, and then polyadenylation was started by ATP addition. The reactions were stopped after 15 min at 37 °C, and the RNA was analyzed by gel electrophoresis. The amount of PABPN1 LALA used corresponds to a calculated 1.3- and 2-fold saturation on L3preA_{280} and -A_{300}, a 5- and 8-fold saturation on L3preA_{110}, and a 38- and 57-fold saturation on L3pre-A_{15} as judged by gel mobility shift analyses.

Stoichiometric Binding of PABPN1 Is Required for Tail Length-dependent Loss of CPSF Stimulation—The experiments described so far reveal that termination of processive polyadenylation is due to the inability of CPSF to stimulate poly(A) polymerase when the two proteins are separated by a long poly(A) tail covered by PABPN1. Loss of the interaction between poly(A) polymerase and CPSF is due to two properties of PABPN1. First, on long tails, the protein no longer supports the interaction between poly(A) polymerase at the 3′ end and CPSF bound to the AAUAAA sequence, as inferred from the length-dependent inhibitory effect of the LALA mutant. Second, because PABPN1 covers the poly(A) tail, it restricts CPSF to the AAUAAA sequence, where it can no longer interact with poly(A) polymerase. This interpretation predicts that the stimulation of poly(A) polymerase by CPSF should be prevented only when the long poly(A) tails are completely covered by PABPN1. The prediction was tested with L3pre RNA carrying an A_{300} tail. Mutant PABPN1 was titrated in a polyadenylation assay; approximately 3.6 pmol of PABPN1 LALA were required for maximum inhibition of CPSF stimulation (Fig. 7*A*). In parallel, RNA binding of the protein was assessed directly in a gel shift experiment, and a similar amount of PABPN1 LALA was required to saturate the RNA (Fig. 7*B*). Thus, the disruption of the CPSF-poly(A) polymerase interaction by PABPN1 requires the poly(A) tail to be covered completely. This also suggests that the length of the poly(A) tail at which the termination of processive elongation occurs is counted by PABPN1.

The Length Control Mechanism Counts Only A Residues—Processive elongation always generates a total poly(A) tail length of ~ 250 nucleotides, independently of the length of the tail initially present. Thus, the length control mechanism is based on a determination of the total length of the poly(A) tail

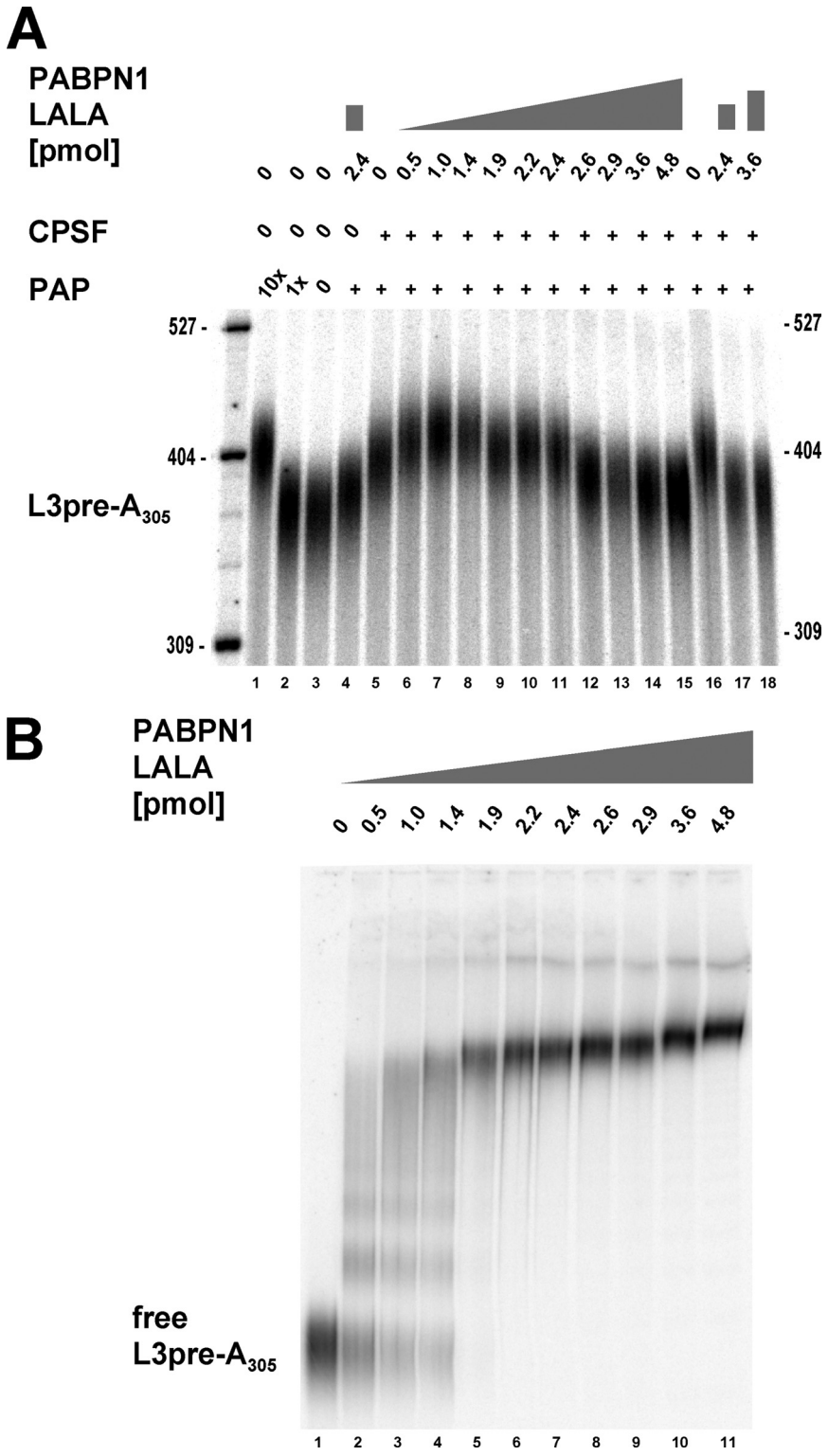

FIGURE 7. **PABPN1 has to coat long poly(A) tails to inhibit CPSF stimulation of poly(A) polymerase.** *A*, titration of the inhibitory effect of PABPN1. 80 fmol of radioactive L3pre-A_{305} was incubated in a 20-μl reaction volume with 1.25 μg of competitor tRNA, 10 fmol of PAP, CPSF approximately equimolar to the RNA, and increasing amounts of PABPN1 LALA as indicated. After 5 min of preincubation at 37 °C, polyadenylation was started by ATP addition and continued for 60 min at 37 °C. Some of the reactions were carried out twice (*lanes 5* and *15* and *lanes 4* and *16*). A control reaction with a 10-fold amount of poly(A) polymerase was also carried out as explained under "Experimental Procedures." RNA was analyzed by gel electrophoresis. The sizes of DNA markers are indicated on both sides. *B*, titration of PABPN1 LALA in a gel mobility shift assay. 80 fmol of radioactive L3pre-A_{305}, 1.5 μg of tRNA, and increasing amounts of PABPN1 LALA were incubated in 20-μl reaction volumes under polyadenylation conditions except that ATP was left out. After 30 min of incubation at room temperature, complexes were resolved on a native polyacrylamide/agarose composite gel and detected by phosphorimaging.

and not of the number of nucleotides added during the reaction or the time poly(A) polymerase spends elongating the tail (45) (see also Fig. 2). Which yardstick is used to measure tail length? PABPN1 is a likely candidate, and this is supported by the experiment shown in Fig. 7. Manipulation of the RNA substrate provides an additional way to test the hypothesis.

Three different RNAs were compared in processive polyadenylation reactions. One was the regular polyadenylated L3pre RNA, L3preA_{15}. The second RNA, L3pre$N_{49}A_{15}$, had 49 nucleotides of additional sequence of mixed composition inserted before the beginning of the poly(A) tail. The third RNA, L3pre$A_{15}N_{63}A_{15}$, had 63 nucleotides of mixed composition inserted between two stretches of 15 adenylate residues each (Fig. 8*A*). Gel shift experiments showed that these RNAs bound PABPN1 in proportion to the oligo(A) sequences independently of their positions (Ref. 41 and data not shown). The non-A sequences inserted into the RNAs do not bind PABPN1 (41). Upon incubation in the presence of CPSF, PABPN1, and poly(A) polymerase, a relatively small fraction of L3preA_{15} RNA received poly(A) tails of up to ~250 nucleotides within 30 s, in agreement with the processive nature of the reaction. Upon longer incubation, most of the remaining RNA was also polyadenylated, and mature tails continued to grow at a lower rate, reflecting largely distributive elongation beyond 250 nucleotides (44, 45). After 5 min, the center of the length distribution corresponded to ~380 nucleotides or slightly more than 300 nucleotides of poly(A) tail length (Fig. 8, *A*, *middle panel*, and *B*). Appropriate controls showed that the processive reaction depended on the simultaneous stimulation of poly(A) polymerase by both CPSF and PABPN1 (Fig. 8*A*, *middle panel*). The substrate L3pre$N_{49}A_{15}$ behaved very much like L3preA_{15}, but at any time point products were ~50 nucleotides longer (Fig. 8, *A*, *left panel*, and

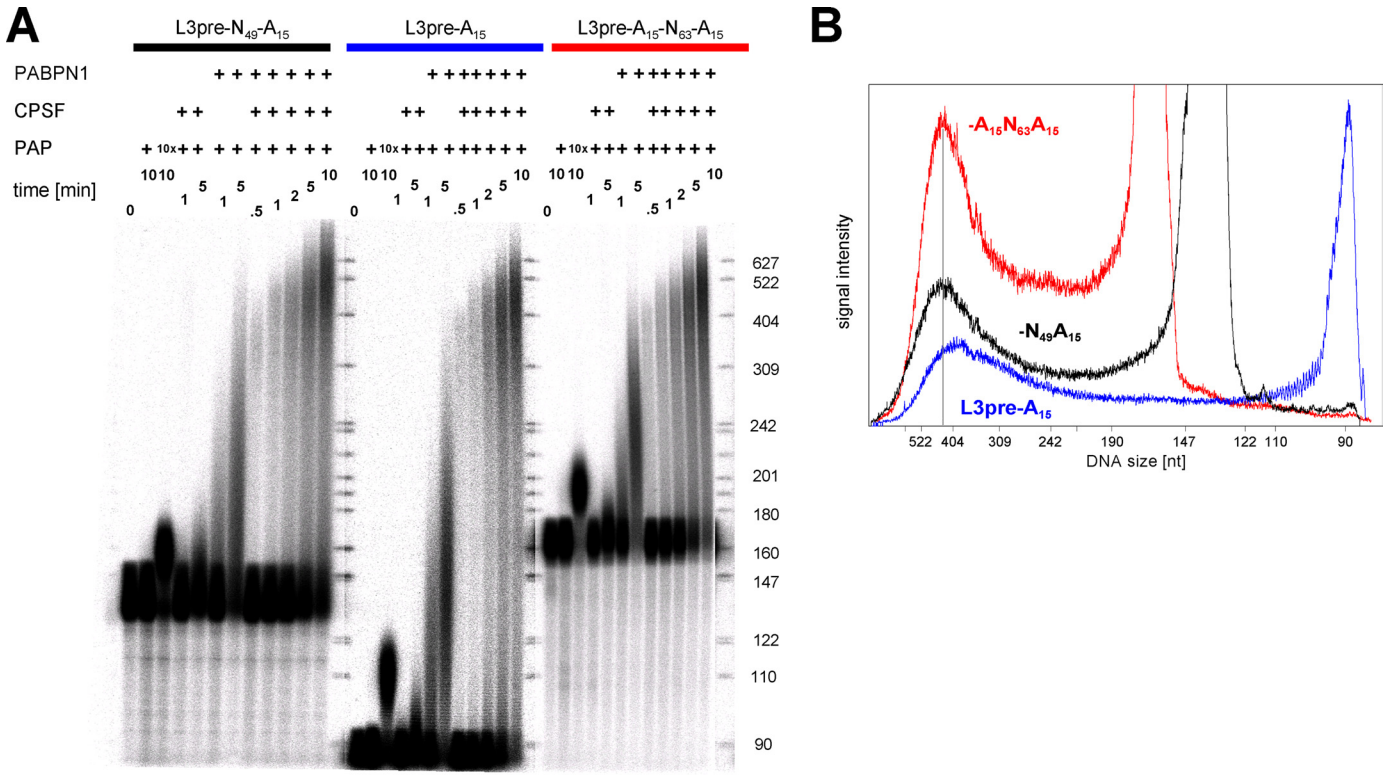

FIGURE 8. **Only poly(A) is counted as part of the tail: elongation of mixed tails.** *A*, reaction mixtures were assembled on ice containing, per 25-μl unit volume, 80 fmol of RNA, 1.25 μg of tRNA, 10 fmol of PAP, 1200 fmol of PABPN1, and an amount of CPSF approximately equimolar with RNA. Control reactions with a 10-fold amount of PAP were done in parallel. The mixtures were preincubated for 2 min at 37 °C, and the reactions were started by the addition of ATP. Aliquots were withdrawn at the time points indicated. The reaction products were analyzed by gel electrophoresis. Unreacted substrate RNA is shown in the first lane of each of the three sets of reactions. The size of DNA markers (in nucleotides) are indicated on the *right*. *B*, *line traces* showing the RNA size distributions from polyadenylation reactions in *A* at the 5-min time points. The *vertical gray line* to the *left* of the 404-nt marker indicates the peak of the product lengths obtained by extension of L3pre-N_{49}-A_{15} and L3pre-A_{15}-N_{49}-A_{15}.

B). Thus, the additional 49 nucleotides upstream of the poly(A) tail were not counted as part of the length control mechanism. In other words, the polyadenylation complex counts poly(A) length and not the distance between the 3′ end and the CPSF-binding site. The substrate L3pre$A_{15}N_{63}A_{15}$ was also elongated processively like L3preA_{15}, but again at any time point the products were ~50 nucleotides longer (Fig. 8, *A*, *middle panel*, and *B*). Although the limited precision of the length control mechanism does not allow us to conclude whether the A_{15} tract separated from the rest of the tail by 63 nucleotides of heterogeneous sequence was counted as part of the tail or not, the N_{63} sequence definitely was not counted. Together, the experiments show that only poly(A) is counted as part of the tail; other sequences are ignored even when they are surrounded by poly(A). Because this matches the binding specificity of PABPN1, the results suggest that binding of PABPN1, possibly even contiguous binding, is required for poly(A) counting and supports the idea that a regular poly(A)-PABPN1 complex forms the basis of the counting mechanism.

DISCUSSION

Based on the data reported here and previously, the polyadenylation reaction can be described as follows. In the normal situation, when the RNA substrate is first cleaved and then polyadenylated, CPSF participates directly in the cleavage reaction, with the 73-kDa subunit acting as the endonuclease (51), and poly(A) polymerase is thought to be part of the cleavage complex as well. In the uncoupled reaction investigated here, CPSF associates with the AAUAAA sequence of the precleaved RNA and recruits poly(A) polymerase, and poly(A) tail synthesis begins. The first molecule of PABPN1 binds to the RNA when the poly(A) tail has reached a length of 10–12 nucleotides (29). Additional copies bind as the tail gets longer. PABPN1 binding has three effects. First, PABPN1 prevents CPSF binding to the poly(A) tail and restricts this protein to the AAUAAA sequence. This was shown both by elongation (Figs. 1 and 2) and by direct binding assays (Fig. 4). Second, PABPN1 facilitates the interaction between CPSF bound to the AAUAAA sequence and poly(A) polymerase at the 3′ end over the distance of the poly(A) tail separating the two proteins, as shown by a constant rate of poly(A) tail elongation in the presence of both CPSF and PABPN1 (45) in contrast to a pronounced distance dependence of elongation in the absence of PABPN1 (Fig. 3). An interaction between CPSF and poly(A) polymerase can be inferred from the CPSF dependence of polyadenylation and has previously been shown by pull-down experiments (38). Presumably, PABPN1 facilitates this interaction because the RNA has to fold back on itself for the contact to be maintained during the elongation reaction, and PABPN1 may promote this structure (49). Third, a direct contact between PABPN1 and poly(A) polymerase provides additional stabilization of the polyadenylation complex (41). The simultaneous interaction of poly(A) polymerase with both CPSF and PABPN1 endows the complex

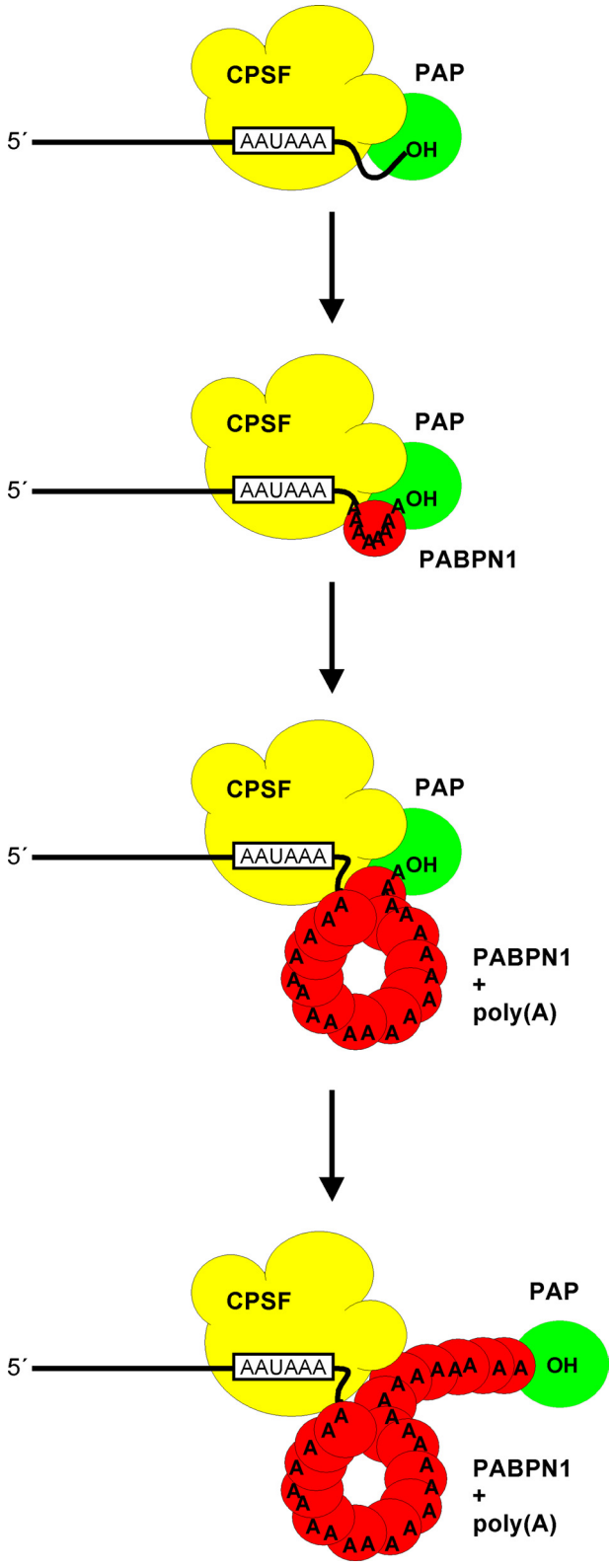

FIGURE 9. **Model of length control mechanism.** CPSF binds the polyadenylation signal AAUAAA and recruits PAP. The first PABPN1 molecule joins the complex once the oligo(A) tail has reached a length of about 12 nucleotides. Additional PABPN1 molecules cover the growing tail. Formation of a tight, spherical PABPN1 particle on the growing poly(A) tail facilitates folding back of the RNA, which is required to maintain a contact between CPSF and poly(A) polymerase. Thus, the enzyme is held in the complex by cooperative interactions with both CPSF and PABPN1 and can synthesize the entire poly(A) tail in a processive manner. When the poly(A) tail exceeds a critical length of about 250 adenylate residues, additional PABPN1 molecules can no longer be accommodated in the spherical RNA-protein complex, and the contact between poly(A) polymerase and CPSF cannot be maintained. Thus, during further elongation of the poly(A) tail, poly(A) polymerase is held in the complex only by PABPN1; elongation becomes poorly processive and therefore slow.

with sufficient stability that a complete poly(A) tail is synthesized in a single processive event. Once the poly(A) tail has reached a length of ~250 nucleotides, the structure of the poly(A)-PABPN1 complex changes such that it disrupts or no longer supports the interaction between CPSF and poly(A) polymerase (Fig. 5). Loss of this interaction terminates processive polyadenylation. Subsequent elongation relies only on the stimulation by PABPN1 and is therefore poorly processive and relatively slow. Because the rate of poly(A) tail elongation beyond 250 nucleotides is very similar in the presence of PABPN1 or CPSF or both, it was previously not possible to determine which of the two stimulatory interactions is lost. Only the mutation that inactivated PABPN1 as a stimulator of poly(A) polymerase uncovered the disruption of the CPSF-poly(A) polymerase interaction. The model of the polyadenylation reaction is summarized in the cartoon in Fig. 9.

An alternative model dissociation or displacement of CPSF upon completion of the poly(A) tail-was excluded by gel mobility shift and UV cross-linking assays, which showed that CPSF remains bound to the RNA after termination of processive elongation. A slight reduction in binding evident in both types of assays is consistent with the model because the binding of CPSF to the RNA is stabilized by its interaction with poly(A) polymerase, which no longer takes place under these conditions.

The length control mechanism measures the length of the poly(A) tract and ignores other sequences, even if they are inserted between stretches of poly(A). Because PABPN1 is the only protein in the reconstituted reaction that specifically recognizes poly(A) and is used at concentrations sufficient to cover the available poly(A) completely, it is hard to escape the conclusion that this is the protein that counts the nucleotides of the tail. This model is also supported by the observation that PABPN1 disrupts the poly(A) polymerase-CPSF contact only when it covers the poly(A) tail completely. However, there is a caveat in the interpretation of this experiment, because complete coverage of the poly(A) tail by PABPN1 is also necessary to prevent illegitimate binding of CPSF to the tail. Thus, the experiment by itself does not permit an unambiguous conclusion about what is required to prevent the interaction between poly(A) polymerase and CPSF when the latter is restricted to the AAUAAA sequence.

Additional support for a role of PABPN1 in measuring the length of the poly(A) tail comes from the examination of PABPN1-poly(A) complexes by electron microscopy, which revealed the existence of compact, spherical particles (49). These particles appeared self-limiting, never exceeding a diameter of 21 nm; on very long poly(A) molecules, several such particles of uniform size were arranged in a beads-on-a-string pattern. Experiments with size-fractionated poly(A) showed that one particle could accommodate 200–300 nucleotides of poly(A); shorter poly(A) supported the formation of smaller,

presumably incomplete particles. We suggest that the spherical particles represent the folded back structure of the poly(A) tail, which is necessary to permit the interaction between CPSF and poly(A) polymerase to be maintained throughout chain growth. Poly(A) beyond a length of ~250 nucleotides can no longer be accommodated in the particle; thus poly(A) polymerase acting at the end of this RNA molecule cannot interact with CPSF anymore, and processive elongation stops (Fig. 9).

Inherent size limits for linear structures like nucleic acids or repetitive nucleic acid-protein complexes are hard to envision. A finite size is much more easily achieved with a globular structure. Therefore, coiling up of the linear poly(A)-PABPN1 complex into a spherical complex seems like an attractive model for length measurement. A much better understood case of length control of a linear polymer by its accommodation in a globular protein structure of defined size is the so-called "headful" mechanism of phage genome packaging into virions. One capsid can accommodate slightly more than one genome length of DNA. Concatemeric phage DNA is spooled into an empty capsid until no more DNA can be accommodated, and then cleavage of the genome is induced (52, 53). In contrast to the stable and well defined virion structures, the spherical poly(A)-PABPN1 complexes appear to be quite unstable (49). It will be a challenge to learn more about their structure.

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