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Evidence for specific RNA/protein interactions in the differential segment of the W sex chromosome in the amphibian *Pleurodeles waltl*

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Pleurodeles exhibits a ZZ/ZW system of GSD (genotype sex determination). However, the Z and W sex chromosomes appear to be morphologically identical. A short RNA sequence is described that was specifically bound to lampbrush loops in the differential segment of the sexual bivalent IV. The distribution of these labeled loops in experimentally produced ZZ and WW females enabled us to demonstrate that such labeled loops were perfectly correlated with the W chromosome. Therefore, this RNA sequence constitutes an excellent marker for the W differential segment. Furthermore, analysis of the labeled loops under various experimental conditions suggested that their labeling is caused by specific interactions between this RNA sequence and lampbrush loop-associated proteins (RNA/protein interactions). North-western assays revealed that nuclear polypeptide(s) of 65 kDa could be responsible for such binding.

Key words: amphibian, *in situ* hybridization, north-western assays, RNA-binding proteins, sex chromosome.

Introduction

Despite the lack of a detectable heterochromosome in the mitotic karyotype of the amphibian *Pleurodeles waltl*, male homogamety (ZZ) and female heterogamety (ZW) have been clearly demonstrated by sex reversal and breeding experiments (Gallien 1950). Indeed, in this species as well as in many other non-mammalian species, sex differentiation may be modified by epigenetic factors (temperature or sex steroids), leading to total phenotypic sex inversion. Thus, functionally phenotypic females of sexual genotypes ZZ and WW or functionally phenotypic males of sexual genotypes ZW can be experimentally produced (Gallien 1950; Collenot 1975; Dournon & Houillon 1984). Because of their large size and their finely structured transcriptional organization, lampbrush chromosomes in the oocytes of female animals offer an original high resolution tool for analyzing chromosome heteromorphism. At the cytological level, heteromorphic sex chromosomes Z and W were indirectly identified at bivalent IV in the oocyte karyotype of *Pleurodeles waltl* (Lacroix *et al.* 1990). Indeed, heterozygous loops were either heat-induced or labeled by monoclonal antibody in bivalent IV. A clear correlation was thus established between the heterozygosity of these loops and heterogametic ZW females, and between the homozygosity of these loops and homogametic experimentally produced WW and ZZ females. Heteromorphism in bivalent IV was also confirmed at the molecular level by an asymmetrical DNA amount and AT/GC base content of the differential sector of the *Pleurodeles waltl* sexual bivalent (Loones *et al.* 1994).

In the present article, we describe a short RNA sequence that specifically labeled lampbrush loops located in the differential segment of the sex chromosome W of *Pleurodeles waltl*. This RNA sequence was issued from a molecular clone that had been recovered by microcloning lampbrush loop DNA of *Pleurodeles* (Penrad-Mobayed *et al.* 1991). Surprisingly, this clone exhibited striking sequence similarity with *Escherichia coli* DNA. Analysis of the labeled loops under various experimental conditions suggested that their labeling was caused by specific interactions between this RNA sequence and lampbrush loop-associated proteins (RNA/protein interactions). Furthermore, north-western assays provided evidence for nuclear polypeptide(s) that display specific binding affinities for the same RNA sequence. The RNA binding to lampbrush loops as well as to nuclear polypeptides was found to be thermosensitive. The importance of this RNA sequence as a sexual marker and the potential interest of these RNA-binding proteins in the field of sex determination are discussed.

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Materials and Methods

Collection of oocytes

Female newts of the species *Pleurodeles waltl* were raised in our laboratory in water at 20°C. For control experiments, oocytes were isolated from these females. For heat-stress experiments, females were placed in water at 34°C for 18 h (Rodriguez-Martin *et al.* 1991). For cold stress, females were placed in water at 8°C for 7 days (N'Da & Angelier 1990). Removal of ovaries and all manipulations involving oocytes from control or stressed animals were carried out at the same temperature as that of the corresponding treatment: 20, 34 or 8°C.

Subcloning and DNA sequencing

Phage and plasmid DNA extractions were carried out following standard procedures (Sambrook *et al.* 1988). The restriction map of clone λPw130 was constructed from double and partial enzymatic digests. Different fragments were subcloned either in pGEM 3Z(f +) or pGEM 7Z(f +) vectors (Promega Corp., USA). Dideoxy DNA sequencing (Sanger *et al.* 1977) of both strands of the different subclones was performed using T7 DNA polymerase and (^{35}S) dATP according to the manufacturer's instructions (Pharmacia Biotech, SW).

Sequence analysis was performed using the BISANCE (Base Informatique Sur les Acides Nucléiques pour les Chercheurs Européens) software package (Dessen *et al.* 1990), provided by CITI2 (Centre Interuniversitaire de Traitement de l'Informatique).

Lampbrush chromosome preparation

Lampbrush chromosomes were prepared as previously described (Gall 1954). Germinal vesicles of oocytes at stages V–VI (Bonnanfant-Jaïs & Mentré 1983) were manually isolated in 75 mmol/L KCl, 25 mmol/L NaCl, pH 7.2, 0.01 mmol/L $MgCl_2$ and 0.01 mmol/L $CaCl_2$ (Angelier *et al.* 1986). The nuclear envelope was removed and the nuclear content was centrifuged (30 min, 1500 *g*) onto the coverslip. Chromosome preparations were 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 for lampbrush chromosomes was performed as previously described (Penrad-Mobayed *et al.* 1991). ^{35}S-labeled RNA probes (SA: 3×10^8 cpm/μg) were synthesized *in vitro* from either DNA strand of the different subclones with T7 or with SP6 RNA polymerases. Each experiment involved at least 50 lampbrush chromosome preparations from 10 different females.

Hybridization was carried out in 40% formamide, 4 × SSC, 0.1 mol/L Na_3PO_4, 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, lampbrush chromosomes were stained with Coomassie blue R according to Gall *et al.* (1981). The staining time varied from 10 min for lampbrush chromosomes of control oocytes (20°C) to 30 min for those of heat-treated oocytes (34°C) and 2 min for those of cold-treated oocytes (8°C).

For control experiments using proteinase K, some preparations were pretreated before *in situ* hybridization with 0.1 μg/mL proteinase K at 42°C for 30 min (Wu *et al.* 1991). In order to verify the quality of lampbrush loop RNA after such treatment, some oocytes were pre-incubated for 24 h at room temperature with 250 μCi/mL of 5,6-^{3}H-uridine (Amersham, Buckinghamshire, UK) in Barth's medium (MBS; Gurdon 1976). Chromosome preparations were then treated with proteinase K and processed for light microscopy autoradiography as previously described (Penrad-Mobayed *et al.* 1986).

For control experiments using RNase A, chromosome preparations were treated after *in situ* hybridization with 20 μg/mL of the pancreatic RNase A (diluted in washing buffer) for 15 min at 37°C.

Chromosome identification and mapping

Lampbrush chromosomes were identified according to the *Pleurodeles waltl* map (Lacroix 1968). The position of the labeled loops (indice) corresponded to the ratio of the distance between the loop and the left telomere to the overall length of the homolog. The number of loops studied was greater than 50 for each treatment.

Protein extraction and SDS-polyacrylamide gel electrophoresis

Oocytes were defolliculated with collagenase as previously described (Moreau & Boucher 1981).

Nuclear proteins were obtained from nuclei of oocytes at stages V–VI. Germinal vesicles were manually isolated in Tris-ethanol (6 mmol/L Tris, 3 mmol/L $MgCl_2$, ethanol 70%). They were then precipitated overnight at − 20°C in ethanol-glycerol (6 mmol/L Tris, 45% ethanol, 45% glycerol). The precipitate was recovered by 5 min centrifugation at 12 000 *g* and treated for electrophoresis.

Cytoplasms from enucleated oocytes were homogenized in Tris-EDTA buffer (Chen & Stumm-Zollinger 1986) containing 0.1 mg/mL pancreatic ribonuclease A

and 10 mmol/L Pefabloc (Interchim, USA). The homogenate was centrifuged for 10 min at 10 000 *g*. Proteins of the supernatant were precipitated overnight at − 20°C by 9 volumes of ethanol. The precipitate was recovered by 30 min centrifugation at 5000 *g* and treated for electrophoresis.

One-dimensional sodium dodecylsulfate (SDS)-10% polyacrylamide gel was performed as described by Laemmli (1970).

North-western assay

Oocyte proteins separated by SDS gel electrophoresis were electrophoretically transferred to Hybond membranes (Amersham) according to Towbin *et al.* (1979). After several washes in phosphate-buffered saline (PBS), membranes were incubated overnight in 5% non-fat dry milk, 0.5% Tween, 0.2% azide in PBS, at 4°C. The filters were then probed with ^{32}P-labeled RNA (800 Ci/mmol/L) for 1 h at room temperature with gentle agitation. The filters were washed with the same buffer, air-dried and autoradiographed with X-O-MAT AR films and an intensifying screen at − 80°C.

Results

Procaryotic origin of the clone λPw130

Among clones we had recovered by microcloning lampbrush loop DNA of *Pleurodeles* (Penrad-Mobayed

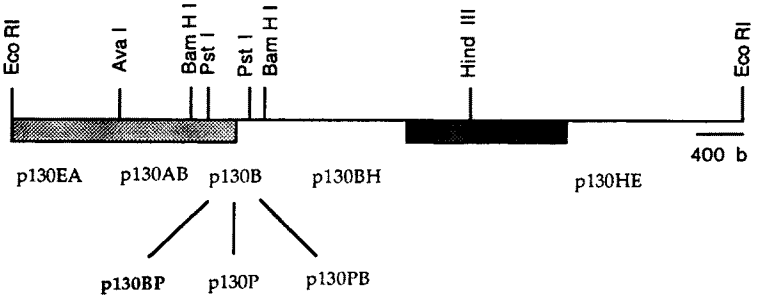

Fig. 1. Restriction map of the cloned sequence λPw130. Subclones corresponding to the restriction fragments are displayed. The frame with black dots represents DNA sequence similarity with the orfT locus of *E. coli* (Cartwright *et al.* 1993). The frame with white dots represents DNA sequence similarity with the hip locus of *E. coli* (Black *et al.* 1991). The nucleotide sequence of subclone p130BP (bold characters) is given.

Bam HI

50
GGATCCTGACTCTTAACAACGATTCCGCGGCGTATCAGGGTACGACGGAT
51 100
ATCGTGGGGGGGGAAATTGCTTTCGGTTCCGACTCTGCCATTAATATGGC
101 150
AAGTCAACACATTAATATCCATAACAGCGGTGTGATGTCGGGAAATGTCA
151 159
CCACTGCAG

Pst I

Table 1. Results of *in situ* binding of RNA derived from the orfT gene of *E. coli* (Cartwright *et al.* 1993) to lampbrush loops of *P. waltl.* BP6, BP7: SP6 or T7-derived cRNA probes issued from subclone p130BP; P6, P7: SP6 or T7-derived cRNA probes issued from subclone p130P; PB6, PB7: SP6 or T7-derived cRNA probes issued from subclone p130PB

Subclone	cRNA probe	Hybridization signal	Chromosomal location
p130BP (0.159 kb)	BP6 (sense RNA)	+	Heterozygous loops on the sex bivalent IV (Fig. 2A)
	BP7 (antisense RNA)	−	−
p130P (0.3 kb)	P6 (sense RNA)	+	Several loops scattered on different autosomes (bivalents VII, IX, X) (see Penrad-Mobayed *et al.* 1991)
	P7 (antisense RNA)	−	−
p130PB (0.166 kb)	PB6 (sense RNA)	−	−
	PB7 (antisense RNA)	−	−

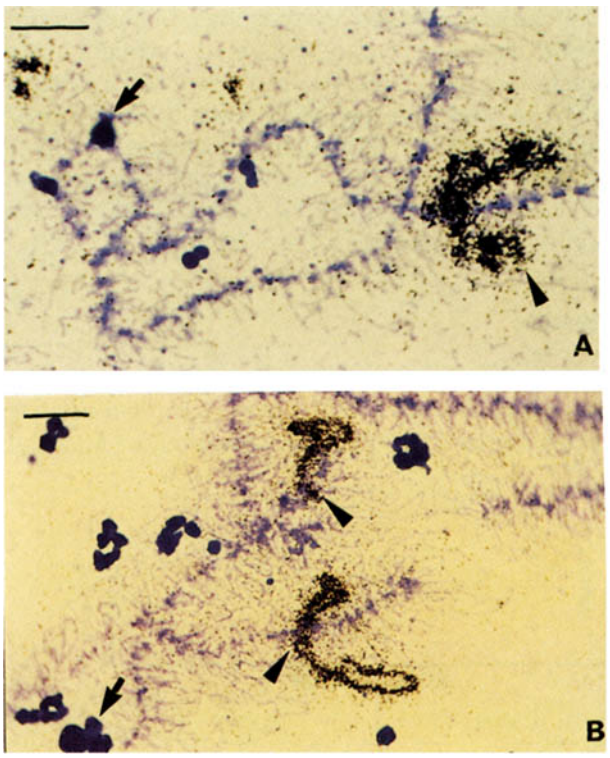

Fig. 2.

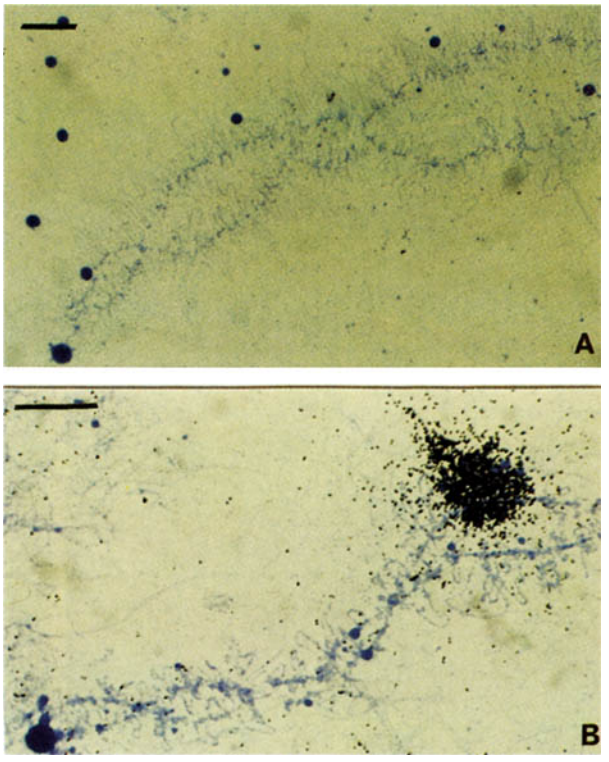

Fig. 3.

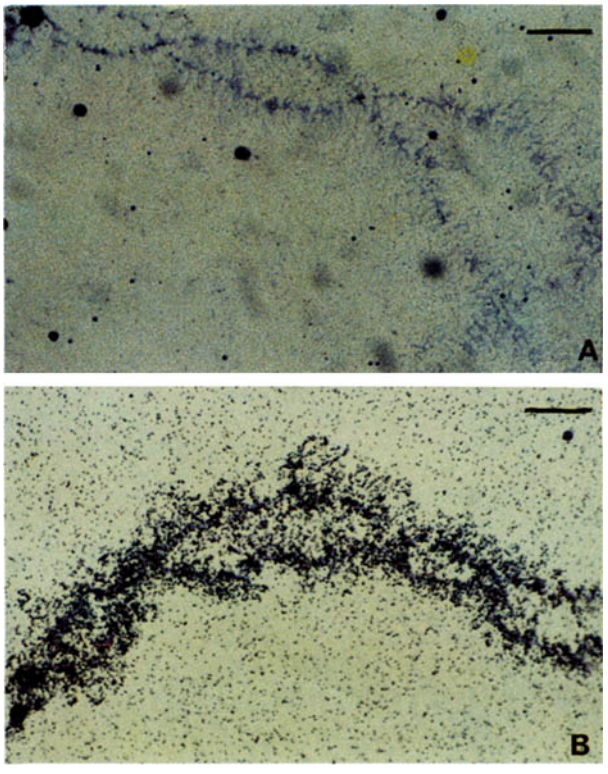

Fig. 4.

Fig. 2. A marker for the differential segment of the sex chromosome W. Binding of the ^{35}S-cRNA probe (BP6) to lampbrush loops from (A) ZW standard females and (B) a WW experimentally produced female. (A,B) Autoradiographs of part of the bivalent IV that is easily recognized because of the presence of a sphere at one of its extremities (arrow). (A) In the ZW karyotype: strongly labeled loops are observed on only one homolog (arrowhead), no labeling signal is observed on the other homolog. (B) In the WW karyotype: labeled loops are observed on the two homologs (arrowheads). Exposure: 4 days. Bars, 20 µm.

Fig. 3. Binding of the BP6 cRNA probe to lampbrush loops is thermosensitive. (A,B) Autoradiographs of parts of the bivalent IV from (A) a heat or (B) a cold-treated female. (A) Note the absence of the labeled loops after heat treatment. (B) Despite the retraction of most lampbrush loops, note the strong signals in the marker loops. Exposure: 4 days. Bars, 20 µm.

Fig. 4. Binding of the BP6 cRNA probes to lampbrush loops is protein-dependent. Autoradiographs of a part of the bivalent IV from proteinase K-treated lampbrush chromosomes (0.1 µg/mL). (A) *In situ* hybridization of ^{35}S-cRNA BP6 probe to proteinase-K-treated lampbrush chromosomes. No labeling signal is observed. (B) RNA synthesis study. Autoradiograph of part of the bivalent IV from proteinase-K-treated lampbrush chromosomes issued from oocytes that were previously incubated with ^{3}H-uridine (250 µCi/mL). Note that lampbrush loops are strongly labeled: proteinase K treatment does not affect the nascent RNA of lampbrush loops. Bars, 20 µm.

et al. 1991), one termed λPw130 (6.6 kb), seemed to be of particular interest for further investigations related to the origin of heteromorphism in amphibian sex chromosomes. Indeed, as described later, this clone enables the identification of the differential segment of chromosome W in *Pleurodeles* oocytes. One of its subclones, termed p130B (0.65 kb) (Fig. 1), was characterized by sequencing. At that time, the search within genomic databases yielded no significant similarities with any known DNA sequence (Penrad-Mobayed *et al.* 1991).

Sequencing of the other subclones of λPw130 was then carried out. Subsequent search within the genomic databases provided evidence for an unusual similarity between most of the λPw130 subclones, including p130B and *E. coli* DNA. This similarity was very high, as 98% identity for 1690 bases (from residue 1–1690) was found with the orfT locus of *E. coli,* a gene showing a potential ancestral relationship to others for the mitochondrial import site proteins ISP42 and MOM38, (Cartwright *et al.* 1993). Such a similarity overlapped the following subclones: p130EA, p130AB and the two restricted fragments of subclone p130B: p130BP and p130P. Furthermore, 97% identity was also found for 1300 bases (from residue 3180–4486), which constituted parts of subclones p130BH, p130HE and the hip locus of *E. coli,* an operon that affects lethality (Black *et al.* 1991). These sequence similarities are reported on the λPw130 map (Fig. 1). Such a similarity could not be explained by an evolutionary relationship, as it is normally found between highly conserved regions of homologous genes in related species. These data therefore seem to indicate that p130B, as well as the entire λPw130 clone, derived from *E. coli* and not from *P. waltl* DNA, as previously believed. This clone probably originated from inadvertent cloning of DNA from minute amounts of micro-organisms contaminating the chromosome spreads, as already reported in other microcloning experiments (Scalenghe *et al.* 1981; Röhme *et al.* 1984).

The cRNA probe BP6 bound to lampbrush loops specific to sex chromosome W

We had previously shown, by photon and electron microscopy that the cRNA probe issued from subclone p130B (Fig. 1) *in situ* bound to the nascent transcripts of lampbrush loops of standard ZW females. Strong, reproducible labeling signals were observed in defined loops scattered on different autosomes, as well as on one of the two chromosomes of the sexual bivalent IV (Penrad-Mobayed *et al.* 1991; Bonnanfant-Jaïs *et al.* 1993) (Fig. 2A). Thus, heterozygous labeling of the sexual bivalent from ZW females exclusively involved either the Z or W sex chromosomes.

In order to determine with which heterochromosomes the labeled heterozygous loops of sexual bivalent IV were correlated, we carried out *in situ* hybridization experiments on lampbrush chromosomes of experimentally produced ZZ and WW females. Indeed, as previously mentioned, Z and W chromosomes could not be recognized by their morphology. In all 50 oocytes issued from 10 different WW females, labeled loops of bivalent IV were homozygous (Fig. 2B). In contrast, in the 50 oocytes from 10 different ZZ females, no hybridization signal was ever observed on the two partners of bivalent IV (data not shown). The precise position of these loops was determined according to Lacroix (1968; see Materials and Methods). They were located at site 50, flanked by two groups of globular loops, B48 and B59, and hence emerged on the differential segment of heterochromosomes (site 36–59; Lacroix *et al.* 1990) Thus, the distribution of these labeled loops showed an absolute correlation with the differential segment of the W sex chromosome.

In order to determine whether the whole 0.65 kb sequence of p130B was involved in labeling of the clustered loops of chromosome W, *in situ* hybridization experiments were carried out using cRNA probes issued from the different subclones of p130B: p130BP, p130P and p130PB (Fig. 1; Table 1). Only SP6-derived RNA from p130BP (0.159 kb; BP6 probe corresponding to sense RNA in *E. coli*) labeled the clustered loops of sex chromosome W. SP6-derived RNA from clone p130P (P6 probe corresponding to sense RNA in *E. coli* [0.3 kb]) labeled loops scattered on different autosomes (data not shown). No labeling was observed with either SP6- or T7-derived RNA (PB6, PB7 probes) of subclone p130PB (0.166 kb). These observations suggested that only the 0.159 kb of p130BP, which corresponded to probe BP6, labeled the lampbrush loops specific to chromosome W.

Binding of cRNA probes to lampbrush loops was thermosensitive

As sex differentiation of *Pleurodeles waltl* gonads has been shown to be thermosensitive (Dournon & Houillon 1984), expression of p130BP (BP6 probe) was investigated under thermal conditions that had been previously defined (Angelier *et al.* 1990). Under these conditions, *in vivo* heat treatment (34°C, 18 h) had been shown to cause progressive disorganization of RNP matrices of lampbrush loops (Rodriguez-Martin *et al.* 1991). *In vivo* cold treatment (8°C, 7 days) had been shown to induce striking structural changes that resulted in numerical and size reduction of most lampbrush loops of normal types (N'Da & Angelier 1990).

In situ hybridization experiments were carried out using lampbrush chromosomes from such heat- or cold-treated females. When these chromosomes were probed with the ^{35}S-labeled BP6 RNA probe, no labeling signal was observed (Fig. 3A). This process was reversible: labeling signals were recovered when the same females were placed back at normal temperature (20°C; data not shown). *In situ* hybridization of the same cRNA probe with lampbrush chromosomes isolated from such cold-treated females led to enhancement of the labeling signals on these loops (Fig. 3B). Similar results were obtained with the ^{35}S-labeled P6 RNA probe (not shown). After heat treatment, no labeling signal was detected. In contrast, after cold treatment, the labeling signals on lampbrush loops of the different autosomes were considerably enhanced.

It is noteworthy that such thermal treatments induced variations in the intensity of lampbrush chromosome Coomassie blue staining. Indeed, very weak staining was observed after heat treatment, whereas strong staining was observed after cold treatment. Thus, to obtain correct staining of lampbrush chromosomes, we had to modulate the staining time in relation to the corresponding thermal treatment (see Materials and Methods). As Coomassie blue is a general protein dye, variations in staining intensity might be related to modifications in proteins associated with lampbrush chromosomes.

Thus, heat and cold treatments induced modifications in the intensity of both labeling signals and lampbrush chromosome-associated-proteins.

Binding of cRNA probes to lampbrush loops was protein-dependent

The results reported here suggested that the labeling signals resulted from cRNA-probe/loop–protein interactions rather than from cRNA-probe/loop-RNA hybridizations. In order to test this hypothesis, we carried out two control experiments using proteinase K-pretreated chromosomes or post-treated lampbrush chromosomes with RNase A, after *in situ* hybridization experiments. This endoribonuclease specifically attacks single-stranded RNA and thus removes non-hybridizing regions. The experiment using RNase-pretreated chromosomes was not undertaken because the introduction of trace amounts of this endoribonuclease during hybridization experiments would also affect the cRNA probes used.

When lampbrush chromosomes were treated with proteinase K and then probed with either ^{35}S-labeled BP6 or ^{35}S-labeled P6 RNA probes under hybridizing conditions, no labeling signal was detected: RNA binding did not occur (cf. Fig. 4A for BP6 probe). Similar results were observed with the P6 RNA probe (data not shown). In order to verify that the absence of a labeling signal was not caused by degradation of lampbrush loop RNA induced by such treatment, we carried out autoradiographic studies of proteinase-K-treated chromosome preparations issued from oocytes previously incubated with ^{3}H-uridine. As shown in Fig. 4B, all lampbrush loops were strongly labeled; tritiated RNA remained on lateral loops after proteinase K treatment. Thus, despite the presence of RNA in lampbrush loops, no labeling signals were observed when cRNA probes were hybridized with proteinase K-treated lampbrush chromosomes.

When lampbrush chromosome preparations were probed with the ^{35}S-labeled BP6 or P6 RNA probes and then washed with RNase A after hybridization, no labeling signal was detected (data not shown). Thus, neither BP6 nor P6 cRNA probes hybridized to RNP transcripts, as they were attacked by this enzyme.

cRNA probe BP6 specifically bound to nuclear polypeptides in a thermosensitive manner

In order to identify proteins of the oocyte that were believed to interact with cRNA probes on the labeled

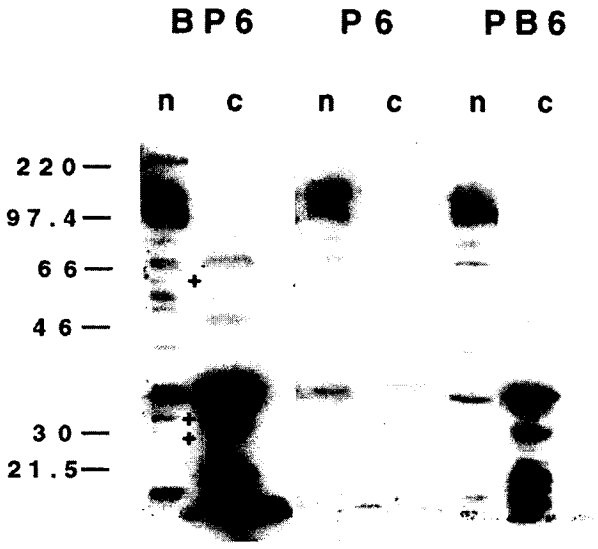

Fig. 5. North-western assays of BP6, P6 and PB6 ^{32}P-labeled probes with proteins of the oocyte. Autoradiographs resulting from RNA binding by proteins transferred to Hybond membrane after electrophoresis on a monodimensional polyacrylamide gel. (n) Proteins from 15 germinal vesicles, (c) proteins from five cytoplasms. Nuclear and cytoplasmic polypeptides of different molecular weight bind to the three probes. Some nuclear polypeptides (+) specifically bind to the BP6 probe. Molecular mass designations on the left are in 10^3 *Mr.*

Table 2. Analysis of north-western results of BP6, P6 and PB6 RNA probes to nuclear proteins of oocytes issued from control, heat- or cold-treated females. Polypeptides of different molecular weight bind to the three probes (+). In bold characters, the polypeptides that specifically bind to the BP6 probe (p65, p35, p30). Circled spots (p65, p60, p53) correspond to polypeptides for which binding to the cRNA probe is affected after heat treatment

Probes	Labeled nuclear proteins (molecular weight in kDa)											
	p185	p97	p74	p69	p65	p60	p53	p43	p40	p35	p30	p16
BP6	+	+	+	+	⊕	⊕	⊕	+	+	**+**	**+**	+
P6	+	+	+	+		+	+	+	+			
PB6		+	+	+		+	+	+	+			+

Table 3. North-western results of BP6, P6 and PB6 RNA probes to cytoplasmic proteins of the oocyte. Polypeptides of different molecular weight bind to the three probes (+)

Probes	Labeled cytoplasmic proteins (molecular weight in kDa)					
	p69	p46	p40	p35	p21	p16
BP6	+	+	+	+	+	+
P6	+	+	+			
PB6	+	+	+	+	+	+

loops, we carried out north-western assays. Proteins from either hand-isolated germinal vesicles (GV) or the corresponding enucleated cytoplasmic fractions were separated by mono-dimensional electrophoresis on polyacrylamide gels and transferred to Hybond filters. They were then probed with either the ^{32}P-labeled BP6 probe, the ^{32}P-labeled P6 probe that labeled loops scattered on different autosomes or PB6, which did not label any lampbrush loops (see Table 1) considered as controls. As shown in Fig. 5, these three probes bound more strongly to nuclear than to cytoplasmic polypeptides. The size of these polypeptides varied from 16–185 kDa. As summarized in Table 2, most of the nuclear polypeptides seemed to be associated with the three probes, whereas others (p65, p35 and p30) seemed to be specifically associated with the BP6 probe. As summarized in Table 3, most of the cytoplasmic polypeptides bound to the three probes. These results provided evidence for the presence of nuclear polypeptides that bound the cRNA probe BP6 in a specific manner.

In order to investigate whether such RNA-protein binding might be thermosensitive, north-western assays were performed with the BP6 probe and nuclear or cytoplasmic proteins extracted from oocytes of heat-treated (34°C, 18 h) or cold-treated (8°C, 7 days) females. As shown in Fig. 6, no modification occurred in the RNA probe binding to cytoplasmic peptides after thermal treatment. In contrast, after heat treatment, the BP6 probe no longer bound to three nuclear polypeptides (p65, p60 and p53), whereas binding to nuclear polypeptides was not affected by cold treatment (Fig. 6, Table 2). These results provided evidence for the existence of nuclear polypeptides of 65 kDa (p65) that specifically bound cRNA probe BP6 in a thermosensitive manner.

Thus, 0.159 kb of the BP6 probe bound to specific lampbrush loops in the W chromosome differential segment, as well as to specific nuclear polypeptides in the oocyte. However, as shown in Fig. 1, this sequence did not reveal any specific features. There was no evidence of internal or inverted repeats, nor of palindromes. This sequence was no richer in AT or in GC than the other λPw130 sequences.

Discussion

One of the most striking deficiencies reported in the literature on sex-chromosome-linked sequences concerns amphibians. These deficiencies were mainly caused by the lack of any discernible sex chromo-

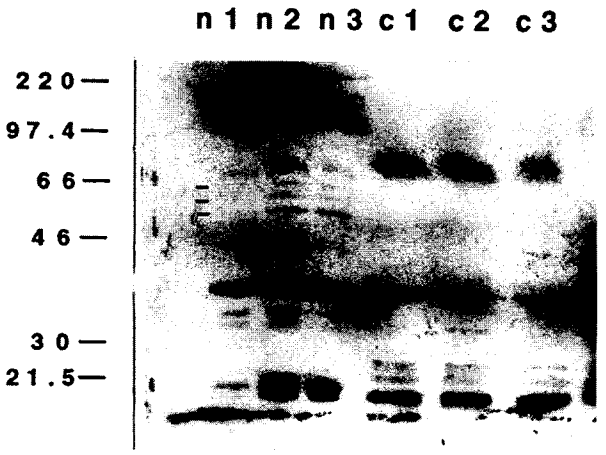

Fig. 6. North-western assays of BP6 ^{32}P-labeled probe with nuclear (n) and cytoplasmic (c) proteins of oocytes issued from heat-treated females (n1, C1); control (n2, c2) or cold-treated females (n3, c3). The heat treatment affects the binding of BP6 with three nuclear polypeptides (−). Molecular mass designations on the left are in 10^3 *Mr*.

somes in most amphibian karyotypes. The present results provide evidence for a specific marker of the differential segment of the W chromosome in *P. waltl*. Contrary to the immunologically labeled loops described by Lacroix *et al.* (1990), which were observed in the sex chromosomes as well as in other autosomes, the labeled loops described here were specifically observed on the W chromosome differential segment. Furthermore, these loops must also be considered as a specific marker of the W segment chromosome at the ultrastructural level. Indeed, as we previously demonstrated, labeling of these loops was also highly specific and reproducible in electron microscopy (Bonnanfant-Jaïs *et al.* 1993). Thus, the W differential segment is now easily recognized at the photonic and electronic levels, and the localization of sex-linked or sex-limited genes transcribed in *P. waltl* can now be envisaged. This species is known to be particularly advantageous for sexual inversion analyses (Dournon & Houillon 1985; Dournon *et al.* 1990). We should emphasize that, among amphibians that exhibited the GSD system (genotype sex determination), *Pleurodeles* is the only genus in which functional sex reversal by temperature was demonstrated (Dournon & Houillon 1984; Dournon *et al.* 1990). Therefore, *P. waltl* can now be used as a model system for undertaking studies of sex differentiation at the molecular level.

It is clearly evident that there is no relationship between the orfT *E. coli* gene and the RNA transcripts of the W sex chromosome. Indeed, as previously mentioned, the BP6 cRNA probe that labeled the W lampbrush loops corresponded to *E. coli* sense RNA. We can assume that the labeling signals we obtained were caused by specific interactions between precise motifs inside BP6 and some proteins associated with the nascent transcripts of the labeled loops (RNA/protein interactions), rather than to partial homology of sequences between this cRNA probe and RNA of the ribonucleoprotein RNP matrix of the labeled loops (RNA/RNA hybridizations). Several arguments exist against the RNA–RNA hybridization hypothesis. The most compelling is provided by results of *in situ* hybridization between cRNA probe and lampbrush chromosomes previously treated with proteinase K. Indeed, binding of BP6 to nascent transcripts did not occur after proteinase K treatment in spite of the presence of RNA in lampbrush loops, as demonstrated by autoradiography. Another argument refuting such a hypothesis is provided by results of experiments in which RNase A was introduced after hybridization. As this endoribonuclease attacks only single-stranded RNA, the disappearance of labeling signals consecutive to such treatment clearly revealed that they corresponded to a non-hybridizing region. Furthermore, results of *in situ* hybridization experiments could not be confirmed by northern experiments: no positive signal was detected with either total RNA, poly (A+), poly (A−) or nuclear RNA (data not shown). Therefore, BP6 do not seem to be transcribed in the oocyte.

In contrast, the RNA/protein interaction hypothesis seems to be more plausible. Thus, according to this hypothesis, the specific labeling signals we obtained might have resulted from high binding affinity displayed by some nuclear proteins associated with lampbrush transcripts of *P. waltl* for a precise motif inside the BP6 cRNA probe derived from *E. coli*, with these proteins being thermosensitive. Hyper- or hypothermic treatment, by modifying these RNA/protein interactions, either decreased or enhanced the corresponding signals. A specific binding activity for a precise RNA motif sequence has already been reported for many known or suspected RNA binding proteins. Thus, the binding of A1 hnRNP protein to an 18-nucleotide sequence was found to be so specific that it was decreased greater than 10-fold by changing a single nucleotide (Swanson & Dreyfuss 1988). It was also shown that the TGATTGGC core sequence in the human immunodeficiency virus type 1 (HIV-1) is the binding site for at least three distinct nuclear proteins present in Jurkat and in HeLa cells (Schwartz *et al.* 1997).

North-western data also supported RNA/protein interaction hypothesis. Indeed, the BP6 cRNA probe bound to nuclear proteins in a sequence-specific manner. Furthermore, such binding was thermosensitive. Dimario *et al.* (1989) previously reported that one or more proteins on most lampbrush loops can bind single-stranded DNA under non-hybridizing conditions, namely at room temperature and low salt concentrations. The present results suggested that, at least for the BP6 cRNA probe we used, specific RNA/protein interactions occurred even under hybridizing conditions. It is noteworthy that a relationship between north-western and *in situ* data could not be established for any cRNA probe used. Indeed, although the *in situ* signals found in autosomes by the P6 probe were also assumed to result from RNA/protein interactions, no specific molecule was detected in north-western assays. In contrast, PB6 cRNA probe that did not label any lampbrush loop PB6 bound to numerous polypeptides in north-western assays.

The present study pointed out the existence of thermosensitive nuclear polypeptide(s) of 65 kDa that display specific binding affinities for the cRNA BP6 probe. Indeed, among the nuclear polypeptides that specifically bind BP6; that is, p65, p35 and p30, only p65 is thermosensitive. As the same probe also hybridized in a thermosensitive manner with lampbrush loops specific to the W differential segment, we assume

that these polypeptide(s) (p65), might be responsible for this binding. The present finding may be related to regulation of sex determination by temperature in non-mammalians. Dorazi *et al.* (1995) have suggested that temperature modulates the expression of peptidase-1, a gene that has been shown to be linked to the W chromosome in *P. waltl* (Ferrier *et al.* 1983; Dournon *et al.* 1988). Thermosensitive factors have also been proposed to regulate the expression of the aromatase gene in *P. waltl* (Chardard *et al.* 1995). This gene is assumed to play a key role in gonadal differentiation (di Clemente *et al.* 1992; Elbrecht & Smith 1992; Wibbels & Crews 1994; Pieau 1996).

The specific RNA/protein interactions for which we have provided evidence raise the issue of the biological significance of both the RNA transcripts and the RNA-binding protein(s) in the differential segment of the W sex chromosome. The existence of RNA painting sex chromosomes that presumed to regulate the expression of sex specific genes, has recently been demonstrated in *Drosophila* (Amrein & Axel 1997; Meller *et al.* 1997). Likewise, many RNA-binding protein(s) have been shown to be implicated in the sex determination of different organisms. Thus, SXL is a female-specific RNA-binding protein that autoregulates splicing of its own transcript in *Drosophila* (Bell *et al.* 1991). Also, in mammals, the SRY-encoded protein (sex-determining region of the Y chromosome) that contains an HMG box (high-mobility group) (Gubbay *et al.* 1990; Sinclair *et al.* 1990) exhibits sequence-specific DNA binding (Harley *et al.* 1992, 1994; Haqq *et al.* 1993). Identification of the 65-kDa RNA-binding proteins should now be carried out. Furthermore, in the field of animal evolutionary sex determination, it would be of interest, to follow interactions between this RNA sequence and polypeptides originating from other animal species.

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