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Original Article

Expression of GTP-binding protein gene *drg* during *Xenopus laevis* development

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ABSTRACT To study the genes which may play a role in the development of the vertebrate central nervous system (CNS) using a subtraction cloning approach, we previously identified a set of novel genes which are predominantly expressed in the mouse embryonic CNS and down-regulated during development. One of these genes, *drg,* encodes a novel 41 kilodalton GTP-binding protein (DRG), which is highly expressed in the embryonic CNS and shows remarkable evolutionary conservation. To study the biological role of this protein during *Xenopus* embryonic development, we cloned the *Xenopus drg* cDNA *(Xdrg)*. The predicted *Xenopus* DRG protein (XDRG) is more than 95% identical to the mouse DRG. Analysis of *Xdrg* expression by Northern blots, whole-mount *in situ* hybridization and RNA-PCR revealed the presence of varying levels of transcript for this gene in embryos and adult tissues. Among the three mRNA species detected by Northern hybridization, two smaller ones show temporally regulated expression patterns during embryonic development.

KEY WORDS: *drg, GTP-binding protein, embryonic expression, central nervous system*

Introduction

Embryonic development of vertebrates involves an intricate program of gene expression that leads to the establishment of the body plan and organogenesis in a predetermined complex pattern. The development of the vertebrate central nervous system (CNS) starts with the formation of a neural plate from cells derived from the ectoderm. This neural plate invaginates and then folds and fuses to form the neural tube. The neural tube develops and differentiates to form the brain and spinal cord which consist of a multitude of neural and glial cells of various types (for reviews on CNS development see Crelin, 1974; and Jacobson, 1978).

Despite recent advances in vertebrate CNS research, the molecular events which bring about lineage determination and differentiation of CNS precursor cells into neurons and glial cell populations and eventually the enormously complex structure of the brain remain largely obscure (reviewed in McKay, 1989; McKay *et al.*, 1990). It is likely that the temporal and spatial patterns of vertebrate brain are achieved by the sequential expression of a complex array of genes, as has been documented for *Drosophila* (reviewed in Akam, 1987; Scott and Carroll, 1987; Gehring, 1992). A number of the genes studied in the vertebrate CNS have been isolated based on their structural and functional homology to genes known to play roles in *Drosophila* neurogenesis, such as those encoding homeobox-containing and helix-loop-helix-containing transcription factors (reviewed in Kessel and Gruss, 1990; Vaessin *et al.*, 1990). Isolation and characterization of the genes which are expressed during the development of CNS is an important step towards the basic understanding of the process of neural development and differentiation.

We are especially interested in identifying genes that control the early development and differentiation of mouse CNS precursor cells, and to this end we have tried to isolate cDNA clones for mRNA species which are predominantly expressed during the early stages of CNS development. This was facilitated by using a cDNA library that was prepared from CNS precursor cells and subtracted with RNA from postnatal and adult brain (Kumar *et al.*, 1992). Using this strategy, ten independent and novel cDNA clones were isolated which are expressed at much higher levels in the developing and differentiating brain than in the fully differentiated adult CNS, and therefore are likely to play roles in early neurogenesis. Further cloning and characterization of one of the cDNAs revealed that it encodes a 41 kD novel GTP-binding protein which we named DRG (gene= *drg*) (Sazuka *et al.*, 1992b). The DRG aminoacid sequence shows remarkable evolutionary conservation (Sazuka *et al.*, 1992b). *drg* mRNA is expressed in a number of mouse embryonic tissues other than the CNS and its expression is dramatically restricted in post-natal and adult animals (Sazuka *et al.*, 1992a). The interesting structure, expression pattern and high evolutionary conservation of DRG points to its role in some essential cellular function. However, our attempts to study the biology of DRG in cells transfected with *drg* cDNA expression vectors have been unsuccessful (Sazuka *et al.*, 1992a). With the view of using the well established frog system

Abbreviations used in this paper: CNS, central nervous system.

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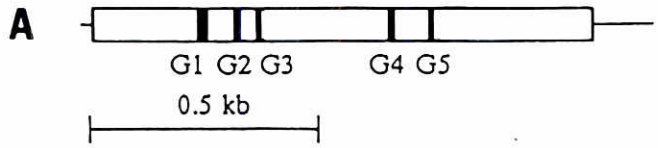

B

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1    GC GTC TTT TGC TGT GAA GCG ACC TAC ACC GGA GGA ACA ATG AGC GGG ACA CTG GCT AGG
1                                                  met ser gly thr leu ala arg

60  ATT GCC GAG ATA GAG GCC GAG ATG GCC CGG ACA CAG AAA AAT AAA GCA ACA GCC TAT CAC
 8  ile ala glu ile glu ala glu met ala arg thr gln lys asn lys ala thr ala tyr his

120 CTG GGT TTG CTG AAA GCT CGC CTT GCC AAG CTG AGA CGT GAG CTC ATT ACT CCA AAA GGA
28  leu gly leu leu lys ala arg leu ala lys leu arg arg glu leu ile thr pro lys gly

180 GGT GGC GGA GGA GGA CCC GGA GAA GGT TTT GAT GTG GCA AAG ACC GGA GAT GCT CGA ATT
48  gly gly gly gly gly pro gly glu gly phe asp val ala lys thr gly asp ala arg ile

240 GGG TTT GTT GGG TTT CCA TCT GTG GGG AAA TCT ACA CTT CTC AGT AAC TTG GCT GGA GTT
68  gly phe val gly phe pro ser val gly lys ser thr leu leu ser asn leu ala gly val
                 G1

300 TAT TCT GAA GTG GCA GCC TAT GAA TTT ACC ACC TTG ACA ACC GTT CCT GGT GTG GTG CGA
88  tyr ser glu val ala ala tyr glu phe thr thr leu thr thr val pro gly val val arg
                           G2

360 TAT AAA GGA GCA AAA ATT CAG TTG CTA GAT CTT CCC GGT ATC ATA GAG GGA GCA AAG GAT
108 tyr lys gly ala lys ile gln leu leu asp leu pro gly ile ile glu gly ala lys asp
                                    G3

420 GGA AAG GGG CGT GGT CGC CAG GTC ATT GCA GTT GCT CGA ACA TGT AAC TTG ATC CTT ATT
128 gly lys gly arg gly arg gln val ile ala val ala arg thr cys asn leu ile leu ile

480 GTT CTG GAT GTA CTG AAA CCA CTC GGA CAC AAG AAG ATC ATA GAG AAT GAA CTG GAG GGA
148 val leu asp val leu lys pro leu gly his lys lys ile ile glu asn glu leu glu gly

540 TTT GGA ATC AGA TTG AAC AAG CAG CCT CCA AAC ATT GGA TTC AAG AAG AAA GAC AAG GGC
168 phe gly ile arg leu asn lys gln pro pro asn ile gly phe lys lys lys asp lys gly

600 GGC ATA AAC CTC ACC GCA ACA TGT GCT CAG AGT GAG CTT GAT AAT GAT ACA GTA AAG AGC
188 gly ile asn leu thr ala thr cys ala gln ser glu leu asp asn asp thr val lys ser

660 ATC CTG GCT GAG TAT AAG ATC CAC AAT GCA GAT ATC ACA CTG CGC AGT GAT GCC ACT GCT
208 ile leu ala glu tyr lys ile his asn ala asp ile thr leu arg ser asp ala thr ala

720 GAT GAC CTG ATA GAT GTA GTA GAG GGT AAT AGG GTT TAT ATT CCT TGC ATC TAT GTG CTG
228 asp asp leu ile asp val val glu gly asn arg val tyr ile pro cys ile tyr val leu

780 AAC AAG ATT GAC CAA ATT TCA ATT GAA GAA CTG GAT ATA ATT TAC AAA GTC CCA CAC TGT
248 asn lys ile asp gln ile ser ile glu glu leu asp ile ile tyr lys val pro his cys
      G4

840 GTG CCT ATC TCT GCC CAT CAC CGC TGG AAT TTT GAC GAC CTT CTT GAG AAG ATC TGG GAT
268 val pro ile ser ala his his arg trp asn phe asp asp leu leu glu lys ile trp asp
                  G5

900 TAC CTT CAA CTT GTG CGA ATT TAC ACC AAA CCG AAG GGT CAG TTG CCT GAT TAT ACA TCA
288 tyr leu gln leu val arg ile tyr thr lys pro lys gly gln leu pro asp tyr thr ser

960 CCT GTA GTG TTG CCG TGT TCT CAT ACT GCA GCA GAA GAC TTT TGC ACA AAG ATC CAT AAG
308 pro val val leu pro cys ser his thr ala ala glu asp phe cys thr lys ile his lys

1020 AAC CTT ATC AAA GAG TTT AAA TAT GCC CTG GTT TGG GGA TCA TCT GTG AAA CAC AAC CCT
328 asn leu ile lys glu phe lys tyr ala leu val trp gly ser ser val lys his asn pro

1080 CAA AAG GTT GGG AAA GAT CAT GTG TTG GAA GAT GAG GAT GTC ATT CAA ATT GTT AAG AAA
348 gln lys val gly lys asp his val leu glu asp glu asp val ile gln ile val lys lys

1140 TAA TTC ACC ATA TAC ATC TGC AAT TGG GCA TCA GTG CAG CAT CTG TCC ATT AAA CAT GTG
     ***

1200 CAT ATT TCA CAG GCA GCA TGC CAA TAA AGC AGC ACG TTT AAT TTT CAG CTG AAA AAA AAA

1260 AAA AAA AAA A
```

Fig. 1. Nucleotide and predicted amino acid sequences of *Xdrg* cDNA. **(A)** *The structure of Xdrg cDNA. The coding region of the cDNA is shown as a box while the GTP-interacting domains of the encoded protein, G1-G5, are shown as dark boxes.* **(B)** *Sequence was obtained from ExoIII nested deletions of the cloned cDNA. The polyadenylation signal is underlined and the GTP-interacting domains, G1-G5, are boxed. The two possible N-glycosylation sites are double underlined.*

to study the role of the *drg* gene during development, in the present study we describe cloning and expression analyses of *Xenopus drg (Xdrg)*.

Results

Cloning and sequence analysis of Xdrg cDNA

To clone the cDNA for the *Xenopus* homologue of mouse *drg* cDNA *(Xdrg)*, we utilized the entire coding region of mouse *drg* cDNA (Sazuka *et al.*, 1992b) as a probe. A directional cDNA library was prepared from a mixture of poly A^+ RNA isolated from *Xenopus* embryonic stages 10, 20 and 30 in λ Uni-ZAP vector. Initial experiments indicated that the mouse probe does not hybridize to *Xenopus* RNA or cDNA clones under stringent hybridization conditions (data not shown). Therefore in further experiments, approximately 300,000 plaques were screened using the mouse probe, under reduced stringency (50°C, 1 M NaCl). After three cycles of screening, three positive plaques were obtained. Analysis of plasmid DNA rescued from these clones indicated that all three clones contained an identical cDNA insert of 1.3 kb. These cDNA inserts were sequenced from the ExoIII generated deletions, and the complete nucleotide sequence of 1269 bp of *Xdrg* cDNA is shown in Fig. 1. The sequence contains a single open reading frame, open at its 5' end and terminating at nucleotide 1140. The 3' tail of the cDNA contains a 19 nucleotide long poly A stretch preceded by an upstream polyadenylation signal, AATAAA. The *Xdrg* nucleotide sequence is 78.8% identical to the mouse *drg* cDNA, the homologous region confined to the coding portion of the sequence. The reading frame of *Xdrg*, starting at the first methionine at nucleotide 39 which is in excellent context for translation initiation (Kozak, 1991), can encode a 41 kD protein of 367 amino acid residues which is identical in size to the mouse DRG protein (Sazuka *et al.*, 1992b). A second in-frame methionine located 39 nucleotides further downstream from the first methionine is also in a good context for translation initiation. Translation of *in vitro* generated RNA from the cloned cDNA using rabbit reticulocyte lysates resulted in an expected size product of 41 kD (data not shown).

Evolutionary conservation of DRG protein

Alignment of XDRG protein with mouse DRG indicated that the two proteins are 95.3% identical (Fig. 2). The GTP interacting domains, G1-G5 (Bourne *et al.*, 1991) of the two proteins are completely conserved. Further alignment of these sequences with the known DRG homologues or DRG-like proteins from various organisms indicated a remarkable degree of evolutionary conservation of this protein (Fig. 2). XDRG is approximately 80%, 54% and 40% identical to the *Drosophila* (unpublished data derived from DNA sequence database entry numbers X58826, S55381 and Petersen, Sommer and Bautz, personal communication), *Schizosaccharomyces pombe* (Hudson and Young, 1993), and *Halobacterium* (Shimmin and Dennis, 1989) putative proteins, respectively. The alignment shown in Fig. 2 also indicates that the *Xdrg* cDNA clone contains the complete coding region.

Expression of Xdrg mRNA

The expression of *Xdrg* mRNA was analyzed by Northern blot analysis of RNA isolated from total embryos at various stages of development (Fig. 3). Using poly A^+ RNA, two major transcripts of 1.8 and 1.3 kb and a less abundant one of 2.8 kb were detected in embryos at stages 12-45 (Fig. 3A). At stage 1, clear signals for the 1.8 kb transcript and much weaker signal for the 1.3 kb species were visible in longer exposures and by more sensitive analysis using a Bas 2000 image analyzer (data not shown, and Fig. 3B). From autoradiographic analysis of Northern blots it is apparent that until blastula (stage 8), the 1.8 kb transcript is the most prominent species, while in the later stages, the expression of the 1.3 kb transcript becomes stronger (Fig. 3A). The EF-1α and *c-src* controls show expected patterns of expression, i.e., EF-1α mRNA rapidly accumulates from the midblastula transition (Krieg *et al.*, 1989),

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Mouse       MSGTLAKIAEIEAEMARTQKNKATAHHLGLLKARLAKLRRELITPKGGGG 50
Xenopus     ------R-------------------Y----------------------- 50
Drosophila  -ITI-EK-SA--S-----------SA-------NV-------LS------ 50
Yeast        M-V-E--Q------R---------EY------GK-----AQLLE- TSKS 48
Halobact.    M-LEED-ESL-E-I-N-PY--S-EA-I-R---K--EQKEK-EAQQS-S- 49

                                 G1                  G2
Mouse       GGPGEGFDVAKTGDARIGFVGFPSVGKSTLLSNLAGVYSEVAAYEFTTLT 100
Xenopus     -------------------------------------------------- 100
Drosophila  -TGEA--E--------V--------------------------------- 100
Yeast       -PK------L-S----VA-I-------------SAITKTK-AT-S------ 98
Halobact.   -  -G-YA-EQH---TVAL---------S-INAMTNAD---G-------N 97

                           G3
Mouse       TVPGVIRYKGAKIQLLDLPGIIEGAKDGKGRGRQVIAVARTCNLILIVLD 150
Xenopus     -----V-------------------------------------------- 150
Drosophila  ----CIK-----------------------------------FM--- 150
Yeast       AI---LE-D--E--M----------SQ-R- ---AVSA---AD---M-FG 147
Halobact.   VN--MLE-R--N-----V--L----AG-R-G-KEILS-I-GAD-VIF--S 147

Mouse       VLKPLGHKKIIENELEGFGIRLNSKPPNIGFKKKDKGGINLTATCPQSEL 200
Xenopus     ------------------------KQ---------------------A---- 200
Drosophila  C--------LL-H----------KK----YY-R--------NSMV----- 200
Yeast       CY-K-PSE-K--Y---QV-----RQ---VTLTI-KN---KFNH-V-LTHM 198
                 [I]
Halobact.   AFEIEQYDRLA- --YNVN--VDAE--SVTVRR-G-D--DVN -SGEL-- 195

                                                         G4
Mouse       DAETVKSILAEYKIHNADVTLRSDATADDLIDVVEGNRVYIPCIYVLNKI 250
Xenopus     -ND---------------I------------------------------- 250
Drosophila  -TDL--T--S--------I---Y---S------I----I------L---- 250
Yeast       -YKMAYN--H--R-----ILI-E-I-V--F--L-M---R--N-L-CYS-- 248
Halobact.   -SD---G--R-RGFI--N--I-GNPSV-R---G-MD----M-SLVTV--V 245

                              G5
Mouse       DQISIEELDIIYKVPHCVPISAHHRWNFDDLLEKIWDYLKLVRIYTKPKG 300
Xenopus     -------------------------------------------------- 300
Drosophila  ---------V-----------------------LM-E--R-Q-------- 300
Yeast       -AV-L--V-RLARL-KS-V--CNMKL-L-F-K-R--EE-N-Y-----R-- 298
Halobact.   -L-EPSYAGTMVSPDDAIF---AEEKGL-V-K-RM-RA-G-I---MDKP- 303
                      [KDALRDHG]

Mouse       QLPDYTSPVVLPYSRTTVEDFCMKIHKNLIKEFKYALVWGLSVKHNPQKV 350
Xenopus     ------------C-H-AA-----------------------S--------- 350
Drosophila  -----N-----HNER-SI----N-L-RSIA---------CS----Q---- 350
Yeast       EM--FSEALII HKGS-I-QV-NR--RT-AEQL-------T-A--S--V- 347
Halobact.   RGV-REE-LIIRRGE- -D-AVQ-LGGT-DER-RF-R-T-P-AQ-DD-Q- 352

Mouse       GKDHTLEDEDVIQIVKK  367
Xenopus     ----V------------  367
Drosophila  -IE-V-N----V-----V 368
Yeast       -LN-RV-EG--VT--T-  364
Halobact.   -R--V------LRV-ARR 370
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Fig. 2. Alignment of DRG protein sequences from various organisms. *The sources of various sequences are as described in the text and in Sazuka et al. (1992b). The sequences identical between mouse and other organisms are indicated by a dash (–). The locations of the putative GTP-interacting domains, G1-G5, are also indicated.*

while *c-src* expression remains relatively invariable during early development (Smith and Harland, 1992). Further densitometric analysis of the signals using *Xenopus c-src* expression as a control for RNA quantity (Smith and Harland, 1992) revealed that the expression of the 1.3 kb mRNA species is up-regulated from stage 1 to around stage 32 followed by down-regulation at stage 45 (Fig. 3B). The 1.8 kb transcript, on the other hand, peaks around stage 12 (Fig. 3B), while the expression of the largest transcript (2.8kb) remains largely invariable between stages 12 and 45 (data not shown). The detection of three transcripts hybridizing to the *Xdrg* probe is different from the results with mouse, where a single transcript of 1.6 kb is detected in all embryonic and adult tissues (Sazuka *et al.*, 1992a,b). The *Xdrg* cDNA isolated and cloned in the present study (Fig. 1) probably represents the 1.3 kb transcript detected in the Northern blot analysis (Fig. 3A).

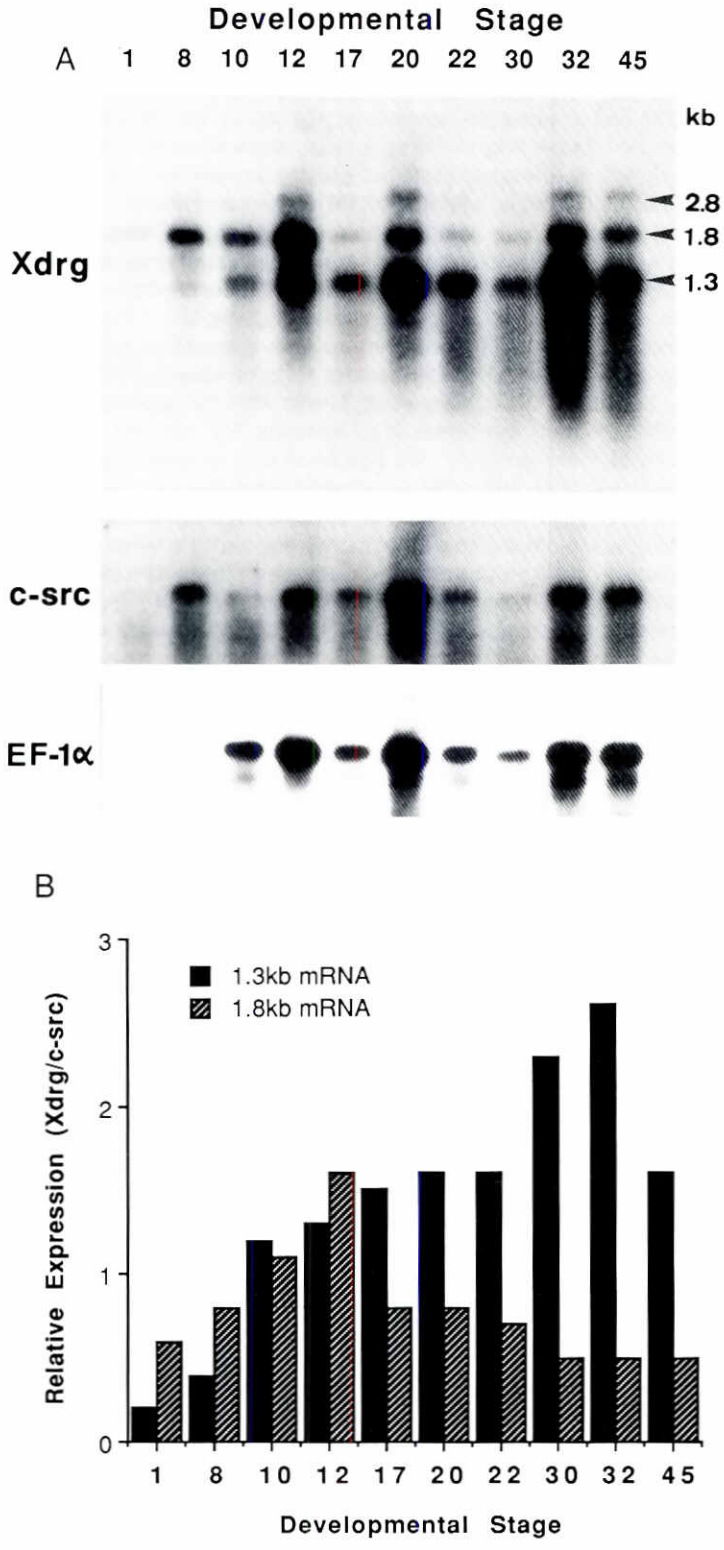
Xdrg expression was further analyzed by whole-mount *in situ* hybridization to stage 10, 17, 22 and 32 embryos (Fig. 4). Using this technique, no signals were evident in stage 10 embryos (data not shown), although clear-cut expression was obvious in stage 17, 22 and 32 embryos. In stage 17 embryos (neurula), *Xdrg* expression can be seen along the neural folds (Fig. 4A). This expression is particularly prominent towards the anterior end of the embryo which gives rise to the brain (Fig. 4B). At stage 22, when the brain has segmented into fore-, mid- and hindbrain regions, the expression can be seen in the brain, developing eye and all along the neural tube (Fig. 4C). In stage 32 embryos, the expression can be seen in the various regions of brain, eye, otic vesicle, branchial arches, somites and all along the neural tube (Fig. 4D,E). Control embryos hybridized with the *Xdrg* sense orientation probe showed no detectable signals at any stage (data not shown). In contrast, the stage 22 embryos hybridized with the N-CAM (Kintner and Melton, 1987) antisense probe as a positive control show strong CNS-specific signals (Fig. 4F). The expression of *Xdrg* in dissected embryos was further analyzed by RNA-PCR to confirm the results of whole-mount *in situ* analysis. Total RNA isolated from various regions of the stage 22 and 32 embryos (Fig. 5A), were reverse transcribed and then amplified using a set of PCR primers for *Xdrg*, *Xenopus c-src* (Steele *et al.*, 1989) and EF-1α (Krieg *et al.*, 1989). After 35 cycles of PCR, *Xdrg* PCR products were seen in all samples (Fig. 5B) and, as no differences were apparent, we assumed that amplification had reached saturation. In further experiments we therefore carried out PCR amplification for 15 cycles, electrophoresed the samples on agarose gels, blotted onto a nylon membrane and hybridized to the [^{32}P]-labeled cDNA probes. Strongest signals were seen for the RNA derived from the head regions followed by the dorsal regions of embryos, at both stages 22 and 32 (Fig. 5C). Taken together, the results of whole-mount *in situ* and RNA PCR analyses reveal that although *Xdrg* mRNA is present in all parts of embryonic body, it is most prominent in the anterior region consisting of brain and other head structures.

Varying levels of *drg* mRNA are present in various adult mouse tissues (Sazuka *et al.*, 1992a). To check if this was also true for *Xenopus*, we analyzed poly A$^+$ RNA isolated from various adult animal tissues by Northern blot analysis (Fig. 6). Strongest signals for the 1.3 kb transcript were seen in ovaries and testes, followed by skeletal muscle, stomach, brain, kidney and liver (Fig. 6). In longer exposures to X-ray films, weak 1.3 kb band was also visible in the RNA isolated from heart (not shown here), which is consistent with our earlier observations with mouse *drg* (Sazuka *et al.*, 1992a). Longer exposures of the blot, shown in Fig. 6, indicated that all three *Xdrg* transcripts were present in the adult brain while all other tissues expressed only the 1.3 and 1.8 kb mRNA. This suggests that the 2.8 kb mRNA species may be specific for the CNS.

Ectopic expression of Xdrg mRNA in embryos

Microinjection of *in vitro* generated mRNA from cloned genes into *Xenopus* embryos has been widely used as an assay system to check biological activities of several genes (reviewed in Vize *et al.*, 1991). To check if ectopic expression of *Xdrg* mRNA can induce developmental abnormalities in embryos, *in vitro* generated mRNA from the full length *Xdrg* cDNA clone was injected into 4-cell stage embryos. As a positive control, we used mRNA synthesized from a carboxyl-terminal truncated version of the *Xenopus* activin receptor (Nishimatsu *et al.*, 1992). The injection of this mRNA results in the formation of a duplicated body axis (Nishimatsu *et al.*, 1992). In several experiments injection of up to 10 ng of mRNA per embryo of a negative control, *Xdrg* sense or *Xdrg* antisense RNA, respectively, failed to induce any clear-cut changes associated with *Xdrg*

ectopic expression over the control RNA (data not shown). Further histological analyses of the representative samples also failed to reveal any specific developmental abnormalities (data not shown). Under similar conditions, injection of mRNA produced from the carboxyl-terminal truncated form of activin receptor caused duplication of body axis in about 15% of the injected embryos (data not shown).



Discussion

In the study described here we have cloned the *Xenopus* homologue of mouse *drg* cDNA and examined its expression in developing *Xenopus* embryos. We have also shown that the encoded DRG protein is remarkably conserved between the two species. The presence of three different species of mRNA hybridizing to the *Xdrg* probe is interesting. The developmentally regulated expression of the smaller species of 1.3 and 1.8 kb suggests that DRG may play some role during development. These three mRNA may either represent closely related transcripts or alternately spliced forms of mRNA originating from a single gene. By comparison with DRG, or DRG-like proteins from other species, it is clear that the *Xdrg* cDNA cloned here contains the complete protein coding region and is the authentic *Xenopus* counterpart. It would be interesting to study the 1.8 and 2.8kb mRNA hybridizing with the *Xdrg* cDNA probes to determine their structures and coding regions. Our attempts so far have, however, resulted only in the isolation of 1.3 kb cDNA.

The results of whole-mount *in situ* hybridization indicate that *Xdrg* transcripts are most abundant in the anterior region of the embryo, followed by the dorsal and posterior regions. The more sensitive RNA PCR technique, however, detected expression in all parts of the dissected embryos. This expression pattern is consistent with results with mouse *drg*, which is expressed in all embryonic tissues at varying levels but is strongest in the CNS (Sazuka *et al.*, 1992a). The signals detected in *Xenopus* embryos most likely represent the cumulative expression of the three transcripts detected in the Northern analysis using the same full length *Xdrg* cDNA probe.

Overexpression of genes by means of injecting *in vitro* generated mRNA into frog embryos has been successfully used for functional analyses of several genes including *Xhox-1A* (Harvey and Melton, 1988), *Xhox3* (Ruiz i Altaba and Melton, 1989), *int-1* (McMahon and Moon, 1989), N-CAM (Kintner, 1988), N-cadherin (Detrick *et al.*, 1990) and others (reviewed in Vize *et al.*, 1991). However, using the *Xenopus* system, several other genes such as *X1H box2, Xhox36, En-2, MyoD*, vimentin, Vg-1, *raf* and N-*myc* fail to show any developmental effects (reviewed in Vize *et al.*, 1991). Likewise, ectopic expression of *Xdrg* mRNA in 4-cell embryos did not cause any developmental defects. The reasons for this are not yet clear. Use of dominant negative mutations of the XDRG protein may show biological effects in embryos, although no such mutations have as yet been obtained.

Fig. 3. Expression of *Xdrg* mRNA in *Xenopus* embryos. (A) *Northern blot analysis. Each lane contained 2-5 μg of poly A⁺ selected RNA isolated from the total embryos at the indicated stage of development. The same blot was sequentially hybridized to Xdrg, c-src and EF-1α probes. The positions and sizes of the three transcripts hybridizing to the Xdrg probe are indicated by arrowheads. The signals shown here were obtained after approximately 48 h (Xdrg), 24 h (c-src) and 3 h (EF-1α) exposures to the X-ray films. Longer exposures (not shown here) reveal the presence of Xdrg and c-src hybridizing bands in stage 1 and EF-1α band in stage 8 lanes also.* **(B)** *Quantitative analysis of Xdrg expression in embryos. Hybridized filters were exposed to Bas 2000 image analyzer (Fuji) plates for 6-10 h and signals quantitated using the software supplied by the manufacturer. The bars represent Xdrg 1.3 kb and 1.8 kb mRNA expression normalized against c-src expression.*

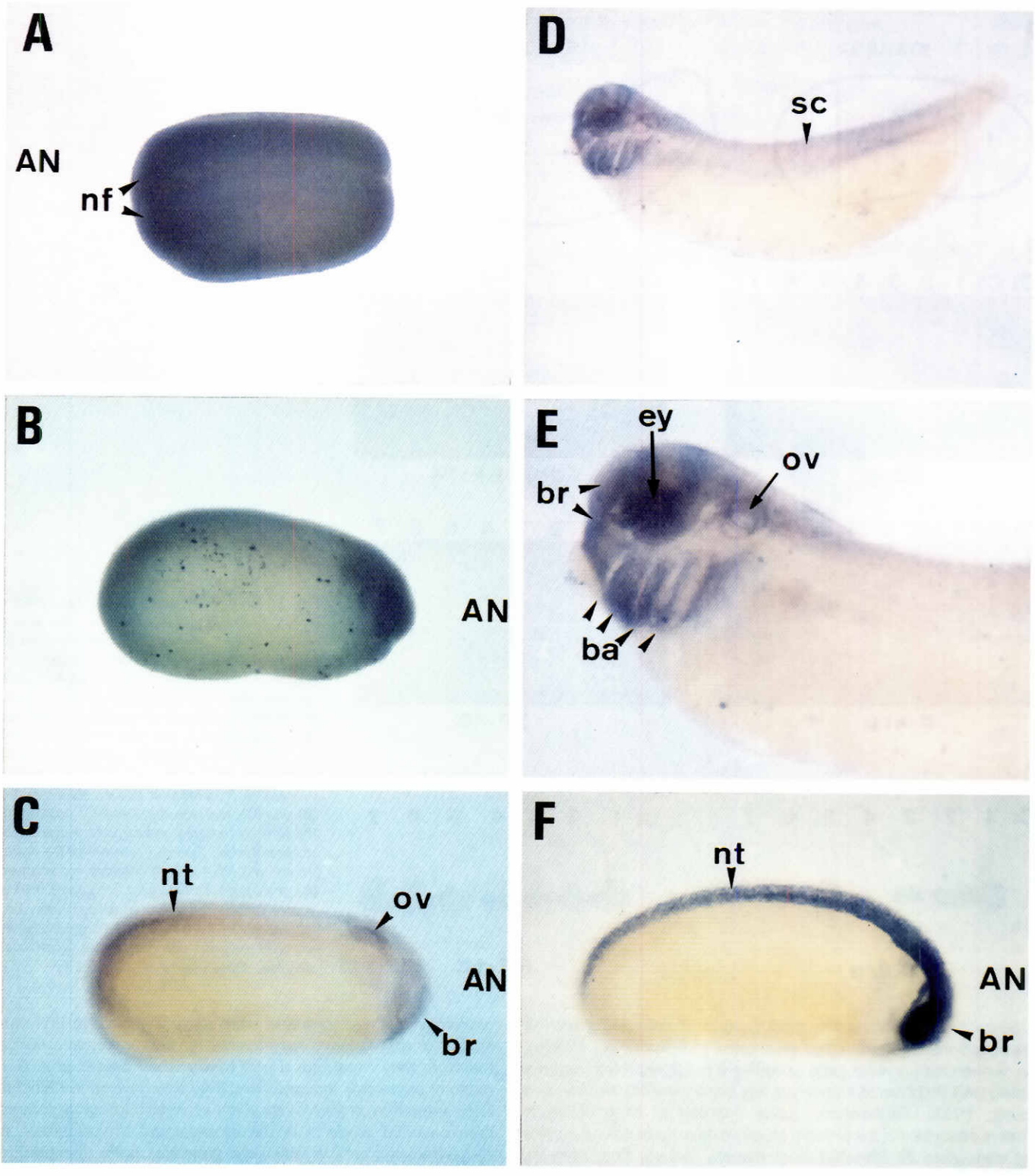


Fig. 4. Whole-mount *in situ* hybridization analysis of *Xdrg* mRNA distribution in *Xenopus* embryos. *Embryos in A through E were hybridized with an Xdrg antisense probe. No hybridization to a control sense probe was detected under similar conditions (data not shown).* **(A)** *Dorsal and* **(B)** *lateral views of the stage 17 (neurula) embryos showing expression in neural folds (nf).* **(C)** *A stage 22 embryo showing expression in developing brain (br), otic vesicle (ov) and the neural tube (nt). Note the strong signals in the anterior and posterior regions of the CNS.* **(D,E)** *A stage 32 embryo showing expression in various head structures (brain, br; eye, ey; otic vesicle, ov), branchial arches-like structures (ba) and spinal cord. As a positive control, in* **(F)**, *a stage 22 embryo was hybridized to an antisense probe derived from the Xenopus N-CAM cDNA (Kintner and Melton, 1987), under conditions similar to those used for the Xdrg probe.*

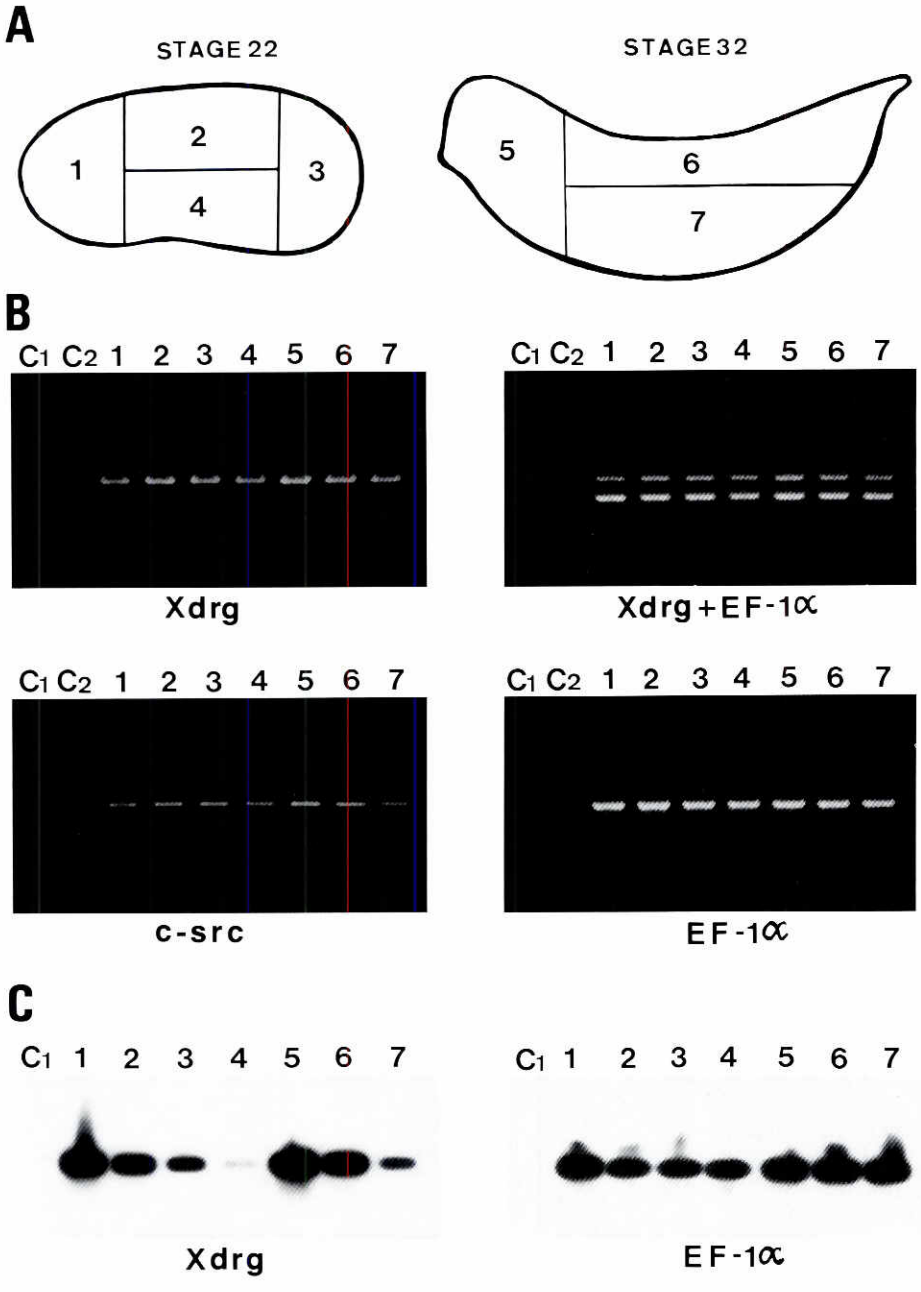


Fig. 5. RNA PCR analysis of dissected embryos. *Stage 22 and 32 embryos were dissected in various sections as shown in* **(A)**. *One μg of total RNA isolated from these dissected embryos was reverse transcribed and subjected to PCR for 35 cycles* **(B)**, *or 15 cycles* **(C)**, *using a set of gene-specific primers as shown underneath each panel.* **(B)** *shows ethidium bromide stained gels, while in* **(C)**, *after electrophoresis, samples were transferred to nylon membranes and hybridized to the respective [^{32}P]-labeled probes as indicated. In* **(B)** *and* **(C)**, *the lane numbers 1-7 correspond to the different stages and regions of the embryos as shown in* **(A)**. *C_1 and C_2 are reverse transcriptase minus and RNA minus controls respectively. In* **(C)**, *the exposure times for Xdrg and EF-1α were approximately 6 h and 2 h, respectively. Longer exposures of Xdrg hybridized membrane in* **(C)** *showed the presence of positive signal in lane 4 also (not shown here).*

Since first describing the identification of mouse *drg* and its possible homologues in other organisms (Sazuka *et al.*, 1992b), the sequence of a yeast gene encoding a putative protein approximately 54% identical to mouse *drg* has been reported (Hudson and Young, 1993). Furthermore, using the pair of oligonucleotide primers described in the present study we have been able to amplify *drg* transcripts by RNA-PCR from human, mouse, frog, chicken, insect and yeast cells (S. Kumar, unpublished observations). It is therefore reasonable to assume that all eukaryotes express *drg* homologues. The growing list of *drg*-like genes in different organisms from mammals to archaebacteria, and their remarkable evolutionary conservation, point to an essential role for their encoded products. So far, however, no evidence of the physiological function for the members of this protein family has emerged. The homology of these proteins with other GTP-binding proteins is limited to the domains responsible for interaction with GTP and therefore they constitute a new family. Their presence in a wide range of organisms indicates that they may have varied functions. The information on the biological role of these interesting genes and their encoded products in the development of vertebrates may come from mutants in which the gene has been disrupted, and efforts are underway along these lines.

Materials and Methods

Eggs and embryos

Eggs of *Xenopus laevis* were obtained by injecting the animals with human chorion gonadotropin (Gestron, Denka Seiyaku Co., Japan). After

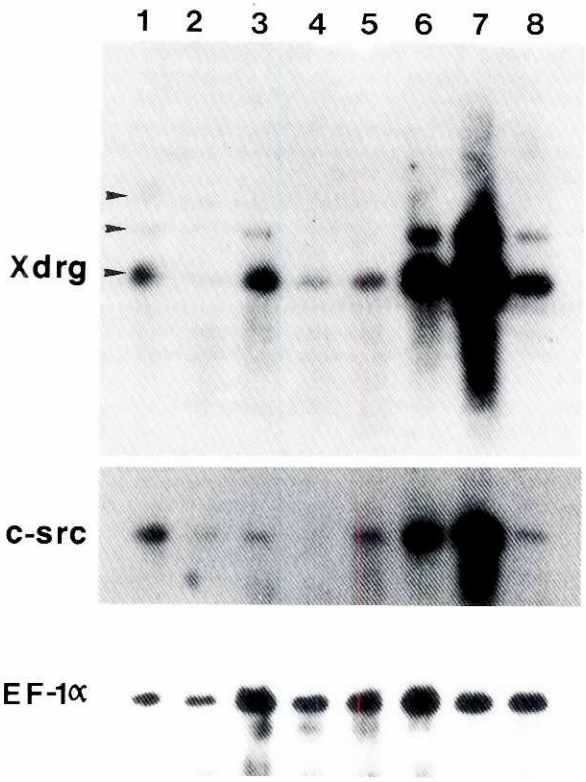


Fig. 6. Expression of *Xdrg* in adult tissues. *Approximately 5 μg of poly A⁺ RNA isolated from various adult tissues was electrophoresed on agarose/formaldehyde gel, transferred to nylon membrane and sequentially hybridized to Xdrg, c-src and EF-1α probes respectively. The exposure times for Xdrg, c-src, and EF-1α were approximately 50 h, 24 h and 4 h, respectively. In longer exposures of Xdrg hybridized membrane, a faint 1.3 kb signal in lane 2, 1.8 kb signals in all except lane 2, and a 2.8 kb signal in lane 1, were visible (not shown here). The positions of 2.8, 1.8 and 1.3 kb transcripts (from top to bottom) are indicated by arrowheads. Lanes 1-8 contain RNA from brain, heart, stomach, liver, kidney, testis, ovary and skeletal muscle respectively.*

dejellying in 4.5% cystein hydrochloride in Steinberg's solution (pH 7.4), the fertilized eggs were transferred to culture dishes containing Steinberg's solution. Embryos were staged according to Nieuwkoop and Faber (1967).

Cloning of Xdrg cDNA

Poly A⁺ RNA was isolated from total embryos at various stages of development using a Fast Tract mRNA isolation kit according to the instructions supplied by the manufacturer (Invitrogen). Two μg of Poly A⁺ RNA each from stages 10, 20 and 30 were used to prepare a unidirectional cDNA library in λ Uni-ZAP vector (Stratagene) as described before (Kumar *et al.*, 1992). A total of approximately 600,000 primary clones were obtained. Approximately 300,000 plaques were screened under reduced stringency hybridization conditions (50°C, 1 M NaCl), using a probe derived from the entire coding region of the mouse *drg* cDNA (Sazuka *et al.*, 1992b). After three cycles of hybridization, three positive plaques containing identical cDNA inserts of approximately 1.3 kb were recovered. Plasmid DNA from these were rescued according to the instructions supplied by the manufacturer (Stratagene), and sequenced from the ExoIII-generated nested deletions using Sequenase kit (USB). The nucleotide sequence reported here will appear in the DDBJ, EMBL and GenBank databases under accession number D13865.

RNA hybridization analyses

Poly A⁺ RNA were isolated from total embryos at various stages of development by Fast Tract kit (Invitrogen), electrophoresed on 1.2% agarose, 2.2 M formaldehyde gels, transferred to nylon membranes (Biodyne, Pall) and hybridized to high specific activity probes labeled to approximately $3x10^9$ CPM/μg DNA by random priming (Sambrook *et al.*, 1989). For *Xdrg*, full length cDNA released as an Eco RI/Xho I fragment was used as a probe. For control probes, *Xenopus c-src* (Steele *et al.*, 1989) and EF-1α (Krieg *et al.*, 1989) fragments generated by RNA-PCR (see below) were used. PCR-generated products were partially sequenced according to standard protocols (Sambrook *et al.*, 1989) to check their authenticity. Quantitative analysis of hybridization signals on Northern blots, was performed by using an image analyzer (Fuji, Bas 2000). The *Xdrg* signals were normalized against *c-src* signals.

RNA-PCR

Xenopus embryos at stages 22 and 32 were dissected into various pieces as depicted in Fig. 5A. Total RNA from quick-frozen pieces of embryos were isolated using RNAzol B (Cinna/Biotecx Laboratories) according to instructions supplied by the manufacturer. One μg of total RNA was reverse transcribed with a mixture of oligo dT and random primers, and subjected to PCR using the reagents of the RNA-PCR kit (Perkin Elmer Cetus). PCR reactions were carried out for various numbers of cycles, each cycle consisting of one minute incubations each at 92°, 58° and 72°C. One tenth of each PCR reaction was electrophoresed on 1.2% agarose gel. The following gene-specific primers were used for PCR: *c-src* (sequence derived from Steele *et. al.*, 1989), forward primer (corresponding to base numbers 947-967), 5'-TTCGGCATGAGAAGCTGGTAC-3', reverse primer (corresponding to base numbers 1491-1471), 5'-GCACTGGAACATCATATCATG-3'; EF-1α (sequence derived from Krieg *et al.*, 1989), forward primer (corresponding to base numbers 232-252), 5'-ACAAACTGAAGGCCGAGCGTG-3', reverse primer (corresponding to base numbers 748-728), 5'-CAAGAGCTTCCA-GCAGGGTAG-3'; *Xdrg* (sequence as shown in Fig. 1B), forward primer (corresponding to base numbers 199-218), 5'-GGAGAAGGTTTTGATGTGGC-3', reverse primer (corresponding to base numbers 875-856), 5'-TCAAAATTCCAGCGGTGATG-3'. The expected PCR products for *c-src*, EF-1α and *Xdrg* are 545, 517 and 677 bp long, respectively. For hybridization analysis of PCR products, after electrophoresis, DNA was transferred to a nylon membrane (Biodyne, Pall) and hybridized to probes labeled with [^{32}P] dCTP by random priming according to standard protocols (Sambrook *et al.*, 1989). The probes used were as described for Northern analysis above.

Whole-mount in situ hybridization

Sense and antisense digoxiginin (DIG)-labeled single-stranded RNA probes were prepared from linearized *Xdrg* cDNA clone in pBluescript (SK-) vector, using the DIG RNA labeling kit according to the instructions provided by the manufacturer (Boehringer Manheim). For a positive control an Eco RI fragment of *Xenopus* N-CAM cDNA cloned into pBluescript (Kintner and Melton, 1987) was used. The probe size was reduced to approximately 200 bp by limited alkaline hydrolysis. Whole-mount *in situ* hybridizations were performed essentially as described (Harland, 1991). Briefly, embryos were fixed for 2 h in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$ and 3,7% formaldehyde), followed by dehydration in methanol. After rehydration, proteinase K treatment, acetylation and refixation, embryos were hybridized for 16 h at 60°C with the labeled probes, treated with RNase A and T1 and washed. For the chromogenic reaction, embryos were incubated with alkaline phosphatase conjugated anti-digoxigenin antibody (Boehringer Manheim, 1:2000 dilution), and reacted with a solution containing 340 ng/ml NBT and 175 ng/ml BCIP. After 30-90 min the reaction was stopped in MEMFA.

RNA microinjection

Xdrg cDNA in pBluescript (SK-) was linearized using either XhoI (sense) or BamHI (antisense) and RNA was synthesized in the presence of 5'-7mGpppG-3' cap analog using T3 (sense) and T7 (antisense) RNA polymerases, respectively (Krieg and Melton, 1984; Wormington, 1991). For negative control RNA preparation, pBluescript digested with XmnI was transcribed with T3 RNA polymerase. The production of carboxyl-terminal truncated activin receptor mRNA used as a positive control has been described previously (Nishimatsu *et al.*, 1992). Reactions were treated with DNase to remove plasmid DNA, extracted twice with phenol: chloroform,

purified by three cycles of ethanol precipitation, washed twice with 80% ethanol, dried and dissolved at 1-3 mg/ml of RNase-free water. The quality of each batch of RNA was checked by gel electrophoresis and cell-free *in vitro* translation analyses. Jelly-free eggs (stage 3, with vitelline membrane) were put on stainless steel mesh in 5% Ficoll containing Steinberg's solution. mRNA samples diluted in microinjection buffer containing 88 mM NaCl, 1 mM KCl and 15 mM Tris-HCl (pH 7.5), were injected into two ventral blastomeres (Gurdon, 1976). The embryos were cultured in the Steinberg's solution for 3 days, observed by light microscopy, fixed in Bouins Fluid, dehydrated through ethanol series, and sectioned at 6 μM. The sections were stained with hematoxylin-eosine.

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