Poly(A) Nuclease Interacts with the C-terminal Domain of Polyadenylate-binding Protein Domain from Poly(A)-binding Protein

Received for publication, February 12, 2007, and in revised form, June 26, 2007 Published, JBC Papers in Press, June 26, 2007, DOI 10.1074/jbc.M701256200

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The poly(A)-binding protein (PABP) is an essential protein found in all eukaryotes and is involved in an extensive range of cellular functions, including translation, mRNA metabolism, and mRNA export. Its C-terminal region contains a peptide-interacting PABC domain that recruits proteins containing a highly specific PAM-2 sequence motif to the messenger ribonucleoprotein complex. In humans, these proteins, including Paip1, Paip2, eRF3 (eukaryotic release factor 3), Ataxin-2, and Tob2, are all found to regulate translation through varying mechanisms. The following reports poly(A) nuclease (PAN) as a PABC-interacting partner in both yeast and humans. Their interaction is mediated by a PAM-2 motif identified within the PAN3 subunit. This site was identified in various fungal and animal species suggesting that the interaction is conserved throughout evolution. Our results indicate that PABP is directly involved in recruiting a deadenylase to the messenger ribonucleoprotein complex. This demonstrates a novel role for the PABC domain in mRNA metabolic processes and gives further insight into the function of PABP in mRNA maturation, export, and turnover.

In yeast and higher eukaryotes, PABPC1 (referred to here as PABP)3 can shuttle between the nucleus and cytoplasm exhibiting different functions depending on its location in the cell (1, 2). In the nucleus, yeast PABP is involved in multiple steps of poly(A) maturation. Specifically, it associates with components of cleavage factor I, which, together with cleavage factor II, are required to prepare the 3′-end of mRNA for addition of adenosine nucleotides by poly(A) polymerase (3). PABP is not directly involved in the cleavage reaction but plays a direct role in poly(A) length control through its association with poly(A) nuclease (PAN) (4).

PAN is a PABP-dependent exoribonuclease consisting of two subunits PAN2 and PAN3 (5). PAN2 contains the 3′–5′ poly(A) nuclease activity, and PAN3 serves as the binding interface for PABP, which together are positive activators for PAN2 activity (4, 6). Initially, poly(A) tracts are synthesized to default lengths but are then trimmed to appropriate or message-specific lengths, which are important for efficient PABP-dependent mRNA export in yeast (1, 7). Overall, final trimming of the 3′-tail, proper loading of mRNA, and interaction with export factors point to the nuclear function of yeast PABP as a mediator between mRNA biogenesis and export (7, 8). The nuclear functions associated with PABP have been described in yeast but are not as clear in mammalian systems. However, recent work has demonstrated that in mammalian cells, PABP associates with the poly(A) tail of unspliced pre-mRNA, co-immunopurifies with poly(A) polymerase, and remains on the transcript during the pioneering round of nonsense-mediated decay (9). In this context, the nuclear function of mammalian PABP was proposed to be involved in pre-mRNA processing, stability, and quality control.

In contrast to the nucleus, shortening of the poly(A) tail in the cytoplasm is associated with transcript silencing or, more generally, signals the start of mRNA turnover. The enzymes involved in deadenylation in yeast have been identified as the CCR4 (10) and PAN (4). Homologues of these enzymes are found in animals, including an additional poly(A)-specific ribonuclease (PARN) that is not found in yeast (11, 12). An interesting aspect of the deadenylation system is the role of PABP in this process. A principal function of PABP is to protect mRNA by binding to its 3′-poly(A) tail (13) and physically limiting the access and/or directly inhibiting the deadenylases (14, 15).

However, in sharp contrast to CCR4 and PARN, PAN is highlighted by the fact that its activity is stimulated by PABP (4, 5). PAN preferentially degrades poly(A) RNA in the presence of PABP, and its activity is not affected by the 5′-cap on mRNA. In comparison to yeast, where PAN functions in both nucleus and cytoplasm, PAN is exclusively found in the cytoplasm of man-
Interaction between PABC and PAN3

malian cells and therefore is thought to only function in cytoplasmic deadenylation (6).

In the cytoplasm of all eukaryotes, PABP coats the poly(A) tail and simultaneously binds to the translation initiation factor 4G (eIF4G), which is a component of the 5′-mRNA cap binding complex eIF4F. These interactions synergistically increase the affinity for each other and for their substrates (16). This effectively fastens the proteins onto both ends of the mRNA transcripts and contributes to the overall stability of the messenger ribonucleoprotein (mRNP) particle. Thus, PABP is thought to enhance translation in two main ways as follows: first by stabilizing the mRNP initiation complex promoting 40 S and 60 S ribosomal recruitment (17); and second by bringing the 5′ and 3′ mRNA termini into closer proximity, enabling terminating ribosomes to cycle efficiently on the same transcript (18).

Structurally, PABP consists of four RNA recognition motifs at its N terminus and a peptide-binding PABC domain at its C terminus. The N-terminal RNA recognition motif domains associate with the poly(A) tail of mRNA and can also act as a scaffold for binding other proteins (1, 7, 13, 16). The function of PABC is to serve as an interface for proteins containing a specific 15-aminoc acid sequence motif (PAM-2). In animals, PAM-2-containing proteins include PABP-interacting proteins 1 and 2 (Paip1 and Paip2), eRF3 (eukaryotic release factor 3), Ataxin-2, and Tob2. Each modulates translation efficiency via interaction with PABP, other translation factors, or cis-elements within the mRNA transcript (19–23).

The structures of the PABC domains from PABPs were determined in numerous species, including human (24), yeast (25), Trypanosoma cruzi (26), and wheat (27). The domains adopt a comparable fold consisting of four or five α-helices and act in an analogous manner by recognizing a similar PAM-2 sequence. The human PABC-PAM2 complex structure displays the PAM-2 motif binding to specific residues within helices α2, α3, and α5 of the PABC domain (28). Primary sequence alignment of PABC domains shows that these residues are highly conserved indicating an analogous mechanism of peptide recognition throughout animal, parasite, and plant species. An exception to these observations was found in Saccharomyces cerevisiae PABP. The yeast PABC domain (yPABC) contained the greatest structural deviations that we show results in differing sequence specificity.

This study characterizes the interaction between yPABC and the yeast Pan3p subunit by NMR spectroscopy, isothermal titration calorimetry (ITC), and the yeast two-hybrid system. Their interaction occurs through a variation of the typical PAM-2 sequence as observed in higher eukaryotes. Pan3p was the first yeast PABC-binding partner identified. Using NMR, ITC, and GST pulldown assays, we also characterize the interaction between human PAN3 and the human PABC (hPABC) domain demonstrating for the first time their interaction in higher eukaryotes. The PABC-interacting sites in poly(A) nucleases are found throughout eukaryotic and fungal species indicating an evolutionarily conserved interaction with PABP.

EXPERIMENTAL PROCEDURES

Bioinformatics—Multiple sequence alignments of proteins were computed using DIALIGN (29) and ClustalW (30). PABC proteins containing the PAM-2 motif were identified through a Basic Local Alignment Search Tool (BLAST) (31) at NCBI (www.ncbi.nlm.nih.gov/blast) using the search option for short nearly exact matches. The input sequence used was the PAM-2 sequence identified in yPan3p or human PAN3. Default parameters were selected, including a PAM30 matrix that evaluates the quality of pairwise sequence alignment.

GST Pulldown Assay—Full-length human PABP and the PABC domain were cloned into the GST fusion vector pGEX-6P1 (Amersham Biosciences), expressed in Escherichia coli BL21 cells, and purified using methods described previously (24, 32). The mutant full-length PAN3 construct was created from a wild-type human PAN3 plasmid (33) using the Stratagene QuikChange site-directed mutagenesis kit. Full-length human PARN was cloned into pcDNA3.1 as described previously (34). The GST fusion protein, which was immobilized on MagneGST particles, was incubated with the in vitro translated (either wild-type PAN3, PAN3 mutant, or PARN) 35S-labeled protein in the binding buffer. After incubation for 1 h at 4 °C, the GST particles were washed with radioimmune precipitation assay buffer, and the bound fraction was eluted in SDS loading buffer. All buffers were described previously (32). The samples were analyzed by SDS-PAGE and stained with Coomasie Blue to show equal loading of the GST fusion protein and then dried for autoradiography to detect the proteins that were pulled down.

Cloning of the Yeast PAM-2 Site from Pan3p—The PAM-2 site from yeast Pan3p (NP_012900, residues Ser138–Ser162) was amplified by PCR using oligonucleotides yPAM2–5F GCGGATCTATACCAAACC TACCCTTCTCG and yPAM2–3R CCGGAATTCCTTAGGAGGTGTTGAAGACGGAGTGA from a full-length Pan3p template (4). The PCR product was digested with BamHI and EcoRI (sites are underlined in above oligonucleotides) and cloned into a pGEX-6P1. The sequence of the yPAM2 plasmid was verified by capillary electrophoresis DNA-based sequencing (Sheldon Biotechnology Center).

Protein Purification—The yPAM2 plasmid was transformed into BL21(DE3), and expression methods were completed as described previously (26). Peptide samples of unlabeled and isotopically labeled yPAM2 were expressed in either LB or M9 minimal media containing 15NH4Cl (Isotech Inc.). Recombinant GST-yPAM2 was purified by affinity chromatography using glutathione-Sepharose 4B resin (Amersham Biosciences). PreScission protease (2.5 units/mg fusion protein; Amersham Biosciences) was added to the column with PreScission cleavage buffer and incubated overnight at 4 °C. The peptide was collected and further purified by reverse-phase high pressure liquid chromatography. Characterization of yPAM2 by electrospray ionization-mass spectrometry confirmed the presence of the 25-residue yPAM-2 site and 5 additional N-terminal residues (GLPGS) encoded by the expression vector. Protein samples of the PABC domains from yeast (25), human PABP (24), and rat100/HYD (32) PABC domains were prepared as described previously.
Peptide Purification—The region corresponding to putative PAM-2 sites found in yeast Pan3p (GenBank™ accession number NP_012900, yPAM2b residues Ser136–Thr161) and human PAN3 (GenBank™ accession number NP_787050, Lys84–Thr100) was synthesized by Fmoc (N-(9-fluorenylethoxycarbonyl) solid-phase peptide synthesis (Sheldon Biotechnology Center) and purified by reverse-phase chromatography on a Vydac C18 column. The composition and purity of the synthesized peptides were verified by ion-spray quadrupole mass spectroscopy.

Yeast Two-hybrid Assay—Constructs lexA(DB)-PAB1 P-H (yPABC, YER165W, residues 406–577), lexA(DB)-PAN2-(FL) (YGLO94C, residues 1–1115), and GAL4(AD)-PAN3-(FL) (YKL025C, residues 1–679) were generated as described previously (4). Mutant alleles of PAN3 were created by site-directed mutagenesis of plasmid GAL4(AD)-PAN3-(FL) with oligonucleotide primer pairs PAN3–151-1/152-1, PAN3–151–152-1, PAN3–156-1/156-2, and PAN3F3A/1/PAN3F3B2 to generate PAN3–151(FL–A), PAN3–152(N–A), PAN3–156(F–A), and PAN3–151/156(FB–AA), respectively. Yeast strain L40 (yDM61) was transformed with the appropriate plasmid. Transformants were selected on synthetic complete medium lacking leucine and tryptophan (SC – leu, – trp plates). The extent of interaction was determined by the amount of β-galactosidase activity detected in each transformant. Multiple colonies from each transformation were grown overnight in SC – leu, – trp broth and serially diluted on SC – leu, – trp and SC – leu, – trp, – histidine plates containing 0, 5, 10, 20, 40, 60, 80, or 100 mM 3-aminotriazole (3-AT).

Analysis of mRNA Poly(A) Tail Lengths—A DNA fragment bearing PAN3, with 373 nucleotides upstream and 314 nucleotides downstream of the open reading frame, was amplified by PCR from yeast genomic DNA using oligonucleotide primer pairs PAN3SAL5 and PAN3XBA1. This fragment was subcloned into the yeast expression vector pRS316 (35). Mutant alleles of PAN3 were created using the same oligonucleotide primer pairs described in the two-hybrid analysis above. The resulting plasmids were transformed into the pan3Δ strain, yME43 (4). Total yeast RNA was isolated from cell pellets using the hot phenol method (36). Poly(A)+ mRNA was isolated by binding to oligo(dT)-cellulose, as described previously (37), except that the RNA was bound, washed twice with binding buffer and twice with wash buffer, and eluted in batch. Poly(A) tails were analyzed by end labeling with [32P]pCp (Amersham Biosciences) and RNA ligase, followed by digestion of the RNA with RNase A and subsequent fractionation on denaturing polyacrylamide gels (38, 39).

Analysis of NMR Data—For NMR analysis, NMR buffer containing 50 mM NaHPO4, 150 mM NaCl, 1 mM NaN3, and 10% D2O at pH 6.3 was added to lyophilized samples of yPAM2 (Ser1380–Ser162), yPAM2b (Ser146–Thr161), and hPAN3–PAM2 (Lys84–Thr100). NMR titrations were carried out by adding unlabeled PAM2 peptides to an ~0.4–0.6 mM 15N-labeled yPABC, hPABC or HYD-PABC samples at increments of 0.2 mM peptide until saturation was achieved (up to 2:1 molar ratio). The spectra were monitored by observing the change in chemical shifts of amide signals ((Δ1H ppm)2 + (Δ15N ppm × 0.2)2)0.5 on an 15N–1H HSQC spectrum. Because intermediate exchange was observed for some samples, additional assignments of amide resonances in the PABC–PAM2 peptide complexes were completed using 15N–1H edited NOESY and 1H–1H total correlation spectroscopy experiments.

All NMR experiments were acquired at 303 K using standard double and triple resonance techniques on labeled samples (40). Experiments were acquired on either a Bruker Avance 600-MHz spectrometer or Varian Inova 800 spectrometers. For backbone dynamics analysis, 15N–1H heteronuclear NOE data were measured by taking the ratio of peak intensities from experiments performed with and without 1H presaturation. All NMR spectra were processed using either XWIN-NMR software version 3.1 (Bruker Biospin) or NMRPipe/NMRDraw (41). Evaluation of spectra and manual assignments were completed with XEASY software (42). The coordinates of the PABC domains used for analysis were taken from Protein Data Bank entries 1G9L for hPABC, 112T for HYD, and 1IFW for yPABC.

Isothermal Titration Calorimetry (ITC) Measurements—Experiments were carried out on a MicroCal VP-ITC titration calorimeter using the VPViewer software for data acquisition and instrument control (MicroCal Inc., Northampton, MA). NMR buffer (as described above) was used for the ITC experiments. A degassed sample of yPABC thermostated at the desired temperature (15 °C) was stirred (310 rpm) in a reaction cell of 1.4 ml. Titrations were carried out using a 296-µl syringe filled with the peptide solution. 37 injections of 8 µl of peptide were added to the sample with a 5-min interval between injections. Heat transfer (µcal/s) was measured as a function of elapsed time. The experiments were performed with 30–50 µM protein solution in the cell and 300–500 µM of PAM-2 peptide solution in the syringe to ensure a final peptide to protein molar ratio of 2:1 in the reaction cell. The calorimetric data were processed using the software package ORIGIN (version 7.0) provided by the manufacturer. The binding isotherm was fit by an iterative nonlinear least squares algorithm (Marquardt method) to a binding model employing a single set of independent sites. The binding constants and thermodynamic parameters were determined directly from the fitted curve.

RESULTS

Human Poly(A) Nuclease Subunit 3 Associates with the PABC Domain of PABP via a PAM-2 Site—Previous co-immunoprecipitation studies demonstrated that the human poly(A) nuclease (hPAN) complex associates with PABP through its Pan3 (32). Specifically, the N terminus of hPAN3 is required for binding PABP and the C terminus for binding hPAN2 (6). Primary sequence analysis of the N-terminal region of hPAN3 (Fig. 1A) revealed a PAM-2 motif, which suggests that the N terminus of hPAN3 binds specifically to PABC domain of PABP. To test this, a peptide sequence corresponding to the terminus of hPAN3 binds specifically to PABC domain of PABP. To test this, a peptide sequence corresponding to the terminus of hPAN3 binds specifically to PABC domain of PABP.
Interaction between PABC and PAN3 and accompanied by a loss of entropy. This indicates that both hPABC and the peptide become more structured upon binding, thus increasing the overall order of the system. All values are consistent with the thermodynamics and affinity of hPABC-peptide interactions from previous studies (28).

Identification of hPABC Peptide Binding Surface by NMR—A 15N-labeled human PABC sample was titrated with an unlabeled peptide, and the changes in chemical shifts were monitored by 13N-1H HSQC experiments (Fig. 2B). An intermediate exchange regime was observed, as some peaks disappeared or broadened over the course of the titration and re-appeared at different parts of the spectrum at higher peptide concentrations. A three-dimensional 15N-edited NOESY experiment was completed on a peptide-saturated hPABC sample to reassign select residues. The amide chemical shift for each residue in hPABC was determined before and after saturation, and the difference was plotted as function of residue number (Fig. 3A). Residues Met69, Gly72, and Phe75 of helix α2 showed significant shifts between 0.16 and 0.25 ppm. The major shifts, ranging between 0.24 and 0.57 ppm, occur within helix α3, including residues Leu88, Met92, and Glu95. Helix α5 also shows significant shifts from 0.16 to 0.41 ppm, the largest of which is His125.

Mapping of the chemical shift changes on the available solution structure of hPABC (Fig. 3A) clearly illustrates its peptide binding surface for the PAN3 peptide. Overall, the aforementioned residues and helices α2, α3, and α5 define the peptide binding pocket and show that the hPAN3 PAM-2 sequence binds to hPABC in a manner consistent with other PAM-2-containing proteins (28).

GST Pulldown Assays between PABP with PAN3 or PARN—GST pulldown assays were completed to compliment our biochemical assays between PAN3 and PABP. The assay (Fig. 4A) illustrates that full-length PABP can effectively pull down the wild-type PAN3 subunit. Furthermore, the hPABC domain alone can similarly pull down wild-type PAN3. As a control, human poly(A) ribonuclease (PARN) was also tested for binding with GST-PABP and -PABC (Fig. 4B). In this case, PARN only interacts with full-length PABP but not the PABC domain. Altogether, this demonstrates that the PABC domain is sufficient and specifically binds to PAN3. As determined in the hPABC-PAM2 complex structure (28), an essential binding region in the PAM-2 sequence is the C terminus, which contains phenylalanine (Fig. 1A, F11). This residue establishes an important aromatic-stacking interaction with a lone aromatic found in the human PABC domain (28). Given this, a mutant PAN3 subunit substituting an alanine for the phenylalanine residue (F93A) within its PAM-2 site was generated. Full-length PABP and PABC pull down the PAN3 F93A (Fig. 4A) mutant with reduced efficiency confirming that the aromatic residue plays a main role in stabilizing the interaction between both proteins. Overall, these results demonstrate that the PABC domain is an important interacting site for human PAN3.
The PABC Domain from HYD—A unique feature in animals is that a PABC domain is also found in the hyperplastic disk tumor suppressor gene (HYD). Its gene product is a ubiquitin-protein isopeptide ligase that is implicated in developmental and cell cycle regulatory processes (43, 44). The structure of PABC from human HYD (45) is very similar to the domain found in PABP (root mean square deviation 1.55 Å). In addition, both bind PAM-2 motifs found in Paip1, Paip2, eRF3, Ataxin-2, and Tob2 in a similar manner and with high affinity (32). However, in this case titration of PABC-HYD with the human PAN3 peptide showed no binding (10-fold) by ITC or NMR (data not shown). This implies a difference in sequence specificity between the PABC domains from PABP and HYD. Previous studies demonstrated that the C-terminal region of yeast PABP was required to interact with Pan3p (4). Furthermore, yPABC was directly involved in this interaction as mutations within the domain abolished Pan3p interaction (4). This suggested that, similar to the case with human PAN3, a yeast PABC interacting site may exist in Pan3p. Given the structural similarities between PABC domains, it was plausible that yPABC recognizes a variation on the typical PAM-2 sequence. Through sequence analysis of the N terminus of Pan3p, a region similar to the consensus PAM-2 was found (Fig. 1B). A construct was created encompassing this yeast PAM2 site (GenBank access number NP_012900, yPAM2 residues Ser138–Ser162) and its binding properties tested with yPABC by isothermal titration calorimetry (Fig. 5A).

Like the human PABC-PAN3 interaction, the binding of yPABC to the yPAM2 peptide is dominated by favorable enthalpic effects giving rise to an exothermic reaction. In this case, an increase in entropy was found, indicating that desolvation effects most likely dominate conformational ordering upon complex formation. The dissociation constant for the yPABC-yPAM2 interaction was determined to be 150 μM. This affinity is comparatively lower than any of the PAM2-PABC interactions observed in humans (32) but the highest observed for any yeast derived peptide sequences (25). Mangus et al. (4) have shown that a yeast PABP construct consisting of only the C-terminal region (AAA34838, residues 406–577) can alone interact with full-length Pan3p via a yeast two-hybrid screen (4). Our data indicate that this interaction is largely mediated by the yeast PAM-2 motif binding to the yPABC domain; however, additional contacts are likely to occur between both the full-length proteins, which would increase the overall affinity.

Yeast Pan3 Contains a PABC-binding Site—Both animal and plant PABC domains bind to a specific 15-residue PAM-2 sequence that was successfully used to search for in vivo binding partners via bioinformatic methods (19, 20, 46, 47). However, proteins containing a PAM-2 site were never identified in yeast through similarity searches. Yeast PABC was shown to bind to animal PAM-2 sequences with much lower affinity (~1000-fold), which can be explained by the differences in the yPABC structure (25). Altogether, these observations imply that different sequence specificity exists for yPABC. Previous studies demonstrated that the C-terminal region of yeast PABP was required to interact with Pan3p (4). Furthermore, yPABC was directly involved in this interaction as mutations within the domain abolished Pan3p interaction (4). This suggested that, similar to the case with human PAN3, a yeast PABC interacting site may exist in Pan3p. Given the structural similarities between PABC domains, it was plausible that yPABC recognizes a variation on the typical PAM-2 sequence. Through sequence analysis of the N terminus of Pan3p, a region similar to the consensus PAM-2 was found (Fig. 1B). A construct was created encompassing this yeast PAM2 site (GenBank access number NP_012900, yPAM2 residues Ser138–Ser162) and its binding properties tested with yPABC by isothermal titration calorimetry (Fig. 5A).

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Identification of the Yeast PABC Peptide Binding Surface by NMR—A 15N-labeled sample of yPABC was titrated with the yPAM2 peptide and an intermediate exchange regime was...
Interaction between PABC and PAN3

![Diagram](image-url)

FIGURE 4. GST pulldown of human PAN3 WT, hPAN F93A mutant, and hPARN with full-length hPABP and the hPABC domain. Full-length PAN3 WT, F93A mutant (A), and WT PARN (B) were each incubated with MagneGST particle-immobilized GST: GST-hPABP (full-length) and GST-hPABC (residues 498–636). The resulting complexes were resolved by SDS-PAGE followed by Coomassie Blue staining (left panel). PAN3 WT, PAN3 F93A, and PARN inputs are shown in the middle panel. The inputs represent 100% of the sample incubated with GST-PABP or GST-PABC.

Observe the interacting residues within the Pan3p site. A 15N-labeled reverse titration experiment was completed to precisely define observed in this helix (Fig. 3) recognition as demonstrated by the small chemical shifts all contribute to the hydrophobic core. Altogether, the results defined only by helices H9251 and H18528. Consequently, this helix terminates anti-parallel to helix H9251, in particular residues Lys44 and Met48. The chemical shift differences of each residue were mapped on to the solution structure of yPABC (Fig. 3B). In contrast to hPABC, the peptide binding pocket was defined only by helices α2 and α3 with only weak shifts observed for helix α5. In comparison with other PABC structures, a strong bend exists in the last α-helix of yPABC. Consequently, this helix terminates anti-parallel to helix α3, whereas helix α5 of hPABC remains perpendicular. Also unique to yPABC, three aromatic residues are present within helix α5 and all contribute to the hydrophobic core. Altogether, the results show that the final helix of yPABC is less available for peptide recognition as demonstrated by the small chemical shifts observed in this helix (Fig. 3B).

Mapping the Boundaries of the Yeast PABC-binding Site—A reverse titration experiment was completed to precisely define the interacting residues within the Pan3p site. A 15N-labeled yPAM2 peptide was prepared and saturated with unlabeled yPABC. Changes in the 15N-1H HSQC of the 30-residue construct (Fig. 6) showed that the residues interacting with yPABC were located at the C terminus (Tyr146–Ser159). A smaller peptide corresponding to this region (Ser146–Thr161) was synthesized and tested for binding. Both NMR and ITC gave comparable affinities (Fig. 5) to the large peptide indicating that this is the sufficient binding region.

A notable difference is that there is a larger peptide interaction surface in human than in yeast PABC. The 15-residue human PAM-2 peptide contacts span from helices α2 to α5 (28), whereas the 15-residue yeast PAM-2 sequence interact only with helices α2 and α3 of yPABC (Fig. 3). Overall, in contrast to hPABC-PAM2 interactions, our results show that yeast Pan3p contains a sequence variation on the typical PAM-2 motif, and that the peptide-binding site in yPABC is defined only in the second and third α-helices.

Yeast Two-hybrid Assay—Using the data from our NMR titration experiments (Fig. 6, A and B) and our observation that aromatic residues are important for hPABC-PAM2 interaction (Figs. 3–5), we chose several residues to assess their relative contribution to yPABC-PAM2 binding. Accordingly, alanine point mutants were created in the PAM-2 site in full-length Pan3p, and a series of yeast two-hybrid assays were completed. Changing the invariant phenylalanine, Phe156, to alanine resulted in a significant decrease in 3-AT resistance (Fig. 7A), suggesting it plays an important role in PABC-Pan3p binding. Mutations F151A and N152A resulted in only a minor reduction in 3-AT resistance relative to wild type and are unlikely to contribute directly to yPABC-Pan3p interaction. This conclusion is bolstered by the observation that a double mutant at residues 151/156 yielded the same phenotype as mutant F156A alone. Loss of interaction was specific to yPABC-Pan3p because each mutant protein fully maintained interaction with Pan2p (data not shown). These results indicate that aromatic residue Phe156 of Pan3p has an important role in binding the yPABC domain. Previous yeast two-hybrid studies (28) indicated that the aromatic residue, Tyr514 (Y29 in Fig. 3), within helix α2 of yPABC is essential for interaction with Pan3p. Taken together, these results imply that the recognition mechanism for yPABC-PAM2 is likely governed by aromatic stacking between protein and peptide in a manner similar to that observed for the hPABC-PAM2 complex. However, further structural studies between the yPABC-PAM2 complex would be required to clearly define this.

Assessment of mRNA Poly(A) Tail Lengths—pan3Δ strains in yeast exhibit a significant increase in the average steady-state poly(A) tail length of total cellular mRNA (4). This defect is thought to be the result of their inability to properly localize Pan2p to mRNA poly(A) tails and promote trimming. We expressed our PAN3 mutant alleles in pan3Δ strains to determine whether they were capable of controlling poly(A) tail length. When compared with wild-type strains, pan3Δ strains accumulate mRNAs with very long poly(A) tails (Fig. 7B, compare lanes 1 and 2) (4). Strains bearing mutations F151A and N152A both produce mRNA with wild-type length poly(A) tails indicating they are functional (Fig. 7B, compare lanes 3–5). However, strains with the F156A mutation, which showed...
reduced affinity for yPABC, were only able to partially complement the defect (Fig. 7B, compare lanes 3 and 6 and traces). This result and the yeast two-hybrid data indicate that Phe\textsuperscript{156} is an important contact for promoting yPABC-Pan3p interaction and proper PAN function.

**DISCUSSION**

In this study, we characterized the interaction between the PABP and PAN in human and yeast. In both cases, our experiments show that the PABC domain from PABP binds directly to the PAN3 subunit of PAN. Similar to other PABC binding partners in humans (28, 32), the interaction is mediated through a conserved PAM-2 interacting site. Previous work showed that the C-terminal region of yeast PABP was directly involved in binding yeast Pan3p (4). In this study, we mapped precisely the site of yeast PABC interaction on Pan3p. According to NMR chemical shift mapping experiments, the peptide-binding site in yPABC is defined by the second and third \( \alpha \)-helices. In addition, as shown in yeast two-hybrid assays, the interaction between the peptide and protein is likely governed by an important aromatic stacking interaction as observed in the human complex structure (28). In contrast to hPABC and PAM-2 interactions, our results show that yeast Pan3p contains a sequence variation of the typical PAM-2 motif found in animals. The PAM-2 motif in PAN3 is conserved throughout different animal and fungal species. Overall, our results demonstrate that the association of PAN with PABP is mediated by the PABC domain and suggests that it is an evolutionarily conserved interaction.

This interaction provides insight into several PABP-related phenomena that were previously observed (48, 49). Multiple experiments in yeast have shown that PABP mutants lacking the C-terminal region generate mRNA transcripts with poly(A) tails exceeding normal lengths (4, 5, 50–52). This can be explained by the absence of the PABC domain, which provides the docking surface for PAN. Without the PABP-PAN association, the final steps of poly(A) trimming cannot be completed. Furthermore, this suggests that the PABP-PAN complex may be necessary for inhibition or displacement of poly(A) polymerase from the transcript (51). Poly(A) polymerase is the central enzyme responsible for adding adenosine nucleotides to the 3’-tail. Without its displacement, mRNA transcripts result in longer poly(A) tail lengths (3, 53).

Recent experiments have described that trimming of the poly(A) tail to a specific length by PAN is required for efficient PABP-dependent mRNA export to the cytoplasm (1, 7). An incorrectly processed poly(A) tail results in nuclear retention of
of maternal mRNAs during oocyte maturation and early development (59). There is considerable communication between the 5′-cap and poly(A) tail with regard to deadenylation. For instance, PARN is inhibited by PABP but can be stimulated by the 5′-cap (60). However, there are no data to suggest that the 5′-cap modulates the activity of PAN. In yeast, the CCR-CAF1-NOT complex was considered to be the principal deadenylase that initiates mRNA decay and then, along with other 3′-5′-exonucleases, completes the degradation process once the 5′-cap is removed. It was proposed that PAN contributes to cytoplasmic mRNA turnover as an alternative to CCR4 (49). However, PAN cannot shorten tails to lengths below ~50 adenylate residues, and knocking out PAN has little effect on deadenylation in yeast (10), indicating that it is not a principal deadenylase. As shown in a recent study in mammals, deadenylation exhibits biphasic kinetics (33). PAN is thought to initiate mRNA decay followed by further degradation by CCR4 (33). In comparison to the deadenylating enzymes PARN and CCR4, PAN is unique in that its activity is stimulated by PABP. Hence, the current body of research suggests that PAN is recruited to the mRNP complex via PABP to initiate a limited shortening of the poly(A) tail. This process may be a required step to start the rearrangement of a translating mRNP complex into a particle to be silenced or sent to the mRNA decay pathway.

From a structure to function point of view, our previous studies have established PABP as a protein-interacting interface that only binds factors containing the specific PAM-2 motif (28, 32). However, a clear cellular role for the PABC domain has yet to be established. PABP-interacting proteins (Paip) 1 and 2, eRF3, Ataxin-2, and Tob2 are among the proteins identified that contain a PAM-2 sequence. Furthermore, each was demonstrated to interact with PABP in cellular extracts or in vivo (19–23). A common theme among these proteins is their ability to regulate translation through association and/or competition with other translation factors or by mRNA modification. In the context of this study, PAN regulation of the poly(A) tail length of mRNA represents another mechanism for translational control either via transcript silencing or decay. Together, these results clearly establish a role for PABC as a binding site for proteins that modulate mRNA metabolism, translation, and consequently gene expression.

Acknowledgments—We thank Christopher R. Young for assistance with the isothermal titration calorimeter, Dr. Alexey Denisov for assistance on the 600-MHz spectrometer, and Dr. Tara Sprules for operation of the 800-MHz spectrometer at the Quebec/Eastern Canada High Field NMR Facility. The NMR facility is supported by grants from the Canada Foundation for Innovation, the Quebec Ministère de la Recherche en Science et Technologie, and McGill University. We also thank Dr. Allan Jacobson (Professor, Dept. of Molecular Genetics and Microbiology, University of Massachusetts) for support of the experiments performed by David Mangus.

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