Molecular cloning of flounder Xp18, a newly identified highly conserved protein mainly expressed in the ovary

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Abstract: Screening of a flounder ovary cDNA library with a rainbow trout p53 probe led to the isolation of a p53-unrelated cDNA encoding an unknown 161 amino acid protein. In view of its apparent molecular weight and yet unknown function, the deduced protein was named Xp18. Corresponding orthologous cDNAs or expressed sequence tags have been identified in several species including human, rodents, bovine, chicken and zebrafish and a related cDNA has also been isolated in the fruit fly. Deduced amino acid sequences appeared to be extremely well conserved throughout vertebrate evolution. Structure predictions suggested that Xp18 may correspond to an integral protein comprising four transmembrane domains. The charged C-termini of all known vertebrate Xp18-like proteins displayed a characteristic KXKXX motif which is considered as an endoplasmic reticulum targeting sequence. Gene expression, as shown by Northern blot and quantitative reverse transcription-polymerase chain reaction analysis, was significantly higher in the ovary and to a lesser extent in the brain. Xp18 transcripts were also detected by in situ hybridization in most of the circumventricular regions of the brain of adult flounders. The gene encoding the human protein is located on chromosome Xq22.1, a genome region involved in numerous genetic diseases including premature ovarian failure.

Keywords: Platichthys flesus; Teleostean; Human; Ovary; Brain; X chromosome

Introduction

It is now well established that a variety of chemical compounds of anthropogenic origin, can induced long term effects on living organisms including cancers and reproductive defects. When acting on reproduction, pollutants can provoke chromosome abnormalities, reduce success of fertilization, decrease hatching or survival of larvae and can also interfere with hormonal regulations (reviewed in Janssen *et al.*, 1995). In marine environment, these disorders mainly affect benthic fish living in coastal and estuarine areas near industrial and urban centers.

As an evidence, an histological survey of gonads of European flounder specimens conducted in the Seine river estuary (France) has shown that up to 8% of males were intersex e.g. had testes characterized by the presence of entire ovarian structures (Minier *et al.*, 2000).

The European flounder, *Platichthys flesus* (L.), is a model species for pollution monitoring in the north eastern Atlantic. This euryhaline flatfish lives on and in the sediment of coastal waters and estuaries resulting in a strong exposure to river-carried pollutants. In addition, this species seems particularly sensitive to several chemical-induced and infectious diseases (Vethaak and Jol, 1996).

In the Seine estuary, sexual differentiation of European flounders is initiated during their second year of life. At this age, gametogenesis begins since early perinucleolus oocytes are continuously produced and gradually accumulate during a year. Development of oocytes takes place the next year when they enter vitellogenesis and start the maturation process (Janssen *et al.*, 1995). Sexually mature flounders then migrate in January and February to shallow coastal areas for spawning. The annual ovarian cycle ends with the degeneration of post-ovulatory follicles in post-spawning female.

Our purpose was initially to identify *p53*-related genes in this fish species in order to study the molecular basis of carcinogenesis in fish and also to develop new markers for mutation and cancer risk assessment in marine ecosystems. A flounder ovary cDNA library was constructed and screened under low stringency conditions using a rainbow trout *p53* cDNA probe. Two positive cDNA clones were isolated, one of which did correspond to the flounder *p53* cDNA (Cachot *et al.*, 1998). Sequencing of the second clone revealed that the cDNA sequence displayed only 35% homology with trout p53. However, *in silico* screening of genome data bases indicated that this novel cDNA had striking homologies with an unknown cDNA which has recently been identified in human embryos (Isogai *et al.* 2000). In the present paper we describe the molecular cloning and the expression profile of this novel cDNA which was named *Xp18*.

2. Materials and Methods

2.1 Cloning of flounder Xp18

A flounder ovary cDNA library previously constructed in Lambda ZAP® II vector (Cachot *et al.*, 1998) was screened at low stringency with the complete rainbow trout *p53* cDNA. The probe was radiolabeled with (α ^{32}P)-dCTP (Amersham) using a random priming kit (Ready-to-Go, Pharmacia). Hybridization was performed overnight at 55°C in 6x SSC (0.9 M NaCl, 0.09 M sodium citrate), 4x Denhardt's reagent, 0.5% SDS, 2mM EDTA and 1ng.mL^{-1} of radiolabeled probe. The membrane were washed in 4x SSC, 0.5% SDS for 1 hour at 55°C before a 24 hr-exposition to X-ray film (Kodak X-Omat). After five rounds of screening, positive plaques were isolated and inserts subcloned into pBluescript SK(-) vector according to the protocol of the manufacturer (Clontech). Both DNA strands were sequenced on a 373A DNA sequencer according to the Big Dye Terminator procedure (Perkin Elmer).

2.2. Northern blot analysis

Flounders were collected in the Seine bay and anaesthetized in MS222 before tissue collection. Total RNAs were extracted from brain, liver, spleen, intestine, muscle, ovary and testis of adult flounder by using the TRI Reagent^{TM} protocol (Sigma). The total RNAs (20 μg) were separated on a 0.8% formaldehyde-agarose denaturing gel and then blotted onto a nylon membrane (Amersham Hybond-N+). A 1300 bp fragment generated by *EcoRI* digestion of the flounder *Xp18* cDNA was used as a probe. This DNA was labeled with (α^{32}P)-dCTP (Amersham) using a random priming DNA labeling kit (Prime-a-gene, Promega). Hybridization was performed for 16 hours at 42°C in 5x SSPE (0.75 M NaCl, 0.05 M NaH$_2$PO$_4$, 5 mM EDTA) 50% deionized formamide, 1% SDS, 5x Denhardt's reagent, 5% dextran sulfate, 100 μg.mL^{-1} of denatured salmon sperm DNA and 1 ng.mL^{-1} of radiolabeled probe. The membrane was then washed for 1 hour at 65°C in 2x SSPE-0.1% SDS and exposed at -80°C to X-ray films (Kodak X-Omat) for 1 week.

2.3. Real-time quantitative RT-PCR

Total RNAs were extracted with TRI Reagent^{TM} (Sigma) from ten tissues of adult flounders. RNA quality was checked on denaturating agarose gel before used. First strand cDNA synthesis was performed using Rnase free Superscript II (Invitrogen) from 5 μg total RNAs in a 20 μL reaction mixture containing 500 ng oligo(dT)$_{15}$ (Genset), 10 nmol dNTP, 1X first strand buffer, 200 nmol DTT for 50 min at 42°C. The obtained cDNAs were purified on QIAquick PCR purification kit (Qiagen) and eluted with 30 μL of Tris-HCl 10 mM, pH 8.5.
PCR amplifications were performed using FirstStart DNA Master SYBR Green I and the LightCycler system (Roche Molecular Biochemicals) with 0.5 μM of each primers, 4 mM MgCl$_2$ and 2 μL of purified cDNA template. Forward (5'-ACCGACAGACGCTCATCCAGT-3') and reverse (5'-TGACCAGCAGCAGCAAAAAGTT-3') primers for *Xp18* were carefully selected in two neighbouring exons using PrimerPremier3 software (Premier Biosoft International) in order to obtain a unique amplicon of 359 bp. The cycling conditions were as follows : initial denaturation at 95°C for 5 min followed by 40 cycles at 95°C for 15 s, 58°C for 5 s and 72°C for 14 s. The level of the housekeeping gene *β-actin* was used to normalize for the variability of RNA quality and quantity as well as for differences in the efficiencies of the RT-reactions. Forward (5'-TCCTGACCCTGAAGTACCCC-3') and reverse (5'-GGTGCCCATCTCCTGCTCGA-3') primers for *β-actin* were carefully selected in two neighbouring exons (Lee *et al.*, 2000) using PrimerPremier3 software in order to obtain a unique amplicon of 496 bp. The cycling conditions were identical as for *Xp18* except for the hybridization temperature (53°C) and the extension time (20 s). The levels of *Xp18* and *β-actin* cDNAs were obtained from the same RT reaction. All samples were analysed in duplicate. For each experiment a standard curve was performed using a ten-fold serial dilution of cloned *Xp18* or *β-actin* cDNA (Fig. 4). The amplification efficiencies were calculated from the slope of the standard curves using the equation E= $10^{-1/slope}$ and was always greater than 1.92 (96%). Specificity of PCR amplification was checked using both melting curve analysis and agarose gel electrophoresis of PCR products.

Results are reported as the ratio of *Xp18* to *β-actin* number of copies of mRNA. Copy numbers were calculated from the standard curve obtained for each experiment.

2.4. In situ hybridization

Briefly, a 510-bp fragment of *Xp18* cDNA was amplified by PCR (sense, 5'-GCAAGCCCCGGTCCAGAAAC-3' and antisense primer, 5'-GATCTTAGGACTGCTT-3') and was subcloned into pGEM-T vector (Promega). Single stranded sense and antisense Xp18 RNA probes were generated following digestion with either P*st*I or S*ac*II by using a Promega riboprobe kit and (α^{33}P)-UTP as a label. RNA probes were purified by Sephadex G-50 gel chromatography (elution buffer; 10 mM Tris-HCl, pH 7.5, 0.1% SDS). Fish were anaesthetized in MS222 and perfused transcardially with 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). The tissues were postfixed for 3 hr at 4°C in the same solution, transferred into 0.1 M phosphate buffer containing 15% saccharose for 12 hr and frozen in isopentane at –30°C. Sections (15 μm thick) were cut in a cryomicrotome (2800 Frigocut, Leica, Heidelberg, Germany) and collected on 0.5% gelatin/0.05% chrome alum/0.01% polylysine coated slides. Tissue sections were incubated in phosphate buffer saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM, Na_2HPO_4, 1.8 mM KH_2PO_4), treated in PBS containing 0.3% Triton X-100 for 15 min and permeabilized for 30 min in TE buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8.0) containing 1 μg.mL^{-1} proteinase K. Sections were then postfixed for 5 min at 4°C in PBS containing 4% paraformaldehyde. Acetylation was carried out for 10 min in 0.1 M triethanolamine buffer pH 8.0, 0.25% acetic anhydride. The slides were rinsed in 2x SSC, and covered with prehybridization buffer (4x SSC containing 50% deionized formamide). Hybridization was performed under a coverslip overnight at 37°C in 40% deionized formamide, 10% dextran sulfate, 1x Denhardt's solution, 4x SSC, 10 mM dithiothreitol, 1mg/ml denatured Salmon sperm DNA containing the purified antisense or sense Xp18 RNA probe. Slides were then washed twice in 2x SSC at 37°C. A series of 4 washes was performed at 37°C in 0.1x SSC. The slides were finally dried in ethanol and exposed either onto films (BioMax MR, Kodak) for 2 weeks or overnight in a phosphoimager (Molecular Dynamics).

2.5. Computer and statistical analyses

Sequence analyses were performed by using the GCG package hosted by the server Infobiogen of INSERM-CNRS. Multiple alignments were carried out using ClustalW (Thompson *et al.*, 1994). Homology searches were performed on the sequences listed in EMBL/GenBank/DDBJ databases with FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.*, 1990).

Statistical analysis of *Xp18* to *β-actin* mRNA ratio in the ovary, brain and testis were determined by the unpaired two-tailed Student's *t* test. Differences were considered statistically significant for $p < 0.05$. Mean values are given with their standard deviation (SD).

2.6. Nucleotide sequence accession number

The EMBL accession number of flounder *Xp18* cDNA is AJ302671.

3. Results and Discussion

3.1. Cloning of flounder Xp18

After five rounds of screening of the flounder cDNA library, two distinct positive clones were isolated. The longest clone contained an insert of 2279 bp in length which corresponded

to the cDNA encoding the p53 protein (Cachot *et al.*, 1998). However, the 1682 bp insert of the second clone had only 35% overall homology with trout *p53* cDNA. This insert included a 5'-untranslated region of 90 nucleotides, followed by an open reading frame comprising 486 nucleotides (Fig. 1A). The 3'-untranslated sequence was 1.1 kb in length and contained two putative polyadenylation signals (nucleotides 677 and 1639) and a polyadenylated tail starting at nucleotide 1665. The open reading frame encoded for a 161 amino acid protein with a molecular weight of 17,927 Da and a theoretical pI of 9.86. In view of its predicted molecular weight, and owing to its unknown function, the protein was coded Xp18. Composition analysis of Xp18 protein revealed a high number of leucine (15.5%) and other hydrophobic residues (37%) in comparison with charged amino acids (25%). Charged residues were mostly clustered in two blocks, the first one encompassing residues 30 to 45 and the second one the last 30 or 35 residues of the C-terminus. Search in several motif databases revealed the presence of 2 putative protein kinase C and 2 casein kinase II phosphorylation sites as well as 2 putative N-myristoilation sites (Fig. 1A). The hydrophobicity plot (Kyte and Doolittle, 1982) revealed the existence of 4 hydrophobic domains spanning the amino-terminus and the major part of the core protein (Fig. 1B). In contrast, the 30 amino acids at the carboxy-terminus appeared to be very hydrophilic. Structural prediction of membrane-bound proteins (Argos *et al.*, 1982) suggested that the Xp18 protein was integrated in biological membranes and contained four transmembrane sections corresponding to the four hydrophobic domains. In addition, prediction of signal peptides and anchor signal by a hidden Markov model (Nielsen *et al.*, 1997) indicated that the first transmembrane domain was likely to correspond to an anchorage region to the endoplasmic reticulum (ER) membrane. Calculation of secondary structures by the PHD network system (Rost and Sander, 1994) indicated the presence of three to four alpha helixes encompassing the four hydrophobic domains of the protein and separated by 2-3 loops. An additional alpha helix spanned the acidic carboxy-terminus. The Xp18 protein contained a characteristic KXKXX motif. This motif is a KKXX related-signal which has been shown to function as an ER localization signal of integral membrane proteins with C-termini oriented on the cytoplasmic side of the membrane (Nilsson *et al.*, 1987). The consensus sequence is recognized by the COPI complex which participates in the formation of coated budding vesicles. Coatomer binding to the C-terminal KKXX signal mediates retrieval of ER membrane proteins (Hong, 1996). These data indicate that Xp18 might correspond to an integral membrane protein of the ER which may be sorted to budding vesicles.

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 -90 aattcgcggccgcgtcgaccgaccgacagacgctcatccagtccggtgagggaaagcgcc

 -30 ctgagctgctgcaagccccggtccagaaacATGGCTTCGCCGAGGACAGTCACCATCGTG
                                  M  A  S  P  R  T  V  T  I  V   10
                                                 •
  31 GCCCTTTCCATCGCCCTGGGCCTGTTCTTCGTGTTCATGGGAACCATCAAACTCACACCG
      A  L  S  I  A  L  G  L  F  F  V  F  M  G  T  I  K  L  T  P    30
                  Anchor                        •           •
  91 AGGCTCAGCAAAGACGCCTACAGTGAAATGAAAAGGGCCTACAAGAGTTACGCCAAGGCA
      R  L  S  K  D  A  Y  S  E  M  K  R  A  Y  K  S  Y  A  K  A    50
 151 CTGCCGGGCCTGAAGAAGATCGGGATCAGCTCAGTCCTGCTCCGCAAGATCATCGGCTCT
      L  P  G  L  K  K  I  G  I  S  S  V  L  L  R  K  I  I  G  S    70
                                      Domain II
 211 CTGGAGGTGGGCTGCGGTGTGGTGCTCACCCTGGTGCCTGGCCGGCCGAAGGACGTGGCC
      L  E  V  G  C  G  V  V  L  T  L  V  P  G  R  P  K  D  V  A    90
                        ◆
 271 AACTTTTTGCTGCTGCTGGTGCATGCTGGCCGTCCTGTTCTTCCACCAGCTAGTGGGAGAC
      N  F  L  L  L  L  V  M  L  A  V  L  F  F  H  Q  L  V  G  D   110
                       Domain III
 331 CCCCTGAAGCGGTACGCCCACGCTCTGGTCTTTGGCATCCTGCTCACCTGCCGGCTGCTC
      P  L  K  R  Y  A  H  A  L  V  F  G  I  L  L  T  C  R  L  L   130
                 Domain IV               ◆           •
 391 ATCGCCCGCCAGACCGACGACCGGCCGGAGAGGAGGACAGCAGAGAGGAACAGCAGGTG
      I  A  R  Q  T  D  D  R  P  E  R  E  D  S  R  E  E  Q  Q  V   150
 451 AACGACCAGGAAAAGAACAAGGTCAAGCAGTCCTAAgatccctctgccactctggtccaa
      N  D  Q  E  K  N  K  V  K  Q  S  *
                                                                 161
 511 gccacaggaatgggaaactgaagcgcctttatggattgtagtaatttgaattttgaagga
 571 aaccgtcactcccagtaataaaagccctcacacatccccccgtctcatccaggtggctga
 631 ctgaataccctcacccactcctctgcaccattaatgtgaaaccaagaacccctgatccat
 691 acgtcctctacatanacatggacaatcttacctttggaaactctgaagagctcagctcgc
 751 tgtaacaatagtctatcccatgttccctatttatttcagtcctacactgtgttaaagtta
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 871 gatttgagaatgctgtttttcatgaaagaatctttgtagcttgtttttttctaatttgttt
 931 ttatactgtatgtaggagagactggtaatttatgaatttcaatatttcaagcatgtttca
 991 tctggtcaagacaacagaacataaagcaaaccgagcactgctgaaagggttttgtactaa
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1171 tttgcagatgtacaaatgaaagccattctcctgaggtcaaatacgctgaattcccctttt
1231 ctcctttttagttcagtgaacacagtgaagttgttaagctggggaaggaccgcagcttctg
1291 cctcaggtgtctgctgcctactcgtacagctgaacatccagctcagtcttttttgtccgtt
1351 aaaattgagcttgacttttgtttttttttaattcacagtttaggattattctctgtctcg
1411 aaacggctctgctcaacccctttggttttaacgttttttcctctgtaaagatttttaatatcct
1471 caggtttggggatgaaatgtcatctaaatattctgtcagatttgagattcaaaatatttc
1531 tgtaaatgatatacttcaaaataaagaattggaagtatgactggca
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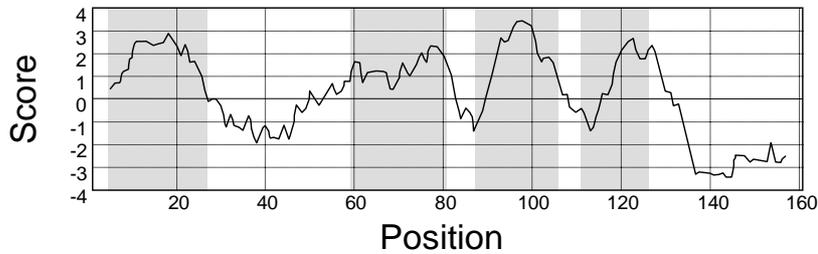
Fig. 1. (A) Nucleotide and deduced amino acid sequences of flounder Xp18. The open reading frame is indicated in capital letters. The two polyadenylation/cleavage sites are underlined. The deduced peptide sequence is represented with the one letter code for amino acids. The anchor domain to the ER is indicated in gray and the three other putative transmembrane domains are boxed in black. The TAA stop codon is marked by a star. Circles and diamonds respectively indicate putative phosphorylation and N-myristoylation sites. (B) Hydrophobicity profile of flounder Xp18 protein (Kyte and Doolittle, 1982). Amino acids are numbered from the translation start site. The four putative transmembrane domains are shaded.

3.2. Comparison of Xp18 orthologuous sequences

Searches in nucleotide databases revealed that the *Xp18* cDNA clone had a high degree of homology with a human cDNA sequenced from whole embryos (Isogai *et al.*, 2000) and with Expressed Sequence Tags obtained from the brain of *Rattus norvegicus* (Bonaldo *et al.*, 1996), *Mus musculus* (Bonaldo *et al.*, 1996, Sasaki *et al.*, 1998) and *Brachydanio rerio* (Clark *et al.*, 2001), as well as from lymphoid organs of *Bos taurus* (Smith *et al.*, 2000) and *Gallus gallus* (Tirunagaru *et al.*, 2000). Comparison of the deduced amino acid sequences from flounder and human cDNAs, zebrafish, mouse, rat, bovine and chicken ESTs revealed a high level of conservation during evolution (Fig. 2A and 2B). Amino acid identity of Flounder Xp18 reached 96%, 83%, 81% and 82% with, respectively, zebrafish, bovine, mouse and human polypeptides. The amino-terminus and the core of the proteins were almost perfectly conserved whereas the last 30 amino acids of the carboxy-terminus were much more divergent. Putative transmembrane segments appeared to be conserved including the anchor signal. Such a high degree of conservation suggests that Xp18 protein plays an important functional role. A substantial identity (38 %) was also observed with a 160 amino acid product of a recently identified gene in the fruit fly, *Drosophila melanogaster* (Adams *et al.*, 2000). However, the fruit fly protein lacked the characteristic KVKXS motif present at the C-terminus end of all vertebrate Xp18 proteins. Extensive Blast and Fasta searches in genome databases did not allow the identification of Xp18 related polypeptides in prokaryotes, in yeast, or in the nematode *Caenorhabditis elegans*.
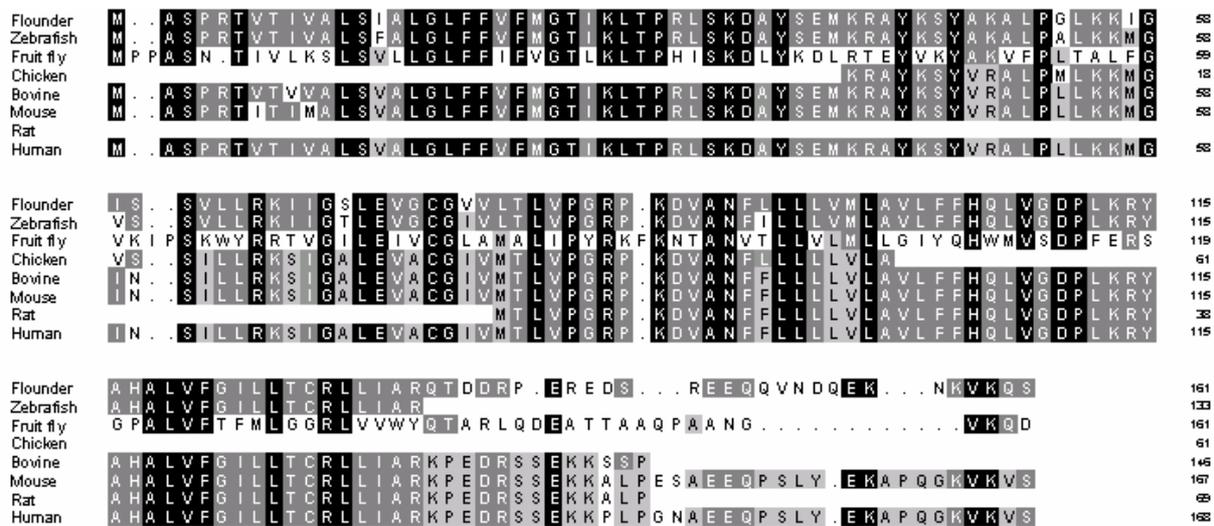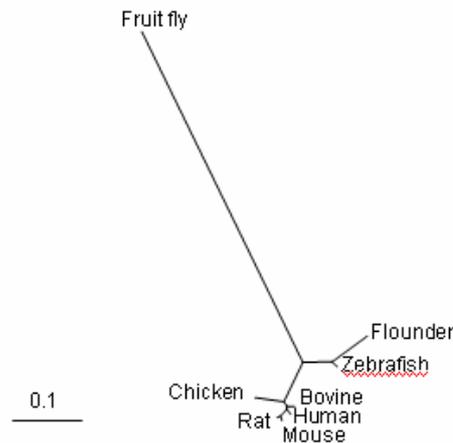
Fig. 2. (A) Sequence alignment of flounder Xp18 deduced protein with zebrafish (Clark et al., 2002), fruit fly (Adams et al., 2000), chicken (Tirunagaru et al., 2000), bovine (Smith et al., 2000 modified), mouse (Bonaldo et al., 1996 and Sasaki et al., 1998), rat (Bonaldo et al., 1996), and human (Isogai et al., 2000) homologues. Multiple alignment was calculated by using ClustalW software (Thompson et al., 1994) and corrected manually. Residues conserved in all species in which the sequence is known are boxed in black. Residues common to flounder and to any other species are shaded in gray. Amino acids common to at least half of the known sequences are shown in light gray background. (B) Cladogramme of Xp18 proteins as calculated from the multiple alignment. Bar corresponds to 0.1 residue substitutions per site.

In silico screening of genome databases with flounder *Xp18* cDNA sequence revealed that the human and mouse Xp18 genes were located on the X chromosome (Sanger Centre, 2002). The human homologue was mapped to Xq22.1, a region implicated in several genetic diseases (OMIM, 2002), including premature ovarian failure (Bione *et al.,* 1998). The human and the mouse *Xp18* genes as were organized as two exons separated by a large intron. The exon-intron boundaries were perfectly conserved between the two species. Exon 1 encoded the first 40 amino acids of the protein while the second exon encoded the last 127 residues. Analysis of the 2,500 bp sequence located upstream from the initiation codon revealed the

presence of multiple potential recognition sites for transcription factors including several putative binding sites for the ubiquitous transcription factor Sp1. The presumed promotor region also contained putative binding sites for the estrogen receptor, Hepatocyte Nuclear Factor-1, MyoD and Nuclear Factor Kappa B. The presence of a potentially functional estrogen responsive element within the promoter region is of particular interest with respect to the high expression of Xp18 in the fish ovary.

3.3 Xp18 expression profile in flounder

Northern blot analysis revealed a strong expression of a single transcript in the ovary. A medium *Xp18* expression was also found in the brain of flounder. No hybridization signal was detected in the other tissues tested (Fig. 3). The transcript was about 1650 bp in length and thus may represent a mature form of the mRNA. Quantitative RT-PCR analysis allowed detection of a basal *Xp18* expression in all tissues tested. However important differences among tissues were measured. A statistically significant higher level of *Xp18* expression was shown in the ovary in comparison with the brain ($P = 0.005$) or in the latter in comparison with the testis ($P = 0.056$). No significant differences were observed between ovary of different developmental stages. In most tissues; e.g. testis, spleen, kidney, gill, heart, intestine and liver, *Xp18* was 10 to 100 fold less expressed than in the ovary (Table 1).

Table 1. LightCycler RT-PCR quantification of *Xp18* to *β-actin* mRNA ratio in different flounder tissues

Tissues	Sex	Number of individuals	Xp18 to β-actin mRNA ratio (mean±SD)	P value (t-test)	
Ovary	F	6	0.789±0.427		
Brain	F	5	0.211±0.110	0.005	
Testis	M	4	0.087±0.013		0.056
Ovary stage II	F	3[a]	0.945±0.482		
Ovary stage III and IV	F	3[a]	0.488±0.220	0.209	
Brain of male	M	2[b]	0.157±0.043		
Muscle	F	2[b]	0.157±0.083		
Spleen	F	2[b]	0.077±0.033		
Kidney	F	2[b]	0.057±0.02		
Gill	F	2[b]	0.036±0.018		
Heart	F	2[b]	0.019±0.006		
Intestine	F	2[b]	0.012±0.005		
Liver	F	2[b]	0.010±0.006		
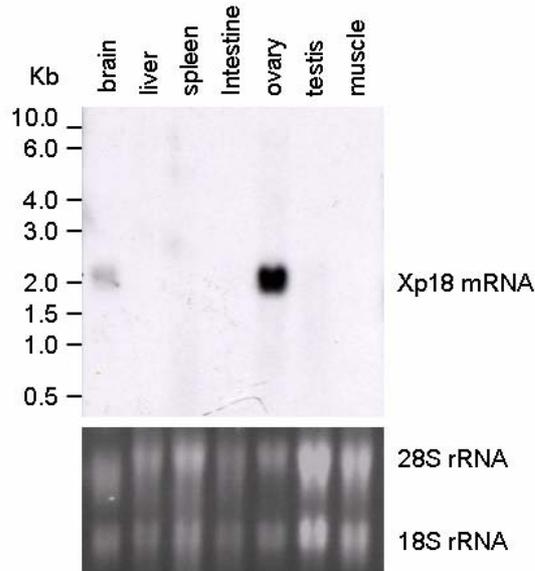
Fig. 3. Northern blot analysis of *Xp18* mRNAs isolated from several tissues of adult flounders. A (α^{32}P)-dCTP-labeled fragment of flounder *Xp18* cDNA was hybridized with 20 µg of total RNA from flounder brain, liver, spleen, intestine, ovary, testis and muscle. Films were exposed for 14 days at −80°C to X-ray films. To illustrate the uniformity of RNA loading, a picture of the ethidium bromide stained gel showing 18S and 28S ribosomal RNAs is shown below the Northern blot.
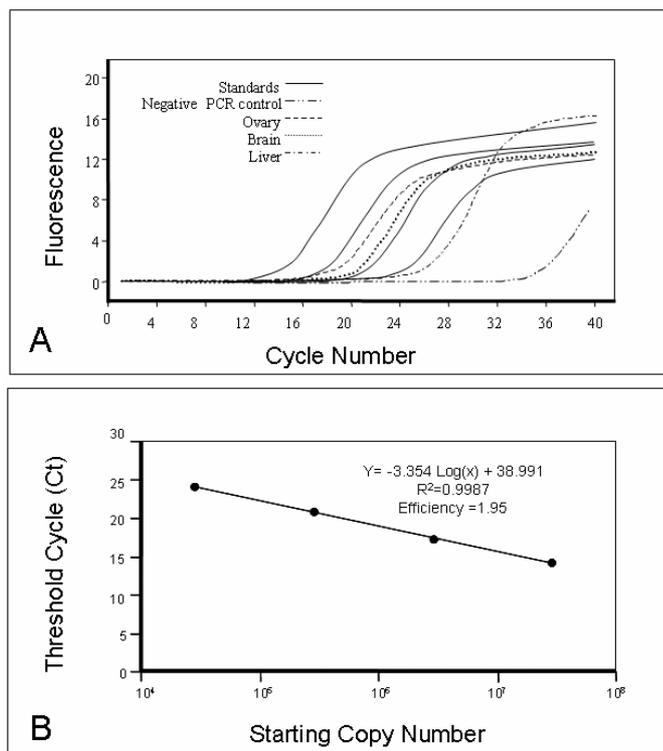
Fig. 4. Real time RT-PCR quantification of *Xp18* and *β-actin* mRNA levels. Quantitative RT-PCR was performed with appropriate primers using LightCycler – FastStart DNA Master SYBR Green I dye (Roche Molecular Biochemicals). (A) Representative amplification plot of standards and samples; and (B) deduced standard curve for *Xp18*. Ct represents the threshold cycle at which fluorescence is first detected above background. A primer dimer signal was detected in the PCR negative control.
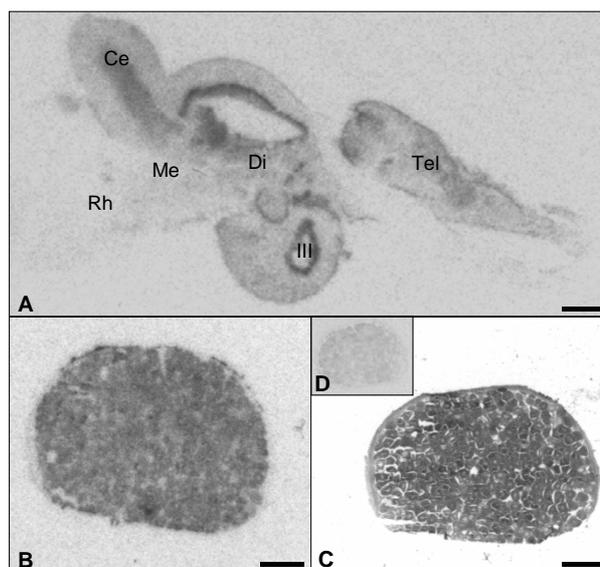
Fig. 5. Expression of *Xp18* gene in the fish brain and ovary. (A) Parasagittal section of the fish brain illustrating the localization of *Xp18* gene expression (Tel, telencephalon; Di: diencephalon; Me, mesencephalon; Ce, cerebellum; Rh, rhombencephalon; III, third ventricle). (B) Autoradiogram of a sagittal section through the ovary of a sexually mature fish labelled by in situ hybridization. The signal was restricted to the oocytes and the nuclei did not appear to be labelled. (C) Section of a fish ovary showing the histological organization of the organ. (D) Autoradiogram of a sagittal control section through the fish ovary hybridized with a sense probe to *Xp18*. Bars represent 1 mm.

In situ hybridization (Fig. 5) revealed that the *Xp18* gene was expressed in discrete regions of the flounder brain. A high hybridization signal was visualized in the circumventricular areas of the fish brain especially in the diencephalon around the third ventricle and in the mesencephalon around the tectal ventricle. In addition, a medium labeling was observed in the palial areas of the telencephalon. In the metencephalon, *Xp18* mRNAs were also detected in the most internal layers of the cerebellum. In gonads, a strong hybridization signal was evenly distributed throughout the ovary whereas only a very weak staining was detected in the testis. Since in sexually mature flounders, the ovary is almost exclusively composed of oocytes, the *Xp18* mRNA probe probably labeled germinal cells.

Data from ESTs suggested that Xp18 was expressed in oocytes of *Xenopus laevis* (Clifton *et al.*, 2001) and in whole embryo of *Oryzias latipes* (Kohara *et al.*, 2001) or fetal pancreas of human (Melton *et al.*, 2002) as well as in several tissues of adults, such as the pancreatic islet in human (Melton *et al.*, 2002), the brain of rodents (Bonaldo *et al.*, 1996, Sasaki *et al.*, 1998) and *Brachydanio rerio* (Clark *et al.*, 2002), and the lymphoid organs of *Bos taurus* (Smith *et al.*, 2000) and *Gallus gallus* (Tirunagaru *et al.*, 2000). All these tissues are characterized by an important protein synthesis and transport associated to endocrine activity and/or cell proliferation.

3.5. Conclusion

Our data indicate that the Xp18 protein identified in flounder has a particularly high level of expression in the ovary. Structural prediction analysis suggests that it may correspond to an integral protein with four transmembrane domains possibly located in the ER

membrane. The biological role of this protein remains to be established. However, the marked expression observed in the ovary supports the view that Xp18 may be involved in specific biological pathways associated with reproduction in females. In fact, cytogenetic studies have defined the X human chromosome long arm region Xq13.3-q26, where the *Xp18* gene is located, as a critical region for normal ovarian function (Therman *et al.*, 1990). Among the syndromes which have been mapped to this region of the X chromosome, premature ovarian failure (POF) is a defect of ovarian development characterized by primary or secondary amenorrhea with elevated levels of serum gonadotropins or by early menopause. POF candidate genes seem to be involved in normal ovary development and/or oogenesis (Bione *et al.*, 1998). It would thus be of interest to investigate the existence of mutations and/or chromosomal abnormalities within the *Xp18* gene in patients suffering from syndromes associated to ovarian malfunction. In the flounder, *in situ* hybridization studies conducted in intersexed male specimens may provide valuable information concerning the molecular mechanisms underlying the de-differentiation phenomenon that leads to the formation of ovarian structures in the testis.

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