A Zinc-binding Domain Is Required for Targeting the Maternal Nuclear Protein PwA33 to Lampbrush Chromosome Loops

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Abstract. In oocytes of the newt *Pleurodeles waltl*, the maternal nuclear protein PwA33 occurs on the lampbrush chromosomes and in some nucleoplasmic particles of the germinal vesicle. PwA33 is a modular protein and we used site-directed mutagenesis to alter the sequences encoding two metal-binding regions, the C_3HC_4 (or RING finger) and B-box motifs. Several mutant clones were generated and their synthetic transcripts were injected into *Pleurodeles* oocytes for in vivo analysis. In the oocyte, all translation products localized in the germinal vesicle. Proteins encoded by RING finger mutant clones were distributed in a pattern identical to that of the wild type protein, but when His266 of the B-box was mutated, PwA33 failed to localize in the lampbrush chromosomes and the nucleoplasmic particles. Using an in vitro colorimetric assay, we demonstrated that PwA33 is a zinc-binding protein and that mutations in the RING finger and B-Box altered its metal-binding properties. The RING finger motif bound two Zn^{2+} ions and the binding ratios of several mutants were consistent with the tertiary structure recently proposed for this motif. The B-box coordinated one Zn^{2+} and this binding was inhibited by the His266 mutation. The failure of the His266 mutation to bind zinc and to localize properly within the germinal vesicle suggests that an intact B-box is required for normal functioning of the PwA33 protein in the oocyte.

DURING oogenesis, transcripts and/or proteins from many genes are stored in the oocyte for use during later development. The significance of this dual form of maternal information storage is still unknown but it involves common cell components as well as specific regulatory factors (Davidson, 1986). In the oocyte, maternal molecules are localized either in the cytoplasm or in the germinal vesicle (GV)[1], and many possess a specific distribution. The first indication that the GV is a storage compartment came from analysis of the maternal effect of the *ova-deficient* mutation in the axolotl. The wild-type gene controls gastrulation, and its product, probably the encoded protein, is stored exclusively in the GV (Briggs and Cassens, 1966; Briggs, 1968; Briggs and Justus, 1968; Brothers et al., 1976). These results suggested that the GV could act as a specific storehouse for regulatory proteins required in early development.

To test this hypothesis, protein components of the GV were screened using mAbs directed against GV antigens (Dreyer et al., 1981, 1982; Lacroix et al., 1985; Roth and Gall, 1987). Many of the proteins detected were found to have features expected of maternal regulatory proteins: exclusively nuclear during oogenesis, these proteins were transmitted to the egg cytoplasm and reentered the embryonic nuclei with specific distribution patterns (Dreyer et al., 1981, 1982; Abbadie et al., 1987). Interestingly, some regulatory proteins that are stored during oogenesis, like the transcription factors TFIIIA and FRGY2, are also functionally active in the oocyte (for review see Tafuri and Wolffe, 1993). To identify new maternal proteins that are stored in the GV and that might also serve a regulatory role in the oocyte, a more restrictive screen was made by selecting mAbs that stain lampbrush chromosomes (Lacroix et al., 1985; Roth and Gall, 1987). In this way, several chromosomal proteins were characterized. One such protein, PwA33, is associated with most lampbrush chromosome loops of *Pleurodeles* oocytes. The loops are the active transcription regions of the chromosomes and are visible by light microscopy because of their dense RNP matrix, which is composed of nascent RNA chains and associated proteins. The intimate association of PwA33 with the chromosomal loops suggests a role in transcription during oogenesis. PwA33 also occurs in some nucleoplasmic granules and in a soluble form (Bellini et al., 1993). It is transmitted to the egg cytoplasm at the time of GV breakdown and reenters embryonic nuclei at the mid-blastula transition (Abbadie et al., 1987).

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1. *Abbreviations used in this paper*: GST, glutathione-S-transferase; GV, germinal vesicle; PAR, 4-(2-pyridylazo) resorcinol; PMPS, *p*-hydroxymercuriphenylsulfonate.

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To assess a possible regulatory role for PwA33 protein, the corresponding cDNA clone was isolated and characterized from an expression library of *Pleurodeles* ovary RNA (Bellini et al., 1993). PwA33 is composed of at least seven domains: two putative nuclear localization signals, a short sequence that may act as a cytoplasmic retention domain (Li et al., 1994), two possible zinc-binding motifs, a coiled-coil domain involved in dimerization, and a "*ret* finger protein domain" or "rfp domain" that may bind an unknown ligand as suggested by Jack and Mather (1990). This last region is composed of ~175 amino acids well conserved at the carboxy terminus of several proteins (Bellini et al., 1993). It was referred to as "rfp-like domain" by Chan et al. (1991) when describing the similarity between the two carboxy termini of the human SS-A/Ro and rfp proteins. The first zinc-binding motif, referred to as the RING finger (Freemont et al., 1991) or C_3HC_4 motif (Everett et al., 1993; Barlow et al., 1994), is shared by about 50 viral and cellular proteins, all of which have putative regulatory functions (for review see Freemont, 1993). The second zinc-binding motif, termed the B-box (Kastner et al., 1992; Reddy and Etkin, 1991; Reddy et al., 1992; Freemont, 1993) is conserved among eight other nuclear proteins, including the nuclear factor XNF7 from *Xenopus* (Reddy et al., 1991), the mouse rpt-1 regulatory protein (Patarca et al., 1988), the mouse T18 oncogenic protein (Miki et al., 1991), the human 52-kD SS-A/Ro autoantigen (Chan et al., 1991; Itoh et al., 1991), the human rfp protein and the related *ret* oncogene (Takahashi and Cooper, 1987; Takahashi et al., 1988), the human proto-oncogene PML or Myl (de Thé et al., 1991; Kakizuka et al., 1991; Kastner et al., 1992), the human estrogen responsive finger protein efp (Inoue et al., 1993) and the murine TIF1 protein (Le Douarin et al.,1995). To further investigate the role of the two zinc-binding domains in PwA33, site directed mutations were generated and mutants were analyzed in vivo by expression in the oocyte. Here we describe the specific effect of the B-box on the subnuclear localization of PwA33 in the oocyte.

Materials and Methods

Site-directed Mutagenesis

Site directed mutagenesis was performed on the myc-PwA33 clone (Bellini et al., 1993) using the in vitro mutagenesis system from Amersham Corp. (Arlington Heights, IL) under conditions suggested by the manufacturer. Because the myc-PwA33 cDNA was cloned in pBluescript II (Stratagene Inc., La Jolla, CA), the f1 origin of replication allowed the rescue of the sense strand DNA of myc-PwA33 upon coinfection of XL1 Blue bacteria with the helper phage R408 (Stratagene). Purified single-strand DNA was then used as a template for mutagenesis. As a result, all mutants were generated with a myc-tag and were cloned in pBluescript II between the HindIII and EcoRI sites of the polylinker. Three degenerate antisense oligonucleotides (A, B, and C) were designed to introduce mutations at cysteines C177 (A) and C201 (B), and histidine H266 (C). An EcoR1 site was included in each mutant to permit rapid screening of mutant clones. DNA sequencing was performed to confirm the presence of the desired mutations. Double mutations were generated with two oligonucleotides used simultaneously (A and B, A and C, B and C). Oligonucleotides used in this procedure were: (A) 5′ GTT ATG GCC GAA TTC CAG AAT GAC GGG TTC C 3′; (B) 5′ CAA GAC TTC CTT GAA TTC TGG GCA GGA AAA 3′; and (C) 5′ GTG ATT AGA ATT CTT AAG AGA GTC GCG GCA 3′. Nucleotides different from those in wild-type PwA33 are underlined.

RNA Injection into Oocytes

Normal and mutant myc-PwA33 clones were linearized at the unique BamHI site of the pBluescript II polylinker, and were used as templates for RNA synthesis in vitro. Sense-strand RNA was generated with T3 RNA polymerase in the presence of 7MeGpppG. After extraction with phenol, RNA was precipitated with ethanol and redissolved in water at a concentration of 1 mg/ml. 30–50 nl were injected into the cytoplasm of *Pleurodeles waltl* or *Notophthalmus viridescens* oocytes. After injection, the oocytes were held at 19°C for 24 h. Newly synthesized translation products were then analyzed by indirect immunofluorescence on lampbrush chromosome preparations or on immunoblots of extracted proteins.

Spread nuclear preparations were made as described by Gall et al. (1981) and Lacroix et al. (1985). The myc-tagged proteins were detected with mAb 9E10, which is specific for the myc epitope (Evan et al., 1985).

GVs were hand-isolated in buffer N containing 10 mM Tris, 75 mM KCl, 25 mM NaCl, 0.5 mM $CaCl_2$, 10 mM $MgCl_2$, pH 7.2. GV pellet and supernate fractions were generated by centrifugation of sonicated GVs at 15,000 *g* for 30 min. Enucleated oocytes were sonicated and centrifuged to produce a yolk-free cytoplasmic fraction. Total proteins from nuclear and cytoplasmic fractions were separated on a 7–15% SDS PAGE gradient gel, transferred to a 0.45-μm PVDF membrane (Millipore Corp., Bedford, MA) and probed with mAb 9E10 or mAb A33/22 (Lacroix et al., 1985).

Fusion Protein Synthesis and Purification

Two regions of PwA33 cDNA (nucleotides 541–1233 and 768–1233) were PCR-amplified using two sense (D and E) and one antisense (F) oligonucleotides. Purified fragments were subcloned at the unique restriction sites BamHI and SacI, or EcoRI and SacI of the pGEX II vector (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) modified in its polylinker (St. Johnston et al., 1992). After subcloning, fragments should be downstream of the glutathione-S-transferase ORF (GST) and in-frame with it. Sequencing was performed to demonstrate that this was the case and to verify that no mistake had been introduced by the Taq polymerase during the amplification step. Expression of the fusion proteins, named FP50 and FP58, was induced in 300-ml cultures of either JM109 or Xl1 Blue bacteria. Cultures grown overnight in LB with 100 μg/ml of ampicillin were diluted 1/100 in LB with 50 μg/ml of ampicillin and were then induced with 1 mM IPTG when in mid-log phase. Induced cultures were grown 3 h before harvesting the cells. Cells were pelleted, washed twice with 10 vol of buffer P1 (25 mM Hepes pH 8, 75 mM KCl, 25 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol), and then resuspended in 45 ml of P1. Cells were sonicated at 4°C (3× 30 s) and lysates were then centrifuged at 25,000 *g*, 20 min at 4°C. Glutathione agarose beads were prepared as suggested by the manufacturer (Sigma Chemical Co., St. Louis, MO) and equilibrated in an equal volume of P1. Beads were added to the cleared cell lysates (200 μl of beads for 5 ml lysate) and allowed to bind fusion protein for 1 h at 4°C with gentle shaking. After washing (5× 30 ml of P1), fusion proteins were eluted from the beads twice in two volumes of buffer P1 containing 5 mM NaOH and 5 mM glutathione. Fusion proteins were then dialyzed against 10 L of buffer P2 (25 mM Hepes pH 8, 75 mM KCl, 25 mM NaCl, 10% glycerol) at 4°C and stored in P2 buffer at 4°C.

Mutant FP58 clones (C177F, C201F, and H266N) were similarly amplified from mutant PwA33 clones.

Oligonucleotides used in this procedure were: (D) 5′ TGA CGG ATC CGA GGA CTT GAC CTG CC 3′; (E) 5′ GAT AAA GAA TTC AAG CCC AAG GAA AAA TGT G 3′; and (F) 5′ CAG TTA AGA GCT CTA TTG GGT CGC TTT CAT TGA GCC 3′.

Zinc Cation Quantitation

Protein concentrations were determined by a Bradford assay (Bio Rad, Richmond, CA) and were normalized by appropriate dilutions with buffer P2. To 1 ml protein samples containing 100 μM 4-(2-pyridylazo) resorcinol (PAR), a saturating quantity of *p*-hydroxymercuriphenylsulfonate (PMPS) (5 μl of 20 mM PMPS) was added, and the amount of released metal cation was determined by measuring ΔOD at 500 nm (Hunt et al., 1985). $HgCl_2$, $NiCl_2$, $CoCl_2$, $CdCl_2$, $FeCl_2$, and $ZnCl_2$ standard solutions (Sigma) were used to determine which metal ion was bound to the fusion proteins. Various amounts of $ZnCl_2$ in P2, containing 100 μM PAR, were used as standards to measure the instantaneous concentrations of zinc released by PMPS. At 500 nm, $\Delta\Sigma = 6.6 \times 10^4 M^{-1}cm^{-1}$ for $(PAR)_2Zn^{2+}$. PAR and PMPS were purchased from Sigma. All buffers used in this procedure were purified with a Chelex 100 resin to reduce metal ions impurities.

Results

Mutation of a Single Histidine Inhibits the Targeting of PwA33 to Lampbrush Chromosomes Loops

Two cysteine and histidine-rich domains (residues 162–201 and 238–269) are recognizable in PwA33 by comparison with previously described protein sequences (Fig. 1) (Bellini et al., 1993). The first contains the C_3HC_4 or RING finger motif, for which the secondary and tertiary structures have been described (Everett et al., 1993; Lovering et al., 1993; Barlow et al., 1994). Within the second cys/his domain, referred to as the B-box, an additional zinc finger could be formed with a suggested motif $CX_2CX_5HX_2H$ (C_2H_2) (Patarca et al., 1988). Point mutations were made in two cysteines of the RING finger, in each case converting the cysteine to a phenylalanine (C177F and C201F) and in one histidine of the B-box, converting it to an asparagine (H266N).

Using the T3 promoter in the bluescript vector, we synthesized capped, sense-strand transcripts from wild-type and mutant myc-tagged PwA33 clones and injected them into the cytoplasm of *Pleurodeles* oocytes. After a 24 h incubation, GVs were isolated and their proteins assayed on immunoblots with mAb 9E10, which is specific for the myc tag (Evan et al., 1985). Identical results were obtained with the wild-type PwA33 clone and with three single and three double mutant clones. In each case, a unique protein of relative molecular mass of 83 kD was newly synthesized and efficiently accumulated in the GV (Fig. 2 *A*). Uninjected oocytes were negative. As expected, when mAb A33/22 was used for detection, two proteins were seen, the endogenous PwA33 (80 kD) and the newly synthesized mutant protein (83 kD) (Fig. 2 *B*). Roughly equivalent amounts of the two proteins were localized in the GV. These data are summarized in Table I.

The subnuclear localization of the myc-tagged translation products was then determined by indirect immunofluorescence of spread GV contents from injected oocytes. When transcripts from the C177F or C201F mutant clones were injected into oocytes, an intense staining was detected with mAb 9E10 on most of the lampbrush loops (Fig. 3 *B*). Some particles in the nucleoplasm were also labeled. This pattern is identical to that obtained when transcripts from a wild-type PwA33 clone are injected (Bellini et al., 1993). Similarly, protein from the double mutant C177F + C201F had a normal distribution on the lampbrush chromosomes and in the nucleoplasmic particles. Thus neither single nor double mutations of the RING finger motif affected the intranuclear distribution of PwA33 (Table I). In contrast, protein encoded by the H266N mutant clone could not be detected on the lampbrush loops or in nucleoplasmic particles. The same was true for the two double mutants, C177F + H266N and C201F + H266N (Table I).

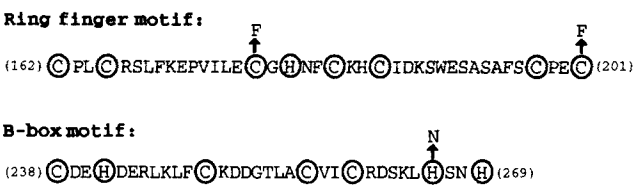

Figure 1. Amino acid sequences of the RING finger or C_3HC_4 motif (162-201) and the B-box (238-269). Highly conserved cysteine and histidine residues are circled. Residues altered by site-directed mutagenesis are indicated by arrows with the corresponding amino acid replacements.

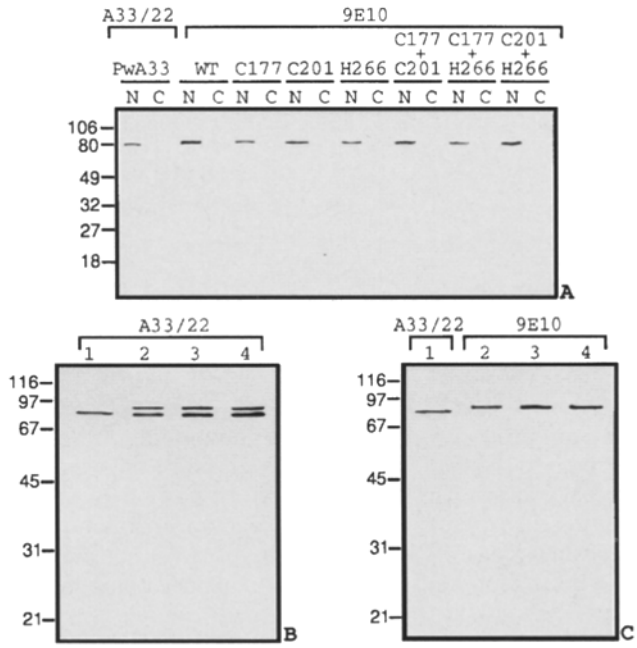

Figure 2. Capped *in vitro* transcripts from normal and mutant myc-PwA33 clones were injected into *P. waltl* (*A* and *B*) or *N. viridescens* oocytes (*C*). Nuclear and cytoplasmic proteins were extracted 24 h later, resolved on a 7–15% polyacrylamide gradient gel, and transferred to an Immobilon-P membrane. (*A*) Newly synthesized myc-tagged translation products were detected with mAb 9E10, which is specific for the myc tag. In each case a unique protein (M_r = 83 kD) is found in the GV (*N*) but not in the cytoplasm (*C*). (*B*) When mAb A33/22 was used for detection, both endogenous (M_r = 80 kD) and newly synthesized proteins (M_r = 83 kD) were detected. GV proteins from uninjected oocytes (lane *1*) or oocytes injected with RNA from mutants H266N, C177F and C201F (lanes *2*, *3*, and *4* respectively). (*C*) Similar results were obtained with oocytes of another newt, *Notophthalmus viridescens*. GV proteins from uninjected oocytes (lane *1*) or oocytes injected with RNA from mutants C177F, C201F, and H266N (lanes *2*, *3*, and *4* respectively).

When GVs from injected oocytes were sonicated and centrifuged at 15,000 *g* for 30 min, conditions that pellet the chromosomes and all other cytologically identifiable structures, the wild-type and RING finger mutant proteins were equally distributed between the soluble and pellet fractions. The H266N mutated protein, however, remained exclusively in the supernatant, suggesting that it is accumulated in the GV in a soluble form only (Fig. 3 *A*).

Similar results were obtained when wild-type and single mutant PwA33 proteins were expressed in oocytes of another newt, *Notophthalmus viridescens*. All four proteins were efficiently translated and translocated to the GV (Fig. 2 *C*), and the H266N mutated protein was the only one not detected on the lampbrush chromosome loops (data not shown).

In summary, in *Pleurodeles* and *Notophthalmus* oocytes,

Table I. Localization of the Newly Synthesized Proteins in Pleurodeles Oocytes

Injected RNA	Cytoplasm	Nucleus		
		Soluble fraction	Particles	Chromosomes
Wild type	−	+	+	+
C177F	−	+	+	+
C201F	−	+	+	+
H266N	−	+	−	−
C177F + C201F	−	+	+	+
C177F + H266N	−	+	−	−
C201F + H266N	−	+	−	−

a mutant protein with the H266N point-mutation was imported into the GV but failed to localize like the wild-type PwA33 protein. This failure indicates clearly that His 266 is required, presumably through a zinc-finger structure, for the association of PwA33 with the transcription units of lampbrush loops and for localization in nucleoplasmic particles.

Effect of Mutations on the Zinc Binding Ability of PwA33

To determine whether the three mutations (C177F, C201F, and H266N) modify the zinc binding properties of PwA33, we used a spectrophotometric method to detect sulfhydryl coordinated heavy metal ions. The assay is based on two reactions. First, zinc bound to the protein is released by oxidation of sulfhydryl residues with the mercurial reagent PMPS. The reaction is reversible upon addition of β-2-mercaptoethanol, and this property can be used to analyze the rebinding of exogenous zinc. Second, the concentration of Zn^{2+} is determined by monitoring the changes in absorbance of the metallochromic indicator, PAR at 500 nm. Because protein overexpressed from the full-length PwA33 clone gave numerous degradation fragments, a PCR-amplified region (nucleotides 541–1233) was subcloned into a modified pGex vector (St. Johnston et al., 1992), and a bacterially expressed fusion protein, FP58, was purified (Materials and Methods). As shown in Fig. 4, FP58 consists of glutathione-S-transferase in-frame with the two cys/his regions and the coiled coil domain of PwA33 (residues 158–388). When PMPS was added to a solution containing FP58 and PAR, a rapid change of absorbance was observed, indicating that metal ions had been released from FP58 (Figs. 5 and 6). When the GST protein encoded by the pGex vector alone was used as a control, only a low background was observed (Fig. 6). A 12-h dialysis was then performed to remove the released metal ions, and the resulting protein sample was divided into several aliquots. Different exogenous cations (Zn^{2+}, Ni^{2+}, Hg^{2+}, Co^{2+}, Cd^{2+}, and Fe^{2+}) were then added, of which only Zn^{2+} was significantly chelated by FP58 in the presence of β-2-mercaptoethanol (Fig. 5). These results strongly suggest that PwA33 is a zinc-binding protein. Using standard zinc solutions to measure the instantaneous concentration of Zn^{2+} at pH 7, we found an average molar ratio of Zn^{2+}/FP58 of 2.84 in five independent FP58 preparations (Fig. 6). We therefore conclude that three zinc cations are chelated by the PwA33 protein.

We used the same spectrophotometric assay to compare the amounts of zinc bound by three point mutants of FP58 and by another construct, FP50, which lacked the RING finger entirely.

FP50 consists of an intact B-box and coiled-coil region of PwA33, but is deleted for all sequences upstream of the B-box, including the RING finger (Fig. 4). When PMPS was used on FP50 protein solutions, an average molar ratio of Zn^{2+}/protein of 0.9 was found. Thus, of the three Zn^{2+} ions bound by wild-type FP58 protein, one can be as-

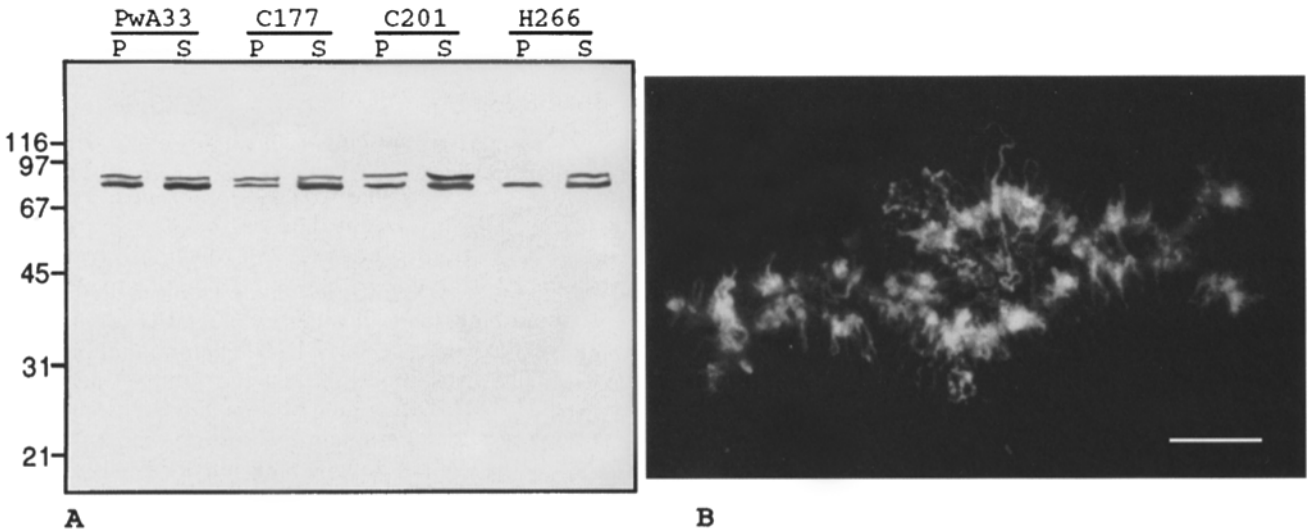

Figure 3. (*A*) Western blots of GV proteins from oocytes injected with RNA from normal and mutant myc-PwA33 clones. GVs were centrifuged to generate supernate (*S*) and pellet (*P*) fractions. Because mAb A33/22 was used as the probe, both endogenous PwA33 and newly synthesized proteins were detected. Both were distributed about evenly between the supernate and the pellet fractions, except for the H266N mutant protein, which was absent from the pellet fraction. (*B*) Immunofluorescence image of lampbrush chromosome X from *P. waltl* after staining with mAb 9E10. The oocyte from which this spread nuclear preparation was made was injected 24 h earlier with RNA from mutant clone C177F. Myc-tagged mutant protein is located on most of the chromosome loops. Bar, 40 μm.

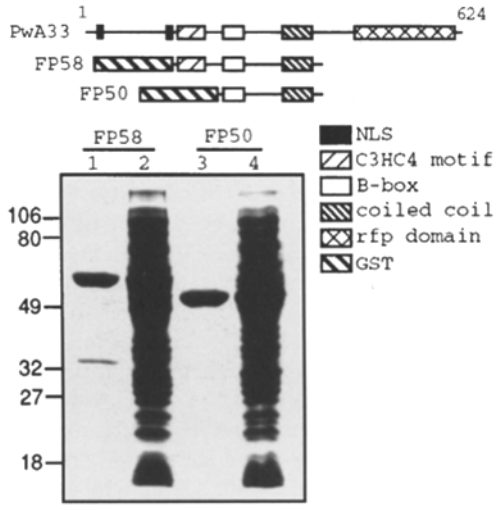

Figure 4. Fusion proteins FP58 and FP50. At the top are diagrams of wild-type PwA33 protein and GST fusion proteins FP58 and FP50 derived from it. Proteins were overexpressed in *E. coli* and purified from cell lysates on glutathione agarose beads. 4 μg purified proteins was resolved on a 7–15% polyacrylamide gradient gel and stained with Coomassie blue (lanes *1* and *3*). Some proteolytic breakdown products appear as minor bands. Total proteins from cleared cell lysates were also run before incubation with glutathione-agarose beads (lanes *2* and *4*). Positions of molecular weight markers in kD.

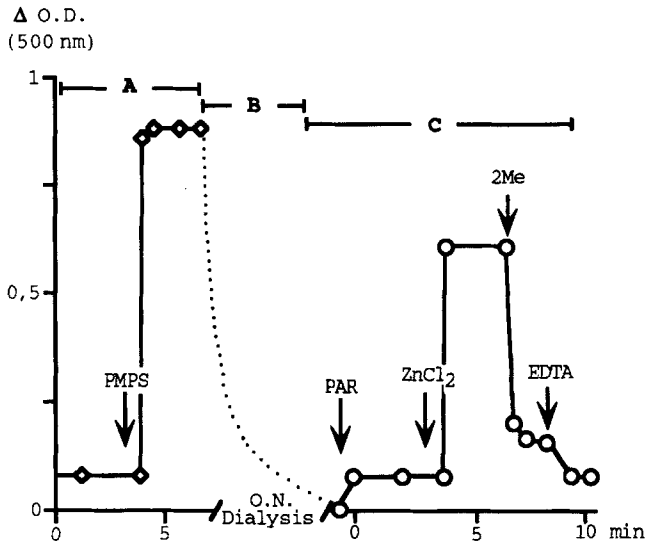

Figure 5. Zn^{2+} binding capacity of fusion protein FP58. Zinc was released from protein FP58 (4 μm) by addition of PMPS, and quantitated by measuring the increased absorption of PAR at 500 nm (*A*). An overnight dialysis was then performed against P2 buffer to eliminate the released Zn^{2+} (*B*). After dialysis (*C*), new PAR (100 μM) and $ZnCl_2$ (8 μM) were added. After OD stabilization, β-2-mercaptoethanol (200 μM) was added to displace the PMPS. The OD_{500} decreased dramatically as exogenous Zn^{2+} was transferred from PAR to the protein. Finally, EDTA was added to eliminate traces of the Zn^{2+}/PAR complex.

cribed to the region containing the B-box and two to the upstream RING finger region.

When histidine 266 in the B-box of FP58 was converted to an asparagine (H266N), the binding assay gave a molar ratio of Zn^{2+}/protein of 1.9 as shown in Fig. 6. This ratio indicates that substitution of asparagine for one of the histidines in the B-box reduced the amount of bound zinc from three ions in the wild-type to two in the mutant protein. A direct participation of H266 in a single zinc chelation is thus demonstrated. Because PMPS reacts with sulfhydryl groups, cysteine residues must be involved as well. Therefore, the B-box domain of PwA33 clearly organizes a zinc-binding structure through cysteine and histidine residues.

When assayed for Zn^{2+} binding, C177F and C201F gave molar ratios of Zn^{2+}/protein of 1.8 and 1.9, respectively (Fig. 6). Thus mutations in two separate sites in the RING finger resulted in loss of one zinc atom each. Surprisingly, a ratio of Zn^{2+}/protein of 1.4 was observed when we tested a double mutant of FP58 containing both C177F + C201F mutations. Because this intermediate value is significantly higher than 1.0, we concluded that some Zn^{2+} was still bound to the RING finger motif.

Discussion

We have shown that the nuclear protein PwA33 contains two cys/his rich regions that bind zinc. The first contains the so-called RING finger motif (C_3HC_4) and binds two Zn^{2+} ions, whereas the second contains a B-box (C_2H_2) and binds a single Zn^{2+} ion. Mutational analysis demonstrates that the normal subnuclear localization of PwA33 in the oocyte depends on an intact B-box but not on an intact RING finger.

Structure of the B-box

Within the second cys/his region of PwA33 is a B-box, which contains four highly conserved cysteines and three histidines that could define one or more zinc fingers (Patarca et al., 1988). Several other amino acids are characteristic of this region and on the basis of multiple alignments a consensus sequence can be derived (Fig. 7).

When this domain was assayed for zinc binding using the FP50 fusion protein, a single Zn^{2+} ion was found to be coordinated. This value indicates clearly that only one zinc finger (or zinc finger-like structure) is formed. This conclusion agrees with recent data on the *Xenopus* nuclear factor XNF7 (Borden et al., 1993), which also contains a B-box. In this case a synthetic peptide corresponding to the B-box bound tetrahedrally a single Zn^{2+} ion.

The substitution of asparagine for His266 in PwA33 eliminated the zinc-binding ability of the domain, indicating that this residue participates in zinc chelation. Because the PMPS titration is based on modification of sulfhydryl residues, at least one cysteine must also be involved. Therefore, the metal binding motif proposed by Patarca et al. (1988), $CX_2CX_5HX_2H$ is consistent with the combined data from PwA33 and XNF7. This motif falls within the arrangement of cysteine and histidine residues defined by Berg (1988, 1990) as a potential metal-binding domain, $CX_{2\text{-}4}CX_{2\text{-}15}aX_{2\text{-}4}a$ in which "a" is either Cys or His. However, because seven putative metal ligands are highly conserved in the domain, we cannot rule out the possible for-

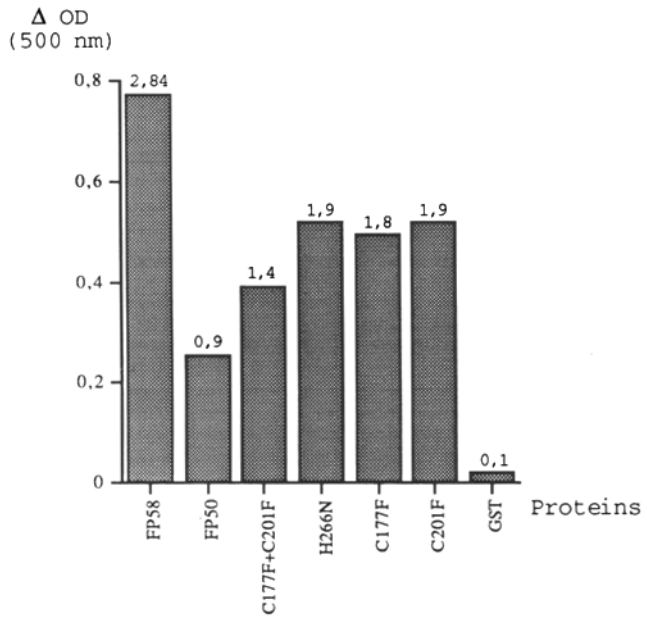

Figure 6. Quantitation of zinc released from fusion proteins FP50, FP58 and various FP58 mutants. In each case measurements were made on 5 independent protein samples. Columns represent the average ΔOD_{500} when PMPS was added to a solution of 4 μM protein and 100 μM PAR. Above each column is the ratio of Zn^{2+}/protein. The GST protein was used under the same conditions to determine the background level.

mation of a nonstandard zinc finger structure. Studies of the three-dimensional structure would be helpful in clarifying the role of the other Cys and His residues in this domain.

An Intact B-box Is Required for Targeting of PwA33 to the Lampbrush Chromosome Loops

The mutation of His266 had a dramatic effect on the intranuclear distribution of PwA33. When RNA from the H266N mutant clone was injected into oocytes, the newly synthesized protein was efficiently accumulated in the GV but was no longer addressed to the transcription units of the chromosome loops or to small particles in the nucleoplasm. It seems probable that the effect of the mutation is due to the lack of a zinc cation within the mutated Cys/His domain. Recent NMR and CD studies on the corresponding region of XNF7 indicate that this domain is structured only in the presence of zinc (Borden et al., 1993). We assume that the H266N mutated domain, being unable to bind zinc, is improperly folded and thus loses its ability to interact with other molecules on the chromosomes and in the nucleoplasmic particles. Alteration of the zinc-binding domain, however, has no effect on the translation of the protein or its importation into the nucleus.

Additional evidence that zinc is essential in vivo for the normal distribution of PwA33 came from the use of PMPS on lampbrush chromosomes. When GV contents were spread in the presence of PMPS, PwA33 could not be detected on the transcription units or in the nucleoplasmic particles by immunofluorescence (data not shown). This observation supports the view that localization of the protein depends on metal binding.

Immunofluorescence shows that PwA33 is precisely colocalized with the matrix of the loops of the lampbrush chromosomes (Fig. 3 *B* and Bellini et al., 1993). The matrix consists of nascent RNA transcripts with associated hnRNP proteins and processing factors, in particular the splicing snRNPs (Wu et al., 1991). Thus targeting of PwA33 to the loops could involve interaction of the B-box with either RNA or protein. At least three previously characterized proteins in the *Xenopus* oocyte bind directly to RNA through C_2H_2 zinc finger motifs: TFIIIA, p43, and XFG5-1 (for review see Mattaj, 1993). The $CX_2CX_5HX_2H$ motif proposed for zinc co-ordination within the B-box might then represent a new example of a C_2H_2 zinc finger with RNA-binding properties. Equally likely, PwA33 could be associated with the nascent transcripts through protein-protein interactions, as is true for a variety of hnRNP and snRNP proteins (Birnstiel, 1988; Piñol-Roma et al., 1988; Bandziulis et al., 1989).

The H266N mutation simultaneously eliminates the association of PwA33 with the chromosome loops and with particles in the nucleoplasm. It is probable, therefore, that the mechanism of binding is the same in the two cases. The particles might be hnRNP granules resulting from chromosome activity or they might represent sites for preassembly of molecules ultimately destined for the transcription units.

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                      *   *       *     * *     * *
PwA33      (236) EKCDE  H DERLK LFCKDDGTLACVICRDSKL HSNHNF (271)
XNF7       (222) EKCSE  H DERLK LYCKDDGTLSCVICRDSLK HASHNF (257)
Rpt1        (94) NICAQ  H GEKLR LFCRKDMMVICWLCERSQE HRGHQT (129)
SSARo       (90) ERCAV  H GERLH LFCEKDGKALCWVCAQSRK HRDHAM (125)
RFP         (94) GVCEK  H REPLK LYCEEDQMPICVVCDRSRE HRGHSV (129)
T18(B2)    (235) VFCPF  HKKEQLK LYCETCDKLTCRDCQLL E HKEHRY (269)
efp(B2)    (155) RKCSQ  H  NRLREFFCPEHSECICHIC LV E HKTCSP (188)
TIF1(B2)   (221) VFCPF  HKKEQLK LVCETCDKLTCRDCQLL E HKEHRY (256)
PML(B2)    (187) IFCSNPNHRTPTLTSIYCRGCSKPLCCSCALLDSSHSELKC (228)

Consensus        --C----H-zEbLb-LΦCzzD--aaChaCz-S-z-Hb-H-h
```

Figure 7. The B-box of PwA33 and related proteins. Conserved cysteine and histidine residues are indicated with a star. In the consensus sequence, charged, basic, aliphatic, hydrophobic, and aromatic residues are represented by z, b, a, h, and ϕ, respectively. See text for references to each protein.

The RING Finger Motif

In PwA33 two zinc atoms are bound to each C_3HC_4 motif. This finding is consistent with recent data from the C_3HC_4 motif of the human RING 1 protein (Lovering et al., 1993) and the viral protein Vmw110 protein (Everett et al., 1993; Barlow et al., 1994). A structural model was proposed on the basis of two-dimensional NMR analysis and three-dimensional crystallographic data. According to this model two Zn^{2+} ions would be tetrahedrally coordinated in PwA33, the first by three cysteines (C177, C201, C198) and one histidine (H179), and the second by four cysteines (C162, C164, C18,2 and C184). Therefore the double mutation of C177 and C201 should interfere with the binding of only one Zn^{2+}. The PMPS releasable zinc titrations obtained with PwA33 are in partial agreement with such a structure, although less than one Zn^{2+} (0.4) was chelated by the doubly mutated C_3HC_4 motif. This deficiency in binding could be caused by the substitution of phenylalanine for C177 and C201, which introduces large aromatic residues that might lead to structural changes. Thus the doubly mutated

C_3HC_4 motif might be improperly folded, resulting in a lowered zinc affinity for the four unaltered cysteine residues.

Unlike the B-box domain, neither single nor double mutations of the RING finger motif affect the intranuclear distribution of PwA33, as determined by immunofluorescence observations on GV contents and on embryonic nuclei. Whatever is the exact structure of the RING finger, it is clear that this motif need not be in its normal configuration for proper localization of PwA33, and thus interaction of PwA33 with the nascent transcripts or their associated proteins is likely to occur independently of the RING finger.

PwA33 Belongs to a Family of Regulatory Proteins

Analysis of the primary amino acid sequence of PwA33 indicates that it shares several domains with a subset of putative regulatory proteins from the "Ring finger" family defined by Freemont (1993). These proteins are XNF7 (Reddy et al., 1991), rfp (Takahashi and Cooper, 1987), the 52-kD SS-A/Ro protein (Chan et al., 1991; Itoh et al., 1991), and efp (Inoue et al., 1993). They all possess a RING finger motif, one or two B-box motifs, a coiled coil region and an "rfp" domain. On the basis of the RING finger and B-box, it has been suggested that these proteins might be DNA-binding regulatory proteins. However PwA33 is clearly associated with the RNP matrix of the lampbrush chromosomes in oocytes of *Pleurodeles* (Bellini et al., 1993), and the same is true for the closely related protein XNF7 in *Xenopus* (Reddy et al., 1991). An interesting possibility is that PwA33 and XNF7 might be regulatory proteins in somatic cells, but with an additional function as RNP proteins in the oocyte. Such a dual role has been demonstrated for two other *Xenopus* proteins, the transcription factor TFIIIA and FRGY2 (reviewed by Tafuri and Wolffe, 1993).

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