Distinct functions of maternal and somatic Pat1 protein paralogs

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ABSTRACT

We previously identified *Xenopus* Pat1a (P100) as a member of the maternal CPEB RNP complex, whose components resemble those of P-(rocessing) bodies, and which is implicated in translational control in *Xenopus* oocytes. Database searches have identified Pat1a proteins in other vertebrates, as well as paralogous Pat1b proteins. Here we characterize Pat1 proteins, which have no readily discernable sequence features, in *Xenopus* oocytes, eggs, and early embryos and in human tissue culture cells. xPat1a and 1b have essentially mutually exclusive expression patterns in oogenesis and embryogenesis. xPat1a is degraded during meiotic maturation, via PEST-like regions, while xPat1b mRNA is translationally activated at GVBD by cytoplasmic polyadenylation. Pat1 proteins bind RNA in vitro, via a central domain, with a preference for G-rich sequences, including the *NRAS* 5′ UTR G-quadruplex-forming sequence. When tethered to reporter mRNA, both Pat proteins repress translation in oocytes. Indeed, both epitope-tagged proteins interact with the same components of the CPEB RNP complex, including CPEB, Xp54, eIF4E1b, Rap55B, and ePAB. However, examining endogenous protein interactions, we find that in oocytes only xPat1a is a bona fide component of the CPEB RNP, and that xPat1b resides in a separate large complex. In tissue culture cells, hPat1b localizes to P-bodies, while mPat1a-GFP is either found weakly in P-bodies or disperses P-bodies in a dominant-negative fashion. Altogether we conclude that Pat1a and Pat1b proteins have distinct functions, mediated in separate complexes. Pat1a is a translational repressor in oocytes in a CPEB-containing complex, and Pat1b is a component of P-bodies in somatic cells.

Keywords: 3′ untranslated region; MS2 tether function assay; 4E-T(ransporter); rck/p54

INTRODUCTION

Gene expression control at the level of translation and mRNA stability is vital in early development, during differentiation and in neuronal function. It is principally exerted by RNA-binding proteins and/or microRNAs (miRNAs) that interact with specific sequences in 3′ untranslated regions (Sonenberg and Hinnebusch 2009; Jackson et al. 2010).

Xenopus laevis oocytes, eggs, and early embryos have been extensively used to examine the control of gene expression at the level of translation since transcription is shut down during oocyte maturation. Any new protein synthesis during meiotic maturation or early mitotic cleavage stages hence relies on the activation of stored, silenced maternal mRNAs. Elements in the 3′ untranslated regions (UTR) of these mRNAs, and the corresponding *trans*-acting factors, dictate their translational activity in early development. The well-characterized 3′ untranslated region element, called the cytoplasmic polyadenylation element (CPE) in conjunction with its binding factor CPEB, is a critical regulator of translation. Indeed, CPEB serves a dual function; it represses translation of CPE-containing mRNAs in oocytes and activates their translation via cytoplasmic polyadenylation in eggs (Richter 2007; Radford et al. 2008; Standart and Minshall 2008). It is important to note that in oocytes, eggs, and early embryos, unlike in yeast or human somatic cells, mRNAs with very short or no poly(A) tails are stable due to lack of decapping activity until zygotic transcription resumes

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(Gillian-Daniel et al. 1998; Zhang et al. 1999). CPEB is a component of a very large ~3 MDa RNP complex in oocytes, which also includes Xp54 RNA helicase, Rap55B, an oocyte-specific cap-binding protein eIF4E1b, its interacting partner 4E-T, as well as CPE-containing mRNAs (Minshall et al. 2007). When tethered to the 3′ UTR of reporter mRNA, Xp54, Rap55, and 4E-T repress its translation (Tanaka et al. 2006; Minshall et al. 2007).

Many of the protein components found in the CPEB maternal RNP are also found in cytoplasmic RNP complexes variously called germinal granules, P-granules, Balbiani-bodies, neuronal RNP granules, and P(rocessing)-bodies in fly, worms, and mammalian germline as well as somatic cells (Kotaja et al. 2006; Roper 2007; Boag et al. 2008; Noble et al. 2008; Swetloff et al. 2009; Flemr et al. 2010). The conservation of components across species emphasizes the importance of these RNP complexes in translational control. P-bodies were first described in yeast, as distinct constitutive cytoplasmic foci involved in mRNA decay. Human cell P-bodies, the best characterized metazoan RNP, harbor RNA-binding proteins, nucleases, translational repressor proteins, and miRNP components. P-bodies exclude ribosomes and apparently only have one translation initiation factor, eIF4E, and 4E-T, rather than eIF4G. Silenced mRNAs resident in P-bodies may undergo decay or return to translation (Bhattacharyya et al. 2006; Eulalio et al. 2007a; Kedersha and Anderson 2007; Parker and Sheth 2007; Standart and Minshall 2008).

P-bodies increase in number and size upon accumulation of nonpolysomal mRNA. In mammals, this is observed when polysomes are disrupted with puromycin (Wilczynska et al. 2005) or when degradation is compromised by Xrn1 silencing (Cougot et al. 2004). Altogether, these data support a role for P-bodies in mRNA degradation, mRNA storage, and si/miRNA silencing. Yet, direct evidence is still lacking, as none of these functions are markedly affected in cells where visible P-bodies have been depleted (Decker et al. 2007; Eulalio et al. 2007b; Serman et al. 2007; Sweet et al. 2007; Stalder and Muhlemann 2009). The emerging model envisages initial mRNP control being exerted in smaller "mini-P-bodies" not readily visible by light microscopy. In line with this model, several P-body components are critical for silencing of cellular mRNAs, including p54 RNA helicase and GW182 (Chu and Rana 2006; Beilharz et al. 2009; Ding and Grosshans 2009; Fabian et al. 2009; Zekri et al. 2009), as well as in regulating viral mRNAs (Beckham and Parker 2008; Chable-Bessia et al. 2009; Nathans et al. 2009), though their detailed function is not understood.

A conserved component of P-bodies, granules, and CPEB RNP is the protein called Pat1p in *Saccharomyces cerevisiae*, with proposed roles in RNA degradation and translational repression. Pat1 proteins lack any clear sequence features and are relatively poorly characterized, particularly in vertebrates. Pat1p-deletion strains show defects in decapping (Bouveret et al. 2000; Tharun et al. 2000; Wyers et al. 2000). Moreover, efficient translation repression during glucose deprivation and P-body assembly requires Pat1p together with Dhh1p (the yeast homolog of Xp54 helicase), while Pat1p overexpression leads to a global repression of translation and accumulation of mRNAs in P-bodies (Coller and Parker 2005). Interestingly, these functions are mediated by distinct Pat1p regions, one promoting translational repression and P-body assembly (residues 422–763) and a second domain (residues 244–422) promoting mRNA decapping after assembly of the mRNA into a P-body mRNP (Pilkington and Parker 2008).

In *C. elegans*, PATR-1 (Pat1-related) is a unique marker for P-bodies in embryonic and somatic cells, while the related mRNA storage bodies in oocytes have low or undetectable levels of PATR-1 (Boag et al. 2008; Gallo et al. 2008). HPat, the *Drosophila* homolog, is an essential component of P-bodies in S2 tissue culture cells and has been shown to promote deadenylation and decapping and may also play a role in miRNA-mediated silencing (Eulalio et al. 2007c; Haas et al. 2010).

The distantly related *Xenopus* homolog of yeast Pat1p (19% identity), called P100, was originally characterized as an oocyte-specific, cytoplasmic, ssDNA-binding protein (Rother et al. 1992). More recently, *Xenopus* P100/Pat1 was identified by mass spectrometry as an abundant partner of CPEB and of Xp54 RNA helicase in oocytes, in coimmunoprecipitation and gel filtration analyses (Tanaka et al. 2006; Minshall et al. 2007).

Here we characterize the two *Xenopus* homologs of yeast Pat1p, which we name xPat1a (formerly P100, Pat1, PATL2) and xPat1b (formerly PatL1), to reflect their order of expression, and in agreement with other investigators (Nakamura et al. 2010; Ozgur et al. 2010). We examined xPat1a and xPat1b expression, their RNA-binding capacity, their ability to repress translation of reporter mRNA when tethered, and their protein interaction partners in oocytes and compared the localization of mammalian Pat1a/1b in tissue culture cells. We show evidence that Pat1a and Pat1b mediate distinct functions in oocytes and in somatic cells.

RESULTS

Vertebrates possess two proteins related to yeast Pat1

S. cerevisiae Pat1p (Pilkington and Parker 2008) and *X. laevis* P100 (Rother et al. 1992), called xPat1a here, were used as queries in BLAST-P (NCBI). All the entries found were eukaryotic protein sequences. The phylogenetic tree shows that vertebrate Pat1 proteins have evolved into distinct groups, representing two paralogous gene families, Pat1a and Pat1b (Fig. 1A). However, there is only one Pat1 protein in yeast and invertebrates, *S.c.* Pat1p, *D.m.* HPat, and *C.e.* PATR-1 (Eulalio et al. 2007b; Boag et al. 2008; Gallo et al. 2008; Pilkington and Parker 2008; Haas et al. 2010). Pat1 proteins range in size from ~530 amino acids (mouse Pat1a)

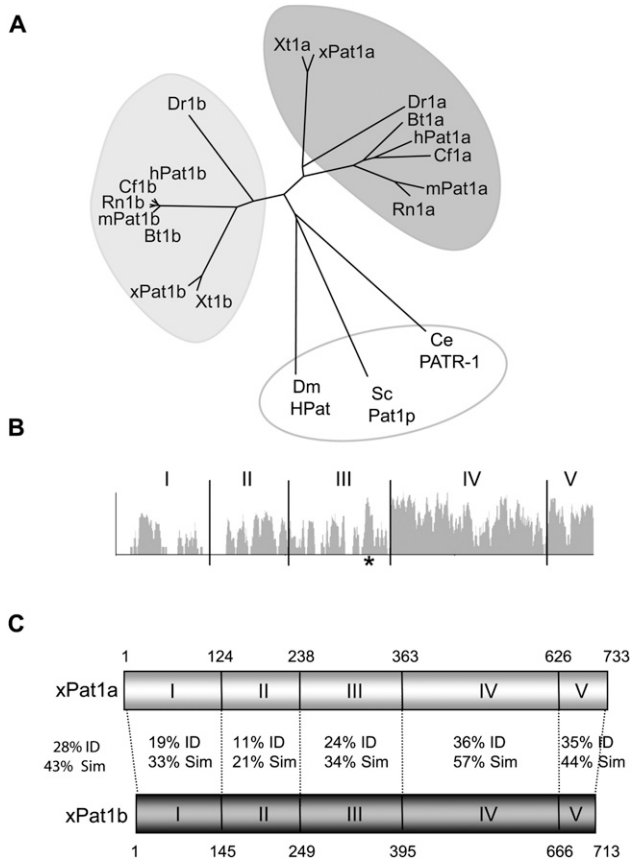

FIGURE 1. Identification of Pat1a and Pat1b proteins. (*A*) Unrooted phylogenetic tree of Pat1 family proteins was assembled using ClustalW (http://workbench.sdsc.edu/). The abbreviations and accession numbers are as follows: Sc: *S. cerevisiae* (NP_010002), Sp: *Schizosaccharomyces pombe* (NP_595976), Ce: *Caenorhabditis elegans* (NP_496514), Dm: *Drosophila melanogaster* (NP_650592), Bt: Bos *taurus* Pat1a (XM_868851), *B. taurus* Pat1b (XP_877785), Cf: *Canis familiaris* Pat1a (XP_851448), *C. familiaris* Pat1b (XP_877785), Hs: *Homo sapiens* Pat1a (NP_001138584), *H. sapiens* Pat1b (NP_689929), Mm, *Mus musculus* Pat1a (AAI45647), *M. musculus* Pat1b (AAH58941), Rn: *Rattus norvengicus* Pat1a (EDL80016), *R. norvengicus* Pat1b (NP_001101990), Dr: *Danio renio* Pat1a (XP_683261), *D. renio* Pat1b (NP_001076497), Xl: *Xenopus laevis* Pat1a (NP_001085311), *X. laevis* Pat1b (AAH98995), Xt: *Xenopus tropicalis* Pat1a (NP_001135679), *X. tropicalis* Pat1b (Xt7.1-TTbA027g22.3.5, which is derived from assembled scaffolds of the Gurdon Institute *X. tropicalis* full-length database). Light gray circle denotes vertebrate Pat1a proteins, dark gray circle vertebrate Pat1b, and white circle invertebrates and yeast. (*B*) Similarity plot of the alignment of eight pairs of vertebrate Pat1 proteins using the Align X module of Vector NTI (Invitrogen) showing the five delineated regions of xPat1 proteins, where the height of the peak indicates degree of similarity. The asterisk in region III indicates the conserved helix called "helix x." (*C*) Schematic representation of *X. laevis* Pat1a and Pat1b protein regions (not to scale). The identity (ID) and similarity (sim) scores between xPat1a and xPat1b are also indicated (calculated with the Ebi, EMBOSS pairwise alignment tool).

to ~970 amino acids (*Drosophila* HPat). Using the Vector NTI alignment tool with eight pairs of vertebrate protein sequences (Fig. 1B), we note that Pat1b proteins are conserved throughout their sequence, whereas the conserved portions of Pat1a proteins are largely confined to their C-terminal halves (Supplemental Figs. 1A, 2).

Our study focuses on the two *Xenopus laevis* Pat1 proteins. xPat1a and xPat1b share 28% identity and 43% similarity, and both are equally distant to *S. cerevisiae* Pat1p (Fig. 1A; Supplemental Fig. 1B). Five regions (RI-V) were delineated based in part on the previously delineated regions of yeast Pat1p (Pilkington and Parker 2008), as well as on the secondary structure predictions for xPat1a and xPat1b, and on the amino acid sequence similarities of eight vertebrate protein pairs (Fig. 1C). The most conserved regions between xPat1a and xPat1b proteins are regions IV and V which share 36% and 35% identity, respectively, whereas region II is the least conserved region with only 11% identical residues (Fig. 1C). Region I of Pat1 proteins is acidic in nature and rich in glutamate and aspartate residues accounting for 30% of the amino acids in that region, while regions II–III are proline rich (~25%) (Supplemental Figs. 1C, 2).

xPat1a expression is confined to oocytes, whereas xPat1b is newly synthesized in eggs

To examine xPat1a and xPat1b expression in early development, we raised specific peptide antibodies (Supplemental Figs. 2, 3) for Western blotting of samples of oocytes, eggs, and embryos. On SDS-PAGE gels, xPat1a does not migrate according to its predicted size (83 kDa), but as a 100-kDa protein, as noted previously (Rother et al. 1992), apparently due to aberrant SDS-binding (see Fig. 3B, below). Though similar to xPat1a in length, xPat1b migrates according to its size of 75 kDa (Supplemental Fig. 3).

xPat1a is expressed throughout oogenesis but is not detectable in eggs, embryos (Fig. 2A), nor in adult tissues (data not shown; Rother et al. 1992). In contrast, xPat1b only starts to be expressed in late oogenesis (stage IV) and is relatively abundant in eggs and embryos, peaking at embryonic stages 12–20, corresponding to gastrula and neural fold stages (Fig. 2A). Both xPat1 proteins are cytoplasmic in oocytes (Fig. 2B). At stage 42, which corresponds to the tadpole-like stage, xPat1b is found in neuron-rich tissues such as eye and brain (Fig. 2C).

In oocytes, eggs, and embryos, the mobility of xPat1b in SDS-PAGE varies, implying that it undergoes modification (Fig. 2A). As detailed in Supplemental Figure 4, we concluded that the upper doublet band in oocytes corresponds to xPat1b phosphorylated at Ser_{62}, that additional site(s) are phosphorylated in eggs and that in embryos xPat1b is (likely) only phosphorylated at Ser_{62}. While of interest here to understand the migration of xPat1b in SDS-PAGE, we note that this serine site is not conserved in vertebrate Pat1b proteins (Supplemental Fig. 2). Indeed the migration of human Pat1b in HeLa cell lysates in SDS-PAGE is insensitive to λ-phosphatase (Supplemental Fig. 4D). Using different antibodies to xPat1 proteins, Nakamura et al. (2010) show a similar pattern of expression and phosphorylation in oocytes and eggs.

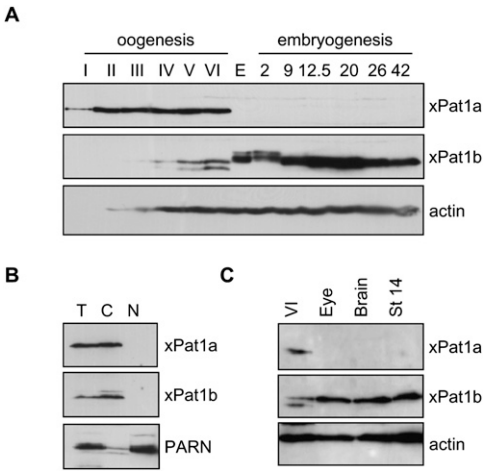

FIGURE 2. xPat1a and xPat1b expression profile in oogenesis and embryogenesis. (*A*) The expression levels of xPat1a and xPat1b were analyzed by Western blot using stage I–VI oocyte, egg, and different embryonic stages lysates (the stages of embryogenesis correspond to two-cell embryo; stage 9, midblastula; stage 12.5, gastrula; stage 20, neural fold closure; stage 26, tail-bud stage; stage 42, tadpole-like stage). Two cell equivalents were loaded. Actin was used as a loading control. (*B*) Both xPat1a and xPat1b are cytoplasmic proteins. Two cell equivalents of total (T), cytoplasmic (C), and nuclear (N) fractions from stage VI oocytes were analyzed by Western blot. PARN was used as nuclear control (Copeland and Wormington 2001). (*C*) xPat1b is expressed in eyes and brains of stage 42 embryos (tadpole-like stage). Actin was used as a loading control. One stage VI oocyte, 10 eyes, five brains of stage 42 embryos, and one stage 14 embryo were analyzed by Western blot.

Overall, based on our and others' data (Rother et al. 1992; Scheller et al. 2007), we conclude that maternal Pat1a is replaced by Pat1b in embryos and adults.

xPat1 protein expression switch in meiosis results from xPat1a degradation and cytoplasmic polyadenylation of xPat1b mRNA

We next examined the timing of the xPat1a/b expression switch in more detail in meiotically maturing eggs. Meiotic maturation is marked by nuclear or germinal vesicle breakdown (GVBD) in meiosis I, which is scored (as a percentage) by the appearance of a white spot at the animal pole. Between 25% and 50% GVBD, xPat1a became undetectable, at about the time that CPEB was degraded, whereas xPat1b levels rose abruptly (Fig. 3A). We delineated the regions responsible for xPat1a proteolysis by injecting stage VI oocytes with in vitro–transcribed mRNAs encoding portions of xPat1a fused to N-terminal MS2 coat protein, and subsequently maturing these oocytes with progesterone. xPat1a protein fragments expressed in oocytes and stable in eggs were detected by Western blotting with MS2 antibodies. Proteins resistant to degradation in eggs include those containing amino acids 332–460, 461–733, and 332–733. In contrast, any fragment containing the first 331 amino acids (the full length [FL], 1–331, and 1–461) was unstable in eggs (Fig. 3B). Interestingly, two predicted PEST sequences (rich in proline [P], glutamate [E], serine [S], threonine [T], and to a lesser extend to aspartate [D]) are located in the xPat1a N terminus, amino acids 1–52, and 135–176 (Supplemental Fig. 2). PEST sequences decrease the half-life of proteins which are targeted to the proteasome (Rechsteiner and Rogers 1996).

In contrast, xPat1b expression increased at GVBD (Fig. 3A). We noted that xPat1b mRNA has a putative CPE element overlapping the hexanucleotide element in its 1505-nucleotide (nt) long 3′ UTR (data not shown). To test whether xPat1b mRNA is polyadenylated during maturation, we injected a ^{32}P-[UTP]–labeled RNA containing the last 180 nt of xPat1b 3′ UTR into stage VI oocytes and matured them into eggs. We used the cyclin B1 3′ UTR as a control, which undergoes extensive polyadenylation (~200–250 nt; Fig. 3C) upon maturation (Sheets et al. 1994). Since a similar shift of ~100-150 nt was observed in the case of xPat1b 3′ UTR (Fig. 3C), we conclude that xPat1b synthesis at GVBD results from the polyadenylation of its mRNA.

In summary, upon meiotic maturation xPat1a is degraded through its N-terminal (PEST) sequences, whereas xPat1b is newly synthesized at or just after GVBD via cytoplasmic polyadenylation of its mRNA.

xPat1a and xPat1b bind RNA in vitro

Xenopus P100 (xPat1a) was initially described as a protein capable of binding single-strand but not double-strand DNA (Rother et al. 1992). Moreover, yeast Pat1p binds poly(U) RNA in vitro (Pilkington and Parker 2008). To determine whether vertebrate Pat1 proteins bind RNA, we added in vitro–translated ^{35}S-methionine–labeled proteins to the four RNA homopolymers bound to agarose beads, and to beads alone (to assess the extent of nonspecific binding), similar to the assays used to detect RNA binding of CPEB and ePAB (Hake et al. 1998; Voeltz et al. 2001). We used CPEB, which interacts with the U-rich CPE, as a control. We observed that both xPat1a and xPat1b bind RNA homopolymers with a preference for poly(G)>poly(U), and to a lesser extent to poly(A), while CPEB, as predicted, binds poly(U)>(polyG) (Fig. 4A). In the case of xPat1b, a portion was phosphorylated in the reticulocyte lysate as it is the *Xenopus* oocyte (Supplemental Fig. 4), and both phosphorylated and unphosphorylated forms bound RNA (Fig. 4A). We also tested the human Pat1b, and, like its *Xenopus* counterpart, hPat1b also bound preferentially poly(G)>poly(U) (Fig. 4A). All Pat1 proteins bound RNA with approximately the same efficiency as CPEB in this assay.

Why Pat1 proteins show higher affinity for poly(G) than for the other homopolymers, and whether this has significance in vivo, is unclear. It is well established that certain G-rich nucleic acid sequences have a propensity to form noncanonical four-stranded structures, called G-quadruplexes, which contain guanine tetrads held by reverse Hoogsteen base pairs (Neidle

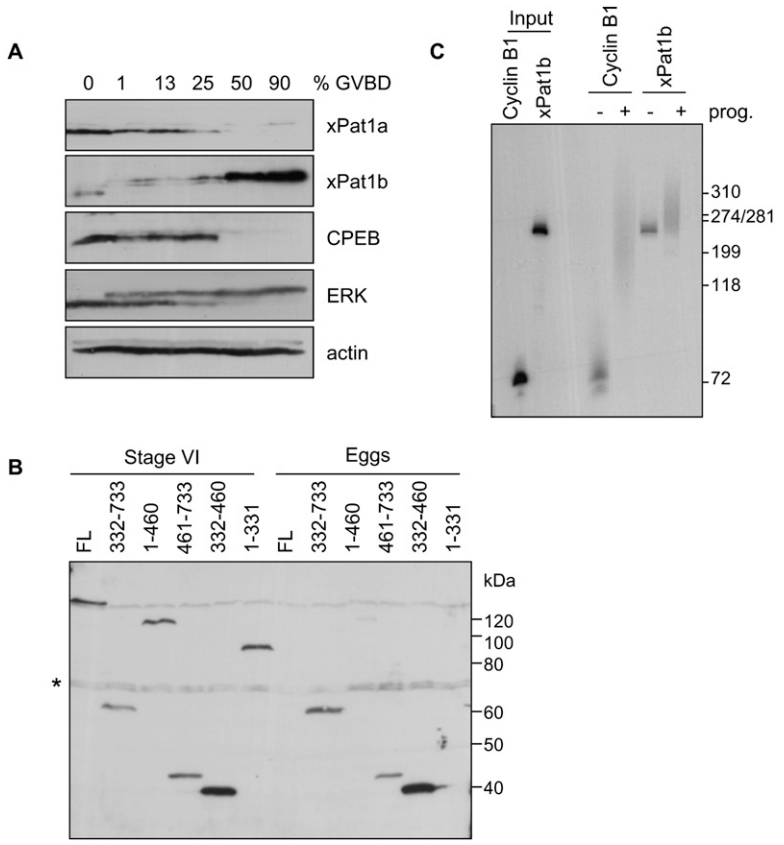

FIGURE 3. xPat1a is degraded whereas xPat1b is newly synthesized upon meiotic maturation. (*A*) Meiotic maturation time course scored as percentage of GVBD. Groups of stage VI oocytes treated with progesterone were sampled at different times, corresponding to maturation status scored in percentage of GVBD. The oocytes were analyzed by Western blot and probed with the indicated antibodies. (*B*) The N-terminal 1–331 amino acids mediate xPat1a degradation. mRNAs encoding for different MS2-tagged portions of the protein as indicated were injected in stage VI oocytes, subsequently matured into eggs by the addition of progesterone. Lysates were prepared from oocytes and eggs and analyzed by Western blotting with an MS2 antibody. Note that region 1–331 runs aberrantly in the SDS-PAGE gel. (*)Nonspecific band, serving as a loading control. (*C*) xPat1b mRNA is polyadenylated upon meiotic maturation. The ^{32}[P]-labeled 3′ proximal 180 nt of xPat1b 3′ UTR and the 3′ proximal 65 nt of cyclin B1 3′ UTR were injected in stage VI oocytes (−) and subsequently matured into eggs (+) by the addition of progesterone. Input lanes contain uninjected RNA. ^{32}[P]-labeled ØX174-HindIII fragments served as size markers.

and Balasubramanian 2006). We thus explored whether Pat1 proteins have affinity for G-quartet containing RNA structures, as is the case for FMRP, an RNA-binding protein involved in translational control (Darnell et al. 2001; Schaeffer et al. 2001; Zanotti et al. 2006). To this end, we used a synthetic RNA G-quadruplex–forming sequence, previously identified within the 5′ UTR of the *NRAS* proto-oncogene as a repressor of translation (Kumari et al. 2007), or a mutant sequence, which does not support G-quadruplex formation, in competition assays with xPat1a and xPat1b. Strikingly, in both cases, the wild-type *NRAS* RNA G-quadruplex, but not the mutant sequence, competed efficiently for poly(G) binding (Fig. 4B).

Next we delineated the region of xPat1a and xPat1b responsible for RNA binding, and, initially, the proteins were divided into two halves composed of region I–III and region IV–V. Region I–III, but not IV–V, bound RNA as well as the full-length protein, and further removal of region I and I–II did not impair their RNA-binding capacity (Fig. 4C). Indeed, we noted that the central region III had the highest affinity for RNA, exceeding that of the full-length proteins (Fig. 4C). We therefore conclude that region III encloses a potential novel RNA-binding domain of vertebrate Pat1 proteins. Of note, region III in xPat1 proteins contains one consistently predicted secondary structure (Jpred); an α helix of 14 conserved amino acids near its C terminus, that we called helix x (Fig. 1B; Supplemental Figs. 1, 2). We therefore performed additional assays using poly(U) and poly(G) RNA with xPat1 proteins lacking region III (ΔRIII) or helix x (Δhelix x) and found that the loss of region III abolished RNA binding, while the loss of helix x severely reduced xPat1 proteins binding to RNA (Fig. 4D).

Overall, xPat1a, xPat1b and hPat1b bind to poly(U) and poly(G) in vitro. The *Xenopus* proteins bind RNA via their central regions III, in part via the conserved secondary structure, helix x.

Both xPat1a and xPat1b repress translation when tethered to reporter RNA

Yeast Pat1p was previously shown to act as an enhancer of decapping and a general translational repressor (Coller and Parker 2005). xPat1a has also been identified as a component of the CPEB repression complex in oocytes (Minshall et al. 2007). This led us to test whether xPat1 proteins act as translational repressors, using the MS2 tethered function assay (Minshall et al. 2010). The mRNA encoding xPat1a or xPat1b fused to MS2 was injected into stage VI oocytes and xPat1 proteins effect on translation was assessed using a capped and nonadenylated firefly reporter mRNA containing three MS2-binding sites in its 3′ UTR. In these assays, the firefly luciferase reporter mRNA was coinjected with a control *Renilla* luciferase mRNA, which lacks any regulatory elements in its 3′ UTR, and the results are reported as ratios of firefly to *Renilla* luciferase activities, normalized to MS2. *Renilla* luciferase activities varied by <10% (data not shown). As shown in Figure 5A, both xPat1a and xPat1b repressed translation approximately

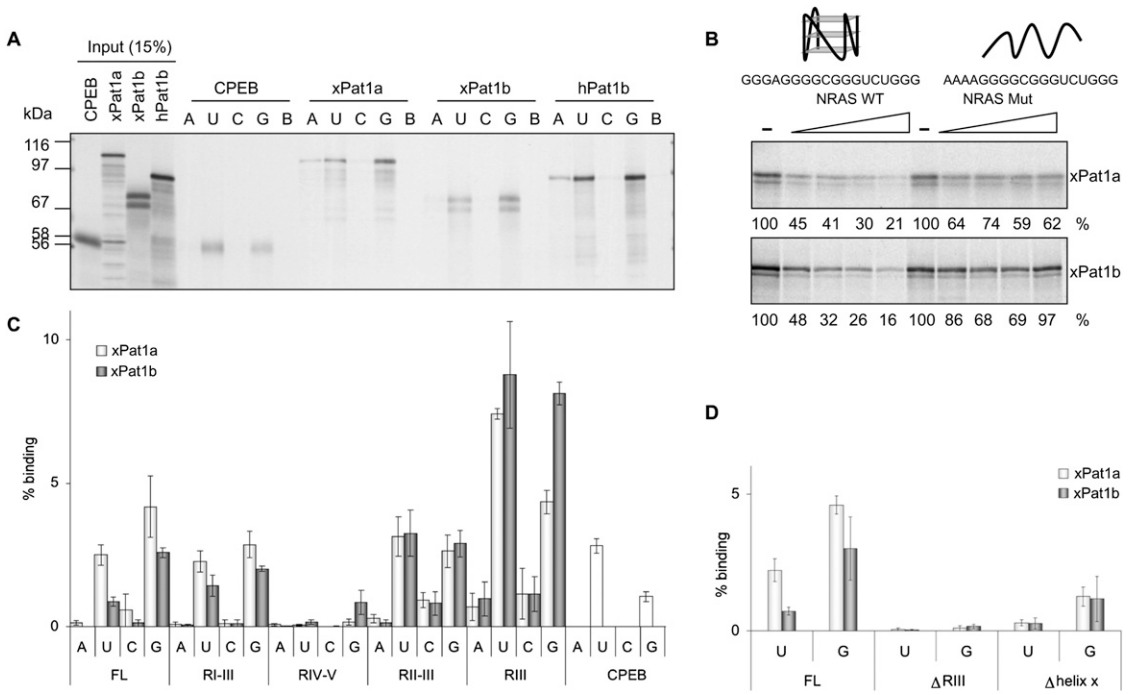

FIGURE 4. xPat1 proteins bind RNA in vitro via a central region. (*A*) xPat1 proteins bind RNA homopolymers in vitro. Autoradiograph of in vitro–translated and ^{35}[S]-Met–labeled xPat1a, xPat1b, hPat1b, and CPEB (control) bound to poly(A), poly(U), poly(C), poly(G), and Sepharose beads (B, as a control for unspecific binding). (*B*) xPat1a and xPat1b poly(*G*) binding is competed by wild-type, but not mutant, *NRAS* G-quadruplex–forming RNAs (Kumari et al. 2007). Approximately 5, 10, 20, and 40 molar equivalents of *NRAS* relative to poly(G) homopolymer were used as competitors, and the levels of Pat1 proteins resistant to competition were quantitated by densitometry. (*C*) xPat1 proteins bind RNA via region III (RIII). Graph of the percentage binding of different regions of xPat1 proteins and CPEB to the RNA homopolymers. (*D*) Helix x in region III is required for RNA binding. Graph of the percentage of binding in full length (FL), constructs lacking RIII (ΔRIII), or helix x (Δhelix x) bound to poly(U) and poly(G) homopolymers. Three independent experiments were performed and standard error bars are shown (*C*,*D*).

two- to threefold, when artificially tethered to a reporter RNA, similar to tethered 4E-T, another component of the CPEB RNP (Minshall et al. 2007). In contrast, tethered ePAB activated translation nearly fourfold, as reported previously (Gray et al. 2000; Minshall et al. 2007). Neither repression nor activation by tethered proteins was detected in the case of reporter RNA lacking MS2-binding sites (Fig. 5A). Moreover, Western blot analysis showed that injected xPat1 and control proteins were expressed in stage VI oocytes (data not shown). Using qPCR, we found that all the injected MS2-tagged protein mRNAs, relative to endogenous GAPDH mRNA, were at approximately equal levels, indicating that the reduction in firefly luciferase expression was due to translational inhibition, not RNA decay (Fig. 5B).

Altogether, we conclude that in oocytes, xPat1 proteins act as translational repressors when tethered to a reporter RNA.

Flag-tagged xPat1a and xPat1b interact with CPEB RNP components in oocytes

Since both xPat1a and xPat1b repress translation when tethered (Fig. 5A), and xPat1a is a component of the CPEB RNP (Minshall et al. 2007), we next assessed xPat1b interactions, using coimmunoprecipitation assays with epitope-tagged xPat1 proteins. mRNAs encoding Flag-MS2-tagged xPat1a or xPat1b were injected into stage VI oocytes, and protein synthesis was allowed to occur before lysate preparation. CPEB antibody coimmunoprecipitated both tagged xPat1 proteins (Fig. 5C). Conversely, using the Flag antibody, xPat1a-Flag coimmunoprecipitated with the CPEB complex, including CPEB, Xp54, ePAB, Rap55B, and eIF4E1b (Fig. 5D). xPat1b-Flag also immunoprecipitated these components of the CPEB complex, to a weaker extent than xPat1a-Flag, possibly due to lower input levels (Fig. 5D; data not shown).

Similar immunoprecipitations were also performed in eggs. As predicted, xPat1a-Flag (and CPEB) was degraded, and therefore no interacting proteins were detected, serving as a control for the immunoprecipitations. Interestingly, xPat1b-Flag still coimmunoprecipitated with Xp54, ePAB, Rap55B, and eIF4E1b in eggs (Fig. 5D) showing that xPat1b does not require CPEB for interacting with other components of the repression complex. We conclude that ectopically expressed xPat1b interacts with the same components of the CPEB complex as xPat1a in oocytes, in line with the tethering results.

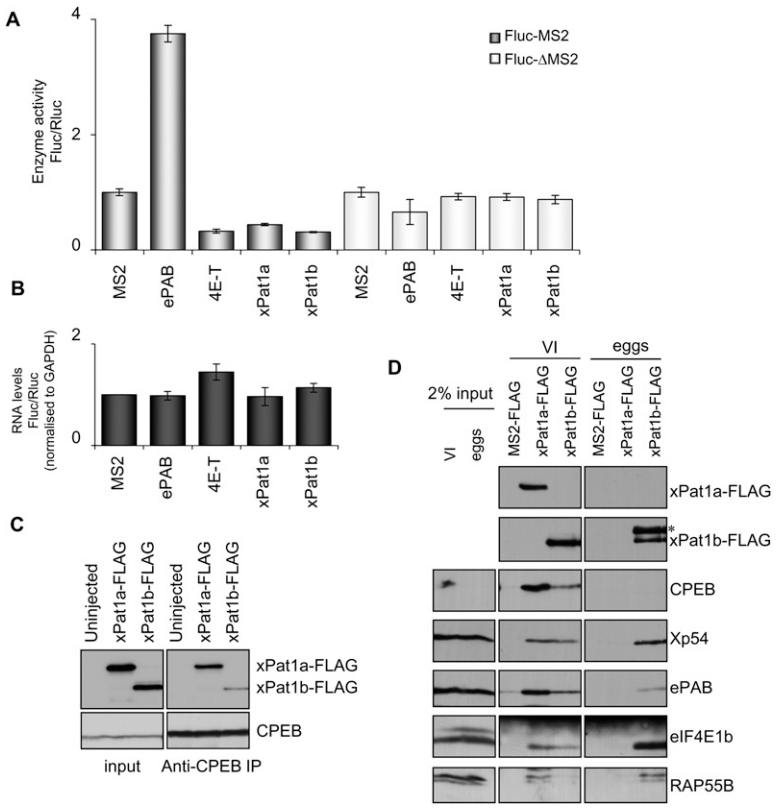

FIGURE 5. MS2-xPat1a and -xPat1b repress translation when tethered and interact with CPEB RNP components. (*A*) xPat1 proteins repress translation when tethered. mRNAs encoding MS2-tagged xPat1a, -xPat1b, and control mRNAs encoding MS2, ePAB, and 4E-T were injected into stage VI oocytes. Six hours after the first injection, firefly luciferase mRNA reporter (Fluc) (m^7GpppG-capped but nonpolyadenylated) containing 3′ UTR MS2 hairpins, was coinjected with *Renilla* luciferase (Rluc), used as an internal control (dark gray). Firely luciferase reporter mRNA lacking the MS2 hairpins was used as a control (light gray). The ratios of the luciferase activities were normalized to the one observed with MS2 alone. Three independent experiments were performed and standard deviation bars are shown. (*B*) qPCR showing equal levels of the injected RNAs. The qPCR was first normalized to GAPDH levels, and the relative ratio between luciferase and *Renilla* reporter mRNAs was subsequently normalized to MS2. Three independent experiments were performed and standard error bars are shown. (*C*) CPEB antibody immunoprecipitates tagged xPat1a and xPat1b. mRNAs encoding the MS2-Flag–tagged xPat1a, xPat1b were injected in stage VI oocytes. The immunoprecipitated proteins were analyzed by Western blotting with the indicated antibodies. (*D*) mRNAs encoding the MS2-Flag–tagged xPat1a, xPat1b were injected in stage VI oocytes, some of which were matured with progesterone into eggs. Immunoprecipitation was carried out using Flag antibody, and the Western blot was analyzed with the indicated antibodies. *Phosphorylated form of Flag-xPat1b. One stage VI oocyte was loaded as an input (2%).

Though xPat1a and xPat1b coexist in stage VI oocytes (Fig. 2), it is also clear that during most of oogenesis Pat1a functions alone, while in embryos (Fig. 2) and adult tissues (Scheller et al. 2007), Pat1b predominates, suggesting that they may have different functions. We therefore asked whether in stage VI oocytes endogenous xPat1a and xPat1b interacted with CPEB, by gel filtration and coimmunoprecipitation assays. Both xPat1a and xPat1b coeluted with CPEB in a large complex of ~3 MDa in a Superose 6 HR10/30 column loaded with stage V/VI oocyte lysate (Fig. 6A; Minshall et al. 2007). However, only xPat1a coimmunoprecipitated with CPEB antibodies (Fig. 6B), and CPEB only coimmunoprecipitated with xPat1a antibodies, and not with xPat1b antibodies (Fig. 6C). While these reciprocal assays are self-consistent, they rely on antibodies which may disrupt interactions, or whose epitopes may be masked in complexes, leading to false-negative data. We therefore sought an independent way to pull down endogenous Pat1-containing complexes, employing either biotinylated wild-type *NRAS* RNA G-quadruplex or the mutant sequence, streptavidin beads, and oocyte lysates. We observed that only xPat1a, but not xPat1b, preferentially binds the wild-type *NRAS* RNA G-quadruplex, as well as CPEB, and eIF4E1b. Xp54 RNA helicase interacted with both wild-type and mutant RNA sequences (Fig. 6D).

Overall, we conclude that in oocytes, only xPat1a but not xPat1b interacts with CPEB and with *NRAS* RNA G-quadruplex, strongly suggesting they are not residents of identical complexes.

hPat1b localizes to P-bodies in HeLa cells

The similarity between the protein components of CPEB RNP and P-bodies (see Introduction) prompted us to examine whether Pat1 proteins localize to P-bodies in HeLa cells. First, we exploited the fact that the antibody raised against *Xenopus* Pat1b cross-reacts with human Pat1b, due to the conservation of the peptide antigen (Supplemental Figs. 2, 4D). Therefore, we used affinity-purified xPat1b antibodies to localize endogenous hPat1b, and Ge-1 protein as a P-body marker (Yu et al. 2005). Approximately 80% of cells contained P-bodies, as seen with Ge-1, in which hPat1b colocalized (Fig. 7A,C). To ascertain whether P-bodies required hPat1b for their formation or maintenance, the protein was depleted with siRNA (Fig. 7B–D). Rck/p54 helicase siRNA acted as a positive control and β-globin siRNA as a negative control for P-body loss (Minshall et al. 2009). In contrast to Rck/p54, which is required for P-body formation, depletion of hPat1b only reduced the number of cells with P-bodies by ~50%, as seen with Ge-1 and Rck/p54 antibodies (Fig. 7B,C; data not shown). Note that the nuclear signal seen with the hPat1b antibody (Fig. 7A) is due to an unspecific protein binding as it was not reduced by hPat1b siRNA (Fig. 7B), nor was it observed in cells transfected with GFP-tagged hPat1b

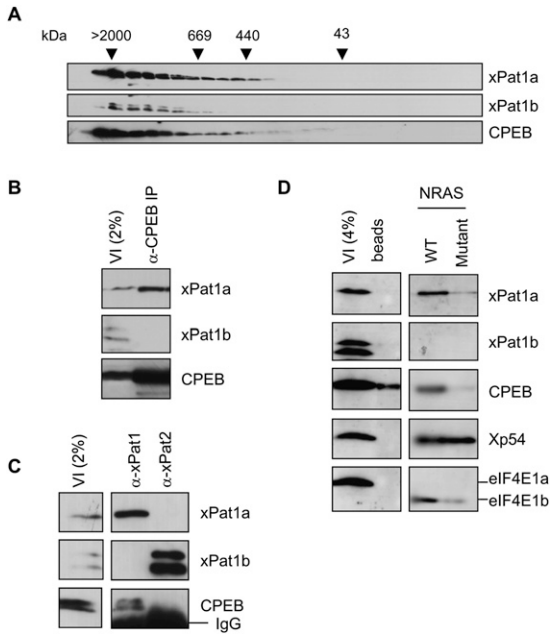

FIGURE 6. Endogenous xPat1a is a component of the CPEB complex, not endogenous xPat1b. (*A*) xPat1a and xPat1b coelute with CPEB in a large complex of ~3 M Da in a Superose 6 HR10/30 gel filtration column loaded with stage V/VI oocyte lysate. Column fractions were analyzed by Western blotting, with the indicated antibodies. (*B*) CPEB antibody coimmunoprecipitates endogenous xPat1a but not xPat1b in oocytes. (*C*) xPat1a antibody coimmunoprecipitates CPEB, whereas anti-xPat1b antibody does not. (*D*) *NRAS* RNA G-quadruplex interacts with the CPEB repression complex. Wild-type biotinylated *NRAS* RNA G-quadruplex interacts preferentially with components of the CPEB complex as compared with a biotinylated mutant sequence.

(Fig. 8A). Altogether we found that endogenous hPat1b is enriched in P-bodies but is not absolutely required for their formation.

Pat1a proteins disperse P-bodies in a dominant-negative manner

Human Pat1a is not expressed in HeLa cells, according to qPCR and Northern blot data (data not shown; Scheller et al. 2007). Hence, to compare Pat1a and Pat1b localization, we transfected cells with GFP fusion proteins encoding mammalian Pat1 proteins. Using confocal imaging, and immunofluorescence with Rck/p54 as a P-body marker, we first showed that hPat1b-GFP localizes to P-bodies (Fig. 8A), as predicted from its endogenous pattern (Fig. 7A) and previous observations (Scheller et al. 2007). Since no full-length human Pat1a clone was available, we used instead one encoding mouse Pat1a, ~65% identical to hPat1a. mPat1a-GFP, in contrast to hPat1b-GFP, displayed both nuclear and cytoplasmic accumulation (Fig. 8A). Moreover, in some cells, mPat1a-GFP showed weak enrichment in P-bodies, while in other transfected cells P-bodies were absent. To examine this in more detail, fluorescence microscopy was used to enable counting of cells with P-bodies in three independent experiments. We noted that the endogenous P-bodies remained in only half of the cells expressing mPat1a-GFP proteins (Fig. 8B, panels a and b, respectively; Fig. 8C). Similar localizations and trends were observed in cells expressing xPat1b-GFP and xPat1a-GFP. xPat1b localized to P-bodies, albeit less efficiently than hPat1b, while xPat1a either weakly localized to P-bodies, or dispersed endogenous P-bodies (Fig. 8C). This dominant-negative effect was even stronger in HEK293 cells, where almost none of the cells expressing mPat1a- or xPat1a-GFP contained P-bodies (Fig. 8C). The differences between Pat1a and Pat1b proteins were not due to differential expression levels, as all four Pat1-GFP proteins were expressed to similar extents (Fig. 8D).

In summary, Pat1b was enriched in P-bodies, whereas Pat1a displayed either weak localization to P-bodies or caused the disassembly of endogenous P-bodies. This dominant-negative effect was stronger in HEK293 cells than in HeLa cells. The distinct behavior of Pat1a-GFP and Pat1b-GFP indicates different protein interactions.

DISCUSSION

Database searches indicate that two Pat1 paralogs are conserved in vertebrates, while fungi, flies, and worms have only one form, raising the question of whether the two vertebrate proteins have evolved to perform different functions, served by one protein in invertebrates.

Our study focused on the characterization of Pat1a and Pat1b proteins in *Xenopus* oocytes and in mammalian cells. We showed that expression of xPat1a and 1b switches during oocyte meiotic maturation. The PEST sequences predicted in xPat1a are likely to promote its degradation at GVBD via proteolysis. Another well-known example of a PEST sequence-containing protein important in oogenesis and meiotic maturation is CPEB, which is degraded at GVBD by the proteasome (Reverte et al. 2001; Thom et al. 2003). On the other hand, xPat1b is newly synthesized via polyadenylation of its mRNA. Cytoplasmic polyadenylation is a well-described mechanism to control translational activation of many mRNAs during meiotic maturation (Radford et al. 2008).

We found that both proteins bind RNA in vitro via their central region III, which involves helix x, a feature confined to vertebrate Pat1 proteins (Supplemental Fig. 2). In xPat1a/b, region III has a high predicted isoelectric point value (12.42 and 12.07, respectively), in part due to its high arginine content (Supplemental Fig. 1C), which may contribute to its ability to bind RNA. While region III and helix x are important for RNA binding in vitro by xPat1 proteins, we do not know whether they mediate interactions with mRNAs in the cell. In yeast, using the same in vitro RNA-binding assay, Pat1p was demonstrated to bind poly(U) RNA via two independent regions, corresponding approximately to regions IV–V of the *Xenopus* Pat1 proteins (Pilkington and Parker 2008). In the case of HPat, the MID domain,

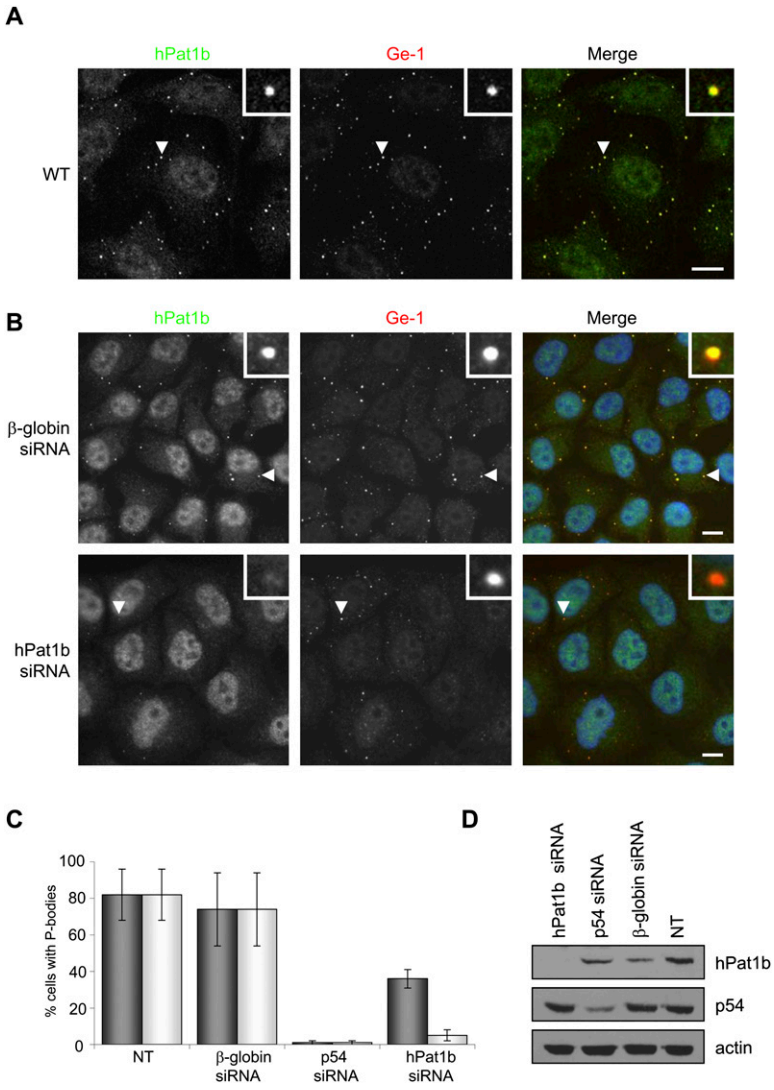

FIGURE 7. Endogenous hPat1b localizes to P-bodies and is not absolutely required for their formation. (*A*) Confocal imaging of HeLa cells stained with Pat1b antibody, with Ge-1 as a P-body marker. The white arrows point to the zoomed P-body (white box). Scale bar, 10 μm. (*B*) Depletion of hPat1b does not prevent P-body formation. hPat1b, p54, and β-globin siRNAs were transfected into HeLa cells, which were fixed and stained with hPat1b and Ge-1 antibodies 48 h post-transfection and visualized by fluorescence microscopy. The white arrows point to the zoomed P-body (white boxes). Scale bar, 10 μm. (*C*) Percentage of cells with P-bodies. The graph represents the percentage of cells with P-bodies as seen with either hPat1b (light gray) or Ge-1 (dark gray) antibodies, in nontransfected (NT) cells, or in cells transfected with control β-globin, p54, or hPat1b siRNA. Three independent experiments were performed and standard deviation bars are shown. (*D*) Western blot of the depletion effect of the different siRNAs used (30 μg protein loaded), with indicated antibodies.

approximating to region IV of *Xenopus*, mediates mRNA binding, directly or indirectly (Haas et al. 2010).

Our results also suggest that xPat1 proteins bind G-quartet containing RNA structures in vitro and in lysates. However, unlike FMRP, a well-characterized RNA-binding protein, which interacts with G-quartet RNA via an RGG box (Darnell et al. 2001; Schaeffer et al. 2001; Zanotti et al. 2006), no such sequences are present in Pat1 proteins. Interestingly, the yeast protein Stm1 also interacts with G-quadruplex, promotes the accumulation of Dhh1 (Xp54 homolog) in P-bodies, and modulates Dhh1 function (Van Dyke et al. 2004; Balagopal and Parker 2009).

Both xPat1a and xPat1b repress reporter mRNA expression in oocytes when tethered (Fig. 5A), extending the list of CPEB complex components that repress bound reporter RNAs, Xp54 helicase (Minshall et al. 2001), 4E-T (Minshall et al. 2007), and Rap55 (Tanaka et al. 2006). Importantly, reporter expression was shown to be down-regulated at the level of translation, not mRNA levels. No particular region of tethered Pat1 proteins could be readily identified as important for repression, though we found that region III was dispensable (data not shown). While region III promotes RNA binding, this function is not required in the tethered function assay to mediate binding to mRNA. In support of the translation data, both ectopically expressed xPat1 proteins interact with CPEB components in coimmunoprecipitation assays. Very recently, it was reported that xPat1a, when tethered to firefly reporter mRNA, represses translation and interacts with components of the CPEB repression complex, in oocytes, in agreement with our findings (Nakamura et al. 2010). However, Nakamura et al. (2010) reported that tethered xPat1a also represses firefly luciferase mRNA lacking 3′ UTR MS2 sites, as well as *Renilla* luciferase mRNA, though not CAT mRNAs, which were all assayed in separately injected oocytes. In our studies, we found that only MS2-bearing mRNA was repressed by MS2-xPat1 proteins in oocytes coinjected with reporter and control mRNAs. The reason for these differences is not known. Moreover, the firefly luciferase reporter mRNA in our assays is not adenylated, and its levels are not altered by the tethered Pat1 proteins. We thus conclude that in oocytes Pat1 proteins repress translation, rather than mediate the coupling of deadenylation and decapping, leading to RNA decay, in contrast to HPat in S2 tissue culture cells (Haas et al. 2010). This distinction is in agreement with reports that oocytes are unusual, possibly unique, cells in which mRNAs with no or a very short oligo(A) tail are stable.

Since xPat1a, whether ectopically expressed or as endogenous protein, interacts with CPEB RNP components and represses translation when tethered, we conclude that it acts

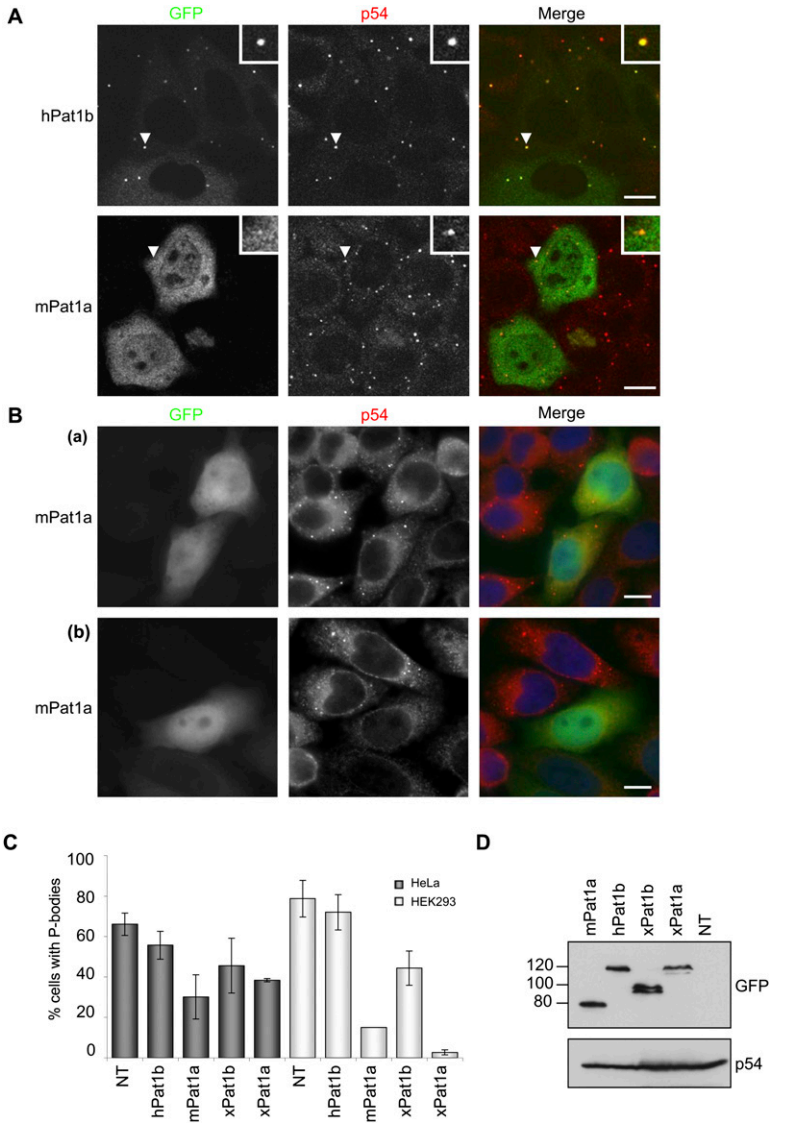

FIGURE 8. Pat1b-GFP localizes to P-bodies while Pat1a-GFP localizes weakly to P-bodies in some cells but exerts a dominant-negative effect on P-bodies in others. (*A*) Confocal imaging of HeLa cells transfected with hPat1b-GFP or mPat1a-GFP. Cells were fixed 24 h post-transfection and stained with p54 antibodies as a P-body marker. White arrow points to the zoomed P-body (white box). Scale bar, 10 μm. (*B*) Fluorescent imaging of HeLa cells transfected with mPat1a-GFP showing two phenotypes: cells with P-bodies (*a*) or lacking P-bodies (*b*). Cells were fixed 24 h post-transfection and stained with p54 as a P-body marker. (*C*) Graph of the percentage of cells containing P-bodies (as seen with p54) when transfected with different GFP constructs in HeLa and HEK293 cells. Three independent experiments were performed and standard deviation bars are shown. (NT) Nontransfected cells. (*D*) The GFP constructs are expressed at similar levels in HeLa cells. Sixty micrograms protein was loaded on a SDS-PAGE gel and analyzed by Western blotting with the indicated antibodies.

as a translational repressor in oocytes. In contrast, while xPat1b is capable of repressing translation and binding CPEB RNP components when overexpressed, it is not detected in immunoprecipitations with endogenous CPEB, nor in pull-downs with *NRAS* RNA. Furthermore, in mass spectrometry analyses of CPEB or Flag-MS2-Xp54 immunoprecipitations in oocytes, we only detected peptides belonging to xPat1a but not xPat1b, though both proteins are expressed at similar levels, (N Minshall, A Marnef, and N Standart, unpubl.). Our data thus strongly suggest that xPat1a and xPat1b are not present in identical complexes in oocytes. We cannot absolutely exclude suboptimal detection of xPat1b in CPEB immunoprecipitations or in pull-down assays. Alternatively, xPat1b, unlike xPat1a, may be masked from both antibody and RNA detection, though this possibility too implies complex differences.

The similarity between the CPEB RNP components and P-body components prompted us to test whether mammalian Pat1 proteins localize to P-bodies. We observed that both the endogenous and GFP-tagged human Pat1b localized to P-bodies in human tissue culture cells. Using siRNA, we found that hPat1b is not essential for normal size P-bodies in all HeLa cells, though the underlying reasons for the variation between cells, possibly linked to cell cycle, are not clear. Other components including GW182 and Rck/p54 are required for P-body formation (Yu et al. 2005; Serman et al. 2007). Furthermore, in contrast to HeLa cells, P-bodies are no longer visible in *Drosophila* S2 HPat-depleted cells (Eulalio et al. 2007b). However in yeast, Pat1p depletion only reduced the size and number of P-bodies but did not eliminate them, while, in *C. elegans*, PATR-1 is required for the recruitment of some though not all P-body components (Boag et al. 2008; Gallo et al. 2008). With the exception of HPat then, Pat1 proteins appear to be dispensable for P-body assembly, though this may vary in cells at different developmental or cell cycle stages.

Mouse Pat1a either localized very weakly to P-bodies compared with hPat1b or dispersed P-bodies in a dominant-negative manner, which was particularly striking in HEK293 cells. These differences in Pat1a and 1b localization, and the Pat1a dominant-negative effect on P-bodies, seen not only with mammalian proteins but also with their *Xenopus* counterparts, presumably reflect their participation in different, though partially overlapping, complexes.

While this paper was in review, two studies on human Pat1 proteins were published. In agreement with our observations, hPat1b localizes in P-bodies, unlike hPat1a, in tissue culture cells. Moreover, hPat1b, like *Drosophila* HPat, was

characterized as a scaffold decay factor which couples deadenylation and decapping via multiple sets of interactions (Braun et al. 2010; Haas et al. 2010; Ozgur et al. 2010; for review, see Marnef and Standart 2010).

Interestingly, maternal and somatic P-body–like RNP possess additional distinguishing component paralogs, including ePab/PABP, Rap55B/A, and eIF4E1a/b. We speculate that examination of their functions may indicate, as in the case of Pat1a and Pat1b, their specialization in cells devoted to translational control rather than predominantly RNA decay with some translational control.

In summary, we present evidence that Pat1a and Pat1b proteins play distinct functions in germ and somatic cells. Pat1a is a translational repressor in oocytes in a CPEB-containing complex, while Pat1b is a component of P-bodies in HeLa/HEK293 cells. Future studies comparing the interactions partners of Pat1a and 1b in oocytes and in human tissue culture cells, as well as assessing their abilities to repress and/or cause decay of mRNAs will be important to understand their specialized functions.

MATERIALS AND METHOD

Xenopus laevis Pat1a/b, mouse Pat1a, and human Pat1b cDNAs

Xenopus laevis Pat1a cDNA was a kind gift from Russell Rother (Alexion Pharmaceuticals, Inc., Connecticut). This clone contains one point mutation compared with the NCBI sequence (NM_001091842) leading to a proline instead of a threonine at position 602. *Xenopus laevis* Pat1b cDNA was available as a full-length I.M.A.G.E. clone in the pCMVSPORT6 vector (clone 5085138: accession number BC098995). Two I.M.A.G.E. clones (clone 5502183: accession number BC111047, and clone 30331777: accession number CD110086) encoding different parts of the human Pat1b ORF were used to construct a full-length hPat1b ORF. The mouse Pat1a cDNA was available as an I.M.A.G.E clone in the pCR4-TOPO vector (clone: 40130949: accession number: BC145646). xPat1a, xPat1b, mPat1a, and hPat1b ORFs were amplified with *Pwo* polymerase using indicated primers (Supplemental Table 1).

xPat1a and xPat1b antibodies

xPat1a and xPat1b antibodies were raised in rabbits against the N-terminal peptides DQESDEEPVKLEDD and EEDEDIDQFNDD, respectively (Sigma-Genosys) (Supplemental Fig. 2). In all experiments, both antibodies were used as affinity purified antibodies. xPat1a-His_6 and xPat1b-His_6 in Pet21-b (Supplemental Table 1) were expressed in *E. coli* BL21* cells for the antibody affinity purification, as previously described (Minshall and Standart 2004).

Xenopus laevis oocytes, eggs and embryo preparations, and gel filtration

Isolation, staging, lysate preparation, and enucleation of *Xenopus laevis* oocytes were performed as previously described (Minshall and Standart 2004). Embryos and eye and brain lysates were a kind gift of Francis van Horck (Department of Physiology, Development and Neuroscience, University of Cambridge). For FPLC gel filtration refer to Minshall and Standart (2004). Samples of 30 μL alternate fractions were separated by SDS-PAGE and visualized by Western blot.

Protein gel electrophoresis and Western blotting

Fifteen percent SDS-PAGE gels and Western blotting were performed as described by (Minshall et al. 2007). Additional primary antibodies and dilutions used were as follows: xPat1a affinity purified (1:200), xPat1b affinity purified (1:200), actin (1:2000) (Sigma), His (1:1000) (Abcam), ERK (1:12,000) (Santa Cruz Biotechnology), Flag (1:2000) (Sigma), affinity-purified MS2 (gift of Chris Smith Lab, Cambridge) (1:500), p54 (1:10,000) (Bethyl Laboratories), and GFP (1:500) (Santa Cruz).

In vitro transcription, translation, and in vitro RNA-binding assay

xPat1a, xPat1b, and hPat1b PGEM4-Z constructs (Supplemental Table 1) were linearized with ScaI and transcribed with SP6 polymerase (Minshall and Standart 2004). RNA was translated in reticulocyte lysate system in the presence of 18.52 MBq/mL [^{35}S]-methionine (Jackson and Hunt 1983). For the RNA-binding assay, 6 μL reticulocyte lysate was added to 300 μL binding buffer (100 mM NaCl, 50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.5% NP-40 [v/v]) with 50 μL 1:1 solution of beads in binding buffer. The different immobilized RNA-homopolymers were poly(A)-agarose (Sigma-Aldrich), poly(U)-Sepharose (Amersham Pharmacia Biotech AB), poly(C)-agarose (Sigma-Aldrich), and poly(G)-agarose (Sigma-Aldrich), which were diluted with Sepharose 4B beads to equivalent milligrams per milliliters of RNA. Binding was allowed for 2 h at 4°C on a rotating wheel. After three washes with binding buffer, the bead-bound fractions were analyzed by SDS-PAGE followed by autoradiography and quantitated using TotalLab software for densitometry. In the competition assay, synthetic *NRAS* RNA oligonucleotides were added to the labeled proteins for 10 min prior to the addition of poly(G)-beads.

Tethered function assay

MS2-xPat1a and MS2-xPat1b (Supplemental Table 1) constructs were linearized with ClaI, and T7 polymerase was used for in vitro transcription. Fifty nanoliters of MS2-xPat1a or -xPat1b fusion mRNA (500 ng/μL) was injected in stage VI oocytes. After 6 h incubation at 16°C, 10 nL of the firefly luciferase reporter mRNA (10 ng/μL), which contains three MS2-binding sites in its 3′ UTR and the internal control *Renilla* luciferase mRNA (0.35 ng/μL), was coinjected. Incubation was continued overnight before harvesting. Five pools of five oocytes were used per experimental point. The luciferase assay was performed as described (Minshall and Standart 2004; Minshall et al. 2010).

RNA extraction and qPCR

For the RNA extraction, five oocytes were lysed in 100 μL TNES (0.1 M Tris [pH 7.5], 0.3 M NaCl, 0.005 M EDTA, 2% SDS) and incubated for 30 min at 50°C with 200 μg/mL proteinase K followed by two phenol/chloroform extractions and one chloroform extraction. DNase-treated RNA (2 μg) was used for reverse transcription

(RT) using AMV-RT (Promega) and oligo-dT, firefly luciferase, and *Renilla* luciferase RT primers. Negative controls lacking reverse transcriptase were carried out. The Rotor-Gene 6000 (Corbett Research) was used for the qPCRs using SYBR green (Sigma). Fold change in mRNA levels was calculated relative to the control and normalized to GAPDH in three independent experiments.

Polyadenylation assay

The 3′ proximal 180 nt of xPat1b 3′ UTR were cloned into the pGEM-1 vector (Promega) (Supplemental Table 1), and the 3′ proximal 65 nt cyclin B1 3′ UTR in PGEM-1 was used as a positive control (gift from M. Wickens [Department of Biochemistry, University of Wisconsin Madison]) for the polyadenylation assay. The constructs were linearized with EcoRI and SP6 polymerase was used for the in vitro transcription in the presence of 3.7 Mbq/mL α-[^{32}P]-UTP. Fifty nanoliters of RNA (5 pmol/μL) was injected in stage VI oocytes, some of which were matured with progesterone for 14 h before harvesting and lysis. The RNA was extracted as described above, and approximately equal counts were loaded on a 5% polyacrylamide-urea gel.

Immunoprecipitation and pull-down assays

Fifty nanoliters RNA (500 ng/μL) encoding Flag-MS2-xPat1a or -xPat1b fusion mRNAs (Supplemental Table 1) was injected into stage VI oocytes. Incubation was continued for 48 h before harvesting. Oocytes were cleared in 1 mL NET buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.5% [v/v] NP-40, 1 mM EDTA [pH 8.0], 0.25% [w/v] gelatin) by spinning at 10,000 rpm for 10 min. Two microliters Flag antibody (Sigma), 1 μL CPEB (Gray et al. 2000; Minshall et al. 2007), 20 μL xPat1a, or 40 μL xPat1b antibody was incubated with 100 lysed and cleared oocytes for 2 h at 4°C followed by the addition of 10 μL protein G–Sepharose beads for 2 h at 4°C. The beads were washed three times in 500 μL NET buffer and eluted in 25 μL boiling 2× SDS sample buffer. Ten microliters (∼50 oocytes) was run on an SDS-PAGE gel for protein detection by silver staining or by Western blot. Four hundred picomoles biotinylated synthetic *NRAS* RNAs was incubated with 75 oocytes in 300 μL binding buffer (100 mM KCl, 10 mM Tris HCl [pH 7.4], 2.5 mM $MgCl_2$, 0.05% NP-40, 5% glycerol) for 1 h at 4°C. Two hundred microliters magnetic streptavidin Dynabeads (Invitrogen) was added for 1 h at 4°C. The beads were washed three times in 500 μL binding buffer and eluted in 25 μL 2× sample buffer. Ten microliters (∼35 oocytes) was run on an SDS-PAGE gel for protein detection by silver staining or by Western blot.

GFP transfection and immunofluorescence

HeLa cells were grown in DMEM and 10% fetal calf serum. Cells were plated on a 20-mm coverslip in a 35-mm diameter dish. Cells were fixed in methanol for 3 min at −20°C. Immunofluorescence was performed as previously described (Ernoult-Lange et al. 2009) using xPat1b (1:100), Ge-1 (1:1000) (Santa Cruz), or p54 (1:1000) (Bethyl Laboratories) antibodies. Cells were observed under a Leica SP1 confocal microscope (Leica) with a 63× 1.32 oil immersion objective, or with a Leica DMR fluorescent microscope (Leica) with a 63× 1.32 oil immersion objective, or with a Zeiss Axioimager M1 microscope and a Plan-Apochromat 100/1.4 Oil DIC objective. Transfections were performed by either a standard calcium phosphate procedure or with 4 μL lipofectamine 2000 (Invitrogen). Two micrograms hPat1b, mPat1a EGFP-C1, xPat1a, and xPat1b EGFP-N1 constructs was transfected, and after 24-h culture the cells were fixed and stained with appropriate antibody. Three micrograms hPat1b (CUAGAAGAUCCAGCUAUUAdTdT; Scheller et al. 2007), β-globin, or p54 siRNAs (si-Glo.1 and si-p54; Minshall et al. 2009) was transfected, and the cells were fixed 48 h later.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at http://www.rnajournal.org.

ACKNOWLEDGMENTS

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