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Localization of specific mRNAs in *Xenopus* embryos by whole-mount *in situ* hybridization

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Summary

We have adapted a non-radioactive technique to detect localized mRNAs in whole-mount *Xenopus* embryos. Synthetic antisense RNA transcribed in the presence of digoxygenin-UTP is used as a probe and is detected *via* an anti-digoxygenin antibody. We show that localized mRNAs can be detected from late gastrula to tadpole stages and that high as well as low abundance RNAs can be detected. The method was tested on muscle actin and α-globin RNAs, whose localization has previously been characterized. In addition, we used the method to determine the distribution of XA-1 RNA, an anterior ectoderm-specific RNA, which we show is expressed in the periphery of the cement gland as well as in the region of the hatching gland. The sequence of an XA-1 cDNA predicts a protein rich in proline and histidine.

Key words: *in situ* hybridization, *Xenopus*, muscle actin, globin, cement gland, hatching gland.

Introduction

Three techniques have been used to analyze localized gene expression in *Xenopus* embryos. Since the embryos are large, microdissected tissues can be assayed biochemically for the presence of specific RNAs (Mohun *et al.* 1984); skilled dissection can yield a high degree of spatial resolution (Hopwood *et al.* 1989*b*) but cannot provide information at the level of single cells. Protein products can be localized by immunological methods, and analysis of embryos by whole-mount immunohistochemistry provides both high resolution and three-dimensional information (Dent *et al.* 1989; Hemmati-Brivanlou and Harland, 1989); however, after isolating a gene of interest it takes considerable time and effort to raise and purify specific antibodies. *In situ* hybridization is a powerful technique for examining the spatial expression of RNAs in embryos, but *in situ* hybridization to sectioned *Xenopus* embryos is laborious and not reproducible. The method has not been sufficiently sensitive to detect rare transcripts, such as those from many homeobox genes, so that only moderately abundant RNAs have been analyzed. Even then an exposure time of weeks (Weeks and Melton, 1987; Sato and Sargent, 1989) or even months (Ruiz i Altaba and Melton, 1989) is often necessary. Since *in situ* hybridization has been carried out on sectioned tissue, two-dimensional information must be reconstituted into three dimensions; this must be done either by the imagination of the investigator or by computer (Wilkinson *et al.* 1987).

Recently a sensitive, non-radioactive *in situ* hybridization method has been developed for the localization of specific RNAs in whole-mount *Drosophila* embryos (Tautz and Pfeifle, 1989). We show that a modification of this technique can be successfully used to detect localized RNAs in embryos of the frog *Xenopus laevis*. In contrast to *in situ* hybridization of radiolabelled probes to tissue sections, the non-radioactive method is rapid, sensitive and allows staining of whole embryos.

As well as confirming that the method works with genes such as muscle-specific actin and α-globin whose transcripts have previously been localized, we have analyzed the expression of the XA-1 gene (Sive *et al.* 1989). From dissection experiments, XA-1 is known to be expressed in the cement gland and non-brain head ectoderm of embryos, but the cellular distribution of this transcript has not been determined.

Materials and methods

The *in situ* hybridization procedure was adapted from Tautz and Pfeifle (1989) and Kintner and Melton (1987).

Embryos

Xenopus laevis were obtained from the Berkeley colony maintained by the laboratory of J. C. Gerhart. Ovulation of females and *in vitro* fertilization were carried out as described by Condie and Harland (1987). Developmental stages were determined according to Nieuwkoop and Faber (1967).

The vitelline membranes of dejellied embryos were loosened by treatment with proteinase K (5 $\mu g\,ml^{-1}$) for 5 to 10 min and were then manually removed. Digestion was monitored under the microscope and when the membranes were seen to lift from the surface the embryos were washed in 1/3MR (1×MR=100 mM NaCl, 1.8 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 50 $\mu g\,ml^{-1}$ gentamycin, buffered to pH 6.9 with 5 mM Hepes). Selected embryos were transferred to a 5 ml screw cap glass vial filled with distilled water. After the embryos settled, the water was removed and the vials were filled to the brim with MEMFA (0.1 M Mops pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde). Embryos were allowed to fix at room temperature for 1.5 to 2 h on a Labquake rotator (Labindustries, Inc). MEMFA was made freshly from a stock of 10× salts and 37% formaldehyde. Surprisingly, the use of paraformaldehyde at this stage led to higher background staining. The fix was removed and replaced with methanol. After a few minutes of equilibration with the methanol, the embryos were stored at −20°C.

Probes

The linearized DNA templates shown in Table 1 were used for *in vitro* transcription reactions in the presence of digoxigenin-11-UTP (Boehringer Mannheim 1209 256) with either T3, T7 or SP6 RNA polymerase (as described by Melton *et al.* 1985). Linearized template DNA (2.5 μg) was transcribed in 50 μl of 40 mM Tris–HCl pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine–HCl, 10 mM DTT, 1 mM ATP, CTP and GTP, 0.33 mM digoxygenin-11-UTP (BMB 1209 256), 0.66 mM UTP, with 10 μCi of ^{32}P-CTP (400 $Ci\,mmol^{-1}$) and 90 units of the appropriate enzyme for 2 to 3 h. After standard DNAseI treatment the reaction was diluted to 100 μl. After taking 1 μl to determine the total counts available, the remainder of the reaction was spun through a 1 ml column of Sephadex equilibrated in 0.3 M sodium acetate pH 5.5, 0.1% SDS. Following ethanol precipitation, the RNA pellet was resuspended in 50 μl of 40 mM sodium bicarbonate, 60 mM sodium carbonate and incubated for 35–50 min at 60°C to generate 200–500 nucleotide RNA probes (Lynn *et al.* 1983). The RNA was again precipitated with ethanol and the final pellet was resuspended in 400 μl hybridization buffer; 50% formamide, 5×SSC, 500 $\mu g\,ml^{-1}$ Torula RNA (Calbiochem), 50 $\mu g\,ml^{-1}$ heparin and 0.1% Tween 20. A second sample was then taken to determine the counts incorporated. The theoretical maximum yield (100% incorporation) is 66 μg.

In situ *hybridization*

Fixed embryos in methanol were gradually rehydrated with ME (90% methanol, 10% 0.5 M EGTA) and PTw (1× PBS+0.1% Tween-20) by 5 min incubations in ME, 75% ME+25% PTw, 50% ME+50% PTw, 25% ME+75% PTw, 100% PTw. They were then washed three times, 5 min each, in PTw. Unless otherwise stated, in all steps of the protocol the vials were filled almost completely with liquid and rocked on a nutator (Clay Adams). The embryos were then incubated at room temperature in 10 $\mu g\,ml^{-1}$ proteinase K (in PTw) on the nutator. The time of incubation must be adjusted for different batches of proteinase K but 15–30 min has given good results. Early stage embryos (prior to stage 20) are more sensitive to damage and should be monitored carefully. Embryos subsequently swell but most stay intact through the procedure. At the end of the proteinase K treatment, embryos were washed twice, 5 min each, in PTw and were refixed for 20 min with 4% paraformaldehyde in 1×PBS at room temperature. Paraformaldehyde yielded marginally better results than formaldehyde. The refix was followed by five washes, 5 min each in PTw. Since the embryos are somewhat delicate after protease treatment, the rocking steps are done by laying the vials horizontally on the nutator and filling the vials to reduce turbulence. Where solutions are more precious and volumes are smaller, the vials are placed vertically in a rack on the nutator.

Hybridization buffer (1 ml) was added to a near empty tube and the embryos allowed to settle and equilibrate for a few minutes. The hybridization buffer was changed and the embryos were prehybridized for 1 h at 50°C. The prehybridization buffer was replaced with fresh hybridization solution containing the probe (5–10 $\mu g\,ml^{-1}$) and embryos were incubated overnight at 50°C. At the end of the hybridization the probe was saved. We find that the probe can be recycled up to 3 times. Hybridization was also tested at 65°C with no obvious improvement in background but with loss of morphology, particularly in later stage embryos where the endodermal mass enlarged in comparison to the other tissues.

The embryos were brought from hybridization buffer to 2×SSC gradually by 1.5 ml changes of the following solutions: 75% hyb+25% 2×SSC, 50% hyb+50% 2×SSC and 25% hyb+75% 2×SSC each wash for 10 min at 37°C in a shaking waterbath at between 0 to 60 revs min^{-1}. This was followed by two washes 20 min in 2×SSC at 37°C. The non-hybridized excess RNA was removed by incubation of the embryos in a solution of 2×SSC containing 20 $\mu g\,ml^{-1}$ RNase A and 10 units ml^{-1} RNAse T_1 at 37°C for 30 min. From 2×SSC the embryos were transferred to 0.2×SSC and washed twice in 0.2×SSC for 30 min, at 55°C; and stepped back to PTw, 5–10 min changes of 75% 0.2×SSC+25% PTw, 50% 0.2×SSC+50% PTw, 25% 0.2×SSC+75% PTw and 100% PTw.

RNA hybrids were detected by immunohistochemistry in a procedure similar to the one described previously (Hemmati-Brivanlou and Harland, 1989). The PTw was replaced with PBT (PBS+2 $mg\,ml^{-1}$ BSA+0.1% Triton X-100), and the vials were rocked at room temperature for 15 min. The PBT was removed and replaced with 500 μl of fresh PBT+10% heat-inactivated goat serum (Gibco; the serum is heated at 56°C for 30 min). This step saturates non-specific immunoglobulin-binding sites. Even though the commercially available digoxigenin antibody is raised in sheep and therefore the optimum blocking serum should be sheep serum, we found that goat serum worked adequately. Vials were rocked vertically at room temperature for 1 h. This solution was replaced with 500 μl fresh PBT+10% goat serum containing a 1/1000 dilution of the affinity-purified sheep anti-digoxigenin coupled to alkaline phosphatase antibody (BMB cat. no. 1093 274). Tubes were rocked vertically overnight at 4°C. To remove excess antibody, the embryos were washed at least 3 times 1 h each with PBT at room temperature (filled up vials and horizontal rocking).

For the chromogenic reaction with alkaline phosphatase, embryos were washed 3 times, 5 min each at room temperature with 100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20 and 1 mM Levamisol added freshly to inhibit endogenous alkaline phosphatase. The last wash was replaced with 1 ml of the same solution and 4.5 μl nitro blue tetrazolium (NBT, Sigma; 75 $mg\,ml^{-1}$ in 70% dimethylformamide) and 3.5 μl 5-bromo-4-chloro-3-indoyl phosphate (BCIP, Sigma; 50 $mg\,ml^{-1}$ in 100% dimethylformamide) were added directly

to the tube. Embryos were rocked at room temperature (it is not critical to protect the reaction from light). The color reaction was visible from 5 min to 60 min after treatment. When satisfactory signal and background were observed, the solution was replaced with PBS. After a total of 2 washes with PBS (5 min each at room temperature), the specimens were dehydrated for 3 min in methanol and mounted in 2:1 benzyl benzoate: benzyl alcohol (BB/BA) for observation. Although satisfactory for intense signals, such as the actin signal shown in Fig. 2A, this medium dissolves less intense stains. Therefore, a limitation to the sensitivity of the method is the solubility of the stain. The actin signal was photographed after 1 month in BB/BA whereas the globin signal faded in one week. For epidermal RNAs, the specimens can be mounted in aqueous media, but for deeper stains, such as those in the nervous system, the embryos must be cleared. If the background associated with a particular probe is not substantial, the NBT/BCIP reaction can be allowed to proceed for 6 h; under these conditions the blue stain remains highly localized and is sufficiently intense to remain insoluble. To circumvent the solubility problem, we have also tested the Vectastain II kit as suggested by Dent *et al.* (1989). This kit gives satisfactory results, though it is not as sensitive as NBT/BCIP and the contrast is inferior. HRP-conjugated secondary antibodies were also tested and are relatively insensitive; however, in cases where mRNA is abundant and NBT/BCIP stains too intensely, the HRP-conjugated antibody is a useful alternative.

In general we have used albino embryos in these experiments. However, the blue stain contrasts well with pigment so that the procedure can be carried out on pigmented embryos (see Fig. 2). We have not attempted to stain embryos bleached with hydrogen peroxide (Dent *et al.* 1989), nor have we tested whether the blue stain is resistant to bleach after the staining is complete.

Northwestern Blot

Radiolabelled and dig-11 UTP labelled synthetic RNA was fractionated by electrophoresis in formaldehyde agarose gels and transferred to a nylon filter. The filters were exposed to X-ray film to estimate the quality and quantity of the synthetic RNA.

The relative efficiencies of incorporation of dig-UTP by SP6, T7 and T3 RNA polymerases were determined as follows: Sense transcription templates driven by the SP6, T7 and T3 promoters and containing the chloramphenicol acetyl transferase gene, (CAT), were prepared. The transcribed sequences were identical except for short polylinker regions. An aliquot of RNA produced from identical transcription reactions was quantitated by ^{32}P-CTP incorporation, and 10 ng of each was run out on a formaldehyde agarose gel. An autoradiograph of the gel was scanned with a Hoefer densitometer and the peaks integrated to determine the relative quantities of RNA loaded per lane.

The incorporation of digoxigenin in each of the bands was estimated as follows: The filter was washed for 15 min in PBS and then washed in PBT for 30 min at room temperature. This was followed by an incubation in PBT+10% goat serum for one hour. The anti-digoxigenin antibody (BMB) was diluted 1:2000 in PBT+10% goat serum and incubated with the filter for 2 h at room temperature. The filter was then washed in PBT for an hour with 4 changes. PBT was replaced by two changes of 100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl and 0.1% Tween 20 for 15 min. 4.5 μl ml^{-1} of NBT and 3.5 μl ml^{-1} BCIP were added to the last wash and the purple color resulting from the chromogenic reaction was detected after 5 min. The wet filter was then scanned and the peaks integrated to quantitate each band; the relative efficiency of dig-UTP incorporation was then determined based on the masses of RNA present in each band. We have not attempted to determine the absolute number of digoxygenin groups per RNA molecule.

DNA cloning and sequencing

An apparently full-length cDNA clone (800 bp) of XA-1 was isolated from a stage 33 *Xenopus* cDNA head library cloned into lambda Zap (Stratagene) employing a probe previously isolated by subtractive cloning (see Sive *et al.* 1989). The plasmid contained within the phage (pBStSK-, into which the original insert was cloned) was excised according to the manufacturer.

Unidirectional exonuclease III deletions of the insert were constructed according to Henikoff (1987). After size selection, the sense strand of individual clones was sequenced (as single-stranded DNA) using the reverse primer and the T7 Sequenase kit (United States Biochemical Corporation). The antisense strand was sequenced after subcloning the entire insert into pBStSK$^+$, employing primers based on the previously sequenced strand.

Sequences were analyzed employing the Genepro program version 4.2 (Riverside Scientific Enterprises). Genbank and PIR databases were searched for significant homologies (lod score of at least 100, requiring a match of 10/30 amino acids).

Results and discussion

Comparison of the efficiency of incorporation of digoxigenin-UTP into synthetic RNA using T3, T7 and SP6 RNA polymerase

Most transcription vectors currently in use contain unique RNA polymerase promoters on either side of a polylinker, allowing the use of experimental antisense probes and control sense probes for *in situ* hybridization. The comparison between the probes is valid only if the relative incorporation of dig-UTP by the RNA polymerases used is approximately equal, and therefore produces probes of equal specific activities.

We have tested SP6, T3 and T7 RNA polymerases on similar templates in identical reactions containing α^{32}P-CTP and dig-UTP. ^{32}P-CTP incorporation was used to quantitate the amount of RNA produced. Samples of each RNA were run on a gel and blotted onto nylon membrane. An autoradiograph of the blot is seen in Fig. 1A. The digoxigenin in each band was detected immunologically and is shown in Fig. 1B. The autoradiograph and the blot were scanned to quantitate the relative amounts of label present, and we find that the different RNA polymerases incorporate dig-UTP with the following relative efficiencies: if SP6 is defined as 1.0, T3 is 1.4, and T7 is 1.7. Thus all polymerases incorporate the nucleotide with similar efficiencies. Furthermore, the RNA products are full length, showing that the modified nucleotide does not cause premature chain termination.

Detection of muscle actin RNA

As a first test of the method we set out to detect muscle-specific cardiac actin mRNA, which is abundant and highly localized in the somites and later in the heart

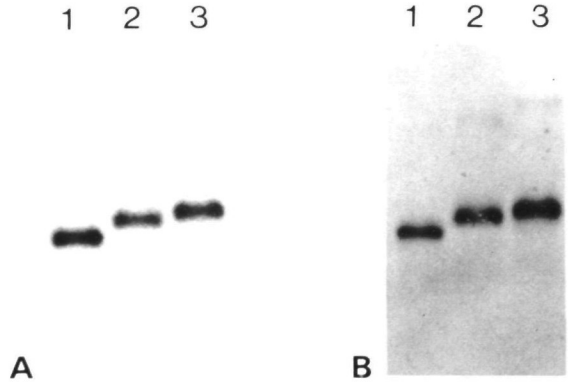

Fig. 1. Comparative efficiency of digoxigenin-UTP incorporation using T3, T7 and SP6 RNA polymerases. (A) Autoradiogram of ^{32}P-CTP labeled CAT RNA transcripts blotted onto a nylon membrane. The RNA polymerases used were SP6, T3, and T7, lanes 1, 2, and 3, respectively, under identical reaction conditions. (B) Anti-digoxigenin-11-UTP antibody detection of the RNA on the same blot as shown in (A). The transcripts are just over 900 bases in length.

(Mohun *et al.* 1984; Dworkin-Rastl *et al.* 1986; Kintner and Melton, 1987; Hopwood *et al.* 1989*a*). The AC100 plasmid (Dworkin-Rastl *et al.* 1986; Kintner and Melton, 1987) was linearized with *Pvu*II (see Table 1) and was transcribed with SP6 RNA polymerase. Fig. 2A shows the pattern of expression of muscle actin message in *Xenopus* embryos from late gastrula to the swimming tadpole stage. The earliest stage in which specific staining was observed was the late gastrula, where two bands of staining correspond to the developing somites (Kintner and Melton, 1987). Some distortion of early embryos often takes place during the procedure; in this case the dorsal view makes the blastopore resemble the neural groove of later embryos, but observation of the embryo in different planes makes identification as a late gastrula stage unambiguous.

The detection of muscle actin RNA at this stage allows us to compare the sensitivities of the two *in situ* hybridization methods. Using radiolabelled probes on sections, Kinter and Melton (1987) detected muscle actin at stage 14. Therefore, the two methods appear to be comparable in sensitivity, detecting 4×10^6 transcripts per embryo. However, where the radiolabelled probe required a 5 day exposure, the muscle actin stain shown in Fig. 2 was developed in 10 min.

A problem with the staining of these early stages can be seen in the background staining of the archenteron floor of the stage 13 embryo. We also see background staining of the blastocoel roof of earlier embryos (not shown); this kind of background has been noted previously by Hopwood *et al.* (1989*a*). Nevertheless, the detection of muscle actin RNA at this early stage illustrates the potential of the whole-mount method. In particular the dorsal view of the stage 13 embryo (Fig. 2A) shows how extensive the presumptive head region is, and how far posteriorly somites are forming at this stage. At later stages the background is usually lower and in the stage 24 embryo the RNA is only detected in the somites. At later stages such as the stage 36 embryo shown here (Fig. 2A), the muscle actin message is also detected in the heart (Hopwood *et al.* 1989*a*) and branchiomeric muscle (Dworkin-Rastl *et al.* 1986). These results show that the whole-mount method is sensitive and has sufficiently low background to apply to other mRNAs. In addition the advantage of observing whole embryos, rather than reconstructing an image from sections, is readily apparent.

Detection of α-globin mRNA

Before proceeding with RNAs whose localization has not been determined precisely, we also tested an α-globin probe. The α-globin message is a good test candidate because it is less abundant than actin at tailbud stages and it has been shown to be localized to a different region of the embryo, the blood islands which are part of the ventral mesoderm. It is also useful to develop a sensitive assay for globin RNA that could be used to investigate the formation of ventral mesodermal cell types in explanted tissues. A cDNA encoding a portion of the *Xenopus* αT1-globin (Widmer *et al.* 1981) was subcloned into pGEM-blue to produce pαGT1dB (M. Bolce unpublished). Transcription with T7 RNA polymerase makes an antisense RNA (see Table 1). Fig. 2B shows the pattern of expression of the globin transcript in a *Xenopus* tadpole (stage 32). In agreement with previous observations, we find this mRNA to be present exclusively in the ventral mesoderm at this stage of development. The blood islands bifurcate anteriorly and are fused posteriorly. The sense probe done in parallel did not show any specific hybridization (not shown).

Spatial distribution of XA-1

The XA-1 gene is expressed in the presumptive cement

Table 1. *Summary of plasmids used with the efficiency of synthesis and amounts used in* in situ *hybridization*

DNA templates	Linearized with	RNA polymerase used	Probe	Amount synthesized (μg)	Concentration used (μg/ml)	References
SP65-CAT	*Bam*HI	SP6	Sense	12	N.A.	Harland and Weintraub, 1985
T3-CAT	*Xba*I	T3	Sense	23	N.A.	Brown Unpub. data
T7-CAT	*Hin*dIII	T7	Sense	12	N.A.	Brown Unpub. data
AC 100	*Pvu*II	SP6	Antisense	18	18	Kintner and Melton, 1987
pαGT1dB	*Hin*dIII	T7	Antisense	8.5	5	Bolce Unpub. data
pαGT1dB	*Sma*I	SP6	Sense	12.6	6	Bolce Unpub. data
XA-1	*Not*I	T7	Antisense	3.5	7	Sive *et al.* 1989
XAG-1	*Not*I	T7	Antisense	6.3	12.5	Sive *et al.* 1989

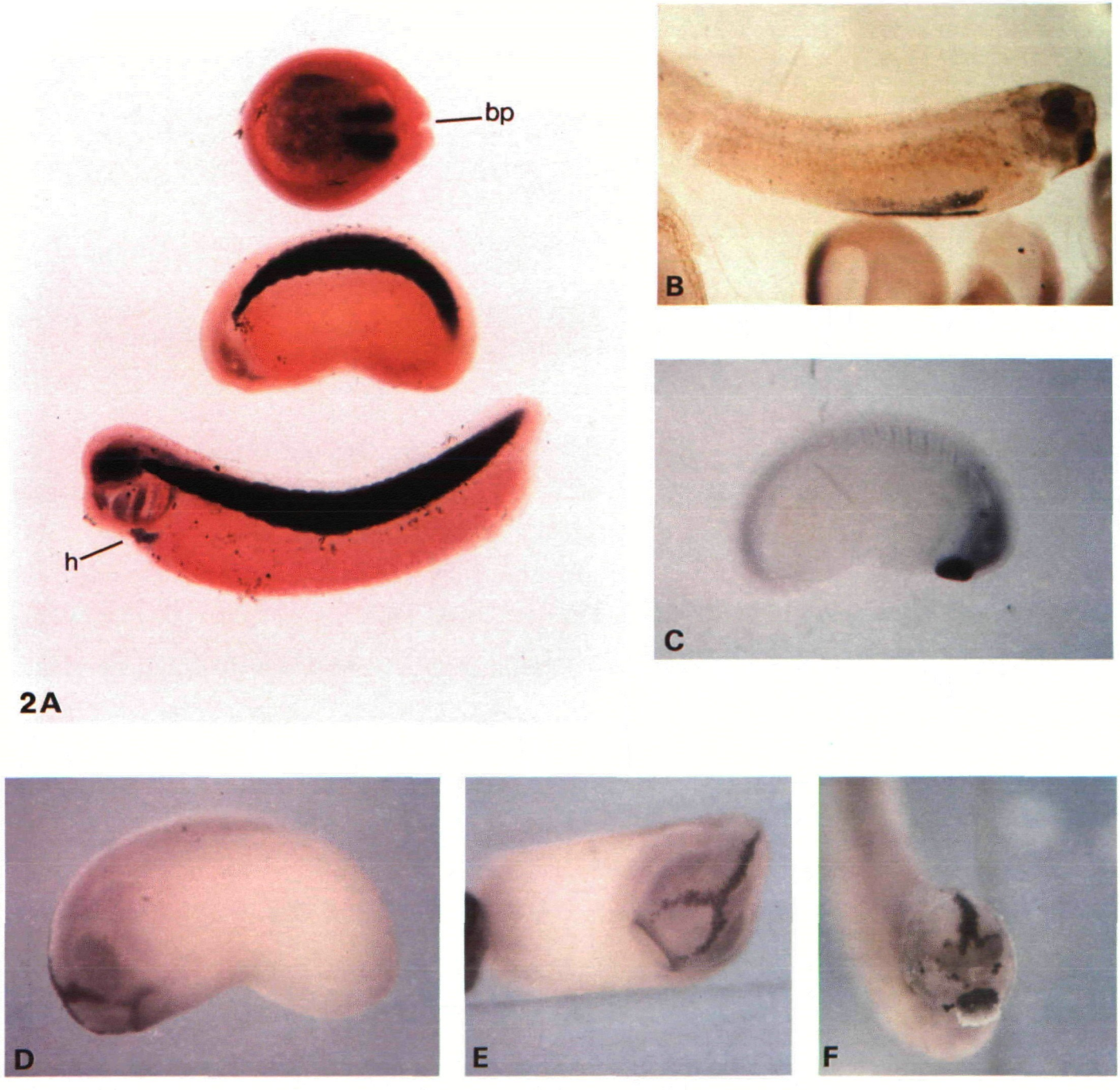

Fig. 2. Detection of specific mRNAs by whole-mount *in situ* hybridization. (A) Detection of muscle actin at different stages of *Xenopus* development. Embryonic stages 13 (dorsal view), 24 and 36 (lateral views) are presented from top to bottom. The blastopore of the stage 13 embryo is arrowed (bp). In addition to somites the heart (h) of the stage 36 embryo is stained. (B) Localization of the globin mRNA in the blood islands of a stage 32 tadpole. The staining is detected on the ventral side of the embryo and is bilaterally symmetrical along the anteroposterior axis. (C) Distribution of the XAG-1 transcript in the cement gland of a stage 23 embryo. Note that under saturating conditions of staining some non-specific staining is also detected on the dorsal side of the embryo. (D–F) Localization of the XA-1 transcript. (D) and (E) represent two different views of a stage 23 embryo; (D) lateral view, (E) anterior view. (F) A stage 36 tadpole viewed in the same plane and magnification as (E). All embryos presented in this figure are albinos with the exception of (B) where a pigmented embryo was used. (A,B and C) have been cleared in benzyl-benzoate/benzyl alcohol to see deep staining; (D,E and F) have not been cleared.

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AAG CAA TCC TCG GCA TTT GAA GAG AGA TAG ACG GGA TTT CCA GCC          45

ATG TTT TTC TAC GTT CTT CTG CTT GCT CTA ATG GCC CAG GGC TGG          90
Met Phe Phe Tyr Val Leu Leu Leu Ala Leu Met Ala Gln Gly Trp         (15)

AGC CTT CCA CAA GGG AAG ACA GGA GAA GAT TCT CCA GTC TTC AGG         135
Ser Leu Pro Gln Gly Lys Thr Gly Glu Asp Ser Pro Val Phe Arg         (30)

CCT CCT TCT CCA CCC ATG GGC CCT AGC CTT CCT CCA CCT GTC AGC         180
Pro Pro Ser Pro Pro Met Gly Pro Ser Leu Pro Pro Pro Val Ser         (45)

CAT GAC CTG CAC AGG CCA TCG GGT CAC CCT GAG GAG TTC AGA ACT         225
His Asp Leu His Arg Pro Ser Gly His Pro Glu Glu Phe Arg Thr         (60)

GGA GCA TCC TTA CCT CCA AAA GAA ACC CCC AAT GAA CCG AGG CAT         270
Gly Ala Ser Leu Pro Pro Lys Glu Thr Pro Asn Glu Pro Arg His         (75)

GGC AGG CCT AAG AGA GAT CTT CAT CAT GGG AAA GTT GTC CCC ACT         315
Gly Arg Pro Lys Arg Asp Leu His His Gly Lys Val Val Pro Thr         (90)

GGA GTC CCT CAT CAC ACA GGA GAA GTC CTA CAT CAC ACA GAC TGT         360
Gly Val Pro His His Thr Gly Glu Val Leu His His Thr Asp Cys        (105)

TCT TCT AAT ACC CAC AAG TCA CAT GAA GAA AAC AGA CCT AAA GGG         405
Ser Ser Asn Thr His Lys Ser His Glu Glu Asn Arg Pro Lys Gly        (120)

TTC AGG ACT GGA CGT CCC TTG CTA CCC ATA AAA CCT GAG CAT GGC         450
Phe Arg Thr Gly Arg Pro Leu Leu Pro Ile Lys Pro Glu His Gly        (135)

AGG CAC AGG AGA GAT CTT CAT CAT GGG AAA GCT GTC CCT ACT GGA         495
Arg His Arg Arg Asp Leu His His Gly Lys Ala Val Pro Thr Gly        (150)

GTC CCT CAT CAC ACA GAA AAA TTC CAC AAC GGC AGC AAT GGA AAA         540
Val Pro His His Thr Glu Lys Phe His Asn Gly Ser Asn Gly Lys        (165)

AGC CAT CCT CCC CGA CCA GGC CAT TCC ACC TCT GCC CAC AAT GAC         585
Ser His Pro Pro Arg Pro Gly His Ser Thr Ser Ala His Asn Asp        (180)

AAC TCA AGT GAA GAA AAG AGA CCA AAA CAT GGT CAG GAA CAA GGA         630
Asn Ser Ser Glu Glu Lys Arg Pro Lys His Gly Gln Glu Gln Gly        (195)

AAG AAG CAT TAG TAA ACA TAA AAA GAA GAA AAT TAA AGA GGA AAC         675
Lys Lys His

CGA GTA CAT TGG ATG GAA TTA TTA ATA AGT TAT GGC AAA ACC AAT         720

AAA GTG ATA ATG AT                                                  734
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Fig. 3. Sequence of XA-1. Nucleotide and conceptual protein sequence of the longest open reading frame are shown. Nucleotides and amino acids (in parentheses) are numbered at the end of each line. The first ATG, the first stop codon, the poly A addition signal (AATAAA) and a 24 amino acid repeated sequence (see text) are underlined. Two potential glycosylation sites are present in the carboxyl domain, at amino acids 169 (*N*GS) and 181 (*N*SS). The poly A tail begins immediately after the last nucleotide shown.

gland and non-brain head ectoderm from late gastrula to post-hatching stages (Sive *et al.* 1989; Sive *et al.* 1990). XA-1 is an interesting gene in that its expression marks the presumptive head region of the late gastrula (stage 12) before any morphological boundary is manifest (Sive *et al.* 1989). This gene has also been useful in allowing dissection of the hierarchy of inductions that occurs during anteroposterior axis formation (Sive *et al.* 1990).

XA-1 encodes a poly(A)+ RNA of approximately 800 bases. An apparently full-length cDNA clone was sequenced on both strands (see Materials and methods) (Fig. 3). The longest open reading frame specifies a protein of 198 residues, M_r approximately 22 000. There are 3 in-frame methionine residues, with the first in the most favorable context for translation initiation (GCC ATG T). The projected protein is rather hydrophilic, except for an 18 residue stretch at the amino end. This may be a signal peptide, suggesting that XA-1 may be a secreted or integral membrane protein. The protein is very proline- (15 %) and histidine- (12 %) rich, and on this basis can be further divided into a more proline-rich amino domain and a basic carboxyl domain. The high proline content throughout the protein makes it unlikely that XA-1 can form long stretches of unbroken alpha helix. An interesting motif in this gene is a twice-repeated 24 amino acid sequence, beginning at residues 73 and 132, with 20/24 identical residues between the repeats. No striking similarities to previously identified open reading frames were apparent (see Materials and methods).

The localization of XA-1, determined from dissection and RNA blot analysis was of low resolution. We therefore used the whole-mount *in situ* hybridization technique to localize the transcripts at single-cell resolution. Antisense RNAs were hybridized to embryos of different stages, and after visualizing the hybridized RNA with either NBT/BCIP or the vectastain II kit, the embryos were cleared for observation. Since no deep staining was apparent, the embryos were remounted in PBS for observation of the ectodermal staining. Figs 2D and E show two views of a stage 23 embryo. The transcript is present in cells that form a ridge starting behind the otic vesicle, extending along the dorsal midline, bifurcating in front of the eyes and reaching the cement gland. The periphery of the cement gland expresses XA-1, in contrast to other genes, which are expressed in the main mass of cement gland tissue (Jamrich and Sato, 1989). Since it was possible that the peripheral staining of the cement gland was due to failure of the probe to penetrate cement gland tissue, we carried out a control hybridization with XAG-1 (Sive *et al.* 1989). XAG-1 is a suitable candidate since dissection experiments show that it is almost exclusively expressed in the cement gland at stage 23 (Sive *et al.* 1989). The *in situ* hybridization shown in Fig. 2C confirms this, and shows that the whole of the cement gland can be stained intensely after hybridization. This result, coupled with the detection of actin transcripts in deep tissues, illustrates that the RNA probes readily penetrate tissues in frog embryos. A potential limitation to the technique is also illustrated in Fig. 2C. In addition to the cement gland, some non-specific staining is seen in the dorsal structures. From the analysis of RNA in dissected animals (Sive *et al.* 1989), it is unlikely that this signal could be due to XAG-1 RNA. Various sense and antisense probes show variation in the amount of such background, in a probe-dependent way; it may therefore be necessary to optimize the fragment of DNA used to generate the probe (as is the case with RNAse protection) in order to minimize background.

The distribution of XA-1 in the head ectoderm of a stage 36 embryo is shown in Fig. 2F. As development proceeds the continuous lines of cells that expressed the gene at stage 23 (Fig. 2D,E) break up into patches. The ridge of cells in the dorsal midline is still continuous and extends to a point behind the otic vesicle (not shown), but the facial expression has broken up into patches of a few cells. Expression is excluded from the nasal pits.

The distribution of XA-1 RNA in the head ectoderm partially overlaps the distribution of UVS.2 protein (Sato and Sargent, 1989) and tyrosine hydroxylase (Drysdale and Elinson, 1989). Both these markers appear to be expressed in the hatching gland. However,

XA-1 and UVS.2 are clearly distinct genes, with different temporal distribution and RNA size.

The representation of XA-1 cDNA clones in libraries indicates that the RNA is rare (less than 1 in 10^5 embryo mRNAs; Sive *et al.* 1989). However, since the expression is clearly highly localized it is difficult to assess the number of mRNA copies per cell, and hence the absolute sensitivity of the method. It is however, encouraging to us that rare transcripts such as XA-1 and *engrailed* (*Xenopus En-2*, Hemmati-Brivanlou *et al.* manuscript in preparation) can be detected with this method.

In summary, we have developed a sensitive and reliable method for *in situ* hybridization in whole *Xenopus* embryos. The successful detection of the localized RNAs, muscle actin and α-globin, shows that the method is specific and reproducible, and a comparison of our results to *in situ* detection of the muscle actin gene with a radioactive probe shows that the non-radioactive method is as sensitive. As with other methods, there are problems with background staining, particularly at early stages. Though the background currently limits the sensitivity of the technique, further efforts will improve the specificity of staining. The solubility of the stain in the currently available mounting media also limits the sensitivity, but this difficulty also should be overcome with further experimentation. The use of a non-radioactive assay reduces the time required to develop the signal from weeks to minutes; in addition the visualization of RNAs in whole embryos greatly facilitates the spatial characterization of gene expression. Even where detailed examination of sectioned material is required, sectioning is more easily carried out after the hybridization procedure than before it. Finally, the hybridization and staining can be carried out on batches of embryos permitting the generation of many pieces of information in one experiment. Thus, the sensitivity, specificity and convenience of this new method make it a powerful and highly useful tool for the detailed study of the expression of individual RNAs throughout development. The same approach should also prove successful in other vertebrate embryos.

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