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Original Article

Ets-1 and *Ets-2* proto-oncogenes exhibit differential and restricted expression patterns during *Xenopus laevis* oogenesis and embryogenesis

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ABSTRACT ***Xenopus* Xl-*ets-1* *and Xl-ets-2* are maternally expressed. From late oogenesis to early embryogenesis their transcripts are localized to the animal pole and the intermediate zone, suggesting a function in the differentiation of animal blastomeres and future mesoderm. Their presence at the level of germ plasm suggests also a role in the differentiation of the germinal lineage. Both zygotic genes are expressed ubiquitously beginning at MBT, and then restricted to a circumblastoporal collar. In neurula and tailbud stages, *ets-1* and *ets-2* transcripts are detected in neural crest cells and their derivatives. Specific transcription can also be observed for *ets-1* in the hemangioblastic precursors, in endothelial cells of the forming heart and blood vessels. *Ets-2* is itself specifically expressed in the putative pronephros and in the forming pronephric tubules and extending pronephric duct. Like another member of the *ets*-gene family (Xl-*fli*), both genes are transcribed in regions of the embryo undergoing important morphogenetic modifications, especially in migrating cells and/or along their migration pathways. We postulate that these genes orchestrate modifications of cellular adhesion. Changes in the expression of cadherins and integrins repertories would be consistent with such a role and could account for the phenotypes we reported earlier for Xl-*fli* overexpression. Such a role would be critical for tumor cell dissemination, in addition to the one already ascribed to *ets-1* in the expression of proteases specific for the extracellular matrix.**

KEY WORDS: *localized maternal transcripts, neural crest cells, cranial ganglia, endothelial cells, pronephric duct cells*

Introduction

Embryogenesis is a complex mechanism requiring the temporal and cell-specific regulation of numerous genes to build an entire organism from a single-cell zygote. Among them, the *ets*-gene family appears to have a key importance in the transcriptional regulation of many genes. The *ets* oncogene (v-*ets*) was discovered as a part of a fusion protein with gag and myb ($p^{135\delta\ gag\text{-}myb\text{-}ets}$) expressed by the E26 avian erythroblastosis virus (E26 transformation-specific) (Leprince *et al.*, 1983; Nunn *et al.*, 1983). It has been shown to be transduced from a cellular gene. Until now, more than 30 related genes have been characterized in various species ranging from *Drosophila* to human (Brown and McKnight, 1992; Watson *et al.*, 1992; Wasylyk *et al.*, 1993). Indeed, in the vast majority of cases, proteins of the ETS family in general, and ETS-1 and ETS-2 in particular, act as transcription positive regulators (Bosselut *et al.*, 1990; Gunther *et al.*, 1990; Ho *et al.*, 1990). However, a transrepressor function has also been demonstrated for ETS-1 (Prosser *et al.*, 1992; Wotton *et al.*, 1993), as well as for NET, another member of this family (Giovane *et al.*, 1994).

The cellular *ets-1* and *ets-2* genes are highly conserved in a wide range of species. They have been characterized in mouse and human (Watson *et al.*, 1988), chicken (Boulukos *et al.*, 1988; Leprince *et al.*, 1988), sea urchin (Chen *et al.*, 1988), and *Xenopus laevis* (Stiegler *et al.*, 1990; Wolff *et al.*, 1990). Another member of the Ets family, the Xl-*fli* gene has been sequenced in *Xenopus* (Meyer *et al.*, 1993).

Like all ETS proteins, ETS-1 and ETS-2 share a particularly well conserved region encompassing 85 amino acids: the ETS domain.

Abbreviations used in this paper: DTT, dithiothreitol; EBS, ETS-binding sequence; GTP, guanosine-5' triphosphate; MBT, mid-blastula transition; MEMFA, Mops, EGTA, Magnesium, FormAldehyde; M-MLV, murine Moloney Leukemia Virus; MMR, Marc's Modified Ringer Solution; MOPS, (3-[N-morpholino] propane-sulfonic acid); PCR, polymerase chain reaction; PND, pronephric duct; RT/PCR, reverse transcription followed by polymerase chain reaction; SSC, sodium saline citrate; Taq, Thermus aquaticus; WD, Wolffian duct.

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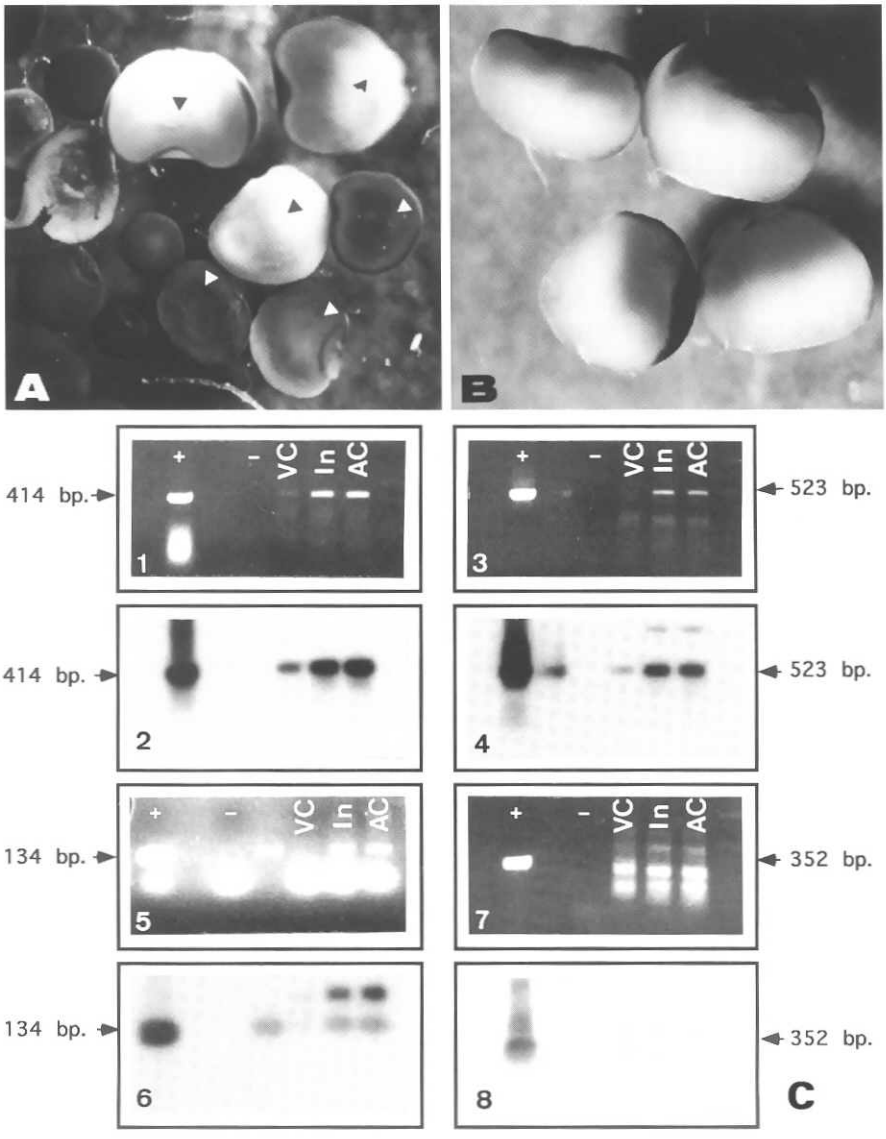

Fig. 1. Whole-mount *in situ* hybridization for *ets-1* and *ets-2* transcripts; RT/PCR quantification in the different regions of the oocyte. (A) *ets-1 transcript detection in oocytes of different developmental stages, using a digoxigenin labeled antisense riboprobe. Note the apparent uniform distribution of the staining in small oocytes (white arrowheads) and the confining of the transcripts to the animal pole of larger oocytes (red arrowheads).* **(B)** *ets-2 transcripts detection in shed eggs, cut into halves prior to hybridization with a digoxigenin-labeled antisense ets-2 probe. Note the confinement of the signal to the animal pole.* **(C)** *RT/PCR analysis of ets-1 and ets-2 transcripts in the animal pole, intermediate zone and vegetal pole of the oocyte. (1,2) ets-1 detection; (3,4) ets-2 detection; (5,6) control on 18S rRNA; (7,8) control on Xl-Twi mRNA. Odd numbers correspond to ethidium bromide staining; even numbers correspond to southern hybridizations with [^{32}P] labeled specific probes. AC, RT/PCR carried out on total RNA extracted from animal caps. (thickness≈ 1/3 of the oocyte diameter). In, RT/PCR on total RNA extracted from the intermediate zone (thickness≈ 1/3 of the oocyte diameter); VC, RT/PCR on total RNA extracted from the vegetal pole (thickness≈ 1/3 of the oocyte diameter); (+), positive control; (-), negative control (PCR without template DNA).*

This domain was recently shown to adopt a helix-turn-helix configuration (Donaldson *et al.*, 1994; Liang *et al.*, 1994a,b) and is able to bind DNA on a short core motif (GGAA/T) defining the Ets binding site (EBS; Karim *et al.*, 1990). This nucleotide core target is present in the promoters or enhancers of various cellular and viral genes (see reviews by Macleod *et al.*, 1992 and Wasylyk *et al.*, 1993). Besides the DNA binding region, transcription factors ETS-1 and ETS-2 are composed of several functional domains involved in activation or DNA-binding (for review see Wasylyk *et al.*, 1993). In fact, the binding specificity and efficiency of the ETS proteins can be achieved through a complex mechanism involving protein-protein interactions with other transcription factors (Thompson *et al.*, 1991; Brown and McKnight, 1992; Dudek *et al.*, 1992; Pongubala *et al.*, 1992; Janknecht and Nordheim, 1993), phosphorylation or oxidation status of internal regulatory domains (Mallery *et al.*, 1991) and sequences flanking the EBS (Ascione *et al.*, 1992). The ETS DNA-binding domain had not been suggested to be structurally related to any other DNA-binding motif. A recent report has shown that there is a motif common to both the heat-shock factor and the ETS family of proteins (Landsman and Wolffe, 1995).

Many studies have shown that *ets-1* is implicated in chicken embryonic development (Pardanaud *et al.*, 1989; Vandenbunder *et al.*, 1989; Desbiens *et al.*, 1991; Pardanaud and Dieterlen-Lièvre, 1993a,b; Quéva *et al.*, 1993). In mouse, both *ets-1* and *ets-2* genes are transcribed in diverse tissues (Kola *et al.*, 1993; Maroulakou *et al.*, 1994) and differentially regulated during development. In *Xenopus*, Xl-*fli* has been found mainly in territories invaded by migratory neural crest cells and in developing vascular structures (Meyer *et al.*, 1993, 1995).

Screening a stage I/II oocytes cDNA library with a *c-ets2* chicken probe we have isolated several *c-ets1* and *c-ets2* homologous cDNAs from the African frog *Xenopus laevis* (Stiegler *et al.*, 1990; Wolff *et al.*, 1990). *Xenopus laevis* possesses two different sets of *c-ets1* and *c-ets2* genes which only differ in a few variant nucleotides and are both expressed. By Northern blot analysis, we showed that *c-ets1* is mainly transcribed as two major mRNAs of 4.4 and 7.5 kb respectively (Stiegler *et al.*, 1993), and *c-ets2* encodes two transcripts of 1.7 and 3.2 kb respectively (Wolff *et al.*, 1991). The expression of both *c-ets1* and *c-ets2* appears to vary considerably during *Xenopus laevis* embryonic development as well as in different adult tissues.

In the present work, we have studied by *in situ* hybridization the spatial and temporal expression of *ets-1* and *ets-2* protooncogenes during *Xenopus laevis* oogenesis and embryogenesis. We showed that the maternal *ets-1* and *ets-2* transcripts are localized during late oogenesis and early embryogenesis to the subcortical region of the animal pole and marginal zone, suggesting a role in early embryogenesis. Both transcripts are also detected in early blastula embryo at the level of the germ plasm, suggesting a role in germinal lineage differentiation. Later in embryogenesis, *ets-1* and *ets-2* transcripts are observed in regions of the embryo affected by important

TABLE 1

RT/PCR ANALYSIS OF *ETS-1* AND *ETS-2* TRANSCRIPT DISTRIBUTION IN THE ANIMAL POLE (AP), INTERMEDIATE ZONE (IZ) AND VEGETAL POLE (VP) OF *XENOPUS* OOCYTES

gene	region	relative volumes	amounts of transcripts*	background	corrected amounts of transcripts#	% of message	corrected amount of transcript per unit of volume$
	AP	1	64		64	47	64
ets-1	IZ	2	54	0.4	53	39	27
	VP	1	19		18	14	18
	AP	1	29		28	44	28
ets-2	IZ	2	30	1.7	29	46	15
	VP	1	7		6	10	6
	AP	1	17		16	36	16
Control	IZ	2	16	0.7	15	34	8
Xl-*Twi*	VP	1	14		13	30	13
	AP	1	17		16	47	16
Control	IZ	2	15	0.7	14	41	7
18S rRNA	VP	1	5		4	12	7

*arbitrary units; #signal minus background; $signal minus background/relative volume of the region under consideration. The amounts of transcripts were measured by image plate analysis. Levels cannot be compared from gene to gene, because amplifications were carried out for different numbers of PCR cycles. Comparisons can only be made, for a given gene, between the different regions of the oocyte.

morphogenetic reorganizations. Particularly, both genes are expressed in a variety of migratory cells and along their migration pathways as already observed for the *fli* gene. These properties are discussed in terms of a possible regulation of the cellular adhesion properties and/or expression of guidance cues along cell migration pathways.

Results

Expression of ets-1 and ets-2 during oogenesis by conventional in situ hybridization on oocyte sections

As reported earlier for *ets-1* (Stiegler *et al.*, 1993), we used ^{35S}UTP labeled probes. Using both *ets-1* and *ets-2* specific probes, we detected an expression of the cognate gene(s) in oocytes. In both cases, the labeling was intense in stage I/II oocytes, then appeared weaker in stage III/IV and insignificant in stage V/VI (not shown). A strong labeling by the *ets-2* probe was also observed in oogonia included in the immature ovary of a stage 62 female during metamorphosis (Fig. 10). The specificity of these hybridizations is assessed by the absence of signal when using sense riboprobes (not shown).

Expression of ets-1 and ets-2 during oogenesis by whole-mount hybridization using digoxigenin-labeled probes

The method applied earlier to the detection of the *fli* transcripts during *Xenopus* embryogenesis (Meyer *et al.*, 1995) proved once more very sensitive and reliable for the detection of *ets-1* and *ets-2* mRNAs. This sensitive technique allowed us to detect additional expression in late stages of oogenesis for both genes, when the radioactive technique mentioned above failed to detect these transcripts (Fig. 1A and B). This signal is clearly specific, as staining was never observed when using a sense riboprobe (for illustration see Fig. 3A). Even more surprising was the observation that whereas *ets-1* and *ets-2* transcripts appeared evenly distributed in early stage oocytes (stage I to IV), they were found localized to the animal pole in stage V/VI oocytes. To ensure that this picture was not an artifact resulting from a poor penetration of the anti-digoxigenin antibody in the late stage oocytes, the experiment was reproduced on stage V/VI oocytes cut into halves prior to the hybridization reaction. The expression pattern observed was indeed restricted essentially to the animal pole.

This localization of *ets-1* and *ets-2* transcripts to the animal pole was confirmed by RT/PCR experiments carried out on total RNA extracted from the animal, intermediate and vegetal regions (these different regions corresponded respectively to roughly 1/3 of the oocyte diameter, yielding relative volumes of 1, 2, 1). Figure 1C shows that equivalent signals are observed for the animal and intermediate regions, whereas the vegetal region only gives rise to a very faint signal, at the limit of detection. The relative amounts of *ets-1* and *ets-2* transcripts were determined by image-plate analysis after blotting and hybridization with cognate radioactive probes. Two controls were carried out by RT/PCR using primers specific for 18S ribosomal RNA and Xl-*Twi* transcripts. Table 1 summarizes the respective percentages of the different transcripts in the three oocyte slices. These results confirm the highest concentration of *ets-1* and *ets-2* messages in the animal cap.

Expression of ets-1 and ets-2 during embryogenesis

ets-1 and ets-2 maternal transcripts are essentially restricted to the subcortical layer of the animal pole in cleavage stage embryos

The presence of *ets-1* and *ets-2* transcripts in the animal cap of a stage 4 embryo is confirmed in Figure 2A,B. These transcripts appear even more restricted to the subcortical layer, as shown in Figure 2C for *ets-1* mRNA, in a two-cell stage embryo cut into halves before hybridization to avoid possible artifacts due to a poor penetration of the anti-digoxigenin antibody. This confinement is maintained throughout blastulation, as shown for *ets-1* in Figure 2D and E. After the mid-blastula transition (MBT), nuclei resuming

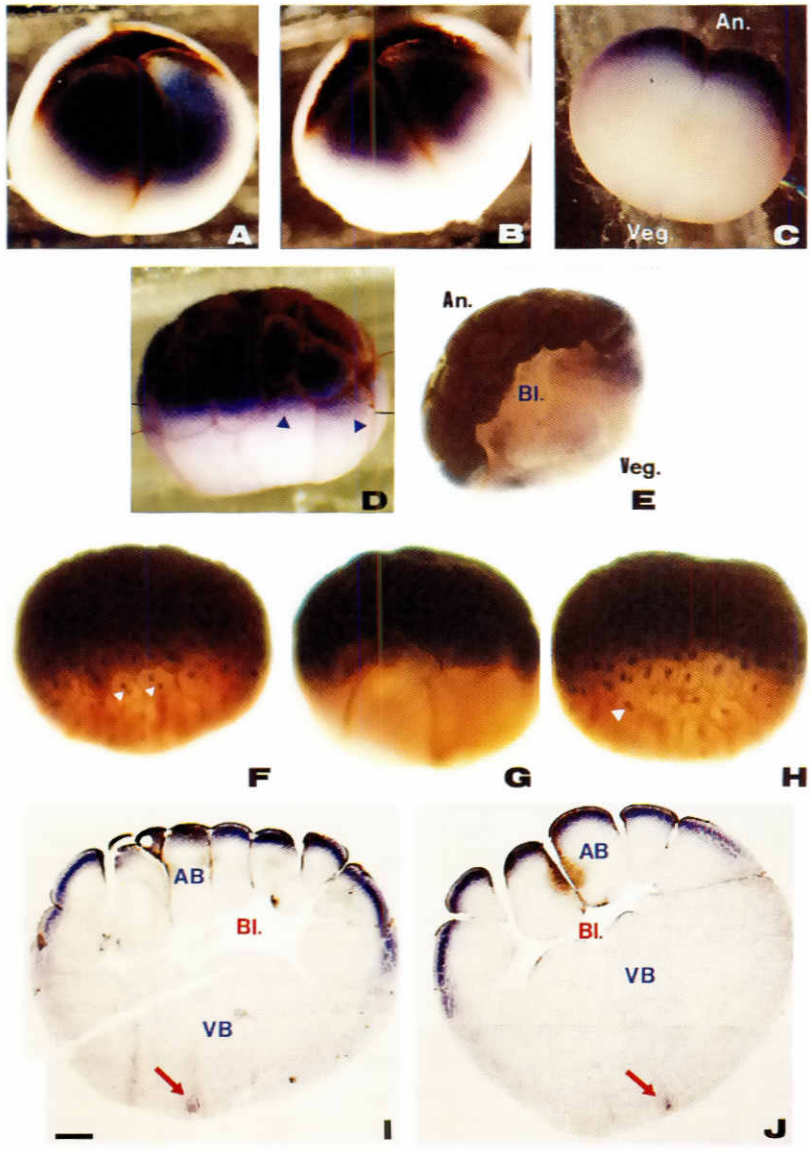

Fig. 2. Whole-mount *in situ* hybridization for *ets-1* and *ets-2* in early embryogenesis. **(A)** *Localization of ets-1 transcript in the animal pole of a 4-cell embryo. The cleavage furrows are clearly indicated by residual dark-brown pigmentation.* **(B)** *Same experiment conducted for ets-2 transcripts.* **(C)** *Control experiment in which a 2-cell stage embryo was cut into halves prior to hybridization to exclude possible artifacts resulting from a poor penetration of the anti-digoxigenin antibody (ets-1 antisense probe). An, animal pole; Veg, vegetal pole.* **(D)** *ets-1 mRNA localization in a 32-cell stage blastula, showing the presence of transcripts in the animal blastomeres and in the upper part of vegetal blastomeres (blue arrowheads).* **(E)** *Same experiment carried out on a 32-cell stage embryo cut into halves prior to hybridization. Note the presence of the transcripts (dark-blue staining) in the roof of the blastocoel and in the marginal zone. An, animal; Veg, vegetal; Bl, blastocoel.* **(F,G,H)** *Whole-mount in situ localization of ets-1* **(F)** *and ets-2* **(G,H)** *in stage 8* **(F and H)** *and stage 6* **(G)** *embryos. In addition to the animal pole cytoplasmic localization of the transcripts, notice the nuclear signal which can be observed in stage 8 embryos, at the level of the vegetal part, in the almost colorless cytoplasmic background. The specificity of this signal is assessed by the fact that no nuclear staining is observed in embryos prior to MBT (compare F and H with G) and by the fact that a sense probe does not give any signal (not shown). The proliferative activity of these cells is demonstrated by the presence of duplicated nuclei (white arrowheads in F) and a mitotic figure (white arrowhead in H), in cells which have not yet undergone cytokinesis.* **(I,J)** *10 μm thick sections of whole-mount hybridizations on a 32-cell stage (ets-1) and 16-cell stage (ets-2) embryos respectively. The transcripts are essentially located below the cortex of the animal blastomeres, but a significant level of both ets-1 and ets-2 mRNAs is also observed at the level of the germ plasms (red arrows). AB, animal blastomere; Bl, blastocoel; VB, vegetal blastomere. Bar, 100 μm.*

zygotic transcription of *ets-1* and *ets-2* genes are clearly detected in the almost colorless background of cells belonging to the intermediate zone and vegetal pole (Fig. 2F,H). These cells are actively dividing, as shown by the presence of duplicated nuclei and mitotic figures in cells which have not yet completed cytokinesis (see Fig. 2F and H). No nuclear staining is observed in a younger blastula (Fig. 2G) or when using a sense riboprobe (data not shown).

However, faint but significant amounts of *ets-1* and *ets-2* transcripts can be detected at the level of the germ plasm which is thought to play a role in the determination of the germ cells (Fig. 2 I,J).

ets-1 and ets-2 transcripts are restricted to a circumblastoporal collar during gastrulation

Whereas the early zygotic transcription of both genes appeared ubiquitous, at stage $10^{1/2}$ both transcripts are restricted to a circumblastoporal collar, as illustrated in Figure 3A for *ets-1*. The section of Figure 3B clearly shows that the transcripts are present in the lateral involuting marginal zone, essentially in the superficial layer, and to a lower extent in the inner layer. The cells rolling over the dorsal blastoporal lip appear however not to contain *ets-1* or *ets-2* transcripts, in agreement with the presence of a thin colorless cordon, surrounding the forming blastoporal lip in the embryos of Figure 3A.

ets-1 and ets-2 are expressed in the neural crest cells, at premigratory and migrating stages

As shown in Figure 4A, *ets-1* expression is conspicuous on both sides of the neural plate, as segregated neural crest cell masses on the external side of the elevating neural fold (see Sadaghiani and Thiébaud, 1987) and corresponding from anterior to posterior, to the future mandibular, hyoid and branchial segments (Beaumont *et al.*, 1994). In stage 15 embryos, *ets-1* is also expressed in a thin line at the internal side of the neural fold and later along the dorsal midline of the closing neural tube (see the corresponding labeling

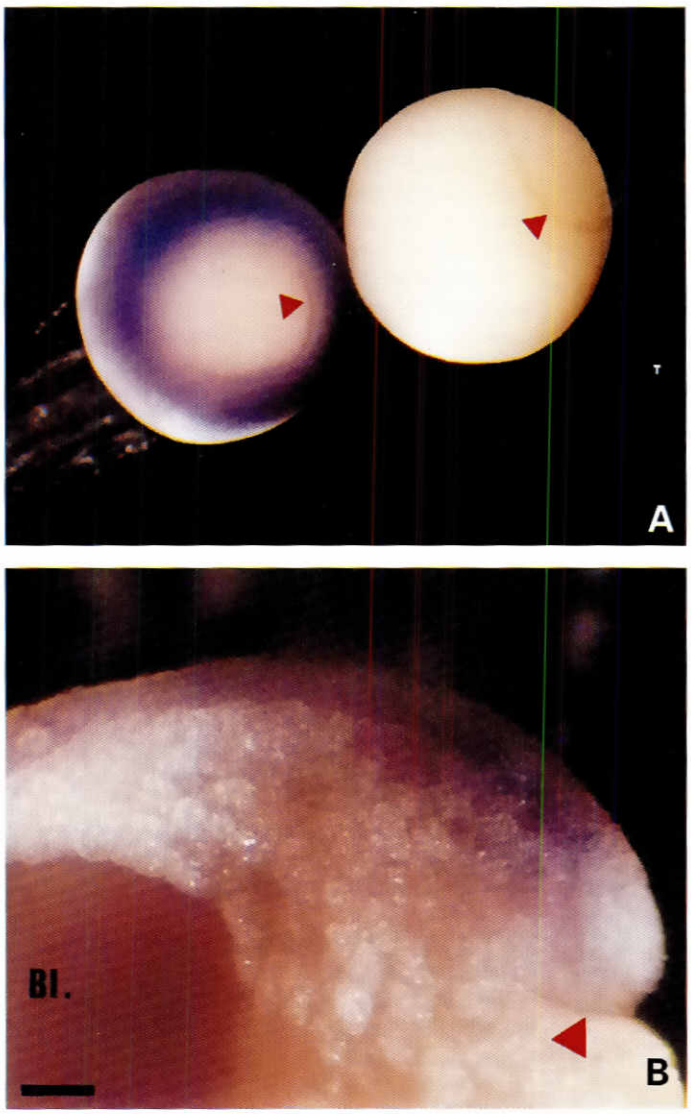

Fig. 3. *Ets-1* transcript localization in *Xenopus laevis* gastrula (stage $10^{1/2}$). (A) *Whole-mount in situ hybridization with antisense and sense probes (left and right embryo respectively). Ets-1 transcripts are observed as a circumblastoporal collar (blue staining in the left embryo), whereas no signal is observed with the sense probe.* **(B)** *Section of a stage $10^{1/2}$ embryo showing the dorso-blastoporal lip (red arrowhead) and the blastocoel (BL). The ets-1 staining is mainly observed in the upper epithelial layer of the marginal zone, and to a lesser extent in the deep marginal zone. However, cells rolling around the dorsoblastoporal lip do not seem to be labeled, in agreement with the absence of staining in a narrow cordon immediately adjacent to the blastoporal lip in the left embryo* **(A)**. *Bar, 100 μm.*

on stage 18 embryos). On stage 18 embryos, a diffuse and faint blue staining is also visible on both sides of the embryo, at the level of the lateral plate mesoderm. The above observations are confirmed by the section of Figure 4B, which clearly shows the labeling of the segregated neural crest cell masses and of the lateral plate mesoderm.

Noteworthy, a faint *ets-1* signal can also be observed in the most anterior region of the neural plate, although the latter is devoid of neural crest cells (Couly and Le Douarin, 1988) and corresponds to the hypophyseal anlage (see Fig. 5A,B and C).

From stage 19/20 *ets1* was detected in the three cephalic neural crest segments (Fig. 5A). The first segment (mandibular crest segment) which originates from the mesencephalon lies dorso-caudally to the eye primordium then moves and curves ventrally to the optic vesicle. The second (hyoid) and the third (branchial) crest segments originate from the rhombencephalon (Sadaghiani and Thiébaud, 1987). At stage 22 cranial neural crest cells migrate around the otic vesicle and descend into the visceral arches which include the mandibular and hyoid arches and the four branchial arches (Fig. 5B,D,E). The observed labeling appears to delineate the rhombomeres, as already described for the *Xslu* gene (Mayor *et al.*, 1995).

Interestingly, at stage 19/20, a faint punctuated hybridization is observed at the border of the pro- and mesencephalon (Fig. 5A, red

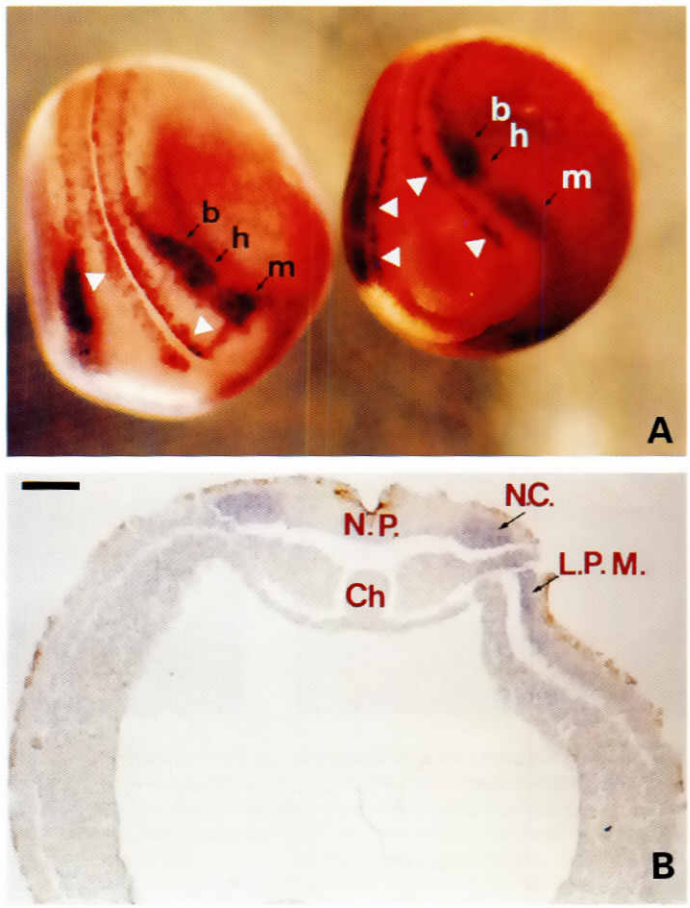

Fig. 4. Whole-mount *in situ* detection of *ets-1* transcripts in *Xenopus laevis* neurula (st. 15/18). (A) *Stage 15 (right embryo) and stage 18 (left embryo). Note the presence of segregated cell masses on the external side of the neural plate, corresponding the mandibular (m), hyoid (h) and branchial (b) segments of neural crest cells. Also note the thin stained line on the internal side of the elevating neural fold (white arrowheads in stage 15 neurula) and the corresponding labeling of the midline in the closing neural tube (white arrowheads in stage 18) embryo.* **(B)** *Horizontal section through a stage 17/18 Xenopus laevis embryo. Due to the curvature of the A/P axis, the section is almost transverse at the level of the neural plate. Segregated neural crest cell masses are conspicuously stained on both sides of the neural plate. A clear staining is also observed in the lateral plate mesoderm. Ch, chord; LPM, lateral plate mesoderm; NC, neural crest; NP, neural plate. Bar, 100 μm.*

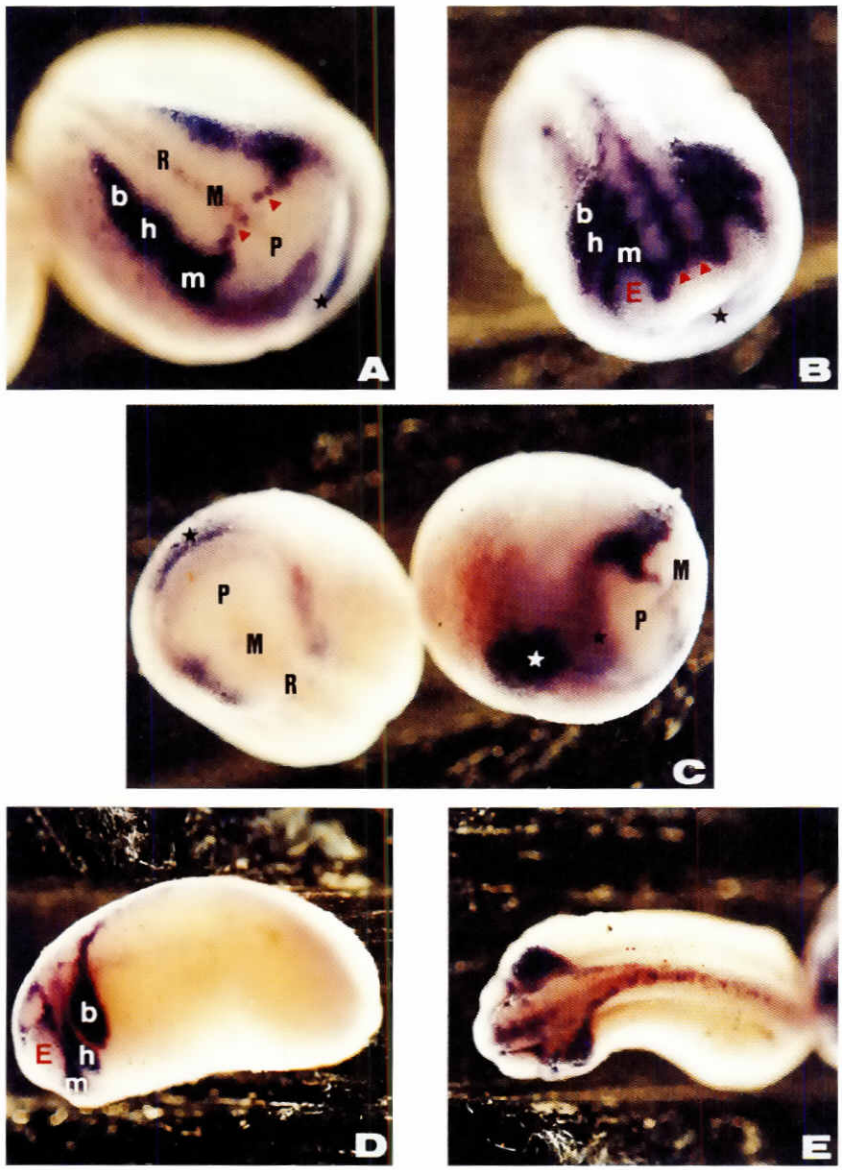

Fig. 5. Whole-mount *in situ* detection of *ets-1* transcripts in pre-migratory and migrating neural crest cells. (A) *Stage 19/20 embryo, antero-dorsal view: the mandibular (m), hyoid (h) and branchial (b) neural crest cell segments are strongly labeled; also note the punctuated label at the level of the border between prosencephalon and mesencephalon (red arrowheads). The black star shows a diffuse faint labeling in front of the most anterior part of the neural tube, normally deprived of neural crest cells and which could correspond to the hypophyseal anlage.* **(B)** *Stage 21/22 embryo, antero-dorsal view: the neural crest cells have now started their migration towards the ventral side of the embryo. The mandibular segment starts to surround the forming eye vesicle (E). Note the increase in the staining at the border between pro- and mesencephalon (red arrowheads), with respect to* **(A)**. *Also notice the strong labeling at the midline of the neural tube.* **(C)** *Stage 19/20 embryo, antero-ventral view (right side embryo) and antero-dorsal view (left side embryo). In addition to the labeling at the periphery of the neural tube already depicted (left side embryo), a strong staining is observed in the ventro-anterior region (white star on the right side embryo), in the putative area for heart and blood island. The faint and diffuse labeling in front of the neural tube is still clearly observed (black star).* **(D,E)** *Stage 22/23 embryos, lateral and dorsal views respectively: the neural crest cells are now populating the mandibular hyoid and branchial arches* **(D)**. *The dorsal view clearly shows a labeling at the level of the vagal and truncal neural crest cells* **(E)**. *b,h,m, branchial, hyoid and mandibular neural crest cell segments or arches; P,M,R, pro-, mes- and rhombencephalon, respectively. E, eye vesicle.*

arrows) which becomes much more intense around stage 22 (Fig. 5B, red arrows). The midline of the neural tube, which was scarcely labeled at stage 19/20 is strongly reactive at stage 22 at the level of mes- and rhombencephalon (Fig. 5B), in agreement with the observation that about stage 25, crest cells reappear on the mesencephalon as a sheet of cells, which will cover the brain to form the meninges (Sadaghiani and Thiébaud, 1987).

By stage 23 (Fig. 5D) mandibular crest cells have encircled the optic vesicle and by stage 26/28, they have become interspersed with cells of the maxillary mesoderm (Sadaghiani and Thiébaud, 1987). From stage 26/27 to 30/31 (Fig. 6A,B), *ets-1* mRNA expression coincided with the migration of mandibular crest cells to ventral position, with hyoid crest cells in the hyoid arch and with branchial crest cells in the anterior and posterior branchial arches. Sections through the mandibular and hyoid arches of animals at stage 26/27 and 29/30 showed that, as reported earlier for the *fli* gene (Meyer *et al.*, 1995), *ets-1* expressing cells formed a ring around the mandibular and hyoid visceral arch; they originated from neuroectoderm. A labeling was observed in the mesoderm of branchial arches. No signal was observed in cells from endoderm origin. From stages 33/34 to 37/38, only the mesoderm lineage derived-cells of visceral arches showed hybridization.

The expression pattern of the *Xl-ets-2* gene was found identical to the one of *ets-1*, with a somewhat lower intensity.

ets-1 and ets-2 are expressed in the cranial and dorsal-root ganglia

From stage 26/27 to 35/36, both *ets-1* and *ets-2* were found expressed in cranial sensory ganglia. These are the ganglion of nerve V (trigeminal) with its ophthalmic and maxillary-mandibular branches and the acoustico-facialis ganglion (nerves VII and VIII) located in front of the ear (Fig. 6A,B). In stage 35/38, the ganglionary vagus complex (glossopharyngeal IX and vague X), just posterior to the ear was also labeled (Fig. 6C). This was obvious on whole-mount *in situ* hybridizations and on sections of embryos hybridized to antisense *ets-1* and *ets-2* probes (Fig. 6F,G).

A strong expression is also observed in the vagal and trunk neural crest cells (Fig. 6A,B). The punctuated pattern observed is evocative of the future dorsal root ganglia (Fig. 6D and E).

From stage 26/27, a clear *ets-2* signal was also visible in the lens, which originates as a placodal thickening of the head ectoderm (see Fig. 6B). This labeling disappeared in stage 37/38 embryos (Fig. 6C).

ets-1 is expressed in the ventral blood islands and forming heart and blood vessels

In stages 26/27, *ets-1* transcripts were observed in the heart presumptive area, as well as in

angioblastic blood islands situated in the ventral region of embryos (Figs. 5C, 6A). These angioblastic islands are the precursors of blood cells which are enclosed by peripheral endothelial cells. The first step in the formation of embryonic vessels is the emergence of some endothelial cells within the mesodermal layer, and thereafter these cells organize into tubular structures. In stages 29/30 when the embryonic heart is formed, *ets-1* signal is no longer detectable. From stage 28/30 to stage 38, *ets-1* was expressed in other developing vascular structures. From stage 33/34 to 38, the signal was mainly evident in the vascular structure of the eye (hyaloid artery, see Fig. 6A), in dorsal aorta, in aortic arches (Fig. 7A), in intersomitic arteries (Fig. 7B) and in vitelline vessels. Frontal sections through the arch region showed a weak signal in their central portions suggesting that *ets-1* is associated with endothelial cells within the mesodermal layer where capillaries and blood vessels are developing. Expression of *ets-1* in embryonic vascular structures appeared during their exponential growth as a transitory state; no signal was observed in later stages when vessels are maturated.

This expression in the forming heart and vascular system appears to be restricted to the *ets-1* gene, as no convincing signal could be observed for *ets-2* in the same regions.

ets-1 and ets-2 are expressed in the dorsal fin and posterior end of the embryo

From stage 29/30, both *ets-1* and *ets-2* mRNAs were found expressed in dorsal fin originating from trunk neural crests (Fig. 7C) (Sadaghiani and Thiébaud, 1987; Collazo *et al.*, 1993). The mesenchyme of developing caudal extremity was also labeled (Fig. 7D).

ets-2 is expressed in the forming pronephric tubules and pronephric duct

As shown in Figure 8A, *ets-2* is strongly expressed in the pronephric tubules and in the growing pronephric duct (WD), from stage 26/27 to 36/37. This expression is reminiscent of that of the *fli* gene (Meyer *et al.*, 1995), but occurs later, when the pronephros and pronephric duct (PND) are actually forming. The difference is emphasized by the observation that *fli* is expressed in the dorso-lateral plate mesoderm as a diffuse band (Meyer *et al.*, 1995) at the base of the somites, whereas *ets-2* gives a strong signal in the epithelia of the pronephric tubules and duct (Fig. 8B and C). Under the same conditions, whole-mount hybridizations revealed only a faint expression of *ets-1* in the above structures (data not shown).

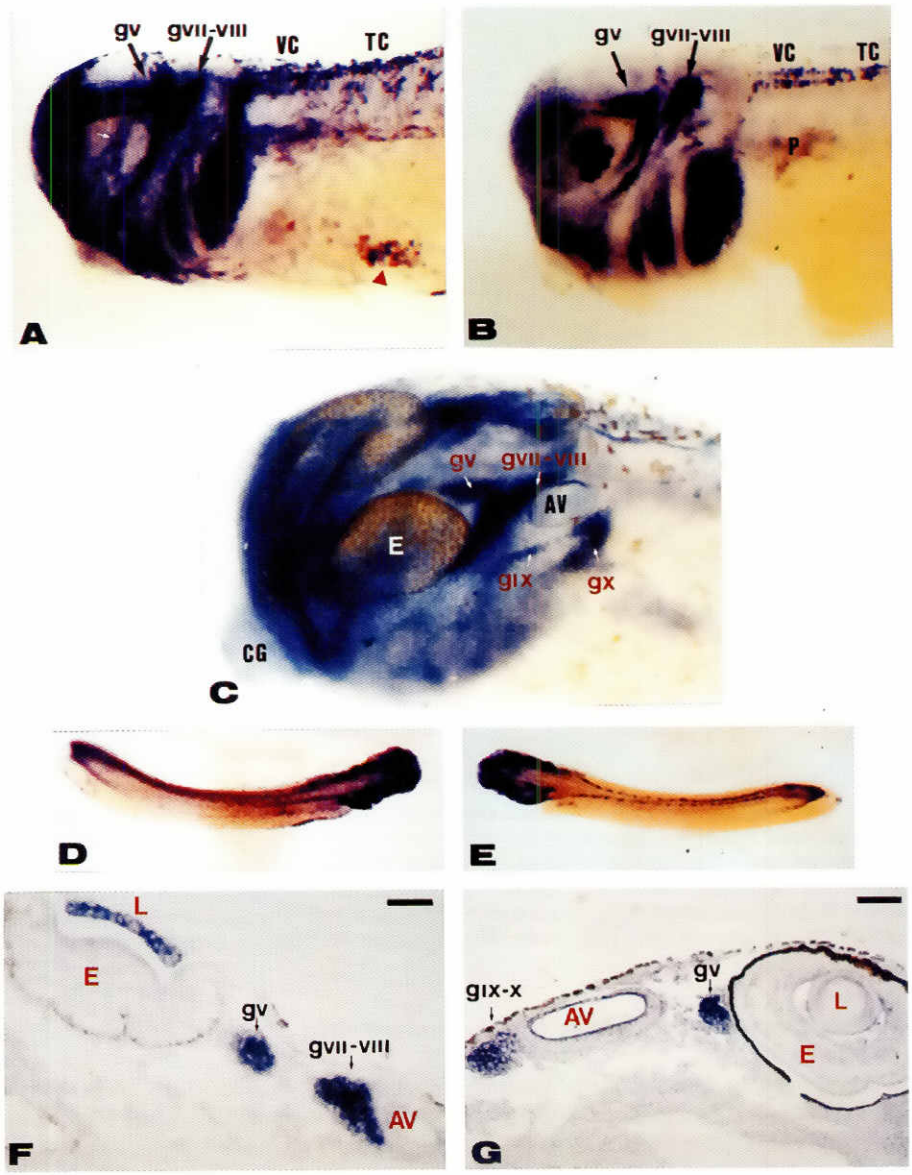

Fig. 6. *Ets-1* and *ets-2* are expressed in the cranial, vagal, and dorsal root ganglia. (A) *Whole-mount hybridization with an ets-1 probe on stage 29/30 embryos showing a conspicuous expression of ets-1 in the cranial ganglia and in the vagal and truncal neural crest cells (respectively VC and TC). Also note the staining at the level of the condensing pronephros, in the hemangioblast precursors, in the ventral region (red arrowhead), and in the forming hyaloid artery (white arrow).* **(B)** *Whole-mount hybridization with an ets-2 probe on stage 29/30 embryos showing the same expression in cranial ganglia, vagal, and truncal neural crest cells. The punctuated signal in the trunk neural crests is strongly evocative of the future dorsal-root ganglia (DRG). Also note the presence of ets-2 transcripts at the level of the forming pronephros rudiment and in the forming lens. At that stage, ets-1 is not expressed in the forming lens* **(A)**. **(C)** *Whole-mount hybridization with an ets-2 probe on a stage 36/37 embryo, showing that in addition to gV and gVII-VIII, the vagal complex (gIX and X) is now labeled.* **(D,E)** *Whole-mount hybridization on stage 29/30 embryos (dorsal view) with ets-1 and ets-2 probes respectively, showing the conspicuous expression at the level of the future DRGs.* **(F,G)** *Horizontal sections of whole-mount hybridizations with an ets-2 probe, on stage 29/30 and 36/37 embryos respectively, showing the intense labeling of the cranial ganglia and vagal complex. Note that the lens, which is labeled at stage 29/30, is no longer stained at stage 36/37. In (A to E) embryos were cleared in Murray's reagent. AV, auditory vesicle; CG, cement gland; E, eye; gV, fifth cranial ganglion (trigeminal or gasserian); gVII-VIII, seventh and eighth cranial ganglia (geniculate and auditory); gIX, ninth cranial ganglion (glossopharyngeal); gX, tenth cranial ganglion (vagus or pneumogastric); L, lens; P, pronephros. Bars, 100 μm.*

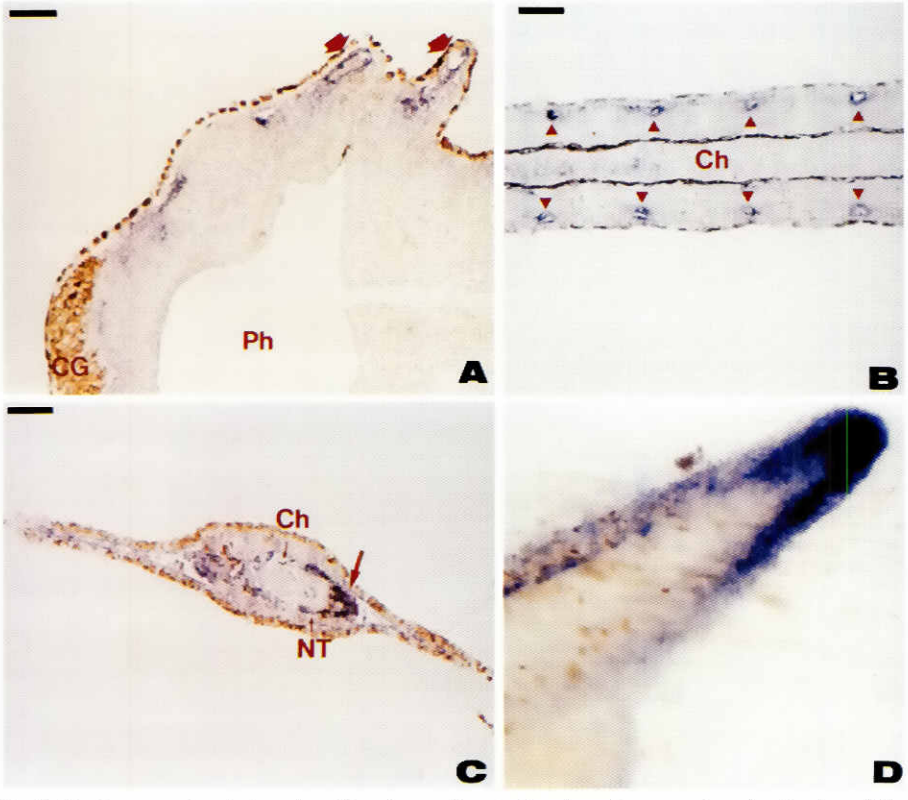

Fig. 7. Ets-1 expression in forming blood vessels; *ets-1* and *ets-2* expressions in the dorsal fin and posterior end of the embryo. (A,B) *Horizontal sections of stage 39/40 embryos showing the expression of ets-1 in forming blood vessels: aortic arches (large arrows in A) and intersomitic arteries (small red arrowheads in B).* **(C,D)** *Presence of ets-2 transcripts in the dorsal fin (large red arrow in the coronal section of C) and in the tip of the tail of a whole-mount stained embryo (stage 35/36)* **(D)**. *CG, cement gland; Ch, chord; NT, neural tube; Ph, pharynx. Bars, 100 μm.*

Expression of ets-1 and ets-2 in late developmental stages and in adults

No signal was detected using ^{35}S-labeled antisense *ets-1* probe during late larval development. *Ets-2* mRNA was found expressed at stages 56/57, 60 and 62 in the different portions of digestive tract: stomach, anterior part of small intestine, intestine and rectum. All these organs were labeled in their muscular layer and more slightly in their epithelia (Fig. 9). Control hybridizations with sense probe gave no signal above background (Figs. 9 and 10).

Throughout these stages of metamorphosis, the digestive tract undergoes histolysis in its epithelial portion only, whereas both the muscular and connective layers are growing. Thus, *ets-2* mRNA was expressed in proliferating tissues. It was also found in epithelial cells of adult intestine which are constantly being replaced.

Notable levels of *ets-2* transcripts were observed in the differentiating tubules of kidney from stage 45 through adult (Fig. 10). *Ets-2* mRNA was found in muscle and spinal ganglion situated at the level of developing forelimb bud from stage 56/57 to stage 62 (Fig. 10).

Discussion

In the present study we have shown that both *ets-1* and *ets-2* genes are expressed throughout the developmental stages of *Xenopus laevis*. In several tissues, their expression domains overlap but sometimes with different temporal patterns; each gene is also expressed at unique sites. These conclusions are similar to earlier reports of Maroulakou *et al.* (1994).

As we and others reported earlier (Chen *et al.*, 1990; Stiegler *et al.*, 1990, 1993; Wolff *et al.*, 1990, 1992), both *ets-1* and *ets-2* genes are transcribed maternally in *X. laevis*. Their transcripts are found in the ovary of the adult female and in developing oogonia of immature gonad in the stage 62 female. We could also detect *ets-1* and *ets-2* transcripts in the most vegetal part of the cleavage embryo, at a location reminiscent of the germ plasm. This observation would be consistent with the expression of these two genes in the germinal lineage. This is in contrast with the report of Rowe and Propst (1992) who showed by *in situ* hybridization that in the adult mouse ovary, *ets-1* and *ets-2* are predominantly expressed in cells of the theca interna and ovarium interstitium, but not in oocytes. However, in a more recent work, Maroulakou *et al.* (1994) reported an expression of *ets-2* in the developing genital ridge primordia, but did not detect *ets-1* transcripts in the same region. Such discrepancies can be due to differences in the sensitivity of the technique used and/or to variations in the level of transcripts. They could also reflect different reproduction processes between mammalians and lower vertebrates. An alternative explanation would be that although ETS-1 and ETS-2 proteins are transcriptional regulators, they serve another function in *Xenopus* early embryogenesis. Indeed, the zygotic transcription only starts after the 12th division in *Xenopus* embryo, whereas it begins at the 2-cell stage in the mouse embryo. ETS-1 and ETS-2 could be required during the rapid cleavage period in *Xenopus* embryos and should therefore be encoded by maternal mRNAs. It has already been shown that the ETS-2 protein was indispensable for the meiotic maturation in *Xenopus* (Chen *et al.*, 1990).

Our unexpected finding using whole-mount hybridization with digoxigenin labeled probes is that *ets-1* and *ets-2* transcripts are evenly distributed in the oocyte only up to stage IV and are localized to the animal pole of late stage oocytes. In the shed egg and early embryos, they even appear confined to the subcortical region of the animal pole. These results do not arise from a difficult penetration of the antidigoxigenin antibodies in the oocyte or embryo, as the same pictures were obtained using oocytes or embryos cut into halves prior to the hybridization reaction. Furthermore, the semi-quantitative analysis by RT/PCR of *ets-1* and *ets-2* mRNA levels in animal and vegetal caps as well as in the intermediate region confirms the polarized distribution of the transcripts. Noteworthy, a very similar localization was reported for the mRNA encoding the β-subunit of an oligotrimeric GTP-binding protein (Devic *et al.*, 1996). The localization of *ets-1* and *ets-2* transcripts at the animal pole of the early embryo would

plead for a major role of ETS-1 and ETS-2 proteins in embryonic development, as important morphogenic information was shown to be confined to the animal quartets by the third cleavage plane (Chung *et al.*, 1994). However, *ets-1* and *ets-2* transcripts are also observed in the upper region of the vegetal blastomere, in agreement with the later expression of both genes in mesodermal derivatives.

The localization of maternal *ets-1* and *ets-2* transcripts essentially to animal blastomeres should also be considered in the light of earlier observations of Grunz (1977, 1994) and Li *et al.* (1996), showing that isolated dorsal animal blastomeres at the 8-cell phase are capable of forming mesodermal derivatives. The authors pointed that the fate of animal blastomeres could be dependent on the presence of localized maternal factors (including transcription factors). The observation that *ets-1* and *ets-2* maternal transcripts are restricted to the subcortical layer of the shed egg and appear to be shifted to the dorsal intermediate zone by cortical rotation could fit with this idea. Alternatively, ETS-1 and ETS-2 transcription factors could be involved in maintaining the competence of animal blastomeres to respond to the inductive signal emanating from the vegetal blastomeres. A role for *ets-1* in the response to an external signal inducing mesodermal cells in a differentiation pathway, has already been proposed by Vandenbunder *et al.* (1989).

The zygotic transcription of *ets-1* and *ets-2* genes was observed immediately after the MBT. It appears to be ubiquitous. However, transcription of both genes is subsequently restricted to a circumblastoporal region. Such a behavior is reminiscent of the one observed for the *MyoD* genes in *Xenopus*, which are ubiquitously expressed at the onset of zygotic transcription, but are later restricted to the presumptive mesoderm (Rupp and Weintraub, 1991). Similarly, *Xbra* is expressed in the entire marginal zone, as an immediate early response to mesoderm induction, but is later restricted to a circumblastoporal ring (Green *et al.*, 1992).

Later, *ets-1* and *ets-2* are expressed in cephalic neural crests, which are a principal source of mesenchyme in the craniofacial and visceral arch regions of all vertebrates (Sadaghiani and Thiébaud, 1987; Eisen and Weston, 1993; Le Douarin *et al.*, 1993) as well as in the vagal and truncal neural crest cells. It should be noted that *ets-1* is also expressed in front of the most anterior part of the neural plate, which has been shown to be devoid of neural crest cells. This signal could be due to head mesenchyme. But it should be kept in mind that the cells from the most anterior part of the neural fold contribute to the hypophysis (Couly and Le Douarin, 1988), where the Xl-*fli* gene was shown to be expressed (Meyer *et al.*, 1995). Ho *et al.* (1994) have shown that in *Xenopus* embryos cephalic neural crest cells migrate into the arch region and encase the arch mesoderm. Similarly, the Xl-*fli* gene was found expressed in a narrow band of cells of neuroectodermal origin surrounding the mesoderm compartment in the branchial arches (Meyer *et al.*, 1995). Our study demonstrates that *ets-1* and *ets-2* are expressed in visceral arches. Moreover, cephalic neural crests contribute extensively to the cranial nerves and ganglia (Couly *et al.*, 1993). In the *Xenopus* embryo, the cells of mandibular crest segment contribute to the formation of both profondus and gasserian branches of ganglion V, and the anterior part of the geniculate ganglion VII; the acoustico-facial ganglionic complex is made up of cells originating from hyoid crest segment and placode (Sadaghiani and Thiébaud, 1987). In the present work, we show that both *ets-1* and *ets-2* transcripts are located in specific nerve structures arising from cephalic vagal, and truncal neural crests. *Ets-1* and *ets-2* therefore exhibit an expression pattern somewhat complementary to the *fli* gene, which was shown to be expressed in the quail, in a subset of neural crest cells giving rise to mesenchyme, at the exclusion of a subpopulation of neurogenic precursors (A. Grapin and P. Remy, in press).

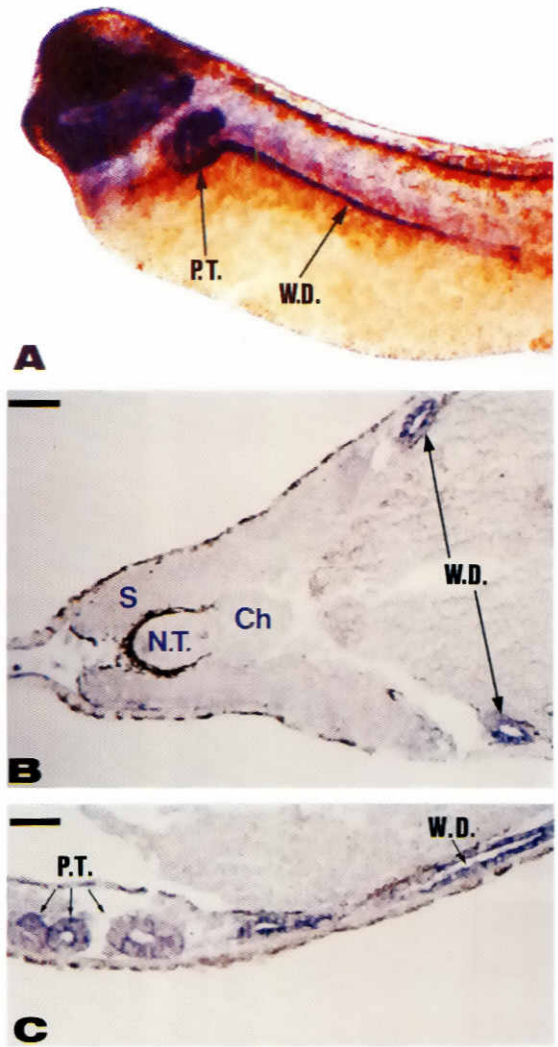

Fig. 8. Ets-2 expression in the pronephric tubules and growing pronephric duct. (A) *Whole-mount in situ hybridization with an ets-2 specific probe, on stage 37/38 embryo. The pronephric tubules (PT) and the Wolffian duct (WD) are strongly labeled.* **(B)** *Transverse section of a stage 37/38 embryo after whole-mount hybridization, showing the strong labeling of the epithelial structure of the Wolffian duct. Dorsal is to the left.* **(C)** *Horizontal section of a stage 37/38 embryo after whole-mount hybridization, showing the labeling of the pronephric tubules and Wolffian duct. Anterior is to the left. Bars, 100 μm. Ch, notochord; NT, neural tube; PT, pronephric tubule; S, somite; WD, Wolffian duct.*

Ets-1 is also actively expressed in the hemangioblastic lineage, as described earlier in the chicken (Pardanaud and Dieterlen-Lièvre, 1993), mouse (Maroulakou *et al.*, 1994) and in forming vessels, as described in humans under normal or pathological conditions (Wernert *et al.*, 1992a). A similar expression had al-

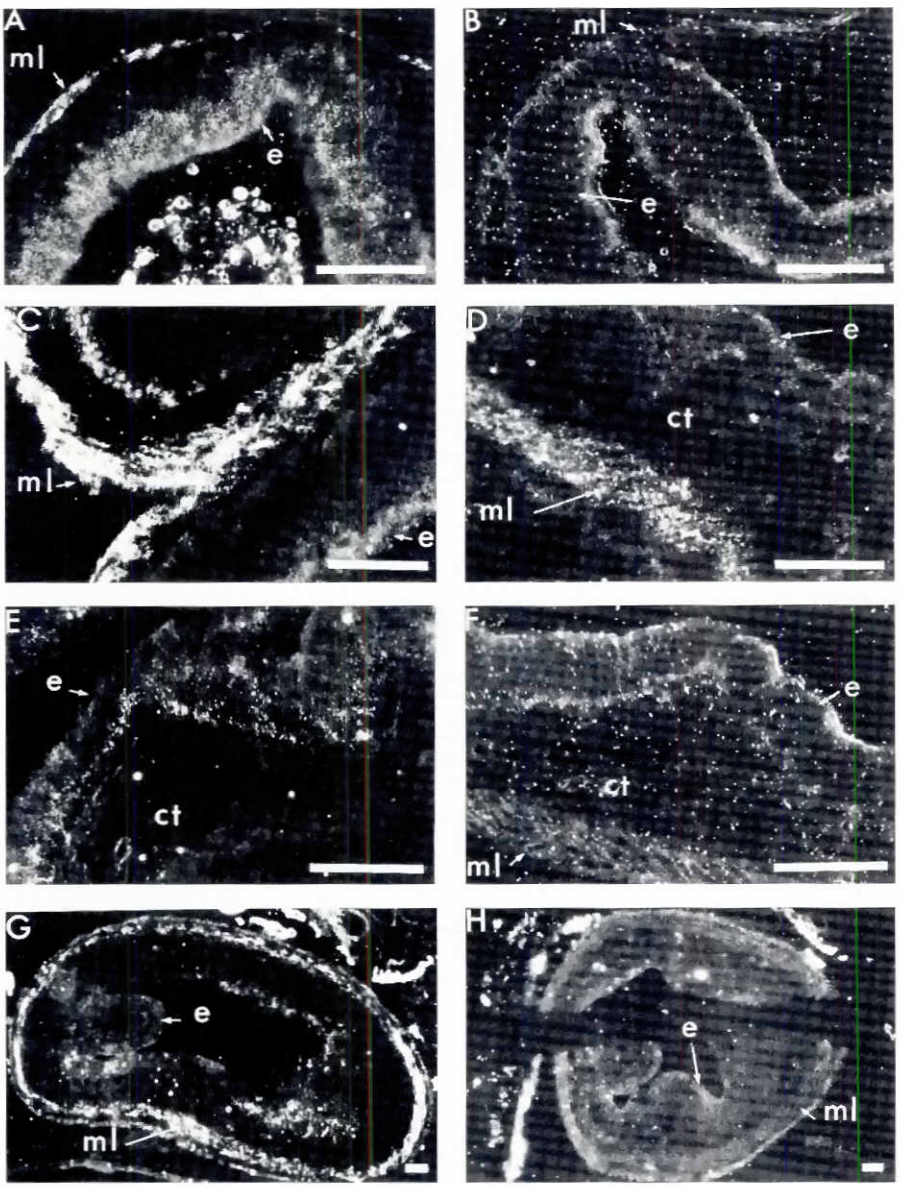

Fig. 9. ***In situ*** **hybridization on sagittal sections of** ***Xenopus*** **gut from late developmental stages by using ^{35}S-labeled** ***ets-2*** **probes. (A,C,D,E,G)** *Antisense probe.* **(B,F,H)** *Control experiments using a sense probe.* **(A,B,C)** *Anterior part of small intestine from stage 60 animal.* **(D)** *Stomach of stage 62 Xenopus.* **(E,F)** *Pyloric portion of stomach from stage 62 tadpole.* **(G,H)** *Rectum of stage 60 animal. Ets-2 transcripts are located in both epithelial and muscular layers. e, epithelium; ml, muscular layer; ct, connective tissue. Bar, 40 μm.*

ready been observed for the Xl-*fli* gene (Meyer *et al.*, 1995) in contrast to the *ets-2* gene, which does not appear to be expressed in the hemangioblastic lineage.

Ets-2 is specifically expressed at high levels in the forming pronephric tubules and pronephric duct, whereas *ets-1* is only detected at low levels in these structures. *Ets-1* and *ets-2* expressions were already reported in the differentiating kidney in the mouse embryo (Maroulakou *et al.*, 1994).

The nature of the signal conveyed by *ets-1* or *ets-2* therefore appears to be essential to development. We have observed that neural crest cells produce *ets-1* and *ets-2* when their migration is starting (stage 19-20), suggesting that their products may facilitate their movement away from the neural tube, possibly by stimulating detachment or migration.

Ets-1 and *ets-2* transcripts are therefore found in regions of the embryo which undergo important structural reorganization, accompanied by cell migrations. A similar observation had already been reported for the Xl-*fli* gene (Meyer *et al.*, 1995). These observations and the effect of *fli* overexpression (Remy *et al.*, 1996), had led us to the hypothesis that members of the *ets*-family could play coordinate roles (cooperation or competition) in the transcriptional control of genes involved in cellular adhesion and/or migratory cell guidance, as already suggested for matrix metalloproteinases by Buttice *et al.* (1996). The results reported here for *ets-1* and *ets-2* genes further support this hypothesis. Some of us indeed showed that the migrations of neural crest cells and pronephric duct cells at least are deeply altered in embryos where Xl-*fli* has been overexpressed (D. Meyer and P. Remy, unpublished results). Such transcriptional control could affect molecules belonging to the integrin and cadherin families, as well as constituents of the extracellular matrix (ECM). For instance, earlier results of our laboratory support a modification of integrins expression upon injection of Xl-*fli* synthetic transcripts in the *Xenopus* fertilized egg and cleavage embryo. Indeed, we reported that it results in the simultaneous absence of mature erythrocytes in the circulating blood at stage 45 and presence of fully differentiated erythrocytes in ectopic vascular structures, not connected to the vascular system. Such a phenotype could be due to a perturbation of β1-integrin subunit expression, as it was shown that in mouse embryos lacking β1-integrin the migration but not the differentiation of erythrocyte progenitors is impaired (Hirsch *et al.*, 1996). A putative control of integrin expression is further supported by recent results of our laboratory, showing that Xl-*fli* overexpression also results in a massive engagement of affected cells in the apoptotic pathway (M. Berardi, unpublished results), in line with many reports suggesting that the failure of the integrins repertory to adapt to the surrounding ECM could result in the triggering of apoptosis (Brooks *et al.*, 1994; Hruska, 1994; Montgomery *et al.*, 1994; Ruoslathi and Reed, 1994). Similarly, we observed that Xl-*fli* overexpression during embryogenesis results in numerous ectodermal blisters (Remy *et al.*, 1996). This could be due to a modification of tenascin expression, as no protein is detected at the level of the blisters whereas neighboring regions appear to express tenascin normally (F. Goltzene, unpublished results).

Furthermore, both *ets-1* and *ets-2* transcripts are found in dorsal fin mesenchymal parts, in agreement with the observations of Collazo *et al.* (1993). They are also observed in the elongating tailbud. A similar observation was reported for the mouse by Maroulakou *et al.* (1994). This could be correlated to the circumferential migration of the neural crest cells. They form an arc from the dorsal to ventral region as reported by Collazo *et al.* (1993). But it should also be kept in mind that this region has been postulated to act as an organizer for the development of the posterior end of the embryo (Gont *et al.*, 1993).

Of course, a function of *ets-1* and *ets-2* genes in regulating cellular adhesion would not be exclusive of a role in the modulation of the expression of proteases specific of the extracellular matrix, as already described (Wernert *et al.*, 1992b; Borchers *et al.*, 1994; Vandenbunder *et al.*, 1994; Calmels *et al.*, 1995).

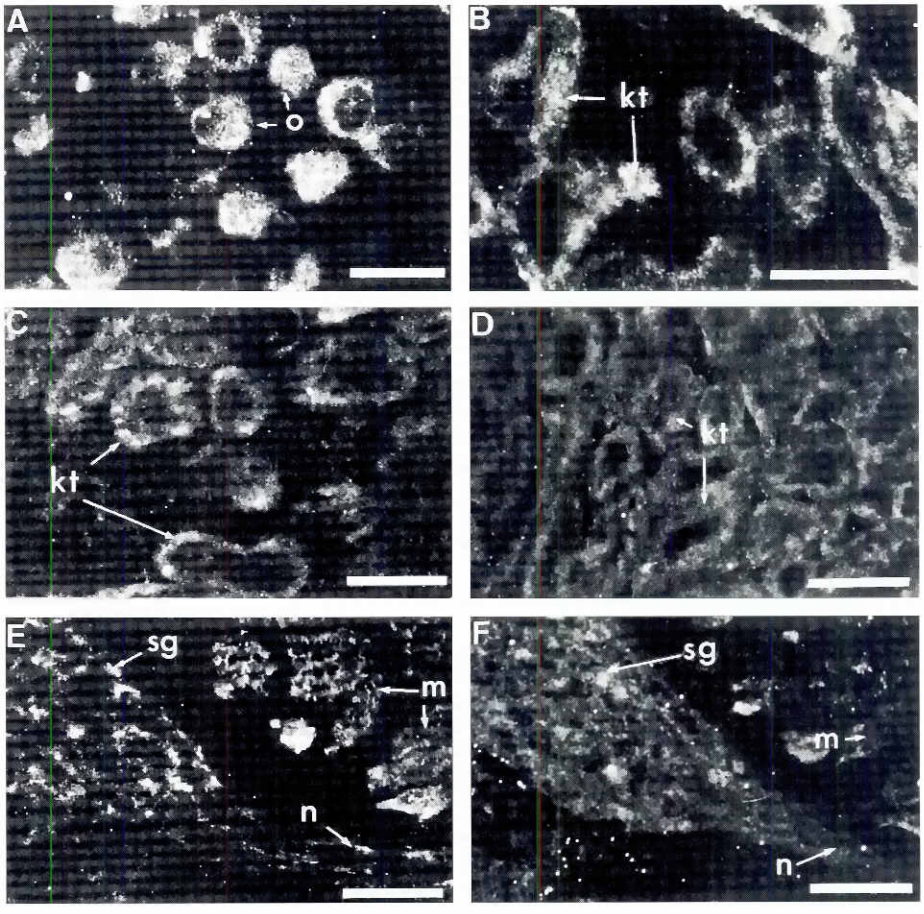

Fig. 10. *In situ* hybridization on sagittal sections of immature ovary and in *Xenopus* adult organs by using ^{35}S-labeled *ets-2* probes. (A,B,C,E) *Antisense probe;* **(D,F)** *control experiments with sense probe.* **(A)** *Ets-2 transcripts are detected in oogonia included in ovary of stage 62 female.* **(B)** *Stage 60 tadpole showing the presence of ets-2 transcripts in the kidney tubules.* **(C,D)** *Ets-2 transcripts are detected in adult kidney.* **(E,F)** *Ets-2 is expressed in muscles and in spinal ganglia at the level of developing forelimb bud in stage 56/57 tadpoles. o, oogonium; kt, kidney tubules; sg, spinal ganglion; m, muscle; n, nerve. Bar, 40 μm.*

Materials and Methods

Animal collection

Eggs obtained from gonadotropin-injected *Xenopus* were fertilized *in vitro* (Moon and Christian, 1989) dejellied manually and cultured in 0.1x Marc's modified Ringer's solution (MMR). Embryo and larval stages were staged according to Nieuwkoop and Faber (1967). *Ets-1* and *ets-2* cDNA sequences are available in GenEMBL databank under the accession numbers X52691/X52692 and X51826/X52635, respectively.

Ets-probes

Two different *ets-2* probes were used: a fragment of 1.1 kb (clone 1121) extending from nucleotide 463 to nucleotide 1589 and containing the Ets-domain, and a fragment of 468 bp (clone 211a) extending from nucleotide 1 to nucleotide 468 lacking the Ets-domain (Wolff *et al.*, 1991). The two *ets-1* probes were: a fragment of 702 bp (clone 112a) extending from nucleotide 1 to nucleotide 702, lacking the Ets-domain, and a fragment of 1237 bp (clone 1522) extending from nucleotide 703 to nucleotide 1940 and containing the Ets-domain (Stiegler *et al.*, 1990). Both types of probes gave similar results for the two genes, showing that even the most conserved regions did not give rise to non-specific detection of the closely related transcripts.

Northern blot analyses

To study the temporal and tissue-specific pattern of *ets-1* and *ets-2* expressions during *Xenopus laevis* development, riboprobes specific for each of these genes were used. To ensure that the probes were indeed specific for their respective genes, Northern blot analyses were performed on *ets-1* and *ets-2* RNAs, transcribed *in vitro*, separated by electrophoresis on agarose gels and blotted onto nitrocellulose. ^{35}S-labeled antisense riboprobes hybridized only with their respective homologous sense RNA (data not shown).

Northern blotting using poly (A)$^+$ mRNAs extracted from embryos up to stage 45, revealed the presence of *ets-1* and *ets-2* transcripts in variable amounts, as reported earlier by Wolff *et al.* (1991) and Stiegler *et al.* (1993) (not shown).

RT/PCR

RT/PCR was carried out on total RNA extracted from animal caps, intermediate zone and vegetal caps according to Lemeur *et al.* (1981). Reverse transcription of *ets-1* and *ets-2* messages was done with the antisense primers TGAGGCGGTCAC *(ets-1)* and GATGTCTTGTGG *(ets-2)*. Controls on 18S rRNA and Xl-*Twi* mRNA were done using the antisense primers CCTTCCTTGGATGTGGTAGCC and GGCTCCC-TGCTGTATAGACTGAGTGTCCCA respectively. The reaction was carried out in a final volume of 20 μl containing 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM dNTPs. The mixture was heated up to 90°C for 2 min and cooled in ice. 200 units of M-MLV reverse transcriptase (Gibco BRL) were added and the reaction mixture was incubated for 10 min at room temperature and 1 h at 42°C. PCR was carried out on 1/10 of the reaction mix using TaqDNA polymerase (Promega) under the conditions prescribed by the manufacturer. The sense primers used were: GTTCTGTATGAGCGGAGC *(ets-1)*, CTGATCCGTTGCAGTGTG *(ets-2)*, GATACATTCGGATGTCTGCC (18S rRNA), CAGGTCTTACAGAGCGACGAGCTGGACTCC (Xl-*Twi*). The antisense primers used were: CATGTTGTCTGGAGTCAC *(ets-1)*, TCCAACAAGAACTGCCAC *(ets-2)*, and the antisense primers used for the RT for the 18S rRNA and the Xl-*Twi* controls. Usually, amplifications were obtained after 27 cycles for *ets-1*, 23 cycles for *ets-2*, 3 cycles for

18S rRNA and 30 cycles for Xl-*Twi*. Amplification products were analyzed by electrophoresis on agarose gel under standard conditions, in the presence of ethidium bromide. Southern blotting for the quantitative analysis of the amplification products was carried out as described in Ausubel *et al.* (1987), using ^{32}P labeled probes. Radioactivity was measured by image-plate analysis.

In situ hybridization

In whole-mount: embryos were collected at different developmental stages. For early embryos, the vitelline membrane was removed by manual dissection. Embryos were then fixed in MEMFA (0.1 M MOPS buffer pH 7.4, 2 mM EGTA, 1.5 mM $MgSO_4$, 3.7% formaldehyde) for 2-6 h at 4°C and processed for whole-mount *in situ* hybridization using digoxigenin-UTP labeled antisense and sense probes as described by Harland (1991). Hybridization was detected with digoxigenin antibody coupled to alkaline phosphatase (Boehringer Mannheim). For the synthesis of *ets-2* probes we used mainly the 1.1 kb fragment (clone 1121). The alkaline phosphatase color reaction was allowed to run for 18 h. Embryos were then post-fixed in MEMFA for 4 h and transferred to methanol. For sectioning, embryos were dehydrated through an ascending ethanol series (70%, 95%, 100%, 10 min each) then transferred to butanol for 24-48 h. Embryos were embedded in Paraplast and sectioned (8-10 μm slides).

On sections: larvae from stages 45 to 62 were frozen in isopentan at -180°C (liquid nitrogen) then fixed in ethanol at -80°C and stored at -80°C. After thawing, the samples were transferred to butanol, embedded in Paraplast and sectioned (7 μm slides). *Ets-1* (702 bp) and *ets-2* (clone 211a, 468 bp) cDNAs were used to synthesize antisense and sense radioactive RNA probes by incorporation of 5' [$\alpha^{35}S$] UTP (400 Ci/mmole, Amersham). Specific activity: 5.10^8 dpm/μg. After deparaffinization and rehydration, samples were post-fixed 20 min with 4% paraformaldehyde in PBS, pH 7.4, washed twice with PBS, and then treated 15 min at 37°C with proteinase K (0.5 μg/ml). The slides were treated twice for 5 min at room temperature with acetic anhydride in triethanolamine buffer 0.1 M, pH 8, washed twice with PBS, and then post-fixed 10 min with 4% paraformaldehyde and washed again. After washing in 2xSSC, prehybridization was carried out for 2 h at 55°C in a solution containing 50% deionized formamide, 0.3 M NaCl, 10 mM TrisHCl pH 7.5, 5 mM EDTA, 1xDenhardt's solution, 10% dextran sulphate, 10 mM DTT, 100 μg/ml yeast tRNA, 0.1% SDS and heated at 80°C for 5 min. Hybridization was performed overnight at 55°C with a probe concentration of 600 pg/ml. Thereafter, slides were washed twice for 60 min at 60°C in 2xSSC (standard saline citrate), 10 mM DTT, then transferred for 60 min at 60°C, in 50% formamide in 2xSSC containing 5 mM EDTA, 10 mM DTT and then treated with RNAse A (20 $\mu g/ml^{-1}$) for 30 min at 37°C. Sections were washed twice for 60 min at 65°C in 2xSSC, 10 mM DTT, then twice for 60 min at 65°C in 0.1xSSC, 10 mM DTT. After dehydration in alcohol containing 300 mM ammonium acetate, slides were coated with Amersham LM1 emulsion (1/1 dilution in distilled water) and stored in the dark at 4°C. Slides were developed with Kodak D19 after 1-2 month exposure and then fixed with Ilford Hypam. Slides were counterstained using 0.1% toluidine blue and mounted in Eukitt (Prolabo). Sections were examined using bright and darkfield optics.

Acknowledgments

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