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Differential RNA-binding activity of the hnRNP G protein correlated with the sex genotype in the amphibian oocyte

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ABSTRACT

A proteomic approach has enabled the identification of an orthologue of the splicing factor hnRNP G in the amphibians *Xenopus tropicalis*, *Ambystoma mexicanum*, *Notophthalmus viridescens and Pleurodeles walt*, which shows a specific RNA-binding affinity similar to that of the human hnRN G protein. Three isoforms of this protein with a differential binding affinity for a specific RNA probe were identified in the *P. walt* oocyte. *In situ* hybridization to lampbrush chromosomes of *P. waltl* revealed the presence of a family of hnRNP G genes, which were mapped on the Z and W chromosomes and one autosome. This indicates that the isoforms identified in this study are possibly encoded by a gene family linked to the evolution of sex chromosomes similarly to the hnRNP G/RBMX gene family in mammals.

INTRODUCTION

Heterogenous nuclear ribonucleoproteins (hnRNPs) are critical regulators of pre-mRNA processing in eukaryotic cells. Accumulating evidence shows that cellular control of mRNA biogenesis is dependent on these proteins even when they are in relatively low abundance in the nucleus. This is the case for the hnRNP G protein, which has emerged in the past few years as an important factor in the regulation of post-transcriptional processes. This protein has been implicated in the alternative splicing of several pre-mRNAs including transcripts of the α_S-tropomyosin, dystrophin, survival motor neuron *SMN2* and *Tau* genes in particular (1–3). In addition to its important functions in pre-mRNA splicing, hnRNP G has been shown to be involved in the tissue-specific regulation of the transcription of the SREBP-1c and GnRH1 genes (4,5). hnRNP G has been reported recently to act as a tumour suppressor in human head and neck cancer cells and a naturally occurring mutation has been identified in the RNA-binding domain of this protein in a tumour-derived cell line (6). hnRNP G is a well conserved protein in vertebrates and knockdown assays show that it is critically required for neural and muscle development in zebrafish and *Xenopus laevis* embryos (7,8).

In mammals the *RBMX* (RNA-binding motif, X chromosome) gene coding for hnRNP G is located on the X chromosome and has a paralogue on the Y chromosome, the *RBMY* gene, which is important for normal sperm development (9,10). These two genes belong to the multigenic *RBMX*/*RBMY* family, which is linked to the evolution of sex chromosomes because it is presumed to have arisen from an ancient pair of genes on proto sex-chromosomes (11). A comparative sequence analysis of this family in different species suggests that the genes have evolved separately in different mammalian lineages (12). Accordingly, the *RBMY* gene has acquired a male-specific function and became amplified in multiple copies on the Y chromosome, while multiple processed copies of *RBMX*, referred to as *RBMX*-like (*RBMXLs*) genes are thought to have been retroposed on different chromosomes. While RBMY is expressed in the testis only, hnRNP G is ubiquitously expressed. One of the retroposed autosomal copies, *hnRNP G-T*, gives rise to a protein, which is expressed predominantly in the testis and is important for male fertility in human and mouse (13–15). Only two other *RBMXLs* genes may be functional: *RBMXL1* is expressed ubiquitously and *RBMXL9* is expressed in the testis and brain but

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contains an early stop codon in its sequence (12). The RBMY and hnRNP G-T proteins are expressed exclusively in the male germinal cells in the testis and are implicated in the splicing of testis-specific exons (15,16). Although the RBMY and hnRNP G-T proteins show 59 and 74% similarity with hnRNP G, respectively, they exhibit different RNA-binding affinities. RNA motifs recognized with high affinity by the RBMY and hnRNP G-T proteins are not bound by the hnRNP G protein (17,18). It has been shown that CCC/A-rich RNAs are preferentially bound by the N-terminal RNA-binding domain (N-ter RRM) of hnRNP G (19). We have reported recently that a stem-(G GAAA)-loop RNA motif present in the W_{Ec} RNA, a 250 nt RNA substrate, formerly called BP6 RNA (20), is a specific binding site for a newly identified C-terminal RNA-binding domain of hnRNP G (21). Altogether, these data suggest that hnRNP G is a sequence-specific binding protein, which recognizes *cis*-acting RNA elements in its targets.

In *Pleurodeles waltl* where the female is heterogametic (ZW) and the male is homogametic (ZZ), functionally phenotypic ZZ and WW females can be experimentally produced (22,23). This enabled us to develop a proteomic approach with the aim of identifying RNA-binding proteins (RBPs) encoded by genes linked to the sex chromosomes. We have previously shown that the W_{Ec} RNA labelled specifically the W chromosome by binding to RBPs associated with transcription units located on three adjacent lampbrush loops in nuclear spreads of *P. waltl* oocytes (20). In the present work, we have used the W_{Ec} RNA as a probe to show the presence in the *P. waltl* oocyte of several isoforms of hnRNP G, referred to as PwhnRNP G, whose differential RNA affinity was correlated with the presence of the Z or W sex chromosomes. We also obtained evidence for a *PwhnRNP G* multigene family whose members are located on the Z and W sex chromosomes and an autosome in *P. waltl*.

EXPERIMENTAL PROCEDURES

Oocytes and cell culture

Ovarian biopsies were performed on adult females *P. waltl* anesthetized in 0.15% tricaine methane sulfonate (MS222, Sigma Chemical, St Louis, MO, USA). Oocytes were defolliculated for 2–3 h in saline buffer OR2 containing 0.15% collagenase type II (Sigma Chemical). Stage IV–VI oocytes were selected and maintained in MBS medium (24) at 18°C. Human cervical carcinoma cells HeLa (ATCC CCL-2) were cultured in RPMI 1640 medium (GIBCO, Invitrogen) supplemented with 5% (v/v) fetal bovine serum and 2 mM L-glutamine. Cell cultures were maintained at 37°C in 5% CO_2 atmosphere.

Nuclear extracts

Oocytes nuclear extracts. Oocytes were incubated in MBS containing 2% TCA (w/v) for 15–20 min at 4°C to precipitate proteins, washed several times in cold MBS and kept at 4°C up to 3 days before being individually dissected in MBS. Germinal vesicles (GVs) were isolated, transferred to microtubes, washed in water, then in 70% ethanol. Air-dried GVs were resuspended in the denaturing electrophoresis loading buffer.

HeLa cells nuclear extracts. Cells were washed twice with cold PBS and resuspended in buffer A: 10 mM Tris pH 8.0, 3 mM $MgCl_2$, 25 mM NaCl, protease inhibitor (Complete Protease Inhibitor Cocktail Tablets, Roche Applied science). After 2 min incubation on ice, cells were disrupted using an Ultra-Turrax homogenizer and centrifuged at 1200*g* for 5 min. Nuclei were resuspended in buffer B: 0.25 M Sucrose, 10 mM Tris pH 8.0, 10 mM $MgCl_2$, 10 mM NaCl, protease inhibitor cocktail. The suspension was layered over modified buffer B (0.88 M Sucrose) and centrifuged for 10 min. Nuclei were resuspended in the denaturing electrophoresis loading buffer.

Gel electrophoresis and staining

For mono-dimensional electrophoresis, samples were denatured by heating in Laemmli buffer for 5 min at 95°C and loaded on a 10% SDS–PAGE gel (Mini PROTEAN II or Protean II xi from Bio-rad). For bi-dimensional electrophoresis, samples were resuspended in DeStreak rehydration solution (8 M urea, 1 M thiourea, 2.5% CHAPS, hydroxyethyl disulfide, GE Healthcare) plus 1% carrier ampholytes mixture PH 6-11 (GE Healthcare). The isoelectric focusing (IEF) conditions were optimized for focalization of basic proteins. After 12 h of strip rehydration, samples were loaded on the anodic side of 13 cm strips that had a linear pH 6–11 gradient (IPG strips, GE Healthcare). The IEF was performed on an Ettan IPGphor (GE Healthcare) at 20°C using the following parameters: voltage was increased gradually to 500 V for 100 Vh then to 4000 V for 3400 Vh and finally to 8000 V for 13 330 Vh. Prior to the second dimension the strips were equilibrated in 6 M urea, 2% SDS, 1% DTT and 30% glycerol. In a second equilibration step the DTT was replaced by iodoacetamide to prevent proteins reoxidation. The second dimension was performed in Ettan Dalt VI (GE Healthcare) or Protean II xi (Bio-rad) vertical electrophoresis apparatuses using 12% SDS–PAGE gels. 2D gels destined for phosphoprotein detection were sequentially stained with fluorescent dyes Pro-Q Diamond and SYPRO Ruby (Molecular Probes, Invitrogen) according to manufacturer's protocols. The Pro-Q diamond stain allows in-gel detection of phosphate groups attached to tyrosine, serine or threonine residues. For individual phosphoproteins, the strength of the signal correlates with the number of phosphate groups and with the protein quantity. The SYPRO Ruby stain is a quantitative stain that reveals all the proteins. The molecular mass indicator PeppermintStick (Molecular Probes, Invitrogen) was used to show the specificity of the Pro-Q diamond dye. The fluorescent gels images were obtained on a Typhoon Imaging System 9200 or 9400 (GE Healthcare).

Nuclear extracts destined to alkaline phosphatase treatment were prepared as follows: aliquots of 60 GVs were extracted by homogenization at 4°C in lysis buffer

[100 mM NaCl, 20 mM Tris–Cl pH 7.6, 1%, Triton, protease inhibitor (Complete Protease Inhibitor Cocktail Tablets, Roche Applied science)] and sonicated 30 min in a sonicator bath at 4°C. Nuclear extracts were incubated with 50 U of Calf Intestinal Alkaline Phosphatase (CIP, biolabs) for 2 h at 37°C. Proteins were precipitated according to Chevallet *et al.* (25) with 7.5% TCA and 0.1% Sodium Lauroyl Sarconisate (NLS) for 2 h at 4°C. After centrifugation (10 000*g*, 10 min, 4°C) the pellet was washed twice with Tetrahydrofuran (THF, pre-cooled in ice), dissolved in Laemmli buffer, sonicated (10 mn) in a sonicator bath and heated for 10 min at 95°C before loading on SDS–PAGE gels.

Mass spectrometry analysis

Protein spots were manually excised from silver stained 2D gels. The excised gels were washed and then digested by incubation with 20 ng/µl Trypsin overnight at 37°C. The supernatants were recovered and the remaining tryptic peptides were extracted from the gels with acetonitrile/0.1% aqueous TFA (1:1, v/v). The combined recovered solutions were concentrated and desalted on ZipTip™ C18 (Millipore) in a final volume of 3 µl. A volume of 0.5 µl of the eluted peptides was spotted with an equivalent volume of a saturated solution of the matrix 2,5-dihydroxybenzoic acid in 0.1% TFA, directly onto the target plate.

Peptide mass fingerprinting. MALDI-TOF spectra of the peptides were obtained with a Voyager-DE STR Biospectrometry Workstation mass spectrometer (Applied BioSystems, Life Technologies Corporation, Carlsbad, CA, USA). Voyager Acquisition and Data Processing Software 5.1 was used, with external calibration. The lists of masses were searched against SwissProt 40.27 database (184988 sequences) with ProFound online version (Prowl, Rockefeller University, http://prowl.rockefeller.edu version 4.10.5) with the following parameters: singly charged monoisotopic ions, up to 2 missed cleavage, accuracy tolerance: ±0.1 Da, partial cysteines carbamidomethylation and methionine oxidization.

LC-MS/MS analysis. The samples (2.5 µl of the eluted peptides) were analysed on an UltiMate capillary liquid chromatography system (LC Packings, Amsterdam, the Netherlands) used on-line with a hybrid nanoESI quadrupole-time of flight mass spectrometer (Q-TOF2; Micromass, Manchester, UK), which is equipped with the MassLynx 4.0 software. The lists of masses were searched against SwissProt database 40.27 with Mascot (MatrixScience, version 1.8) with the following parameters: restriction on eukaryotes, partial cysteine carbamidomethylation and methionine oxidization, monoisotopic tryptic peptide mass tolerance: ±75 ppm, up to one missed cleavage individual ions scores >40 indicated identity or extensive homology ($P < 0.05$).

Northwestern assays

The amphibian GVs and the nuclear proteins extracts of HeLa cells were submitted to northwestern assays (NWA) as described previously (20,21). Briefly, proteins separated in a SDS–PAGE gel were transferred to a nitrocellulose membrane (Hybond-ECL, GE Healthcare). Membranes were incubated overnight in blocking buffer (5% fat-free dry milk, 0.1% Tween-20 in PBS) at 4°C. The membranes were then hybridized with ^{32}P-labelled RNA in blocking buffer for 1 h at room temperature. The unbound RNA was removed by washing several times with blocking buffer. Autoradiograhy of the air-dried membranes was performed using X-ray films (Hyperfilm, GE Healthcare) or storage phosphor screen (rego X-ray GmbH), which were scanned on GS-800 calibrated densitometer (Bio-rad) or Typhoon Imaging System (GE Healthcare), respectively. Membranes were subsequently used for western blot analysis. ^{32}P-labelled single stranded (ss) RNA probes were synthesized *in vitro* from either DNA strand of the P130B clone [described in ref. (20)] with T7 or SP6 RNA polymerases using the Riboprobe *in vitro* transcription system from Promega and the [α-^{32}P] UTP (800 Ci/mmol). All radiolabelled RNAs were purified by gel filtration chromatography using G25 or G50 Sephadex columns (Quick spin columns, Roche Applied science) followed by ethanol precipitation.

Western blots

Membranes were incubated overnight in PBS + 0.1% Tween-20 (PBST) + 5% fat-free dry milk at 4°C and further incubated with a dog anti-hnRNP G autoimmune serum (26) at a 1/2000 dilution in PBS + 0.5% BSA for 1 h. They were then washed in PBST, incubated with an HRP conjugated anti-dog (Jackson ImmunoResearch) diluted to 1/10 000 in PBS + 0.5% BSA for 1 h and washed in PBST. Detection was performed using the ECL kit from GE Healthcare. Membranes were exposed to X-ray films (Kodak BioMax MR films) or scanned in the LAS-3000 imaging system (FUJI). As a control experiment, a western blot was also performed using a serum pre-absorbed with a GST-tagged fragment of hnRNP G bound to a gluthatione column (GS trap™ FF Column, GE Healthcare). This fragment, which contains the major antigenic domain of hnRNP G (26), was produced as previously described (21) from a cDNA clone kindly provided by Dr V. Della Valle.

Nuclear spreads

Nuclear spreads were prepared as described previously (20,27). Preparations destined to immunofluorescence were fixed for 30 min in phosphate buffer saline (PBS) containing 2% *p*-formaldehyde at 4°C and washed with PBS. For *in situ* hybridization usage, preparations were instead fixed in 70% ethanol for 30 min, dehydrated through an ethanol series, washed in xylene to remove paraffin wax and air-dried from acetone.

In situ hybridization

In situ hybridization with nuclear spreads was performed as previously described (20). Hybridization was carried out in 40% formamide, 4 × SSC, 0.1 mol/l Na3PO4, pH 7 and 300 µg/ml of yeast tRNA at 42°C overnight.

After several washes for 1 h in 0.1 × SSC, 10 mmol/l dithiothreitol at 65°C, slides were dehydrated through an ethanol series, dipped in NTB2 emulsion (Kodak; diluted 1:1 with H_2O) and exposed at 4°C for 3–7 days. After development, nuclear spreads were stained with Coomassie blue R. The cDNA used as a template for the synthesis of *RBMX* probes was isolated from the *Xenopus tropicalis* (xthr) library of cDNAs prepared from the head and retinas of advanced limb bud stages (28). The xthr17M05 plasmid contained a partial cDNA of *RBMX* (830 bp) inserted into the pCMVSPORT6-xthr plasmid, a modified version of the PCMVsport6 vector. A small portion of this *RBMX* cDNA (300 bp) was subcloned into the pCR-Blunt II-TOPO vector (Invitrogen). Both of the long and the small cDNAs of *RBMX* were used for the synthesis of the RNA probes. The plasmids were linearized with EcoR1 or BamH1. The sense and antisense *RBMX* riboprobes (SA. 3×10^8 cpm/μg) were generated by *in vitro* transcription using the Riboprobe *in vitro* Transcription system from Promega and [^{35}S] UTP.

RESULTS

Evidence for a 42 kDa polypeptide(s) with sequence-specific RNA-binding activity linked to the sex genotype

The W_{Ec} RNA probe was used in NWA to identify nuclear proteins mediating its binding to the W chromosome. Proteins extracts of GVs of ZZ, ZW and WW oocytes were separated by mono-dimensional electrophoresis in polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were probed with ^{32}P-labelled RNA probes. In parallel to the W_{Ec} RNA, other RNA probes, which did not label the W chromosome lampbrush loops, were used as controls (Figure 1). In repeated NWA experiments, the W_{Ec} RNA probe showed a very similar pattern of binding proteins for the three karyotypes except in the 42 kDa range where strong binding signals were present for the ZW and WW GVs while no signal was observed for the ZZ GVs. The PB6 RNA probe showed the same differential interaction with the 42 kDa polypeptide(s) for the ZW and WW GVs although the binding signals were weaker than the ones

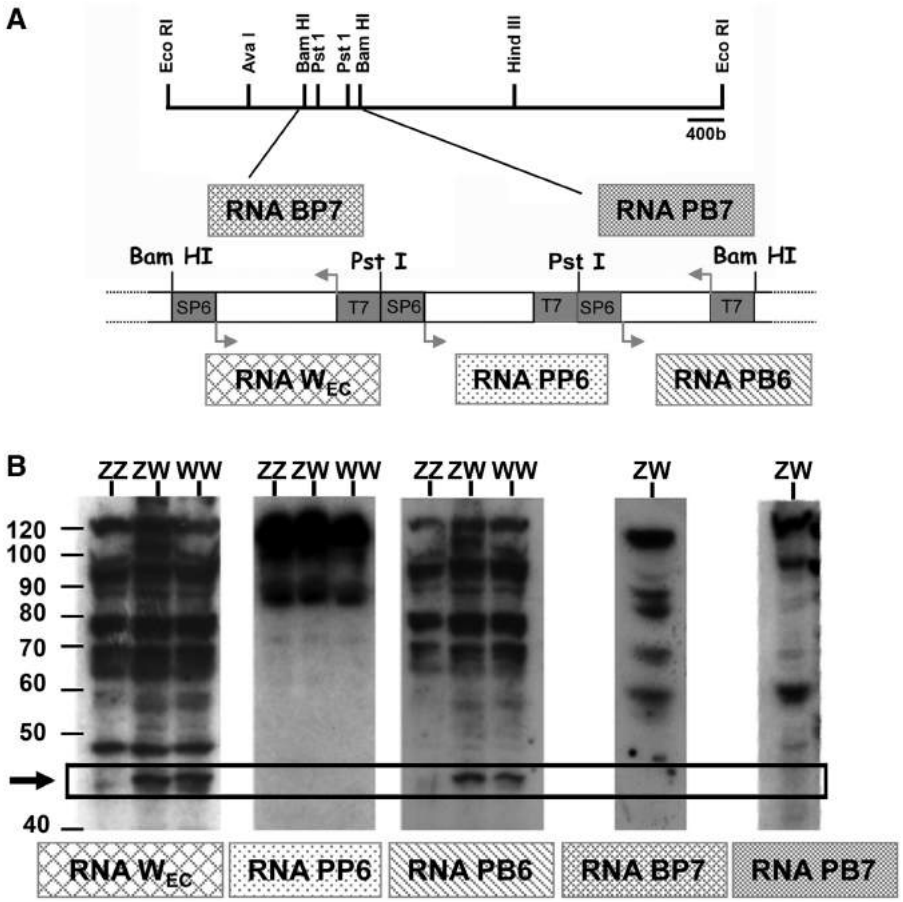

Figure 1. *Pleurodeles waltl* 42 kDa polypeptide(s) bind the W_{Ec} RNA specifically in the ZW and WW GVs. (**A**) Restriction map of the clone λ130 from which the RNA probes were derived by *in vitro* transcription. (**B**) NWA of ^{32}P-labelled RNA probes with nuclear proteins from ZZ, ZW and WW GVs. Nuclear proteins were transferred to nitrocellulose membrane after electrophoresis in mono-dimensional polyacrylamide gels and incubated with the RNA-probes. Note that nuclear protein(s) in the 42 kDa range specifically bound to the W_{Ec} and PB6 RNA probes in ZW and WW karyotypes and not in ZZ karyotypes (arrow and box). No interaction could be detected for these proteins with the other RNA probes tested.

observed with the W_{Ec} RNA. The other RNA probes (PP6, PB7 and BP7) did not bind to these polypeptides although they bound to other polypeptides for all three oocyte karyotypes. These data provided evidence for sequence-specific RNA-binding polypeptide(s) whose expression in the oocyte could be correlated with the presence of the W chromosome in its karyotype.

Characterization of the 42 kDa polypeptide as a homologue of the human hnRNPG/RBMX protein by mass spectrometry

In order to identify the 42 kDa polypeptide(s), GVs proteins from ZZ, ZW and WW oocytes were separated in duplicated bi-dimensional gels. One gel, to be used for preparative purposes, was stained to detect all proteins while the other was transferred to a nitrocellulose membrane, which was incubated with the W_{Ec} RNA probe (northwestern blot) and processed for autoradiography. Two pH ranges of immobilized gradients (IPG) were used for the first dimension. A wide pH range (pH 3–10) of IPGs was used initially, but no obvious difference in the pattern of protein spots labelled with the W_{Ec} probe was detected between the three types of GVs in NWA (data not shown). However, with a pH 6–11 gradient significant differences were found in the basic pI region. As shown in Figure 2, only one labelled polypeptide spot was detected in the 42 kDa/pI 10 region by the W_{Ec} RNA in the case of the ZW and WW GVs. This labelled spot was absent in the ZZ GVs extract contrary to other labelled spots of higher molecular mass, which were present for all three karyotypes, and are used as internal controls. Interestingly, extrapolation of the position of this 42 kDa/pI 10 spot to the matching silver-stained 2D-PAGE gels pointed to at least two closely migrating polypeptides that were present not only in the ZW and WW GVs but also in the ZZ GVs (Figure 2B, lower panels). Each of the corresponding spots was excised separately from the gels and submitted to mass spectrometry analysis. They were identified as homologues of the

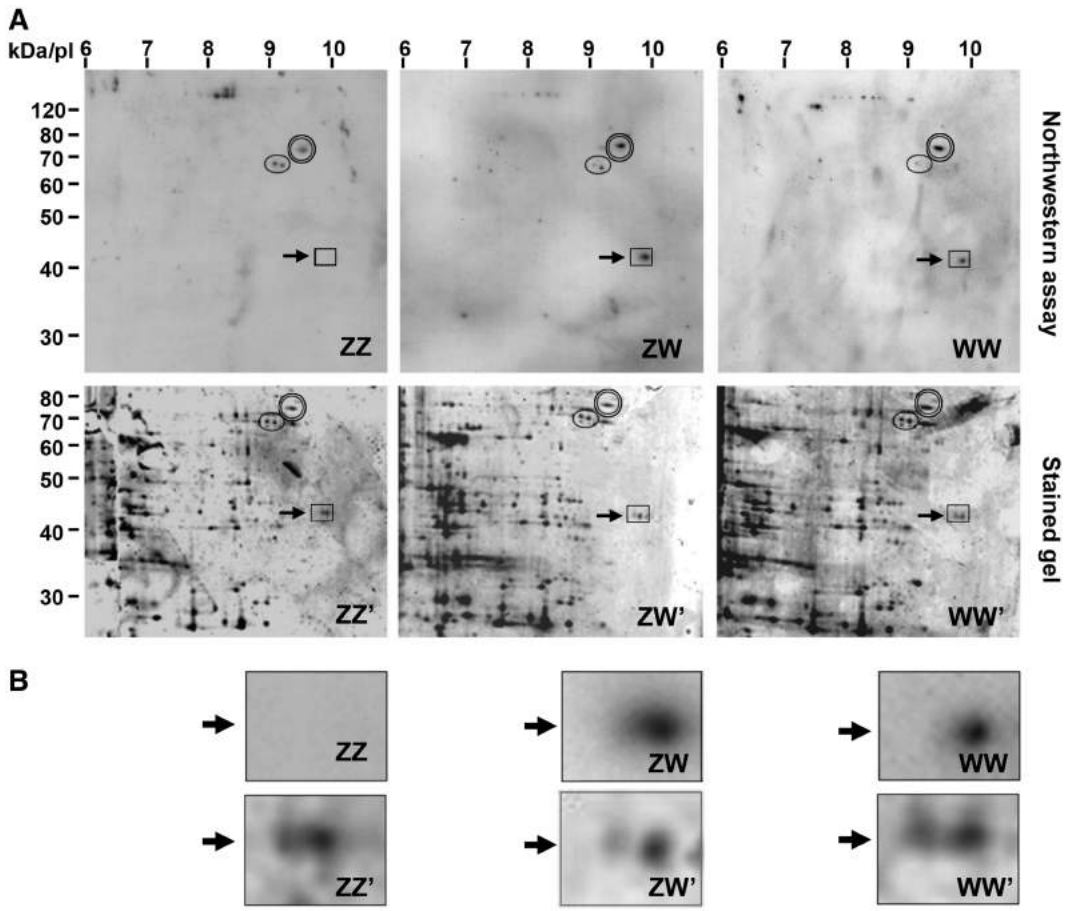

Figure 2. Characterization of the W_{Ec} RNA-interacting 42 kDa polypeptides. (**A**) Six IEF-2D PAGE gels of ZZ, ZW and WW GVs proteins were prepared using 13 cm wide pH 6–11 IEF gel strips. IEF was followed by the separation in the second dimension, which was carried out simultaneously for the six gels. For each karyotype, one gel was processed for NWA using the W_{Ec} RNA as a probe, while the other was silver stained. Note that some spots (circles and double circles) were observed in the ZZ, ZW and WW northwestern blots. The one (double circles) was identified by LC-MS-MS as the DEAD (Asp-Glu-Ala-Asp) box polypeptide 17 (*Homo sapiens*, ATP-dependant RNA helicase DDX17) (Supplementary Table S3). In contrast, the 42 kDa and pI 10 spots (arrows) were observed in the ZW and WW and not in the ZZ northwestern blots although the corresponding polypeptides were visible in the stained gel. The spots of interest (42 kDa, pI 10) were cut out from the gel and processed for mass spectrometry. (**B**) High magnification of the areas corresponding to the spots of interest from the northwestern blots (ZZ, ZW and WW) and the stained gels (ZZ′, ZW′ and WW′).

human hnRNP G/RBMX (accession number P38159) and the mouse hnRNP G (accession number O35479) proteins (Figure 3, Supplementary Tables S1 and S2).

These results suggested the presence in the ZZ, ZW and WW GVs extracts of PwhnRNP G protein isoforms with closely similar molecular masses and isoelectric points but showing differential binding activity for the W_{Ec} RNA probe according to the karyotype of the oocyte.

Further confirmation of the PwhnRNP G identity of the 42 kDa polypeptides by RNA binding and western blot assays

In order to confirm the identification of the 42 kDa polypeptides as the homologues of the human hnRNP G protein, W_{Ec} RNA-binding assays followed by immunodetection assays with canine autoantibodies sera, which had been shown to react monospecifically with the human hnRNP G protein (26), were performed sequentially on blots of proteins from *P. waltl* GVs separated by mono-dimensional electrophoresis. No signal was immunodetected when the serum was pre-absorbed with the human hnRNP G (Supplementary Figure S1). In contrast, the canine anti-hnRNP G autoantibodies serum recognized one major labelled band in *P. waltl* GVs (Figure 4A). This band showed a perfect superimposition with the labelled 42 kDa band present in the corresponding northwestern blots with the ZW, WW GVs and Hela extracts (Figure 4A and B). In the case of the ZZ GVs an immunoreactive band was also detected at the same position but no radioactive signal was observed in the corresponding NWA. This result confirmed the presence of PwhnRNP G proteins in the 42 kDa band, which differentially bound the W_{Ec} RNA in the three *P. waltl* karyotypes.

We further investigated whether hnRNP G homologues from other vertebrate species could also interact with the W_{Ec} RNA by carrying out similar assays on protein extracts from GVs of the amphibians *X. tropicalis*, *Ambystoma mexicanum* and *Notophthalmus viridescens*. As shown in Figure 4B, in all three cases one band of nearly 42 kDa was labelled by the W_{Ec} RNA probe and showed a perfect superimposition with the immunodetected band. Altogether, these results indicated that the affinity of the hnRNP G protein for the W_{Ec} RNA is conserved among vertebrate species.

Correlation of the W_{Ec} RNA-interacting isoforms of the PwhnRNP G protein with the sex genotype of the *P. waltl* oocyte

Northwestern and immunodetection assays performed in parallel on the same 2D western blot further confirmed the identification of the 42 kDa/pI 10 *P. waltl* polypeptides as hnRNPG homologues (Figure 5A). The position of the immunodetected spots corresponded to the polypeptides showed by mass spectrometry to be the homologues of the human hnRNPG protein. Furthermore, the western blot analysis revealed the existence in the oocytes of the three karyotypes, of a basic train of isoforms, ranging from pI 9 to 10 with closely similar molecular weights and present in different amounts. Three major spots were visible, one at pI 9 and the other two at pI 10, which could be distinguished from one another by a slight variation in molecular weight and pI. The superimposition of the autoradiogram with the western blot showed that none of the hnRNP G homologues bound the W_{Ec} RNA probe in the ZZ GVs because no radioactive hybridization signals were detected in the corresponding area of the autoradiogram. In contrast, the most basic hnRNP G polypeptide in the ZW and WW GVs showed an exact match with the radioactive signal detected in the corresponding autoradiogram reflecting its binding to the W_{Ec} RNA probe. This binding was confirmed to be sequence-specific by the lack of hybridization signal with the PP6, BP7, BP7 RNA probes (data not shown). Occasionally and depending on the degree of resolution of the IEF in the basic pI region, three major closely migrating spots were found in the pI 10 area of the silver stained 2D-gels with ZW GVs instead of the two more frequently observed. The two more basic very close spots were of the same molecular mass while the third more acidic spot was of a lower molecular mass. The superimposition of the northwestern blot autoradiogram with the matching immunodetection blot showed that the W_{Ec} RNA probe bound the most basic polypeptide only (Figure 5B). We attempted to achieve a higher IEF resolution to determine whether the pI and molecular mass of the hnRNP G isoforms in the ZZ GVs were slightly different from those in the WW GVs. To this end we used the 2D DIGE fluorescence labelling method to reduce gel-to-gel variations. Samples of ZZ and WW GVs proteins were pre-labelled with different fluorescent dyes and co-migrated in the same gel. The superimposition of the fluorescent images did not reveal any difference for these isoforms between the two karyotypes (data not shown).

Taken together, these results demonstrated that the polypeptides of 42 kDa and pI 10 observed in the ZZ, ZW and WW of *P. waltl* GVs did correspond to three PwhnRNP G isoforms in the ZW GV and two isoforms in the ZZ and WW GVs. Only the most basic isoform bound to the W_{Ec} RNA probe in a sequence specific manner in the ZW and WW GVs.

The phosphorylation of PwhnRNP G is not required for binding the W_{Ec} RNA

The theorical isoelectric point of the human hnRNPG predicted from its primary protein sequence is 10.06. As shown in Figure 5A, the 2D migration profile of its immunodetected homologues in *P. waltl* oocytes extracts showed a train of spots with nearly identical molecular weights and isoelectric points ranging between pI 9 and pI 10. These pI shifts could result from post-translation modifications (PTMs) such as phosphorylation, acetylation or methylation, which are known to play a significant role in the regulation of gene expression by modifying protein–protein and/or nucleic acids–proteins interactions. Phosphorylation is one of the most common PTM that can be detected by a direct in-gel method. Because the hnRNP G isoforms of the ZZ GVs did not bind the W_{Ec} RNA probe and differed in their ability to

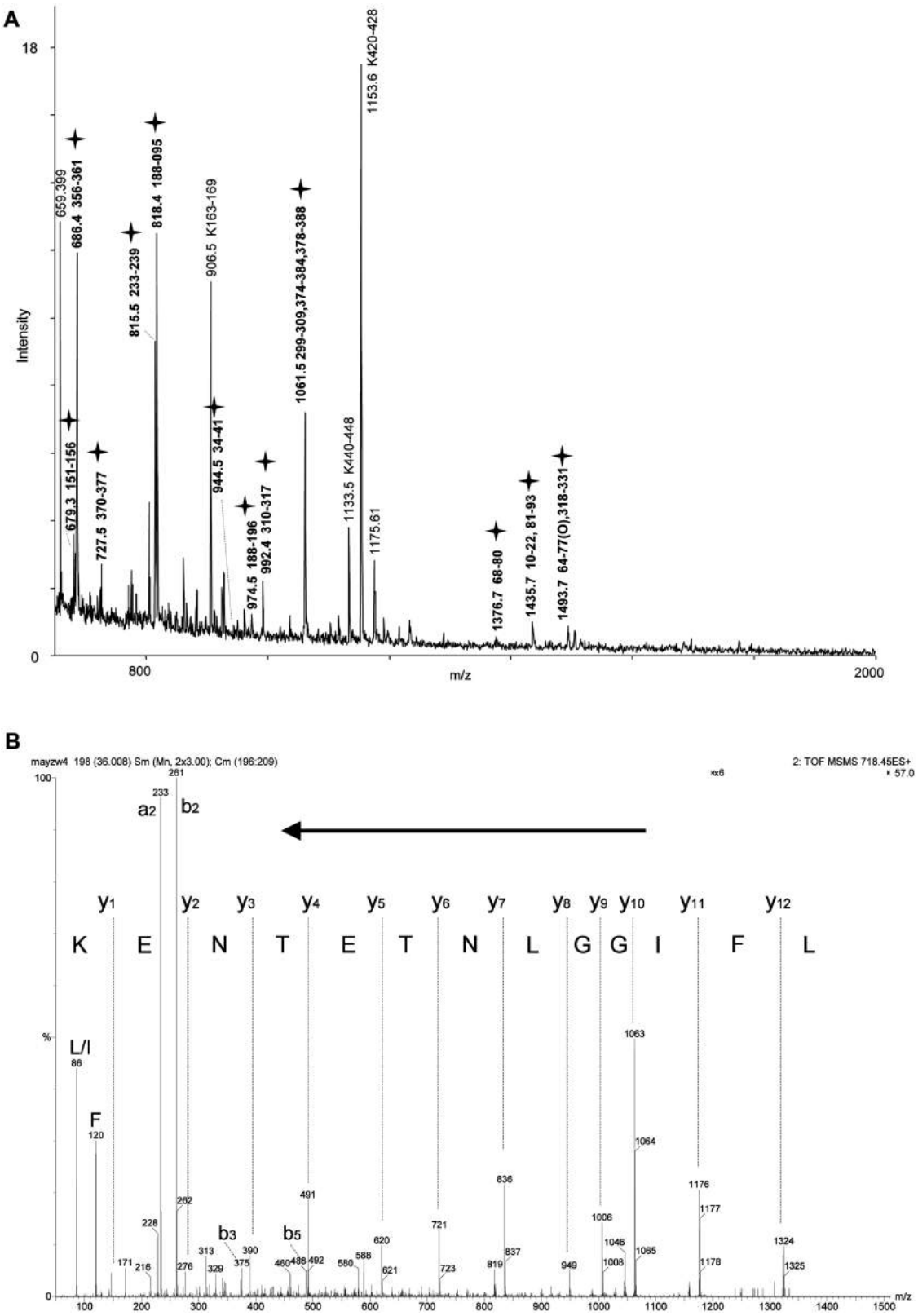

Figure 3. Identification of the 42 kDa polypeptides as the hnRNPG homologues by Mass spectrometry analysis. (**A**) MALDI-TOF MS spectrum of one of the 42 kDa spots. The peaks are labelled with stars when the mass was identified as an hnRNP G peptide. (**B**) The peak at 1435.7 m/z was fragmented for further analysis. The N-to-C sequence of this peptide could be obtained directly by reading the spectrum from right to left (arrow). Thus, the sequence was ^{10}LFIGGLNTETNEK^{22} and corresponded to an hnRNP G-specific peptide. Some a-, b-type and immonium fragments were also present and confirmed this unambiguous identification.

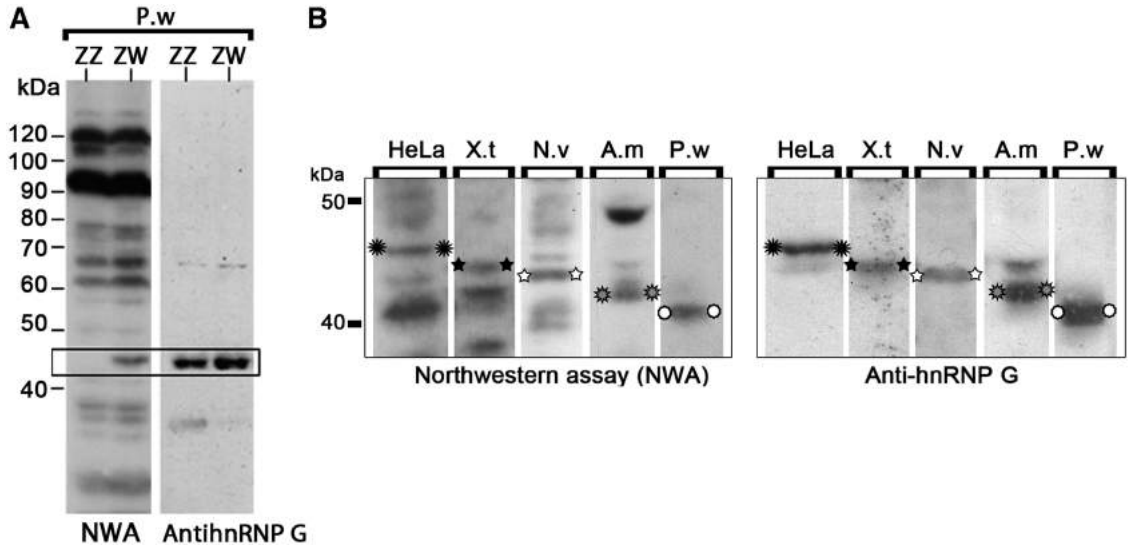

Figure 4. Validation of the identification of hnRNP G and inter-species conservation of hnRNP G interaction with the W_{Ec} RNA. 1D-NWA of the binding of the ^{32}P-labelled RNA W_{Ec} probe with nuclear proteins extracts from different species, followed by immunodetection using the canine anti-hnRNP G serum. (**A**) hnRNPG homologues in ZZ and ZW GVs of *P. waltl*. The 42 kDa labelled band (box) detected by NWA in ZW GVs corresponded exactly to the one detected by the anti-hnRNP G serum. Note that a band corresponding to hnRNP G was immunodetected in ZZ GVs, although no signal was visible in the corresponding blot after NWA. (**B**) hnRNP G from nuclear proteins extracts of human HeLa cells and GVs of the amphibians *X. topicalis* (X.t), *N. viridescens* (N.v) *A. mexicanum* (A.m) and *P. waltl* (P.w, ZW karyotype). Stars and circles indicate the bound hnRNP G.

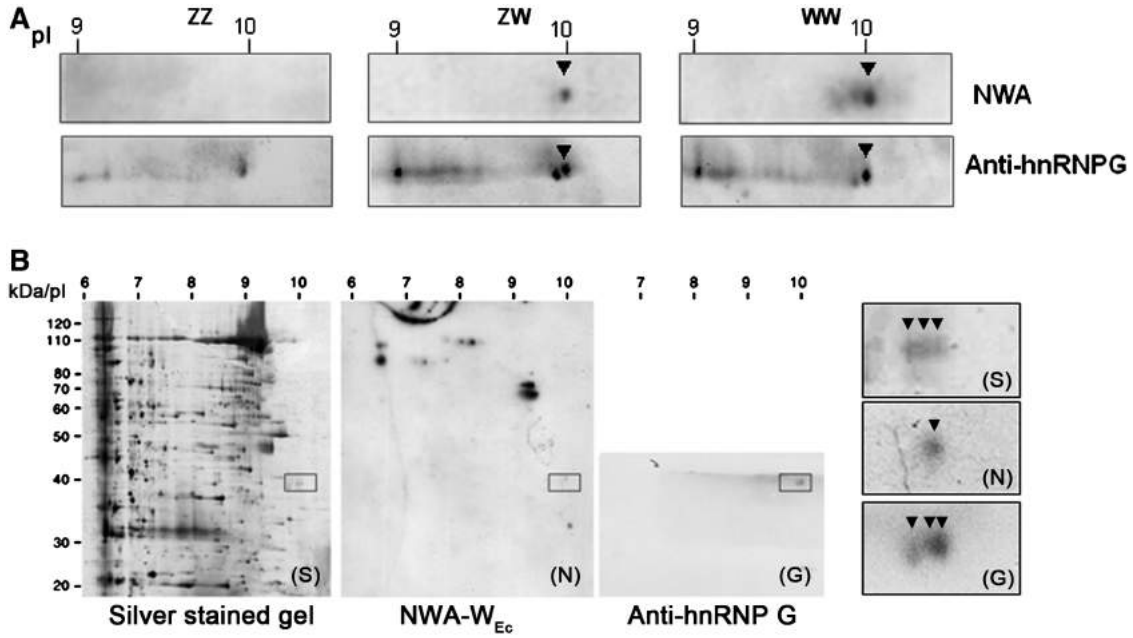

Figure 5. Interaction of the *P. waltl* hnRNP G isoforms with the W_{Ec} RNA. (**A**) 2D-NWA of the ^{32}P-labelled RNA W_{Ec} probe with nuclear proteins from ZZ, ZW and WW GVs, followed by immunodetection using the anti-hnRNP G serum. Note that the homologues of hnRNPG formed a train of basic spots ranging from pI 9 to 10 with two major spots at pI 10. The most basic spot corresponded to the one detected by NWA in ZW and WW GVs. (**B**) An example of isoelectrofocusing of the protein extracts of the ZW GVs showing three spots of hnRNP G at pI 10 (arrowheads) in the silver stained gel (S). Note that only one spot was visible in the northwestern blot (N).

bind the W_{Ec} RNA probe in the WW GVs, we investigated the phosphorylation state of these proteins, which may influence their RNA affinity. Pro-Q diamond phosphoprotein stain was used on 2D gels for a sensitive and direct in-gel detection of phosphoproteins irrespective of the phosphoamino acid ('Materials and Methods' section). The subsequent staining of the same 2D-gels using the total-protein SYPRO Ruby stain provided a measure of the phosphorylation level relative to the total amount of the protein. As seen in Figure 6, the two major spots corresponding to hnRNP G appeared as phosphorylated in ZZ and WW GVs. Nevertheless, the

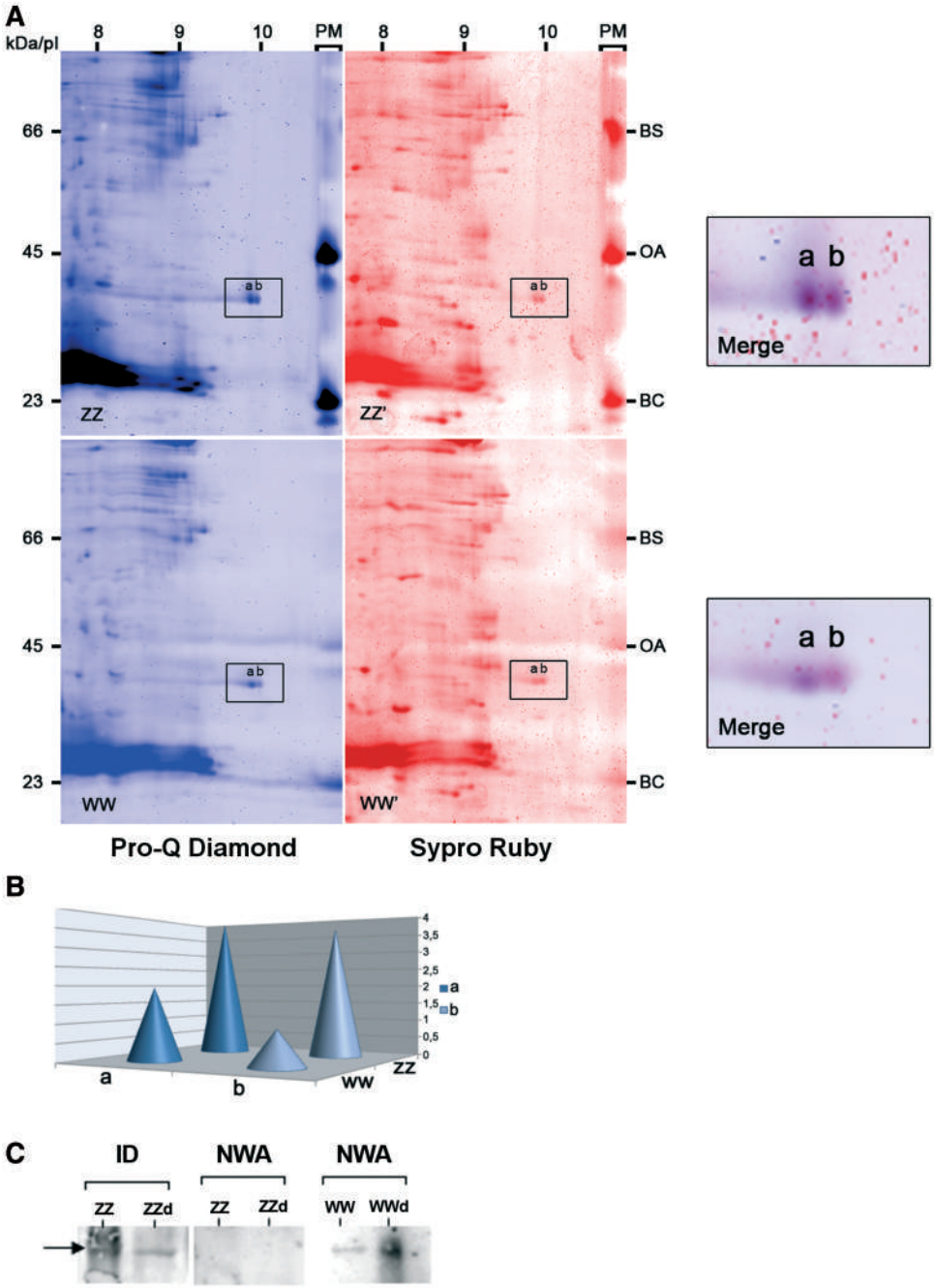

Figure 6. Relationship between PwhnRNPG phosphorylation state and RNA-binding activity. (**A**) Protein extracts of ZZ and WW GVs were separated in 2D-gels and stained with two sequential fluorescent dyes. Pro-Q Diamond (in blue) was specific of the phosphorylated proteins and Sypro Ruby (in red) revealed all proteins. A molecular mass indicator, PeppermintStick (PM), which contained phosphorylated proteins, β-casein (BC), ovalbumin (OA) and an non-phosphorylated protein, bovine serum albumin (BA) was run in parallel with each gel to show the specificity of the staining. The box indicates the major forms (a and b) of PwhnRNP G at pI 10. A high magnification of the merge of the two fluorescent stains is shown on the side of each gel. (**B**) Representative quantification of the phosphorylation degree of the major forms of PwhnRNP G (a and b) based on the intensity of their fluorescent staining by Pro-Q Diamond. Values were normalized relatively to the protein quantity estimated from the Sypro Ruby staining. (**C**) RNA-binding activity of dephosphorylated PwhnRNP G. Dephosphorylated ZZ (ZZd) and WW (WWd) or non-dephosphorylated (ZZ and WW) GV extracts were submitted to monodimensional NWA using WEc RNA probe followed by immunodetection (ID) of PwhnRNP G using the anti-hnRNP G serum. The arrow points to the PwhnRNPG protein. Note that a band corresponding to PwhnRNP G was immunodetected in ZZ GV, although no signal was visible in the corresponding blot after NWA in dephosphorylated or non-dephosphorylated extracts.

degree of phosphorylation of the WW-b isoform spot, which bound the W_{Ec} RNA probe, was lower than that of the WW-a, ZZ-a and ZZ-b isoforms, which did not bind this probe.

In order to study a possible functional relationship between the phosphorylation degree of PwhnRNPG and its ability to bind the W_{Ec} RNA, protein extracts from ZZ and WW GVs were treated with alkaline phosphatase. The

dephosphorylated form of PwhnRNPG in WW GV still interacted with the W_{Ec} RNA, as shown on 1D-NWA while that of ZZ GV did not (Figure 6C) These data demonstrated that hnRNP G phosphorylation was not necessary for its interaction with the W_{Ec} RNA.

Evidence for a PwhnRNPG multigene family in *P. waltl*

In order to determine whether the different PwhnRNP G isoforms expressed in the *Pleurodeles* oocyte corresponded to proteins coded by one or several genes, we carried out *in situ* hybridization of an *RBMX* cRNA probe to RNA transcripts of oocyte lampbrush chromosomes (LBCs). *In situ* hybridization of specific probes to nascent transcripts of lateral loops of LBCs is a powerful approach to tackle this question in amphibians. It yields strong signals due to the binding of the probe to many closely-packed RNA transcripts in these loops [for review, see (27,29,30)]. The chromosomal localization of these hybridizing loops can be precisely determined because LBCs maps were established in many amphibian species thanks to the presence of prominent landmarks, which show a distinctive morphology and are reproducibly observed at constant sites along the axis of each chromosome (31–33). This is well illustrated in the case of *P. waltl* where the ZW sex bivalent chromosomes (bivalent IV) are easily identifiable in the oocyte karyotype contrary to the mitotic karyotype in which sex chromosomes cannot be distinguished (34,20). In addition, the hybridization to the Z or to the W chromosomes can be further confirmed using ZZ or WW GVs. Because the sequence of the hnRNP G/RBMX cDNA is highly conserved among vertebrate species (87% of similarity between *X. tropicalis* and human and 97% of similarity between *X. tropicalis* and *X. laevis*, Supplementary Figure S2), two antisense *RBMX* RNAs from *X. tropicalis* were used as probes. Results clearly indicated that *RBMX* gene expression took place at the level of at least three distinct genomic sites: one on the autosome VI, one on the sex chromosome Z and one on the sex chromosome W (Figure 7). We have named these genes *RBMA*, *RBMZ* and *RBMW* respectively for RBM linked to an autosome or to the Z or W sex chromosomes. As shown in Figure 7, the labelling signal on the Z chromosome was stronger than that observed on the W chromosome, possibly because of the presence of multiple gene copies expressed on at least two adjacent loops. These results suggested that in *P. waltl* the PwhnRNP G protein is encoded by a multigenic family, which we referred to as the *RBMZ*/*RBMW* family by analogy with the *RBMX*/*RBMY* family in mammals. This family would be comprised of several genes located on the Z chromosome (*RBMZs*), the W chromosome (*RBMW*(s) and at least one autosome, chromosome VI, (*RBMA*).

DISCUSSION

The PwhnRNP G protein shows a sequence-specific RNA-binding activity associated with the presence of the W chromosome in the oocyte genotype

hnRNP G is a sequence-specific RNA-binding protein known to be implicated in the splicing of pre-mRNAs. Using an RNA probe that we had previously shown to interact with the human hnRNP G via a sequence-specific motif (21), we provide evidence for the presence of several PwhnRNP G isoforms in the ZZ, ZW and WW oocytes of

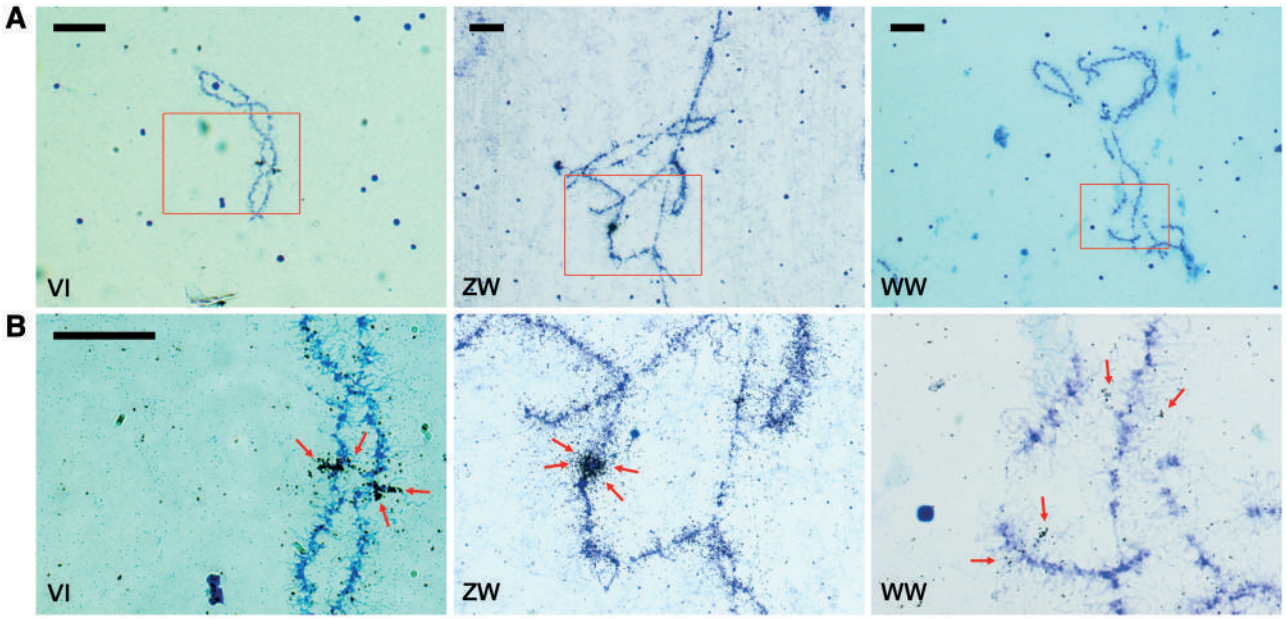

Figure 7. *In situ* hybridization to nascent RNA transcripts on lampbrush chromosomes from *P. waltl* using the ^{35}S-labelled *RBMX* cRNA. (**A**) Autoradiographs of bivalents VI and IV (ZW and WW). (**B**) High magnification of the boxed area of the A autoradiograph. Arrows point to the labelled loops. On bivalent VI, each of the two hybridization sites concerns a uniformly labelled pair of loops (arrows). On ZW bivalent, strongly and uniformly labelled loops are observed on only one homologue (arrows). On bivalent WW, each of the two hybridization sites concerns a partially and lightly labelled pair of loops (arrows). Scale bars: 50 µm.

P. waltl whose differential affinity for this RNA was correlated with the presence of the W chromosome. One of these isoforms in the ZW and WW GVs bound to the W_{Ec} RNA, while none of the isoforms in the ZZ oocytes bound to this RNA. Because the molecular weights and the isoelectric points of these isoforms are very close in the ZZ, ZW and WW GVs, it can be assumed that RNA-binding domains are present in the ZZ isoforms but display a binding affinity for RNA sequences different from the ones that we have used as probes. It has been shown that a small divergence in the amino acid composition of an RNA-binding domain can have a great effect on its affinity for RNAs, as it is the case for the divergence of the N-ter RRM between the human RBMY protein and its mouse orthologue mRBMY (17). The human RBMY RRM recognizes with high affinity stem-loop-structured RNAs that contain the sequence CA/UCAA. Because of a difference of two critical amino acids, the mRBMY RRM is unable to bind efficiently to these RNAs. Therefore, it is possible that a small sequence divergence in the ZZ isoforms or a differential PTM is responsible for their lack of affinity towards the W_{Ec} RNA.

A multigenic RBMZ/RBMW family in amphibians

We also provide evidence for the existence of a multigenic family of PwhnRNP G genes in *P. waltl*. hnRNP G transcripts were mapped by *in situ* hybridization to at least three distinct genomic loci on different chromosomes: *RBMZ* on the Z chromosome, *RBMW* on the W chromosome and *RBMA* on the autosomal bivalent VI. No *P. waltl* genomic sequences are available to test this assumption. A single gene coding for the hnRNP G protein was found in *X. tropicalis* cDNAs databases (35,36). However, the possibility that other copies of this gene are present in the *X. tropicalis* genome cannot be ruled out because low abundance mRNAs are often poorly represented in databases. It is also possible that this gene family has evolved differently in *P. waltl* and *X. tropicalis*. The proposed *P. waltl RBMZ/RBMW* multigene family does not necessarily reflect a general organization of the hnRNP G-encoding genes in other amphibian genomes.

Although sex chromosomes have been intensively studied in mammals, very little is known about the origin of sex chromosomes in amphibians. Genes linked to sex chromosomes could have evolved differently in very closely related amphibians. A recent study proposed that chromosome III is the W chromosome in *X. laevis* because it carries the *DM-W* gene, a candidate for the female-determining gene in this species (37). However, no *DM-W* orthologue was found in the *X. tropicalis* genome (38). Furthermore, eight genes necessary for sex differentiation have different chromosomal locations in *X. laevis*, *X. tropicalis* and *Rana rugosa* suggesting that the W chromosome was acquired independently in *X. laevis* (38).

Correlation between hnRNP G isoforms and RBMX-like genes

Our results raise the question of the relation between the hnRNP G isoforms and the multiple *RBMX-like* genes localized on different chromosomes. At least two possibilities can be considered. The first one is that the *RBMZ*, *RBMW* and *RBMA* genes code for different proteins, RBMZ, RBMW and RBMA. At pI 10, we observed consistently two major hnRNP G isoforms in the ZW, ZZ and WW GVs and occasionally three isoforms in the ZW oocytes, which differed slightly in their pIs, the more basic isoform being the most abundant. We propose that the acidic isoform could correspond to the RBMA protein encoded by the autosomal *RBMA* gene because it is common to the three ZZ, ZW and ZW karyotypes; the basic isoform could correspond to two very closely related proteins, RBMZ and RBMW encoded by separate genes, the RBMW protein being the one binding to the W_{Ec} RNA.

An alternative possibility is that these proteins are the products of a single gene, the *RBMA* autosomal gene, the *RBMZ* and *RBMW* genes being non-functional because of frameshifts or stop codons in their coding sequences. The different pI isoforms could represent splicing variants or result from post-translational modifications regulated by one or several W-linked genes. Phosphorylation is one of the most currently observed modifications of the proteins that regulate their functions. Indeed we noted a significant difference in the phosphorylation level between the *Pleurodeles* hnRNP G isoforms. However, dephosphorylation of hnRNP G in ZZ or WW GV extracts did not affect their sequence-specific binding to this substrate. Other modifications of the protein such as methylation and acetylation that can affect the RNA-binding affinity could also be considered.

Regardless of their origin, it is clear that the PwhnRNP G isoforms show differential RNA-binding activity. Therefore, we can assume that their RNA affinity is directly or indirectly, under the control of the sex genotype, because the only variant that recognizes the W_{Ec} RNA is found in the ZW and WW karyotypes exclusively.

This study demonstrates that the proteomic approach can yield important information concerning differential RNA-binding affinities of RBP isoforms correlated with the sex genotype in animal species not currently subjected to genomic studies. Our findings open the door for further investigations of the role of the hnRNP G gene family in the regulation of RNA processing in a sequence-specific manner in non-mammalian vertebrates.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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