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Molecular Cloning of a cDNA Encoding the Amphibian *Pleurodeles waltl* 70-kDa Heat-Shock Cognate Protein

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We isolated and characterized a cDNA coding for heat-shock protein 70 of the amphibian *Pleurodeles waltl*. This 2212-bp sequence exhibited one open reading frame of 645 amino acids. The predicted amino acid sequence exhibited the three conserved elements of the HSC/HSP70 protein family. Comparison of nucleotide and amino acid sequences between this gene and other *hsc/hsp-like* genes revealed a high identity with the cognate form HSC70. By *in vitro* translation, this gene encoded a 70-kDa protein which was different than the inducible *Pleurodeles waltl* HSP70 protein. This translated protein was recognized by *Pleurodeles waltl* N1 anti-HSC/HSP70 antibody. Heat-inducibility tests showed that this gene was constitutively expressed during oogenesis and embryogenesis, and its expression was not increased after a heat-shock. These results led us to conclude that we recovered a *Pleurodeles waltl* cognate *hsc70* gene.

Heat-shock protein (HSPs), among the most highly conserved sets of proteins, can be grouped in different families according to their molecular mass (HSP100, HSP90, HSP70, small HSPs) [1]. In higher organisms, hsp70 is a member of a multigenic family that includes the transiently stress-inducible form(s) of HSP70 proteins involved in regulation of the cellular response to various types of stress, and one or more cognate protein(s) HSC70 constitutively expressed in normal conditions, during cell-cycle and differentiation. Such constitutive gene expression has been reported in a wide variety of eukaryotic non-stressed cells from yeast, Drosophila, amphibians, and mouse to man [2].

In terms of amphibian development, one inducible *hsp70* and two constitutive *hsc70.I* and *hsc70.II* genes were identified in *Xenopus laevis* oocytes and embryos [3-6]. Recently, we isolated a heat-inducible *hsp70* gene in another amphibian, the Urodele *Pleurodeles waltl*, and our results implied, as previously proposed for *Xenopus* oogenesis [3,7], that an *hsp70* gene strictly inducible in somatic cells is constitutively active during oogenesis and embryogenesis of the amphibian *Pleurodeles waltl* [8,9]. Furthermore, the corresponding inducible protein HSP70 was found to be implicated in the control of transcriptional events [10]. However, we do not know whether such a role is restricted to this inducible form HSP70, or is also extended to the cognate form HSC70. Since the cognate gene was not identified in *Pleurodeles waltl*, we isolated and characterized a constitutive cDNA encoding a 70-kDa protein in this amphibian. Here we describe a full-length 2212-bp cDNA encoding an amphibian HSC70 corresponding to a constitutively expressed mRNA in ovary.

MATERIALS AND METHODS

Pleurodeles waltl embryo maintenance and heat shock. *Pleurodeles waltl* (Amphibian, Urodele) females were raised at 20°C in our laboratory. Ovaries were surgically removed and cut into several pieces. One of the batches was maintained at 20°C and used as control. The others were heat-shocked for 2.30 hr at 34°C [8] or for 15 min at 37°C followed by recovery during 2.15 hr at 20°C [11].

Cloning and sequencing. A 280-bp fragment containing the 5′ coding region (ATP-binding domain) of the *Pleurodeles waltl* inducible Pwhsp70 cDNA [8] was used as probe to screen a *Pleurodeles waltl* tail-bud stage lambda ZAP II cDNA library (kindly provided by D^r J. F. Riou) [12]. Screening was performed according to standard procedures [13]. Two rounds of screening were carried out. Eleven positive clones were recovered. These clones were partially sequenced on both strands by the chain termination method [14] using the T7-Sequencing kit (Pharmacia-Biotech) and synthetic oligonucleotides (Genset). One of these (Pwhsc70, 2212 bp), was entirely sequenced. Sequence comparison were performed within BISANCE databases (Base Informatique Sur les Acides Nucléiques pour les Chercheurs Européens) [15].

RNA extraction and RT-PCR. Total RNA was isolated from heat-shocked or non-heat-shocked tail-bud stage embryos using the RNA-Insta-Pure LS System kit (Eurogentec). The integrity of RNA was controlled by ethidium-bromide staining. 250 ng of total RNA from each sample were used for reverse-transcription reaction with oligo-dT primers [25 $\mu g/\mu l$] according to the manufacturer's instructions

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   1                                    ccc ggg ctg cag gaa ttc ggc acg aga tca gca ggc aca  39
  40 atg tcg aaa gga cca tca gtt gtg ggc gtc ttc cag cat ggc aaa gtt gaa ggc att gat  99
   1  M   S   K   G   P   S   V   G  I   D   L   G   T   T   Y   S   C   V   G   V  20

 100 ctt ggt acc acc tac tcc tgc atc att gcc aac gac cag ggc aac aga acc act cca agc 159
  21  F   Q   H   G   K   V   E   I   I   A   N   D   Q   G   N   R   T   T   P   S  40

 160 tac gtt gcc ttc acg gat acg gag agg ctc att ggc gat gag gca aag aac caa gtt gca 219
  41  Y   V   A   F   T   D   T   E   R   L   I   G   D   E   A   K   N   Q   V   A  60

 220 atg aat cct aca aac aca gtt ttc gat gca aag cgt ttg att ggc cgc aga ttc gaa gat 279
  61  M   N   P   T   N   T   V   F   D   A   K   R   L   I   G   R   R   F   E   D  80

 280 ggt gtt gtt cag tct gac atg aag cac tgg cct ttc aat gtg ata agt gac ggc ggg agg 339
  81  G   V   V   Q   S   D   M   K   H   W   P   F   N   V   I   S   D   G   G   R 100

 340 cca aag gtg gag gtg gac tac aag ggg gaa aac aag tct ttc tac cct gaa gaa att tct 399
 101  P   K   V   E   V   D   Y   K   G   E   N   K   S   F   Y   P   E   E   I   S 120

 400 tca atg gta ctc acc aag atg aaa gag att gca gag gcg tac ttg ggc aag acc gtc tcc 459
 121  S   M   V   L   T   K   M   K   E   I   A   E   A   Y   L   G   K   T   V   S 140

 460 aat gct gtg gtg act gtt cca gct tac ttt aac gac tcc cag cgt caa agt act aag gat 519
 141  N   A   V   V   T   V   P   A   Y   F   N   D   S   Q   R   Q   S   T   K   D 160

 520 gca ggt acc att gca ggt ctc aac gtg ctt cga atc atc aat gaa cca act gct gct gca 579
 161  A   G   T   I   A   G   L   N   V   L   R   I   I   N   E   P   T   A   A   A 180

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 201  G   G   G   T   F   D   V   S   I   L   T   I   E   D   G   I   F   E   V   K 220

 700 tcc aca gca ggc gat act cat ttg gga ggg gaa gac ttc gac aac cga atg gtc aac cac 759
 221  S   T   A   G   D   T   H   L   G   G   E   D   F   D   N   R   M   V   N   H 240

 760 ttt gtg gcc gag ttc aag cgc aac agt aag aaa gac att atc gat aac aag cga gca gtt 819
 241  F   V   A   E   F   K   R   N   S   K   K   D   I   I   D   N   K   R   A   V 260

 820 cag cgc cta cgt cgc aca gcc tgt gaa cgt cgc caa gca aca ctt tca tcc agc act caa 879
 261  Q   R   L   R   R   T   A   C   E   R   R   Q   A   T   L   S   S   S   T   Q 280

 880 gcc agc att gaa atc gat tcc ctc tat gag ggt att gac ttc tac acc tcg atc acc aga 939
 281  A   S   I   E   I   D   S   L   Y   E   G   I   D   F   Y   T   S   I   T   R 300

 940 gcc cgt ttt gag gag ctg aat gct gat ctg ttc aga gga acc ctc gta ccc gtg gaa aag 999
 301  A   R   F   E   E   L   N   A   D   L   F   R   G   T   L   V   P   V   E   K 320

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1060 tcc act cga att ccg aaa atc cag aag ctg ctc cag gat ttc ttc aat ggc aaa gag ttg 1119
 341  S   T   R   I   P   K   I   Q   K   L   L   Q   D   F   F   N   G   K   E   L 360

1120 aac aag agc att aat ccc gac gaa gct gtg gct tat ggt gca gct gtg cag gcg gcc ctt 1179
 361  N   K   S   I   N   P   D   E   A   V   A   Y   G   A   A   V   Q   A   A   L 380

1180 tca gga gac aaa tct gaa aat gtt cag gat ctg ctg ttg ctc gat gtc acc cca ttg tcc 1239
 381  S   G   D   K   S   E   N   V   Q   D   L   L   L   L   D   V   T   P   L   S 400

1240 ttg gga att gag act gcc gga gga gtc tgc cgt ctg gtc aaa aca aac acc acc atc ccc 1299
 401  L   G   I   E   T   A   G   G   V   C   R   L   V   K   T   N   T   T   I   P 420

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 421  T   K   Q   T   Q   T   F   T   T   Y   S   D   N   Q   P   G   V   L   I   Q 440

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1480 aac ggt atc ctt gga aat gtt tct gct gtg gat aag agc acc gga aag gag aac aaa att 1539
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1540 aca atc aca aac gac aaa ggt cgc ctc agt gaa gat att gag cga atg gtc caa gag gct 1599
 501  T   I   T   N   D   K   G   R   L   S   E   D   I   E   R   M   V   Q   E   A 520

1600 gag aaa tac aaa gcg gag gat gaa aaa caa cgc gac aag gtg tcg tcc aaa aac tcc ttg 1659
 521  E   K   Y   K   A   E   D   E   K   Q   R   D   K   V   S   S   K   N   S   L 540

1660 gaa tcc tat gca ttc aac atg aag gca aca gtg gaa gat gag aag ctc caa gga aag att 1719
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1720 atc gat gaa gac aaa cag aag ata ttg gag aaa tgc aac gaa atc att gcc tgg ctg gat 1779
 561  I   D   E   D   K   Q   K   I   L   E   K   C   N   E   I   I   A   W   L   D 580

1780 aag aat cag act gca gag aaa gat gag ttc gaa cat cag cag aaa gag ctg gag aag gtg 1839
 581  K   N   Q   T   A   E   K   D   E   F   E   H   Q   Q   K   E   L   E   K   V 600

1840 tgt aac cca atc att acc aaa ctg tac cag ggt gct ggt gga atg cca ggt ggt atg ccg 1899
 601  C   N   P   I   I   T   K   L   Y   Q   G   A   G   G   M   P   G   G   M   P 620

1900 gga ggg ttc cca agc gca ggc ggc gct cca gct ggc agc ggt ggc tca tct gga ccc act 1959
 621  G   G   F   P   S   A   G   G   A   P   A   G   S   G   G   S   S   G   P   T 640

1960 atc gaa gaa gtc gat taa aag att att tcc gct ggt ttt gtc gtc aaa ggt gat cca aga 2019
 641  I  E   E   V   D   *

2020 cac aca ttt gta aca gta gca tgc tgt ttg aaa aag caa acc cat ttc aca ttg aag ctg 2079

2080 ctg tac aac tac tgg gca tca gag tta ctt gaa tct ggg gcg taa gga gag gac agc att 2139

2140 gca ctt tac tta cca gta act gtt aac caa cgt tga gtc ctg acg aat aaa atc tat tta 2199

2200 att ggc aat tta_n
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FIG. 1. Nucleotide sequence and amino acid sequence (listed below) of *Pleurodeles waltl* 2212-bp cDNA Pwhsc70. Underlined: start codon, stop codon, and polyadenylation signal. Bold characters: three conserved elements; sign 1, IDLGTTYS; sign 2, DLGGGTFD; EEVD motif. Underlined: nuclear localization signal. This sequence will appear in the EMBL nucleotide sequence database under the accession number Y13661.

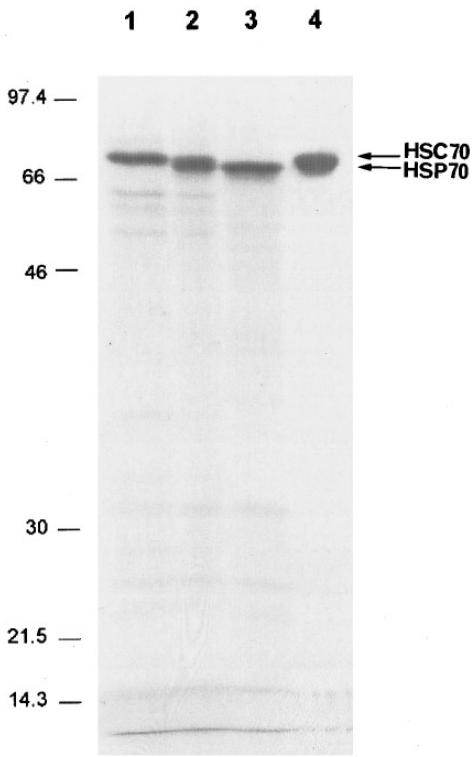

FIG. 2. *In vitro* translation of PwHSC70 and PwHSP70 protein. Monodimensional electrophoresis on SDS 8-15% gradient polyacrylamide gel and immunodetection on Western-blot with the N1 antibody. Lane **1,** *in vitro* translation of PwHSC70; lane **2,** co-migration of *in vitro* translation of PwHSC70 and inducible PwHSP70; lane **3,** *in vitro* translation of inducible PwHSP70; lane **4,** Western blot of *in vitro* translation of PwHSC70 and PwHSP70 incubated with the N1 antibody. Using 8-15% gradient monodimensional electrophoresis, both forms of PwHSP70 and PwHSC70 are clearly distinguishible by their molecular mass. Molecular mass markers on the left are in 10^3 Mr.

(Perkin-Elmer). 692 bp of Pwhsc70 cDNA and 1234 bp of Pwhsp70 cDNA in the 3′ coding region were amplified by polymerase chain reaction with AmpliTaq DNA polymerase (Perkin Elmer); 30 thermocycles were performed for 30 sec at 93°C, 30 sec at 53°C or 58°C, respectively, and 90 sec at 72°C. PCR products were electrophoresed in TBE agarose gel, blotted onto nylon membrane (Nytranplus, Schleicher et Schuell) and hybridized with Pwhsc70 cDNA probe and Pwhsp70 cDNA probe. Hybridization was carried out at 68°C in 6× SSC, 2× Denhardt's, 0.1% SDS. Blots were washed 20 min in 1× SSC, 0.1% SDS at room temperature, and 3 × 20 min at 68°C in 0.2× SSC, 0.1% SDS, then autoradiographed on Amersham MP film.

In vitro translation, Western blot, and immunodetection. *In vitro* transcription and translation were performed from Pwhsc70 cDNA cloned in pBluescript-SK$^+$ expression vector using rabbit reticulocyte lysate (Rabbit Reticulocyte Lysate Systems, Promega) with ^{35}S-methionine. Aliquots of translation products were electrophoresed in monodimensional SDS 8-16% gradient polyacrylamide gel electrophoresis [16]. The dry gel was autoradiographed on Amersham β-Max film. The molecular mass of the translation products were determined by reference to the molecular mass markers. Translation products were electrophoresed in monodimensional SDS gradient 8-16% PAGE and blotted on Immobilon-PVDF membrane (Millipore) [17]. Blot was incubated with the polyclonal antibody N1 which was raised against *Pleurodeles waltl* 70-kDa HSP/HSC70 protein [11]; the N1 epitope is a 16 amino acid sequence 56 residues upstream of the C-terminal domain and is common in HSC70 and HSP70 proteins. We used secondary anti-rabbit antibody coupled to the peroxidase (Amersham Life Science) to reveal the N1 antibody. Immunodetection was revealed with the chimioluminescence enzymatic system (ECL kit, Amersham Life Science).

RESULTS AND DISCUSSION

To recover a constitutive form of the 70-kDa protein, we screened a cDNA library from tail-bud stage embryos of *Pleurodeles waltl* using a 280-bp probe fragment corresponding to the 5′ ATP-binding domain of the inducible *Pleurodeles waltl* Pwhsp70 cDNA (see Materials and Methods) [8]. In fact, among different species, this domain corresponds to the most conserved region between inducible or constitutive genes of the 70-kDa heat-shock proteins. Two rounds of screening were performed. Eleven positive clones were recovered and partially sequenced. One of these clones, 2212 bp in length, showing similarities with Pwhsp70 cDNA, was selected for further analyses and was entirely sequenced. As shown in fig. 1, it exhibited one open reading frame of 645 amino acids that localized the start codon ATG after 40 nucleotides of the 5′ untranslated region (UTR). The 3′UTR contained the polyadenylation signal (aataaa) 209 amino acids downstream from

TABLE 1

Nucleotide and Amino Acid Sequence Comparison between *Pleurodeles waltl* Pwhsc70 and *hsc/hsp70* Genes of Different Species

	HSC70		HSP70	
Species	Nucleotide (%)	Amino acid (%)	Nucleotide (%)	Amino acid (%)
Rattus	78.5	89	72.3	79.8
Homo sapiens	72.7	89	78.5	80
Bos taurus	78.1	88.4	73.2	79.6
Xenopus	hsc70.I:76.1 hsc70.II:77.8	HSC70.I:88.5 HSC70.II:90.5	71.9	80.8
Pleurodeles	—	—	72.5	79.3

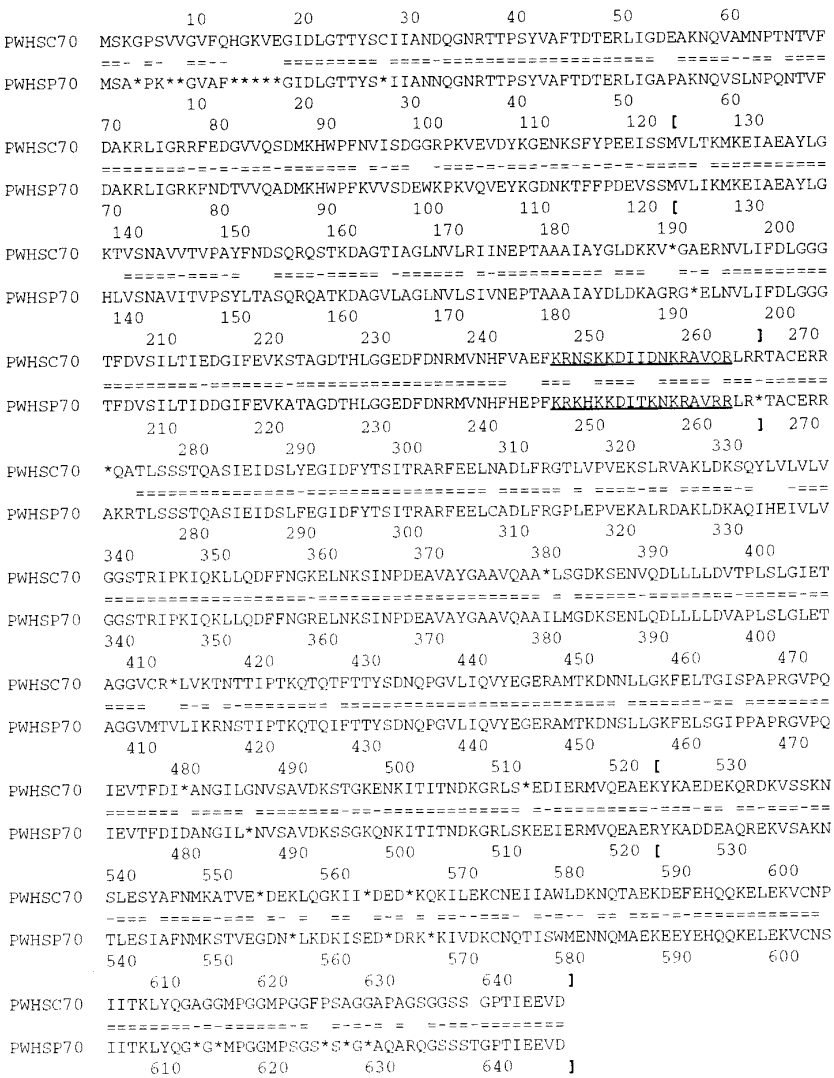

FIG. 3. Amino acid sequence comparison between *Pleurodeles waltl* PwHSC70 and PwHSP70. Brackets: the 5′ ATP-binding domain, from amino acids 122 to 264, and the 3′ peptide-binding domain, from amino acids 521 to 645. Underlined: nuclear localization signal. Asterisks indicate gaps in the amino acid sequence.

the stop codon (taa). The predicted amino acid sequence of this clone exhibited three evolutionary conserved elements typical for 70-kDa heat-shock proteins: the first domain, IDLGTTYS, called "sign 1", from amino acids 9 to 16; the second domain, DLGGTFD, called "sign 2", from amino acids 199 to 206 ; and the third element, EEVD, at the carboxy-terminal end of the coding region. In many different species, these three domains are known to be conserved in most HSP70 and HSC70 proteins [18,19]. A putative nuclear localization signal (NLS), from amino acids 246 to 262 (KRNSKKDII-DNKRAVQR), was found similar to the NLS in *Xenopus laevis* HSC70.I or to the NLS reported in human HSP70-like protein [20,21] (see fig. 3). This 17 amino acids motif would be necessary to the nuclear targeting [22-24].

To validate that this cDNA clone encoded a 70-kDa protein and that its transcript was translatable, we expressed the corresponding protein in an eukaryotic *in vitro* translation system in presence of ^{35}S-methionine. In order to distinguish this form of the protein from that previously isolated [8], the translation products were separated by 8-16% gradient monodimensional electrophoresis. As shown in fig. 2, the molecular mass of the translated protein was found to be approximatively 70 kDa, and migrated at a higher molecular mass than the band of the inducible PwHSP70 protein. Moreover, this translated protein was recognized by the *Pleurodeles waltl* N1 anti-HSC/HSP70 antibody, as expected. These results led us to conclude that we recovered a cDNA that belongs to the *hsc/hsp70* gene family.

A search within the nucleotide BISANCE database (see table 1) provided evidence for 74% identity of this cDNA clone with inducible Pwhsp70, 78.5% with rat hsc71, 72.7% with human hsc71, 78.1% with bovine hsc71 and 77.8% with *Xenopus* hsc70.II. In fact, the identity of this *Pleurodeles waltl* clone was higher with hsc70 of other species than with *Pleurodeles waltl* inducible Pwhsp70. A search within the protein BISANCE database confirmed the results of nucleotide sequence comparison (see table 1). Indeed, the predicted amino acid sequence of this clone revealed a higher identity with constitutive HSC70 of other species: 89% with rat HSC71, 89% with human HSC71, 88.4% with bovine HSC71 and 90.5% with *Xenopus* HSC70.II. The identity was only 79.3% with inducible *Pleurodeles waltl* PwHSP70, 79.8% with rat HSP70, 80% with human HSP70, 79.6% with bovine HSP70 and 80.8% with *Xenopus* HSP70. The 5′ coding region for ATPase activity of this clone, from amino acids 122 to 264, revealed a high identity with constitutive HSC70 of other species: 94.4% identity with constitutive rat HSC71, 92.2% with *Xenopus laevis* HSC70.I or 95% with HSC70.II; it represented only 79.5% identity with *Pleurodeles waltl* inducible PwHSP70 (see fig. 3). Moreover, at the carboxyl-end of the PwHSC70, from amino acids 521 to 645, the identity of the 3′ peptide-binding domain was also higher with the constitutive HSC70 of other species than with the inducible *Pleurodeles waltl* PwHSP70, but at a lower degree: identity with rat HSC71 was 91.1%, 83.4% with *Xenopus laevis* HSC70.I, 86.3% with HSC70.II, and only 66.1% with *Pleurodeles waltl* inducible PwHSP70. As previously described by authors [4], these domains were more divergent in the same species than between different species.

To precise whether the cDNA clone corresponded to a cognate gene, experiments for heat-inducibility in ovary, which includes germinal cells (oocytes) as well as somatic cells (theca and follicular cells), were carried out (see fig. 4). Due to high similarity between the nucleotide sequence of Pwhsc70 and inducible Pwhsp70 (72.5%), RT-PCR assays were performed rather than Northern blots. Indeed, signals detected by Northern blot could not be attributed specifically to a cognate or an inducible gene. RT-PCR experiments using specific primers for Pwhsc70 cDNA were performed in normal conditions and after heat-shock (see fig. 4). To test efficiency of heat-shock conditions, RT-PCR experiments, using specific primers of the inducible form Pwhsp70 cDNA, were considered as control. After heat-shock at 34°C or 37°C, comparing hybridization signal's intensity, the amount of inducible Pwhsp70 mRNA increased whereas the amount of Pwhsc70 mRNA was not affected. These results clearly demonstrated that the *Pwhsc70* gene was constitutively expressed in ovary. Thus, results of sequence comparison and heat-inducibility experiments led us to conclude that we recovered a cognate *Pleurodeles waltl* gene encoding a protein that we named PwHSC70.

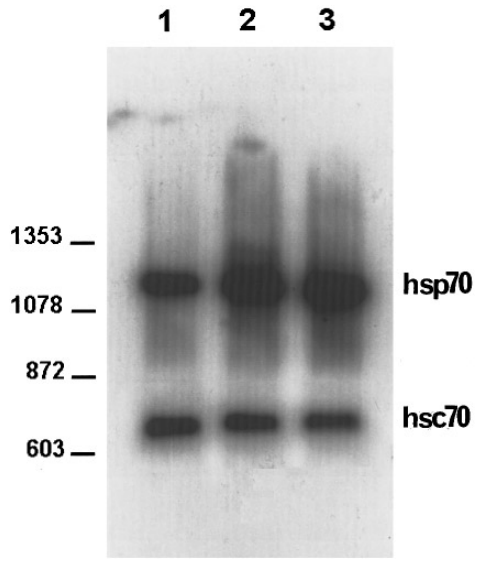

FIG. 4. Heat-inducibility experiments. Autoradiograph resulting from Southern hybridization using Pwhsp70 and Pwhsc70 cDNA probes. RT-PCR assays on total RNA were performed using specific primers for Pwhsp70 (band of 1234 bp) and specific primers for Pwhsc70 (band of 692 bp). Total RNA was extracted from ovary maintained either for 2.30 hr at 20°C (lane **1**), heat-shocked 2.30 hr at 34°C (lane **2**), or heat-shocked 15 min at 37°C followed by recovery during 2.15 hr at 20°C (lane **3**). Molecular mass designation on the left is in bp. Note that the amount of Pwhsc70 mRNA was not affected after a heat-shock treatment, whereas the amount of Pwhsp70 mRNA (inducible form) increased.

Finally, our results pointed out that the identity between *hsc70* gene and *hsp70* gene was lower in the same species than between different species [4]. Such results suggest that the divergence between hsc70 gene and hsp70 gene into the same species occurred very early during vertebrate evolution. This could reveal a highly specific function for one or the other form of the protein. The elucidation of the specific role of the cognate PwHSC70 during oogenesis and embryogenesis in *Pleurodeles waltl* is now in progress.

ACKNOWLEDGMENTS

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