A Novel Nuclear Human Poly(A) Polymerase (PAP), PAPγ*

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Poly(A) polymerase (PAP) is present in multiple forms in mammalian cells and tissues. Here we show that the 90-kDa isoform is the product of the gene PAPOLG, which is distinct from the previously identified genes for poly(A) polymerases. The 90-kDa isoform is referred to as human PAPγ (hsPAPγ), hsPAPγ shares 60% identity to human PAPII (hsPAP II) at the amino acid level. hsPAPγ exhibits fundamental properties of a bona fide poly(A) polymerase, specificity for ATP, and cleavage and polyadenylation specificity factor/hexanucleotide-dependent polyadenylation activity. The catalytic parameters indicate similar catalytic efficiency to that of hsPAPII. Mutational analysis and sequence comparison revealed that hsPAPγ and hsPAPII have similar organization of structural and functional domains. hsPAPγ contains a U1A protein-interacting region in its C terminus, and PAPγ activity can be inhibited, as hsPAPII, by the U1A protein. hsPAPγ is restricted to the nucleus as revealed by in situ staining and by transfection experiments. Based on this and previous studies, it is obvious that multiple isoforms of PAP are generated by three distinct mechanisms: gene duplication, alternative RNA processing, and post-translational modification. The exclusive nuclear localization of hsPAPγ establishes that multiple forms of PAP are unevenly distributed in the cell, implying specialized roles for the various isoforms.

The majority of mammalian mRNAs end with a 200- to 250-adenosine residue tail at their 3'-ends. The function of the poly(A) tail is not fully understood, but studies have highlighted its role in regulating gene expression via translation and mRNA stability (1–4). The mRNA poly(A) tail is added post-transcriptionally, and the biochemistry of mammalian nuclear polyadenylation has been extensively studied (reviewed in Refs. 5–8). Polyadenylation is a multistep and multicomponent reaction and proceeds through two separable steps, pre-mRNA cleavage and adenosine addition. Both reactions are dependent on a highly conserved sequence element, the hexanucleotide AAUAAA. At least six trans-acting protein factors are required for the reaction in vitro (5–8).

Poly(A) polymerase (PAP),¹ is the enzyme responsible for mRNA poly(A) tail synthesis. PAP has been identified and cloned from several eukaryotic species, e.g. yeast, human, mouse, bovine, frog, and chicken (9–19). Interestingly, multiple forms of PAP are present in cell lines and tissues of several species (9, 11, 13–16, 19–21). In HeLa cell nuclear extracts, three isoforms, having apparent molecular masses of 90, 100, and 106 kDa, have been found (16). The molecular mechanisms for generating all these isoforms are still not completely understood. However, molecular cloning has established that at least five isoforms of full-length PAP can be generated by alternative RNA processing (15, 17).² It is also known that phosphorylation contributes to the multiplicity of PAP (9, 16, 20). Recently it has been established that PAP and PAP-related genes are present in the human genome (11, 13, 14, 22). Therefore, so far at least three distinct mechanisms can generate multiple isoforms of PAP: gene duplication, alternative RNA processing, and post-translational modification. These phenomena unexpectedly increase the diversity of PAP and raise questions about the functional significance of multiple PAPs in vivo.

It seems reasonable to hypothesize that different PAPs are responsible for different functions in vivo, because PAP participates in a whole set of different reactions, e.g. RNA cleavage at the poly(A) site and AAUAAA-dependent or -independent poly(A) tail synthesis (23, 24). Biochemical fractionation studies have indicated that different forms of PAP reside at different subcellular compartments (16, 25). A testis-specific PAP has been identified, suggesting that some isoforms of PAP are restricted to certain developmental stages or tissues (11, 13).

In this report we have molecularly cloned the human 90-kDa PAP isoform, previously identified in HeLa nuclear extracts. The 90-kDa isoform is encoded by a distinct locus recently identified as a PAP-related gene (22). The gene has been named PAPOLG, and its product is hsPAPγ. In a recent report (14) the same gene was implicated in monoadenylation of small RNAs. Here, we show that hsPAPγ is a bona fide poly(A) polymerase harboring both nonspecific and CPSF/AAUAAA-dependent polyadenylation activity. hsPAPγ is exclusively localized in the nucleus.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Full-length hsPAPγ and various deletion mutants were molecularly cloned by a standard RT-PCR procedure using the following strategy. A 371-amino acid N-terminal fragment of hsPAPγ was amplified by RT-PCR using HeLa total RNA and primers a and b, subcloned into pGEM-T vector (Promega Inc.), and further subcloned into the pET-32(a) vector (Novagen Inc.) between the NcoI and SacI sites. The resulting clone was called pPAPγ(H1–493), and its product is hsPAPγ. In a recent report (14) the same gene was implicated in monoadenylation of small RNAs. Here, we show that hsPAPγ is a bona fide poly(A) polymerase harboring both nonspecific and CPSF/AAUAAA-dependent polyadenylation activity. hsPAPγ is exclusively localized in the nucleus.

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‡ The abbreviations used are: PAP, poly(A) polymerase; CPSF, cleavage and polyadenylation specificity factor; RT-PCR, reverse transcription-polymerase chain reaction; b-MEOH, β-mercaptoethanol; DTT, dithiothreitol; PBS, phosphate-buffered saline; PFA, paraformaldehyde; kb, kilobase(s); NLS, nuclear localization signal.

¶ C. B. Kyriakopoulou, H. Nordvarg, and A. Virtanen, unpublished results.
was introduced to enable cloning into the pCALc vector and adds two restriction sites for AATCCAAAAGATTGTCTCTGGATAGC-3. Restriction sites for introduction between the TTA and GTACC; (5) GAGACTTCAAGAGATGTCTGCAAACACC-3. Primers used were as follows: (a) 5’-CACCCTTG-GAAAGATGTCTGCAAACACC-3’; (b) 5’-GAGAGGTGTCCACTCTTTTGAAAGAAATTCGGTGG-3’; (c) 5’-GCCCTGTCTGG-GGTACC-3’, (d) 5’-GAGACTTCAAGAGATGTCTGCAAACACC-3’. Proteins were eluted with 0.5 mM oligoA15, and no peptide, 9.6 pmol of U1A di-peptide or U1A mono-peptide, respectively. The reaction mixture was ordered to the specific and nonspecific assays in this study. To normalize the differences in PA kinetics for the deletion mutants the same enzyme concentration was used, however, the primer concentrations were in the 0.5–5.5 μM range. Unthawed recombinant hsPAP and hsPAPII were stabilized by addition of 0.05% Nonidet P-40, 20% glycerol, and 1 mM DTT for the time kept on ice. Reactions were stopped by precipitation of the insoluble polyadenylated product in acid conditions (5% trichloroacetic acid-1% sodium pyrophosphate) in glass fiber filters and washed three times with 5% trichloroacetic acid (35). The U1A inhibition assay of hsPAP was done using the U1A di-peptide (from N termini to N termini; CAAAERDRKREKRKAAAA, Sigma-Genosys) in the nonspecific polyadenylation activity assays were carried out as described previously (21, 29) with modifications optimizing the activity; the reaction mixture (25 μl) contained: 100 mM Tris/HCl buffer, pH 7.5 (measured at room temperature), 40 mM KCl, 0.040 mM EDTA, 10% glycerol, 1 mM DTT, 9 units of RNAase (ribonuclease inhibitor), 0.1% Nonidet P-40, 0.5 mM MnCl₂, 0.5 mg/ml bovine serum albumin, 0.5 mM cold ATP, 1.2 μCi of [α-³²P]ATP (3000 Ci/mmol) and 2 μl oligoA₁₅ (Pharmacia), and the reaction was performed for 20 min at 37 °C. One unit of PAP is defined as the amount of enzyme needed for incorporation of 1 pmol of AMP per min. Reaction rate was measured in a linear range versus PAP concentrations (8–23 nM) and time (10–30 min). Kinetic parameters were determined using oligoA₁₅ in the concentration range 0.0125–2 μM for the full-length hsPAP and hsPAPII. In the case of kinetic estimations for the deletion mutants the same enzyme concentration was used, however, the primer concentrations were in the 0.5–5.5 μM range. Unthawed recombinant hsPAP and hsPAPII were stabilized by addition of 0.05% Nonidet P-40, 20% glycerol, and 1 mM DTT for the time kept on ice. Reactions were stopped by precipitation of the insoluble polyadenylated product in acid conditions (5% trichloroacetic acid-1% sodium pyrophosphate) in glass fiber filters and washed three times with 5% trichloroacetic acid (35).

The U1A inhibition assay of hsPAP was done using the U1A dipeptide (from N termini to N termini; CAAAERDRKREKRKAAAA, Sigma-Genosys) in the nonspecific polyadenylation assay described above but modified to conditions previously described (31, 32). The reaction mixture above was modified to final concentrations: 20 mM Tris/HCl, 60 mM KCl, 10% glycerol, 5 mM DTT, 0.1–0.2 μM oligoA₁₅, and no peptide, 9.6 pmol of U1A di-peptide or U1A mono-peptide, respectively.

The specific polyadenylation activity was carried out as described previously (29, 33) with modifications to normalize the differences in between the specific and nonspecific assays in this study. [³²P]-Labeled and capped RNA substrates (LS954, LS654) were synthesized by in vitro transcription and purified as previously described (34). CPSF partially purified from calf thymus (35) and recombinant hsPAP were used as specified in the figure legends. The reaction mixture (25 μl) contained: 100 mM Tris/HCl buffer, pH 8.3 (measured at room temperature), 40 mM KCl, 0.040 mM EDTA, 10% glycerol, 1 mM DTT, 9 units of RNAase (ribonuclease inhibitor), 0.1% Nonidet P-40, 0.72 mM MgCl₂, 1 mM cold ATP, 2.5% polyvinyl alcohol, 20 mM oligoA₁₅. The reaction was performed for 20 min at 30 °C. The reaction was stopped in Proteinase K buffer, and the incubated RNA product was extracted and resolved in 10% polyacrylamide (acrylamide/bisacrylamide 19:1)‐7 M urea. Immunocytochemical Methods—HeLa cells were grown up to 50–70% confluency on coverslips in the presence of Dulbecco’s modified
Eagle’s medium supplemented with glutamine and 10% fetal calf serum (Life Technologies, Inc.). Coverslips were washed two times in PBS and fixed in 1% paraformaldehyde (PFA) in PBS (pH 7.3) for 3 min, extracted with 0.5% Triton X-100 in PBS for 15 min, and then post-fixed in 4% PFA in PBS for 10 min. For immunofluorescence staining the following antibodies were used: Primary antibodies; 20:14, anti-PAPγ, Y12, and anti-PAPIIE22 in dilutions 1:2, 1:40, 1:10, 1:20, respectively; the respective pre-immune serum was used in the cases of polyclonal antibodies at the same dilutions. Secondary antibodies; species-specific goat anti-mouse IgG coupled to biotin (Amersham Pharmacia) or to Alexa Fluor 488 (emission at green spectrum) (Molecular Probes), species-specific goat anti-rabbit IgG coupled to Alexa Fluor 594 (emission at red channel) (Molecular Probes). All coverslips were mounted in Vectashield (Vector Laboratories) and shielded. Where polyclonal serum was used, a control antibody was used, the cells were washed 4 min with blocking reagent buffer (5% Eliza blocking reagent, Roche Molecular Biochemicals) at room temperature. Subsequently they were incubated for 30 min or 2 h with primary antibodies diluted in blocking reagent and washed 3 × 5 min in PBS. Secondary antibodies were incubated for 30 min or 1 h and washed 3 × 5 min in PBS. In the case where dual staining experiments were performed with monoclonal antibody 20:14 and anti-PAPγ and a biotin labeled anti-mouse secondary antibody was used, the cells were washed 4 × 5 min in PBS and incubated for 1 h with streptavidin coupled to Alexa Fluor 488 (Molecular Probes). All coverslips were mounted in Vectashield (Vector Laboratories) and shielded. Where polyclonal serum was used, a control pre-immune serum was used to subtract the background signal. Fluorescence microscopy was performed in an Axioplan 2 imaging fluorescence microscope, using a 100× objective lens. Image analysis was done by the Axion vision 3 software.

Transfection Methods—HeLa cells, grown up to 50% confluency, were transfected using plasmids pPAPγEGFP1–371, pPAPγEGFP1–506, and pPAPγEGFP1–736, and left to grow for 10 or 24 h. The Superfect transfection reagent (Qiagen) was used, and conditions were optimized for ratio of DNA:transfection reagent, as suggested by the manufacturer. Cells were fixed in 4% PFA (in PBS, pH 7.3), shielded, and analyzed in a fluorescence microscope by excitation at 495 nm and emission at the green spectrum.

RESULTS

Molecular Cloning of Human PAPγ—Monoclonal antibodies NN:2 and 20:14 raised against hsPAPII recognize three isoforms of PAP: 90, 100, and 106 kDa in sizes (16). However, a polyclonal antibody raised against bovine PAPII recognizes only the two larger forms (24, 36). A reason for this discrepancy could be that the monoclonal antibodies recognize a common epitope shared among all three isoforms of PAP, whereas the polyclonal antibody is directed against epitopes not present in the 90-kDa form. This experimental evidence suggests that the 90-kDa isoform of PAP has unique antigenic epitopes unrelated to the 100- and 106-kDa forms, implying that the 90-kDa isoform could be encoded by a separate gene.

To identify potential human PAP-related genes we regularly searched using the BLAST algorithm (37) high throughput (htgs) and non-redundant sequence data bases, while they were being released during the human genome sequencing project (38). During these searches we identified a PAP-related sequence in the human genomic clone (AC011245.6) located on chromosome 2. The same locus has recently been identified as a PAP-related gene and as a small RNA monoadenylating enzyme (14, 22). Further data base searches revealed several overlapping expressed sequence tags. These results combined with 3′-RACE semi-nested RT-PCR allowed us to predict the sequence of an mRNA encoding a potential PAP. The novel human gene was named PAPOLG and its product hsPAPγ. The sequence information was used to molecularly clone cDNAs originating from HeLa cells by RT-PCR. A schematic drawing of the exon/intron organization of hsPAPγ and comparison to the previously reported gene hsPAPII is shown in Fig. 1A. The deduced amino acid sequence of hsPAPγ is presented in Fig. 1B and compared with the bovine and human PAPIL. Structural and functional domains/motifs are also represented. A comparison using the ClustalX algorithmic approach (39) showed that hsPAPγ has an overall identity of 67% at the nucleotide level and 60% identity at the amino acid level.

Organization of the hsPAPγ Gene—The genes encoding hsPAPII and hsPAPγ span 62.5 and 37 kb of genomic sequences, respectively. They both contain 22 exons, and all splice sites obey the GT/AG rule (40). The topology and the sizes of exons 2–16 are shared between the two genes, implicating that they share a common ancestor and arose through gene duplication (41, 42). Sequence comparison (Fig. 1A) revealed that the exons 1 of both genes were unrelated to each other; exons 2–16 were highly conserved both at the amino acid and nucleotide levels, having an overall identity of ~75% at both levels; exons 17–21 were less conserved in their sequences whereas exon 22 exhibits a high degree of identity both at the amino acid and nucleotide levels. The last half of exon 22 encodes a potential U1A protein-interacting region (see also below).

Structural Organization of hsPAPγ—An inspection of known structural and functional motifs/domains in hsPAPII revealed that several of those were conserved in hsPAPγ. These motifs/domains included amino acids important for catalysis, recognition of the ATP substrate, and RNA binding (29, 43) (Fig. 1B). The cyclin-recognition motif and four of the seven consensus and non-consensus phosphorylation sites that have been mapped for cyclin dependent kinases were conserved (44, 45). Two nuclear localization signals (NLS) (46) were conserved between the two PAPs, whereas a third putative bipartite NLS was found in the C-terminal end of hsPAPγ. The sequence encompassing the U1A protein interaction region is highly conserved (14 out of 18 amino acids).

The 90-kDa Isoform Is the Product of the Novel hsPAP Gene—To raise an antisera specific for hsPAPγ, we molecularly cloned the C-terminal region of hsPAPγ splicing amino acids 521–683 into the pET32(a) vector. The recombinant polypeptide was expressed in E. coli, purified to homogeneity, and used to raise an hsPAPγ-specific antisera, named anti-PAPγ. Fig. 2A shows that the obtained serum was specific for hsPAPγ, because it only recognized recombinant versions of hsPAPγ and not hsPAPII. In these experiments C-terminally calmodulin-tagged recombinant proteins were used to exclude recognition of the N-terminal-located tags present in the polypeptide used for immunization. As predicted the monoclonal antibody (20:14) raised against hsPAPII recognized hsPAPγ (Fig. 2B). An analysis of C-terminal deletion constructs of hsPAPγ revealed that the epitope was located in the highly conserved N-terminal region of hsPAPγ and hsPAPII (Figs. 1B and 2B).

To investigate if the anti-PAPγ serum recognized the 90-kDa isoform of HeLa cell PAP, we probed HeLa nuclear extracts. Fig. 2C shows that the serum exclusively recognized the 90-kDa species. An hsPAPII-specific polyclonal antisera, named anti-PAPIIE22, was raised against a synthetic peptide of exon 22 (amino acids 715–726) of hsPAPII. An affinity-purified anti-PAPIIE22 recognized the 100- and 106-kDa mobility species and not the 90-kDa isoform (Fig. 2C). Thus, we conclude that the 90-kDa isoform corresponds to hsPAPγ, the product of the PAPOLG gene.

Properties of hsPAPγ—To investigate if hsPAPγ had polyadenylating activity, we used the nonspecific polyadenylation assay in the presence of Mn(II) and various nucleotide triphosphates. The assay was designed so that the amount of hsPAPγ was provided in excess. Fig. 3A shows that recombinant PAPγ(1–736) exhibited specificity for incorporation of ATP, whereas incorporation of UTP, GTP, CTP, and dATP were inefficient and stopped after the addition of one to two molecules of the respective nucleotide analogue.

PAPII acquires specificity for hexanucleotide containing
mRNAs in the presence of cleavage and polyadenylation specificity factor (CPSF) (33). To investigate whether hsPAP had this classical property, we performed specific polyadenylation assays in the presence of Mg(II) and partially purified CPSF from calf thymus (35). Fig. 3B shows that hsPAP exhibited CPSF/AAUAAA-dependent activity, because the L3(54) RNA substrate was efficiently polyadenylated compared with the hexanucleotide mutated L3G(54) RNA substrate. Furthermore, hsPAP did not exhibit any polyadenylation activity in the presence of Mg(II) when CPSF was omitted. A C-terminal deletion mutant PAP(H1–371) was inactive, as described for bovine PAPII (29).

We conclude that hsPAP exhibits the fundamental catalytic properties for a bona fide poly(A) polymerase, i.e. specific incorporation of ATP- and CPSF/AAUAAA-dependent polyadenylation activity.
Kinetic Parameters and Mutational Analysis of hsPAPγ—To further characterize hsPAPγ we measured the kinetic parameters ($K_m$ and $V_{max}$). Nonspecific polyadenylation activity was measured in the presence of Mn(II) and highly purified oligoA$_{15}$. The $K_m$ for hsPAPγ was shown to be 0.051 μM using a variety of calculation methods. The ratio $V_{max}/K_m$ that represents the real efficiency of the reaction in terms of affinity to the primer and actual catalytic capacity was determined. The kinetic parameters for hsPAPγ (Table I) are in a similar range as for recombinant hsPAPIII and for the reported bovine PAPIII (24, 29). A calmodulin tag at the C terminus of hsPAPγ and hsPAPIII did not significantly alter the kinetic parameters (Table I). Thus, recombinant hsPAPγ has similar kinetic properties as hsPAPIII.

Functional Analysis of C- and N-terminal Deletion Mutants—To identify regions in hsPAPγ important for polyadenylation activity, we constructed C-terminal deletion mutants (see “Experimental Procedures”). In Table I the $K_m$ and $V_{max}/K_m$ for these deletion mutants are shown. The increased $K_m$ of PAPγ(H1–493) and PAPγ(H1–506) compared with full-length PAPγ(H1–736) suggests that hsPAPγ contains a primer binding domain spanning the NLS 1 region, as proposed for mammalian PAPIII (29). Interaction with CPSF has previously been implicated in this region of bovine PAPIII (29). Table II shows the nonspecific and specific polyadenylation activities of deletion mutants and full-length hsPAPγ using the same molar

![Figure 2](http://example.com/figure2.png)

**Fig. 2.** The 90-kDa isoform of human PAPs is PAPγ, A, C-terminally calmodulin-tagged recombinant proteins expressed and purified from *E. coli* were resolved by 6.25% SDS/polyacrylamide electrophoresis, blotted, and subsequently probed with anti-PAPγ polyclonal serum (lanes 1–4, dilution 1:4000) or with preimmune serum (lanes 5–8). Lanes 1 and 5, PAPγ(H1–736C); lanes 2 and 6, PAPγ(H1–683C); lanes 3 and 7, PAPII(H1–745C); lanes 4 and 8, protein purified from *E. coli* containing the calmodulin vector only. B, recombinant proteins purified from *E. coli* were resolved by 7% SDS/polyacrylamide electrophoresis, blotted, and probed with 20:14 monoclonal antibody (dilution 1:20). The position of molecular size markers are indicated in kilodaltons.

![Figure 3](http://example.com/figure3.png)

**Fig. 3.** hsPAPγ is a bona fide poly(A) polymerase. A, specificity for ATP. Polyadenylation activity assays in the presence of Mn(II) were performed as detailed under “Experimental Procedures.” Ribonucleotides and dATP, as indicated, were tested at 0.5 mM. 5′-End-labeled primer oligoA$_{15}$ (300 fmol), and recombinant PAPγ(H1–736) (300 fmol lanes 2, 5, 8, 11, 14), 600 fmol (lanes 3, 6, 9, 12, 15), or 900 fmol (lanes 4, 7, 10, 13, 16) were added to the reactions. The ratio of enzyme to primer was 3:1. Lane 1, no PAP added; lanes 2–4, ATP; lanes 5–7, UTP; lanes 8–10, GTP; lanes 11–13, CTP; and lanes 14–16, dATP. Reactions were incubated at 37 °C for 30 min, and reacted oligoA$_{15}$ was purified and resolved in a 16% sequencing polyacrylamide gel. The resulting gel was exposed and analyzed by a 4005 PhosphorImager (Molecular Dynamics). S and P denote location of oligoA$_{15}$ substrate and polyadenylated product, respectively. B, hsPAPγ exhibits CPSF/AUUAAA-dependent polyadenylation activity. Specific polyadenylation activity was performed as detailed under “Experimental Procedures.” The reaction contained 70 fmol of RNA substrate (13/54) in lanes 1–10 and L3G(54) in lanes 11 and 12), CPSF, PAPγ(H1–736), and/or PAPγ(H1–371) were included as indicated. Lane 3, 130 fmol of PAPγ(H1–371); lanes 4–11, 20, 40, 80, 160, 320, 640, 80, and 80 fmol of PAPγ(H1–736), respectively. The incubated RNA substrates were extracted as described in panel A and resolved in 10% polyacrylamide-7 M urea gel.
Dual staining approach using indirect immunofluorescence tested whether it could be inhibited by U1A. Fig. 4 shows that finally located U1A interaction motif is functional, and hsPAP activity can be inhibited by U1A as previously reported for bovine PAPII. We conclude that hsPAP and hsPAPII have similar organization of functional domains.

Inhibition of hsPAPγ Activity by U1A—The inhibition of PAPII by two molecules of U1A protein is well documented (31, 32). The inhibition requires the last 18 amino acids of PAPII and a region of U1A corresponding to amino acids 102–115. Because hsPAPγ contains a putative U1A interaction motif, we tested whether it could be inhibited by U1A. Fig. 4 shows that hsPAPγ was inhibited by an U1A di-peptide but not by the U1A mono-peptide. hsPAPII was inhibited to the same extent under these conditions (data not shown). In these experiments C-terminally tagged recombinant PAPγ (1–736C) was used, because even a loss of two to three amino acids from the C terminus abolishes the inhibition effect. Thus, the C-terminally located U1A interaction motif is functional, and hsPAPγ activity can be inhibited by U1A as previously reported for bovine PAPII.

Subcellular Localization of hsPAPγ—Biochemical fractionation studies suggested that the 90-kDa isofrom of PAP was nuclear, whereas the 106- and 110-kDa isofroms were both nuclear and cytoplasmic (16). To study whether hsPAPγ localize to the nucleus we used antibodies 20:14 and anti-PAPγ in a dual staining approach using indirect immunofluorescence techniques. Fig. 5E shows that the monoclonal antibody 20:14 gave a nuclear and weak cytoplasmic staining, as previously reported (36). In the same cell, native hsPAPγ exhibited an exclusive nuclear distribution (compare panels E and F, Fig. 5). The polyclonal sera specific for exon 22 of hsPAPII type (anti-PAPIIex22) stained both cytoplasm and nucleus (Fig. 5C). Control antibody Y12, recognizing the Sm epitope of general nuclear splicing factors, showed an expected nuclear distribution (Fig. 5A). Intriguingly, in dual staining experiments using anti-PAPγ and Y12, a high degree of co-localization was observed indicating that hsPAPγ localizes close to structures enriched in general splicing factors (data not shown). Our data show that endogenous hsPAPγ is exclusively nuclear, whereas hsPAPII is both nuclear and cytoplasmic.

The C-terminal Region of hsPAPγ Is Important for Nuclear Localization—To identify regions important for guiding hsPAPγ to the nucleus, we used a transient expression approach with hsPAPγ/EGFP chimeric proteins. Two C-terminal deletion mutants were constructed: PAPγ(EGFP1–506) containing the putative NLS 1 region and full-length PAPγ(EGFP1–736) containing all three putative NLS 1, 2, and 3 regions. Fig. 5 (H–J) shows that PAPγ(EGFP1–736) was exclusively nuclear, whereas PAPγ(EGFP1–506) was both nuclear and cytoplasmic.

### Table I

| Kinetic parameters of human PAPs | $K_m$ | $V_{max}/K_m$ |
|---------------------------------|-------|---------------|
| PAPγ(H1–736)                   | 0.051 | 105.0         |
| PAPγ(H1–736C)                  | 0.032 | 59.5          |
| PAPII(1–745C)                  | 0.045 | 121.0         |
| PAPγ(H1–506)                   | 0.72–2.53 | 10.70       |
| PAPγ(H1–493)                   | 10.8  | 1.35          |

*If the deviation of the parameters was <40%, the average is indicated, otherwise the range is shown. The assays were carried out at least two to five times, using two independent recombinant protein preparations.

### Table II

Deletion analysis of PAPγ

|                        | Non-specific activity | Specific activity |
|------------------------|----------------------|------------------|
| PAPγ(H1–736)           | 100                  | 100              |
| PAPγ(H1–683)           | 75                   | 85               |
| PAPγ(H1–575)           | 102                  | 85               |
| PAPγ(H1–506)           | 65                   | 70               |
| PAPγ(H1–493)           | 14                   | 20               |
| PAPγ(H1–736)           | 108                  | 97               |
| hsPAPγ(H1–371)         | <0.1                 | <1               |
| PET32(a) plasmid       | <0.1                 | <1               |

*Relative incorporation of AMP as measured the non-specific polyadenylation activity in the presence of Mn(II).

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3 S. Gunderson, personal communication.
hsPAP and PAP(EGFP1–736) and PAP(EGFP1–506) have predicted molecular masses of ~115 and 70 kDa, respectively. This makes both proteins too large to passively enter the nucleus (47). Thus, we conclude that NLS 1 can mediate partial nuclear import and that the entire C-terminal region (amino acids 506–735) of hsPAP is required for exclusive nuclear localization. We note that this region contains the putative NLS 2 and 3 elements.

**DISCUSSION**

**Multiple Isoforms of PAP**—Multiple forms of PAP are present in mammalian cell lines and tissues (11, 13, 14, 16, 20, 21). In HeLa cell nuclear extracts three isoforms, having apparent molecular masses of 90, 100, and 106 kDa, have been found (16). In this study, we show that the 90-kDa isoform (hsPAPγ) is encoded by a distinct locus named **PAPOLG**. Similar sequences and exon topology of the hsPAPγ and hsPAPII genes suggest that the two genes arose by gene duplication (Fig. 1A).

In summary, at least three mechanisms can encounter for multiple isoforms of PAP, gene duplication, alternative RNA processing, and post-translational modifications (11, 13, 15–17, 20–22). These phenomena unexpectedly increase the diversity of PAP and provoke questions about the functional significance of multiple PAPs in vivo.

**hsPAPγ Is a Bona Fide PAP**—Several lines of evidence suggest that hsPAPγ is a bona fide PAP. Most importantly, hsPAPγ is specific for the ATP substrate (Fig. 3A), shows hexanucleotide-dependent polyadenylation activity in the presence of CPSF (Fig. 3B), and has similar kinetic parameters as human/bovine PAPI (Table I and Refs. 24, 29). Furthermore, the amino acid sequence of the region required for PAPII catalytic activity and CPSF/hexanucleotide-dependent polyadenylation activity is highly conserved in hsPAPγ, suggesting similar functional and structural properties. The resolved crystal structure of PAP (43, 48) demonstrated that amino acids 365 to 513 of bovine PAP, folds into a compact globular domain topologically similar to the RNA binding domains of several RNA binding proteins. The same region of hsPAPγ contains most likely a similar RNA binding domain and is, in analogy to bovine PAPII, important for CPSF/hexanucleotide-dependent activity (Tables I and II).

hsPAPγ has been implicated as an enzyme responsible for monoadenylation of small RNAs (14). However, the monoadenylation activity is, in contrast to the polyadenylation activity of PAPII (31, 32) and hsPAPγ (Fig. 4), not inhibited by U1A (49). The reason for this inability of inhibition is not known yet. Possibly, the U1A inhibitory effect does not occur unless multiple adenosine residues are incorporated by PAP. Another possibility is that an alternatively processed isoform of hsPAPγ, lacking exon 22, is responsible for the more specialized monoadenylation function in vivo.

**hsPAPγ Is a Nuclear PAP**—In this study we show that hsPAPγ (i.e., 90-kDa isoform) resides exclusively in the nucleus, whereas the 100- and 106-kDa isoforms of PAP are both nuclear and cytoplasmic (Fig. 5), in keeping with our previous cell fractionation studies (16). It has been reported that polyadenylation factors and a subset of PAP are concentrated at sites of RNA synthesis and associated with domains enriched in splicing factors (38, 50). The antibody used in these studies was the monoclonal 20-14, which recognizes both hsPAP and hsPAPγ. It is tempting to speculate that the subset of PAP at sites of RNA synthesis and 3′-end processing is hsPAPγ, because we have observed a high degree of co-localization of hsPAPγ with basal splicing factors (data not shown). These observations suggest that hsPAPγ participates in the nuclear polyadenylation reaction. In support of this we have previously shown that a fraction enriched in hsPAPγ is active both in pre-mRNA cleavage and poly(A) addition (51).

In Fig. 5 we show that the PAPγ/EGFP chimeric hsPAPγ is imported into the nucleus. The molecular mass of this chimera is higher than the size limit for passive diffusion through the nuclear pore. This suggests an active transport mechanism (47, 52). The region important for guiding hsPAPγ to the nucleus must reside in the C-terminal region, because elimination of it (amino acids 507–736) disturbs the observed nuclear pattern of PAPγ/EGFP chimeric protein. It has been reported that the NLS 1 and 2 elements are important for efficiently directing bovine PAPI and PAPII to the nucleus using transfection experiments (46). In our experiments the NLS 1 element of hsPAPγ was needed for partial nuclear localization. A careful inspection of the C-terminal sequence of hsPAPγ revealed a putative bipartite NLS, spanning amino acids 680–714 (NLS 3, Fig. 1B). Two potential phosphorylation sites can be predicted in the C terminus of hsPAPγ, upstream and in close vicinity of the putative NLS 3. There is increasing amount of data suggesting that regulated phosphorylation is a mechanism that modulates recognition of NLSs by components of the nuclear import machinery (52). A detailed site-directed mutagenic analysis of hsPAPγ combined with the fusion of NLS 3 to EGFP constructs would be informative to investigate the interesting possibility that phosphorylation may regulate subcellular distribution of hsPAPγ.

**Phylogenetic Conservation of hsPAPγ—PAPγ is a phylogenetically conserved vertebrate variant of PAP present already in the bony fish branch (Table III). The existence of a goldfish hsPAP orthologue supports the hypothesis that gene duplication was an important event in the evolution of early vertebrates (41, 42). In a newly duplicated gene, mutations are generally selectively neutral because of redundancy of genetic information (41, 42). The rate between degenerative and advantageous mutations can be influenced in the gene’s favor, if the probability of forming novel regulatory interactions with other genes that are evolving in parallel occurs. Once only a new function has been acquired, the duplicated parologue will be retained in the population as an evolutionary change. The unique C-terminal region (amino acids 507–736) of hsPAPγ could be implicated in directing a new function. It is evident that the evolutionary machinery has selected nucleotide sub-

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**TABLE III**

| Organism* | hsPAP** | hsPAPγ** | U1A domain* |
|-----------|---------|----------|-------------|
| Human (testis) PAP | 84 | 59 | – |
| Bovine PAP II | 97 | 60 | + |
| Mouse (testis) PAP | 85 | 60 | – |
| Chicken PAP | 88 | 51 | – |
| Xenopus PAP | 81 | 56 | + |
| Goldfish PAP | 58 | 72 | + |
| Drosophila PAP | 46 | 43 | – |
| C. elegans putative PAP | 43 | 39 | – |
| C. elegans PAP-related | 38 | 36 | – |
| Arabidopsis PAP | 28 | 25 | – |
| Arabidopsis PAP-related | 31 | 31 | – |
| Yeast PAP | 32 | 34 | – |

*The accession numbers are: human PAPII (P51003), human PAPγ (AC011245.6), human testis PAP (Q9NRJ5), bovine PAP (P25500), mouse testis PAP (Q9R1R3), chicken PAP (Q73841), Xenopus laevis PAPI (P51004), goldfish PAP (BAB39139), Drosophila melanogaster PAPI (Q9VMXT), Caenorhabditis elegans putative PAP (Q99956), C. elegans PAP-related (Q9UF23), Arabidopsis thaliana PAP (Q9LXXO), A. thaliana PAP-related (Q9LMT2), yeast PAP (P29468).

**The comparison was done using the multiple sequence alignment mode of the ClustalX algorithmic approach. Percentage of identity compared to hsPAPγ or hsPAPγ is listed.**

**The presence of a U1A-interacting region in the C-terminal end is indicated when present.**

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Nuclear Poly(A) Polymerase
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