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Multiple forms of poly(A) polymerases in human cells

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ABSTRACT **We have cloned human poly(A) polymerase (PAP) mRNA as cDNA in *Escherichia coli*. The primary structure of the mRNA was determined and compared to the bovine PAP mRNA sequence. The two sequences were 97% identical at the nucleotide level, which translated into 99% similarity at the amino acid level. Polypeptides representing recombinant PAP were expressed in *E. coli*, purified, and used as antigens to generate monoclonal antibodies. Western blot analysis using these monoclonal antibodies as probes revealed three PAPs, having estimated molecular masses of 90, 100, and 106 kDa in HeLa cell extracts. Fractionation of HeLa cells showed that the 90-kDa polypeptide was nuclear while the 100- and 106-kDa species were present in both nuclear and cytoplasmic fractions. The 106-kDa PAP was most likely a phosphorylated derivative of the 100-kDa species. PAP activity was recovered *in vitro* by using purified recombinant human PAP. Subsequent mutational analysis revealed that both the N- and C-terminal regions of PAP were important for activity and suggested that cleavage and polyadenylylation specificity factor (CPSF) interacted with the C-terminal region of PAP. Interestingly, tentative phosphorylation sites have been identified in this region, suggesting that phosphorylation/dephosphorylation may regulate the interaction between the two polyadenylylation factors PAP and CPSF.**

The addition of poly(A) tails to the 3′ ends of eukaryotic mRNAs is one of the key events that take place in the nucleus during maturation of mRNA. The polyadenylylation reaction proceeds in two distinct steps: endoribonucleolytic cleavage of the pre-RNA at the polyadenylylation site followed by synthesis of a poly(A) tail at the 3′ end of the upstream cleavage product (1). Both of these reaction steps require a hexanucleotide sequence (i.e., AAUAAA), which is located 10–30 nt upstream of the polyadenylylation site (for review, see ref. 2). However, after addition of ≈10 adenosine residues, the requirement for the AAUAAA sequence element is released (3). Careful biochemical characterization of the components involved in the polyadenylylation reaction has shown that multiple protein factors are assembled together with the RNA substrate during polyadenylylation (for reviews, see refs. 4 and 5). The composition of this complex changes as the reaction proceeds and some of the factors are exclusively required for the endoribonucleolytic cleavage reaction while others are required for both reaction steps. The poly(A) polymerase (PAP) is, as expected, required for the adenosine addition reaction. This reaction also requires the presence of the cleavage and polyadenylylation specificity factor (CPSF). CPSF is the factor that interacts with the AAUAAA sequence element and conveys the dependence on this element for both the RNA cleavage reaction and the initial phase of adenosine addition (6–8). However, PAP is also required for the endoribonucleolytic cleavage reaction at some polyadenylylation sites (e.g., the L3 polyadenylylation site of human adenovirus) (9–11). Thus, PAP is involved in both RNA cleavage and adenosine addition reactions. Taken together, these observations suggest that PAP is involved in several different reactions (i.e., RNA cleavage and AAUAAA-dependent or -independent adenosine addition). However, it is not known yet whether the same PAP participates in all these reactions or whether each or some of the reactions require a particular PAP. The latter possibility is attractive since multiple PAPs have been identified in mammalian cells by chromatographic properties (see refs. 9, 12, and 13 and references therein) and by molecular cloning of alternatively spliced mRNAs (14, 15). Furthermore, some evidence for phosphorylated forms of PAP have been reported (16, 17). Here we present direct evidence, by Western blot analysis, for the presence of multiple PAPs in HeLa cell extracts. We also show that one of these forms is phosphorylated and that PAP is composed of distinct functional domains.§

MATERIALS AND METHODS

Synthetic Oligonucleotides. Synthetic oligonucleotides with cleavage sites for *Nde* I or *Bam*HI at their 5′ ends were prepared. The sequences (given in 5′ to 3′ order) of the oligonucleotides were as follows (underlined nucleotides are numbered according to the bovine PAP mRNA I and II sequences of ref. 14): AA31, CACCATATGCCGTTTCCAGTTACAACACAG (nt 1–24); AA32, CACCATATGTGTGTTGCACCAAGACATGTTGA (nt 350–374); AA35, TGTGGATCCTAGTTGCTTTCTTTTTACATGCAT (complementary to nt 1456–1478); AA36, TGTGGATCCCTCTTCTGTTTTGGTTTTCTTGGG (complementary to nt 1963–1987).

Molecular Cloning and Sequencing of Human PAP mRNA. cDNA primed with pd(N)$_6$ was synthesized using cytoplasmic mRNA (18) prepared from primary human fibroblasts as the template and the first-strand cDNA synthesis kit (Pharmacia; 27-9261-01). cDNAs located between synthetic oligonucleotide pairs AA31–AA35 or AA32–AA36 were amplified by the PCR (19) using AmpliTaq DNA polymerase (Perkin–Elmer/Cetus; N801-0060), cleaved by *Nde* I and *Bam*HI restriction enzymes, inserted between these sites of plasmid vector pET 11 (20), and transformed into *Escherichia coli* strain BL21(DE3) (20). Bacterial clones containing either cDNA were identified and named pPAP4 and pPAP8 (cDNA between primer pairs AA31–AA35 and AA32–AA36, respectively). The nucleotide sequences of the inserted cDNAs were determined by the chain-termination method (21) with synthetic oligonucleotides used as primers. One additional plasmid, pPAP12, was constructed by insertion of the *Xba* I

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Abbreviations: PAP, poly(A) polymerase; CPSF, cleavage and polyadenylylation specificity factor.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. X76770).

and *Kpn* I fragment of pPAP4 between the *Xba* I and *Kpn* I sites of the pPAP8 clone. Thus, the resulting plasmid contained cDNA sequences between primers AA31 and AA36. Restriction enzymes and T4 DNA ligase were purchased from Pharmacia LKB.

Expression and Partial Purification of Recombinant Human PAP. Expression of recombinant human PAP in *E. coli* was done according to Studier *et al.* (20). LB medium (1 liter), at 37°C and supplemented with ampicillin (200 μg/ml), was inoculated with fresh bacterial colonies. Isopropyl β-D-thiogalactopyranoside (Sigma; I 6758) was added at A_{590} = 0.5 to a final concentration of 0.4 mM. After incubation for 60 min at 37°C the cells were harvested, dissolved in 12 ml of lysis buffer (50 mM Tris·HCl, pH 7.9/100 mM NaCl/5 mM EDTA), and sonicated six times for 10 sec. Insoluble material was collected by centrifugation and washed in 6 ml of wash buffer (50 mM Tris·HCl, pH 7.9/100 mM NaCl/10 mM EDTA/0.5% Triton X-100). The obtained pellets were solubilized by rotation for 60 min at 4°C in 12 ml of 6 M guanidine hydrochloride (Gdn·HCl) or in 12 ml of 4 M urea. Soluble material from the Gdn·HCl treatment was renatured for 10 h at 4°C after diluting 1:50 in buffer D [20 mM Hepes·KOH/100 mM KCl/1.5 mM $MgCl_2$/0.2 mM EDTA/0.5 mM dithiothreitol/20% (vol/vol) glycerol, pH 8.2]. Insoluble material from the 4 M urea treatment was collected, solubilized in 8 M urea, and centrifuged. The supernatant was collected and mixed with an equal volume of 2× SDS/gel electrophoresis buffer. Recombinant polypeptides were subsequently purified by SDS/PAGE (22) followed by electroelution (23) of recombinant polypeptides. Eluted polypeptides were dialyzed against phosphate-buffered saline [0.14 M NaCl/2.7 mM KCl/8 mM Na_2HPO_4/1.5 mM KH_2PO_4, pH 7.3 (HCl)] for 24 h and concentrated by centrifugation (Filtron Technology, Northborough, MA; OD 010C40).

Generation of Monoclonal Antibodies. Monoclonal antibodies were obtained, using standard techniques (24), by immunization of BALB/c mice with recombinant PAP gel purified from bacterial cells expressing plasmid pPAP4. Two monoclonal antibodies were isolated and named NN2 and 20:14.

RNA Substrates and Detection of PAP Activity *in Vitro*. *In vitro* transcription and purification of uniformly ^{32}P-labeled RNA substrates L3(54) and L3G(54) (25) was done as described (9).

Detection of specific adenosine addition activity (AAUAAA dependent in the presence of Mg^{2+}) was done as described by Åström *et al.* (9). Nonspecific PAP activity (AAUAAA independent in the presence of Mn^{2+}) using poly(A) (Sigma; P.9403) as primer was detected as outlined by Ryner *et al.* (12).

Preparation of HeLa Cell PAP and CPSF. Nuclear and cytoplasmic (S100) extracts (26, 27) were prepared from HeLa cells and fractionated by DEAE-Sephacel chromatography as outlined by Åström *et al.* (9). Proteins (52.5 mg) in fraction I (2.1 mg of protein per ml) were subjected to heparin-Sepharose chromatography (bed vol, 12 ml; diameter, 1.6 cm; flow rate, 30 cm/h) in buffer D (20 mM Hepes·KOH/100 mM KCl/1.5 mM $MgCl_2$/0.2 mM EDTA/0.5 mM dithiothreitol/20% glycerol, pH 8.2). PAP activity (30.8 mg of protein in 11 ml) was eluted by increasing the KCl concentration to 0.5 M (fraction HS0.5). Proteins (14.0 mg) of fraction HS0.5 were dialyzed against buffer D and applied to a cellulose phosphate P11 column as outlined by Åström *et al.* (9). PAP activity was eluted from this column by a linear gradient of KCl between 0.1 and 1.0 M in buffer D. PAP activities equivalent to activities in fractions IB and IC of Åström *et al.* (9) were identified, pooled, and named P11B (3.0 mg of protein in 6 ml) and P11C (2.3 mg of protein in 6 ml). Proteins (2.5 mg) of fraction P11B and proteins (1.7 mg) of fraction P11C were fractionated by FPLC (Mono S HR 5/5; Pharmacia LKB) after dialysis against buffer D containing 20 mM KCl. The column was eluted (flow rate, 0.5 ml/min; fraction vol, 1 ml) by a 20-ml linear gradient between 20 and 300 mM KCl in buffer D followed by a final wash at 500 mM KCl in buffer D. Fractions containing PAP activities were identified, pooled, and named Mono SB (0.08 mg of protein in 2 ml) or Mono SC (0.38 mg of protein in 2 ml), respectively. Fractions Mono SB and Mono SC eluted around 100 and 180 mM KCl, respectively. During fractionation, PAP activities were identified by *in vitro* reconstitution of specific adenosine addition activity.

CPSF (fraction IIIC) was isolated as described by Åström *et al.* (9).

SDS/PAGE and Western Blot Analysis. SDS/PAGE was carried out according to Laemmli (22) using 4% and 6.25% polyacrylamide in spacer and separation gels, respectively.

Western blot analysis was done according to the protocol of Jareborg and Burnett (28). Detection was done by anti-mouse immunoglobulin, horseradish peroxidase-linked whole antibody (Amersham; NA 931) diluted 1:1000 and the ECL Western blotting detection reagents (Amersham; RPN 2106).

Phosphatase Treatment of HeLa Cell PAP. Fraction HS0.5 (10 μl) was diluted 1:5 in 100 mM Tris·HCl, pH 9.0/10 mM $MgCl_2$, treated by the indicated amounts of calf intestine phosphatase (Boehringer Mannheim; 713023) at 37°C for 1 h, and precipitated by 2 vol of acetone at −20°C. Precipitates were collected, dissolved in SDS sample buffer, and subjected to SDS/PAGE.

RESULTS

Cloning of Human Poly(A) Polymerase. Cytoplasmic RNA, purified from primary human fibroblasts, was transcribed by reverse transcriptase. Subsequently, cDNAs representing human PAP mRNA were amplified by PCR. Two PCR products located between nt 1 and 1478 and between nt 350 and 1987 (numbers according to the sequence of ref. 14) were obtained and subsequently cloned between the *Nde* I and *Bam*HI sites of plasmid pET 11 (20). The obtained cDNA clones were named pPAP4 (nt 1–1478) and pPAP8 (nt 350–1987). The nucleotide sequences of at least three independent clones of each PCR product were determined (21). The deduced nucleotide and amino acid sequences of human PAP were compared to the bovine PAP sequences of Raabe *et al.* (14) and Wahle *et al.* (15). Tables 1 and 2 summarize the differences between bovine and human PAPs. The primary sequence of PAP is extremely well conserved between the two species since only 64 nt differed within the regions that

Table 1. Differences in amino acid sequences of bovine and human PAPs

		Bovine†		Human‡	
nt*	aa*	nt	aa	nt	aa
111	37	G	Leu	A	Val
129	43	G	Val	T	Ile
240	80	A	Arg	T	Ser
1496	499	G	Ser	A	Asn
1549	517	C	Pro	G	Ala
1625	542	T	Met	C	Thr
1732	578	C	Leu	G	Val
1741	581	A	Ile	G	Val
1816	606	T	Ser	C	Pro
1882	628	C	Pro	T	Ser
1900	634	G	Ala	A	Thr
1913	638	A	Asn	C	Thr

*Numbers refer to nt and aa of the bovine PAP sequence (14). A tandem duplication of aa 629–634 in human PAP has not been included.
†Refers to the bovine sequence of Raabe *et al.* (14).
‡This study.

Table 2. Differences in nucleotide sequences of bovine and human PAPs

nt*	Bovine†	Human‡	nt*	Bovine†	Human‡	nt*	Bovine†	Human‡
33	G	A	384	T	C	1491	G	A
42	G	A	459	T	C	1496	G	A
75	C	T	471	C	T	1536	C	T
109	C	G	477	G	C	1542	G	A
111	G	A	531	T	A	1549	C	G
114	C	T	537	A	T	1554	G	C
123	G	A	543	C	T	1560	T	C
126	G	A	549	C	T	1590	C	T
127	G	A	550	T	C	1625	T	C
129	G	T	553	A	C	1638	G	A
135	T	A	570	T	A	1732	C	G
136	C	T	669	C	T	1741	A	G
141	G	A	687	G	A	1815	A	T
147	C	T	855	C	G	1816	T	C
202	T	C	1149	T	A	1851	C	T
240	A	T	1164	A	G	1861	T	C
282	T	A	1212	T	C	1878	A	T
285	G	A	1227	G	A	1882	C	T
309	T	C	1353	T	C	1900	G	A
321	A	G	1440	C	T	1905	G	A
348	A	G	1455	G	A	1913	A	C

*Numbers refer to nt of the bovine PAP sequence (14). A tandem duplication of nt 1883–1900 in human PAP has not been included.
†Refers to the bovine sequence of Raabe *et al.* (14).
‡This study.

were compared (96.7% identity). At the amino acid level, only 12 amino acids differed (97.6% identity; 99.2% similarity). Nine of these 12 amino acids were located in the C-terminal part of the polymerase within a region that has been identified as a serine/threonine-rich region in bovine PAP. In addition to these differences, we found that 18 nt (between nt 1883 and 2000) were tandemly duplicated in human PAP mRNA.

Identification of Multiple Poly(A) Polymerases. We have previously identified at least two different PAPs in HeLa cell nuclear extracts (9). The two PAPs were discriminated from each other by chromatographic properties and heat sensitivity. To investigate the relationship between these PAPs, we prepared monoclonal antibodies against human PAP. We used Western blot analysis to identify two monoclonal antibodies, NN2 and 20:14, that recognized differently truncated variants of recombinant PAP (data not shown), indicating that the monoclonal antibodies were specific. We were not able to find monoclonal antibodies that were useful in detecting soluble PAP by immunoprecipitation. The explanation for this is most likely that gel-purified denatured recombinant polypeptides were used as the antigens. Subsequent Western blot analysis showed that the monoclonal antibodies recognized three polypeptides in HeLa cell extract (Fig. 1; data not shown). The polypeptides had estimated molecular masses of 106, 100, and 90 kDa. To obtain further evidence that these polypeptides represented PAPs, we investigated their cofractionation with PAP activity. Western blot analysis of the obtained fractions (Fig. 1) revealed that the 106- and 100-kDa polypeptides cofractionated with the previously identified heat-resistant PAP activity, while the 90-kDa polypeptide cofractionated with heat-sensitive activity. Thus, we conclude that the polypeptides represent three different HeLa cell PAPs and that multiple PAPs are present in HeLa cells. Based on their estimated molecular masses, we have named them PAP 90 kDa, PAP 100 kDa, and PAP 106 kDa.

The subcellular distribution of the PAPs was investigated. Fig. 2*A* shows that PAP 90 kDa was nuclear, while PAP 100 kDa and PAP 106 kDa were present in both nuclear and cytoplasmic fractions.

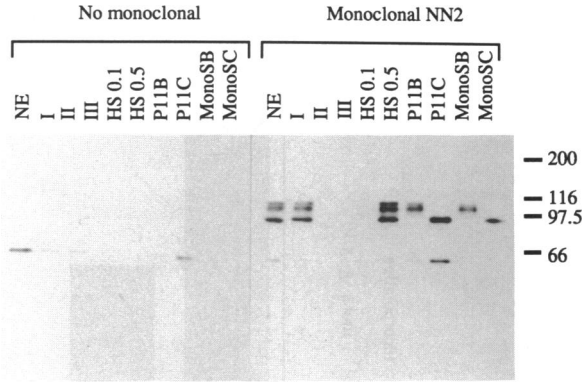

FIG. 1. Detection of human PAPs by Western blot analysis. Equivalent amounts of nonspecific PAP activity obtained from the indicated fractions or HeLa cell nuclear extract (NE) were fractionated by SDS/PAGE, blotted onto nylon membranes, and probed with monoclonal antibody NN2. Immunocomplexes were visualized by immunoglobulin, horseradish peroxidase-linked antibody. The resulting fluorogram is shown. Monoclonal antibody NN2 was omitted before detection in lanes labeled No monoclonal. Numbers on the right (kDa) refer to location of molecular size markers that were separated in an adjacent lane.

PAP 106 kDa Is Phosphorylated. To investigate the relationship between the multiple PAPs, we examined whether any of the PAPs were phosphorylated. We treated PAPs in fraction HS0.5 with increasing amounts of calf intestine phosphatase before fractionation by SDS/PAGE. The PAPs were subsequently visualized by Western blot analysis using monoclonal antibody NN2 as the probe. The resulting fluorogram (Fig. 2*B*) showed that PAP 106 kDa was sensitive to phosphatase treatment while the two other PAPs were not affected by phosphatase. Furthermore, we did not detect an unusual accumulating polypeptide, suggesting that the dephosphorylated PAP 106 kDa polypeptide comigrated with either PAP 90 kDa or PAP 100 kDa. Based on our fractionation data, we propose that PAP 106 kDa is related by phosphorylation to PAP 100 kDa.

Identification of Functional Regions in Human Poly(A) Polymerase. To identify functional regions in human PAP, we investigated the PAP activity of three cloned PAP variants. These variants were obtained by expression of plasmids pPAP4, pPAP8, and pPAP12 in *E. coli*. The expressed polypeptides were purified, denatured, and renatured as

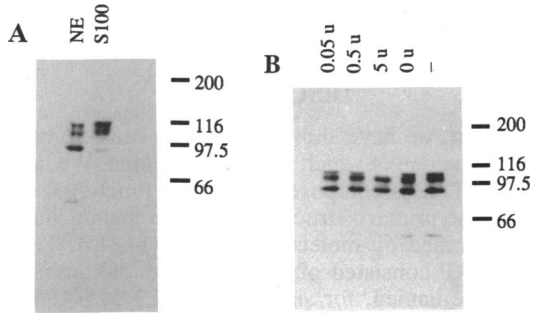

FIG. 2. Subcellular location of PAPs and identification of a phosphorylated form of PAP. (*A*) HeLa cell nuclear extract (NE) and cytoplasmic fraction (S100) were fractionated by SDS/PAGE. PAPs were visualized by Western blot analysis using monoclonal antibody NN2 as the probe. (*B*) Phosphatase treatment of fraction HS0.5. Calf intestine phosphatase (5, 0.5, 0.05, and 0 units, as indicated) and 10 μg of proteins present in fraction HS0.5 were incubated for 30 min at 37°C before fractionation by SDS/PAGE. PAPs were visualized by Western blot analysis using monoclonal antibody NN2 as the probe. Numbers on the right (kDa) refer to location of molecular size markers that were separated in adjacent lanes.

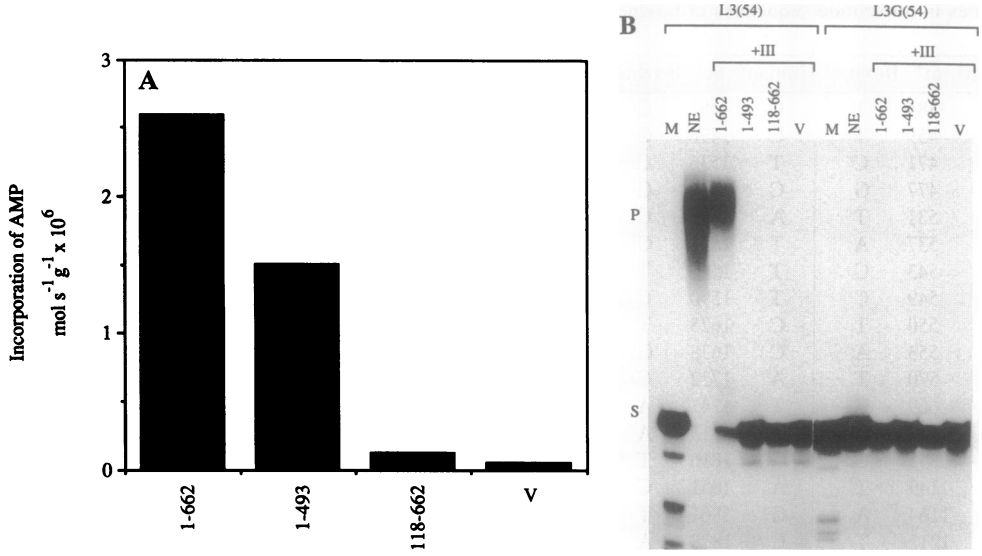

FIG. 3. Identification of functional regions in human PAP. (*A*) Detection of nonspecific PAP activity (AAUAAA independent) by incorporation of radioactively labeled AMP into trichloroacetic acid-precipitable polymers using purified recombinant PAPs. The structures of the different recombinant PAPs are indicated by numbers referring to amino acids of bovine PAP (14) present in each polypeptide. V refers to PAP activity in material obtained from *E. coli* (containing the cloning vector) purified as recombinant PAP. (*B*) Detection of specific adenosine addition activity (AAUAAA dependent). Precleaved RNA substrate [L3(54)] or precleaved AAGAAA mutated RNA substrate [L3G(54)] was incubated under conditions for specific adenosine addition activity together with nuclear extract (NE) or in reconstituted reaction mixture containing CPSF and the indicated recombinant PAPs (see above). The presence of CPSF is indicated by +III. Reacted RNA was purified and subjected to gel electrophoresis. The resulting fluorogram is shown. S and P denote locations of unreacted and polyadenylylated RNA substrates, respectively.

described. Fig. 3*A* shows that nonspecific PAP activity was detected when we incubated polypeptides containing amino acids 1–662 (PAP expressed by plasmid pPAP12) or 1–493 (PAP expressed by plasmid pPAP4) of human PAP. No activity was detected when an N-terminally truncated variant (amino acids 112–662; PAP expressed by plasmid pPAP8) was used. Thus, the N-terminal region of PAP was required for nonspecific polymerase activity while the C-terminal region was dispensable. Fig. 3*B* shows that AAUAAA-dependent polymerization (in the presence of Mg^{2+} and CPSF) was recovered only when the polypeptide containing amino acids 1–662 was used. Notably, we did not recover any AAUAAA-dependent polymerization when we used the C-terminally truncated variant, even though this variant showed high activity in the nonspecific PAP assay (compare Fig. 3*A* with 3*B*). Thus, we conclude that both the N- and C-terminal regions of PAP were required for AAUAAA-dependent polymerization.

DISCUSSION

In this report, we have shown that HeLa cells contain three PAPs, at least one of which is phosphorylated. We have also shown that PAP is composed of distinct functional regions. Recently, the primary structures of three mammalian PAPs were determined by molecular cloning (14, 15). The three forms of PAP consisted of 375, 689, and 740 amino acids. They will be named, for simplicity, PAP375, PAP689, and PAP740 in the rest of this discussion. PAP375 was found in a cDNA library made from HeLa cells, while PAP689 and PAP740 were of bovine origin. Comparison of the deduced amino acid sequences showed that PAP689 and PAP740 shared the first 662 amino acids, while their remaining amino acids (27 and 78 amino acids, respectively) were unique (14). PAP375 shared the first 371 amino acids with the two larger PAPs, while the remaining four C-terminally located amino acids were unique (15). It has been suggested that the two larger PAPs consist of four structural domains. These were an RNA binding domain located between amino acids 62 and 142, a tentative polymerase motif located between amino acids 120 and 230, a nuclear localization signal located around amino acid 500, and a tentative phosphorylation target defined as a serine/threonine-rich region located in the C-terminal part. PAP375, due to its smaller size, contains only the two N-terminally located domains (i.e., RNA binding domain and polymerase motif). The nucleotide sequence of human PAP revealed that it contained all these sequence motifs and that the nucleotide sequences of bovine and human PAPs were 96.7% identical, which translated into 99.2% similarity at the amino acid level (Tables 1 and 2). The changes at the amino acid level were located in the N-terminal part (3 amino acids) and in the C-terminal part (9 amino acids). We also found that 6 amino acids were tandemly repeated in the C-terminal part of human PAP.

The relationship between the three PAPs (PAP375, PAP689, and PAP740) identified by cDNA cloning and the three human PAPs (PAP 90 kDa, PAP 100 kDa, and PAP 106 kDa) that we identified by Western blot analysis is not yet established. The calculated molecular masses of PAP375, PAP689, and PAP740 are 43, 78, and 83 kDa, respectively. Thus, the estimated molecular masses of the three PAPs we detected are larger than the predicted sizes calculated from the amino acid compositions. However, it has been observed that *in vitro* translated recombinant PAP689 migrates as an 85-kDa polypeptide upon SDS/PAGE (14), while *E. coli*-produced PAP740 migrates as 82 kDa (15). Based on these observations, it seems likely that the discrepancies between predicted and estimated molecular masses to some degree are caused by anomalous migration during electrophoresis. It is worthwhile to mention that the sizes of the three human PAPs that we detected were not affected by the use of different protocols for extract preparation. The PAPs we detected were identified in HeLa cell extracts. Thus, these PAPs may have been subjected to posttranslational modification and, indeed, our data showed that PAP 106 kDa was phosphorylated (Fig. 2*B*) and, by chromatographic properties, related to PAP 100 kDa (Fig. 1). Finally, the large difference between the calculated mass of PAP375 and the three PAPs we

detected is most likely explained by the fact that our monoclonal antibodies did not detect a polypeptide corresponding to PAP375. Taken together, the simplest interpretation of these data would be that PAP689 corresponds to PAP 90 kDa while PAP740 corresponds to PAP 100 kDa and PAP 106 kDa. Mammalian PAPs have been purified by several investigators from a variety of different sources and the molecular mass of PAP has been determined to be around 50–60 kDa (12, 14, 15, 17, 29–34). The large difference in molecular mass of PAPs determined in previous studies and this study could be caused by partial proteolysis during extensive purification. In contrast to these studies, we have been able to detect PAP by Western blot analysis in crude extracts, thereby minimizing changes of the molecular masses by proteolysis of PAPs. Interestingly, Wahle *et al.* (15) reported that *E. coli*-produced PAP740 (having an apparent molecular mass of 82 kDa) was cleaved by proteolysis during purification, resulting in a polypeptide of 57 kDa that comigrated during electrophoresis with homogeneously purified bovine PAP. Ryner *et al.* (12) have described two different PAPs, having estimated molecular masses between 50 and 60 kDa, in HeLa cell nuclear extracts (NE PAPs I and II) and one PAP in cytoplasmic extracts (S100 PAP, most likely identical to NE PAP I). With regard to subcellular localization of these PAPs and their elution profiles upon Mono S chromatography, it seems likely that NE PAPI/S100 PAP corresponds to our PAPs 100 kDa and 106 kDa, while NE PAPII corresponds to PAP 90 kDa, in spite of the differences in estimated molecular size.

Our limited mutational analysis of human PAP (Fig. 3) revealed that both the N- and C-terminal regions of PAP were required for proper PAP activity. Deletion of the 118 N-terminally located amino acids resulted in a polypeptide lacking both specific (CPSF and AAUAAA dependent) and nonspecific (AAUAAA-independent polymerization in the presence of Mn^{2+}) PAP activity. This lack of activity was most likely caused by the deletion of the well conserved RNA binding domain motif located in this region of PAP (14, 15). Deletion of amino acids located C-terminally of amino acid 493 resulted in a polypeptide that retained nonspecific PAP activity but lacked specific activity. This shows that the C-terminal region of PAP is required for specific PAP activity and suggests that the C-terminal region of PAP interacts with polyadenylylation factor CPSF. Raabe *et al.* (14) have previously shown that a polypeptide consisting of the first 538 N-terminally located amino acids retained both specific and nonspecific PAP activity. Thus, this observation together with our data suggest that a major determinant for specific PAP activity is located between amino acids 493 and 538.

The functional significance of multiple forms of PAP is not yet known. One obvious explanation could be that different PAPs are functional in the nucleus and the cytoplasm. This conclusion is supported by our observation that PAP 90 kDa was nuclear, while PAPs 100 kDa and 106 kDa were present in both nuclear and cytoplasmic fractions. Another explanation could be that the structure of PAP influences its interaction with the RNA substrate, ATP, or other polyadenylylation factors. Intriguingly, the potential target for phosphorylation, the serine/threonine domain of PAP, coincides with the region of PAP that was required for CPSF and AAUAAA-dependent adenosine addition (Fig. 3). This indicates that phosphorylation/dephosphorylation could be an important mechanism by which the activity and/or substrate specificity of PAP is regulated. The development of monoclonal antibodies will undoubtedly facilitate studies aimed at understanding the functional significance of multiple forms of PAPs.

The completion of this work is dedicated to the memory of A.Å. We thank Drs. U. Pettersson, E. Bridge, and C. Phillips for valuable suggestions and discussions throughout this work. This work was supported by the Swedish Natural Science Research Council and grants from Uppsala University.

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