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Original Article

Whole-mount *in situ* hybridization reveals the expression of the Xl-*Fli* gene in several lineages of migrating cells in *Xenopus* embryos

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ABSTRACT The expression of the Xl-*Fli* gene, which belongs to the *ets* family of transcription factors, was studied by whole-mount *in situ* hybridization during *Xenopus* embryogenesis. Digoxigenin-labeled antisense RNA probes were synthesized by *in vitro* transcription and used in the hybridization reaction. In addition to expression in territories invaded by neural crest cells reported earlier (Meyer *et al.*, 1993), we observed Xl-*Fli* gene expression in a number of regions affected by important cellular migrations and/or epithelium ↔ mesenchyme transitions: in the endothelial cells of the heart, in blood vessels, along the pronephric duct migration pathway and at the level of hypophysis. The possibility that the FLI protein is involved in the expression of guidance cues and/or modification of the cellular adhesion properties is discussed. A screening of a promoter library with a consensus sequence, bound by the FLI protein with a high affinity, revealed the presence of putative FLI response elements in a number of genes encoding adhesion molecules or components of the extra-cellular matrix.

KEY WORDS: *cranial neural crest, pronephric duct, blood islands, hypophysis, FLI binding sites*

Introduction

The Xl-*Fli* gene, a member of the *ets* family of transcription factors, is expressed during *Xenopus laevis* embryogenesis. Our earlier studies showed that contrary to other members of the family, like *ets*-1 or *ets*-2 which are maternally expressed (Wolff *et al.*, 1991; Stiegler *et al.*, 1993), *Fli* is only transcribed from neurulation to late stages of embryogenesis. The expression pattern was found to be consistent with the map of territories invaded by neural crest cells (Meyer *et al.*, 1993). In the adult, the *Fli* gene exhibits an expression pattern intermediate between the highly restricted pattern of *ets*-1 and the almost ubiquitous pattern of *ets*-2 (Meyer *et al.*, 1993). The involvement of an anomalous transcript of the *Fli* gene in the Ewing's sarcoma (Delattre *et al.*, 1992), as well as the restricted expression pattern of the *Fli* gene during embryogenesis, prompted us to investigate in more detail the embryonic area expressing this gene. Whole-mount *in situ* hybridization, using digoxigenin-labeled probes reveals embryonic expression in territories where important cellular migrations occur: the *Fli* gene is expressed in the migrating cranial neural crest cells, along the migration pathway of Wolffian duct cells, in heart endothelial cells and at the level of the hypophysis. Our results on the expression pattern of the *Fli* gene are consistent with the effects observed when overexpressing it in the course of embryogenesis (P. Remy, unpublished results) and could reflect a role of the gene product in controlling the modification of cellular adhesion promoting cell migration. Such a hypothesis is supported by the observation that the promoter regions of several genes encoding adhesion molecules or components of the extracellular matrix contain sequences efficiently recognized by the FLI protein, as determined in a random oligonucleotide selection experiment.

Results

Xl-Fli is expressed in a restricted pattern

Figure 1 shows a typical staining pattern using an antisense Xl-*Fli* probe in a stage 32 embryo. A characteristic and intense localized staining is observed. The specificity of the labeling is assessed by the absence of signal when using a sense probe. In addition to the previously characterized expression pattern which reflects neural crest cell localizations (Meyer *et al.*, 1993), a marked hybridization is observed on lateral parts of the embryo, evocative of the pronephric duct (PND) migration pathway (Lynch and Fraser, 1990). The intensity of the signal is not

Abbreviations used in this paper: Ag, angioblasts; AH, adenohypophysis; AP, alkaline phosphatase; BCIP, bromo-chloro-indolyl phosphate; CNC, cranial neural crest cells; EC, endothelial cells; HNK, human natural killer; NBT, nitro blue tetrazolium; NC, neural crest; PND, pronephric duct.

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Fig. 1. Whole-mount *in situ* hybridization on a stage 32/33 *Xenopus* embryo, cleared in BB/BA. *The antisense Fli probe (upper embryo) gives a strong signal at the level of the mandibular, hyoid and branchial arches (see also Fig. 2B), at the level of the pronephric duct migration pathway, as well as at the level of the dorsal aorta, since small spikes (small arrows) can be observed along the hybridization line at mid-flank position, probably corresponding to emerging intersomitic arteries (see also Fig. 3F). Also note the short capillary-like structures on the flank of the embryo (small open arrowheads). Small islets of cells strongly labeled by the Fli probe are visualized in the peri-anal region (large arrows), which could be recruited in the pronephric duct under formation (see text). E, eye; CG, cement gland. The sense probe (lower embryo) does not give rise to any signal, attesting to the specificity of the hybridization. Upon careful examination, a very faint blue color can however be observed in the head and dorso-lateral regions, which might correspond to a residual activity of the endogenous alkaline phosphatase (AP), despite the presence of the inhibitor levamisole. These regions where the neural crest and pronephric duct cells migrate are indeed particularly rich in endogenous AP (Thibaudeau et al., 1993).*

homogeneous along the hybridizing band and probably reflects the presence of two different neighboring structures, the prospective Wolffian duct and blood vessels, as will be discussed later. It should be noted that tiny blue spikes regularly spaced along the hybridizing line can be observed, which most likely correspond to emerging intersomitic arteries, as visualized in Figures 1 and 3F. Furthermore, Xl-*Fli* transcripts can be detected in the forming heart and merging vessels, as shown in Figure 1.

Xl-Fli is expressed in neural crest cells and in the territories where they migrate

Our previous results (Meyer *et al.*, 1993), obtained by *in situ* hybridization had suggested that the Xl-*Fli* gene was expressed along the migration pathway of the neural crest cells. However, the resolution of the experiment was not sufficient to ascertain that the gene was indeed expressed in the cranial neural crest cells. That this is the case is demonstrated in Figure 2A which shows a horizontal section of a stage 17/19 embryo. As a result of the cephalic flexure, the section appears transverse at the level of the forebrain. Masses of labeled cells can easily be detected on both edges of the cephalic structure, as well as between the optical diverticula and the forebrain, which are typical locations for cranial neural crest cells. The specific expression of the *Fli* gene in the neural crest (NC) cells was also confirmed in quail and chick embryos, where isolated migrating NC cells stained with the anti-HNK1 antibody also hybridize the antisense *Fli* probe (A. Grapin, personal communication). Expression obviously increases in later stages of embryogenesis, as shown in Figure 1 for a stage 32/33 embryo, where the mandibular, hyoid and branchial arches (see Sadaghiani and Thiébaud, 1987) exhibit an intense staining. This specific expression is confirmed by the horizontal section through the ventral part of the visceral arches (Fig. 2B), showing a marked staining at the level of cells originating from cranial neural crest cells, at the exclusion of cells of ectoderm, mesoderm and endoderm.

Xl-Fli is expressed in angioblasts and endothelial cells

In early embryos (stage 16/17) the most evident expression of Xl-*Fli* occurs in the ventro-anterior region (Fig. 3A), under the form of a triangular zone, pointing caudally, evocative of the heart and blood islands presumptive area (Huettner, 1943; Beaumont *et al.*, 1994). Later in embryogenesis, a convincing labeling of the forming heart is observed at the level of endothelial cells, as illustrated in the series of pictures of Figure 3B,C,D,E, clearly showing the fusion of the two procardiac tubes (posterior to anterior sections) (Beaumont *et al.*, 1994). Many other blood vessels are stained, as shown in Figure 3F for intersomitic arteries, in Figure 3G for vitelline veins, in Figure 4D for caudal vein/artery and in Figure 3I for the retinal artery. Intense labeling can also be detected at the level of the anterior cardinal veins, dorsal aorta (not shown). The Xl-*Fli* expression appears to be transient, as suggested by the non-homogeneous labeling observed in Figure 4D for the caudal vein/artery. It appears to progress as a wave, when the vessels extend caudally. The expression of Xl-*Fli* during vascularization is further supported by the observation of short capillary-like structures on the flank of the embryo, labeled by the *Fli* antisense probe (Fig. 1). Xl-*Fli* also appears to be expressed at the level of angioblasts, as shown on the transverse section of Figure 3H, showing strongly hybridizing cells, sticking to the outer surface of the neural tube. This can be brought together with the observations that i) vascularization of the brain occurs primarily by angiogenesis, whereas vascularization of other tissues relies mainly on vasculogenesis (for review, see Bischof, 1995), and ii) that angioblasts differentiate in the vicinity of the neural tube, in a rostro-caudal and ventro-dorsal pathway (Feeney and Watterson 1946; G. Couly, personal communication). Such a differentiation pathway is supported by Figure 3I, showing a horizontal section in the anterior region of a stage 33/34 embryo, where these strongly hybridizing cells are observed in the dorsal region of the forebrain and in the ventral region of the hindbrain.

Xl-Fli is expressed along the pronephric duct migration pathway

As clearly shown in Figure 1, Xl-*Fli* is expressed along the pronephric duct (PND) migration pathway, first slightly caudal to the branchial arches, later lengthening towards the cloaca, which it reaches around stages 30/34 (Lynch and Fraser, 1990). This lengthening is clearly shown by the comparison of the signals in

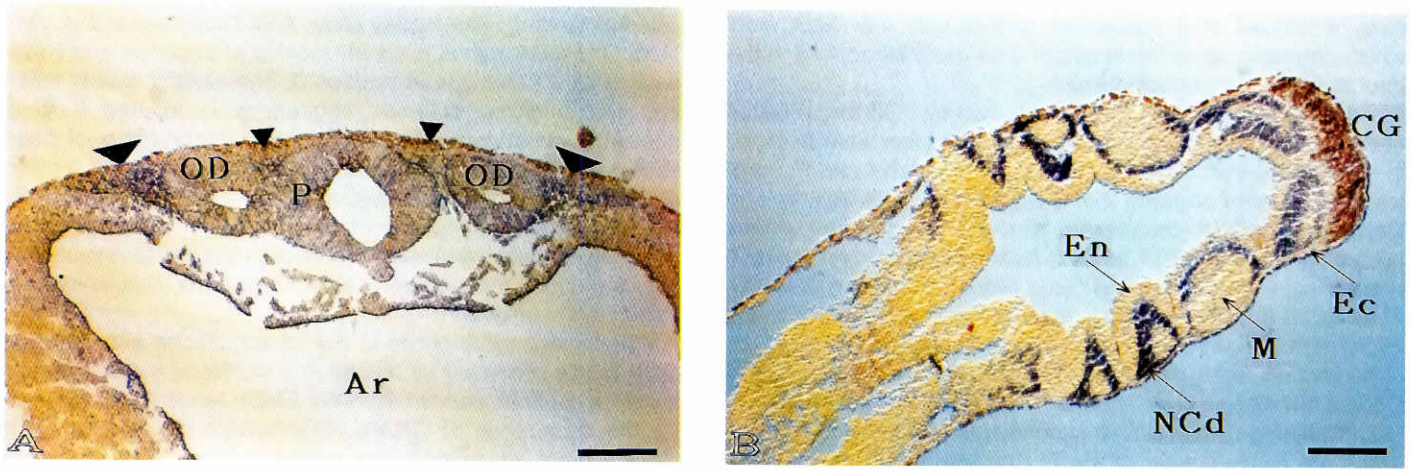


Fig. 2. Xl-*Fli* expression in neural crest cells and neural crest derived layer of the branchial arches. (A) *Horizontal section of a stage 17/19 Xenopus embryo, after whole-mount hybridization. As a result of the cephalic flexure, the section appears transverse at the level of the forebrain. A positive staining by the Fli probe can be observed on both sides of the cephalic structure (large arrowheads), as well as between the optical diverticula and the forebrain (small arrowheads). Anterior and dorsal are to the top. Ar, archenteron; OD, optical diverticulum; P, prosencephalon. Bar, 100 μm.* **(B)** *Horizontal section of a stage 33/34 Xenopus embryo, below the level of the eyes, showing that cells of the visceral arches originating from the neural crest are specifically labeled by the Fli probe. Anterior is to the upper right. CG, cement gland; Ec, ectoderm; En, endoderm; M, mesoderm; NCd, neural crest derived. Bar, 100 μm.*

stage 23 (Fig. 4A) and stage 32 (Figs. 1 and 4C) embryos. Interestingly, hybridization at the level of the pronephros can already be detected in earlier stage embryos (stage 17; data not shown), at a time when even the pronephros rudiment is not yet individualized (Lynch and Fraser, 1990). This could suggest that the expression of the *Fli* gene is involved in marking the migration pathway of the PND cells (see below). The presence of *Fli* transcripts along the PND migration pathway is confirmed on the transverse section of Figure 4B, where a strong hybridization is observed subcutaneously on the flanks of the embryo, at the border between the somite and the endoderm. In some instances, this hybridizing area is resolved in two different patches, probably corresponding to the PND on the one hand, and on the other hand, to the posterior cardinal vein, which lies in close juxtaposition to the PND. The labeling of closely juxtaposed pronephric ducts and blood vessels is further confirmed by Figure 4C and D, which show, on embryos of slightly different stages, the Wolffian duct merging into the cloaca (Fig. 4C) and blood vessels clearly entering the tailbud (Fig. 4D).

Interestingly, Figure 1 shows small groups of cells exhibiting a strong hybridization of the *Fli* probe in the peri-anal region. This has to be put together with the observation by Cornish and Etkin (1993) that the formation of the pronephric duct in *Xenopus* simultaneously involves elongation of a pronephric duct rudiment and a specific recruitment of cells from the lateral plate mesoderm in the posterior region. The labeled islets of cells could therefore correspond to posterior cells which will be recruited to form the PND.

Xl-Fli is expressed in the hypophysis in Xenopus embryos

As shown in Figure 5, a faint but significant labeling is observed in transverse sections of a stage 29/30 embryo, just in front of the extreme tip of the notochord, and along the dorsoventral axis, between the esophagus fingertip and the chord itself, a position expected for the hypophysis. The signal is observed on 5 consecutive sections of 10 μm thickness, which represents an overall diameter of ≈50 μm, consistent with the size of the hypophysis.

The FLI protein preferentially binds to regularly spaced, direct repeats of GGA sequences

As a first step in studying possible target genes of the FLI protein, a search for the sequences preferred by FLI was undertaken with the view of screening libraries of promoter sequences. We used a binding site selection method (Pollock and Treisman, 1990). As the invariant core motif GGA has been found in all the ETS-family protein binding sites identified so far (for review see Wasylyk *et al.*, 1993), we generated a pool of oligonucleotides with a central GGA flanked on each side by 6 randomly specified positions. Sequences bound by *in vitro*-translated FLI protein were isolated by immunoprecipitation and amplified by PCR with primers that flanked the central 15 bp. The selection and amplification procedure was repeated two times. The amplified DNA obtained after two and three rounds of selection was able to form protein-DNA complexes when assayed by gel retardation with FLI protein, whereas the random oligonucleotide pool failed to form such complexes. This indicated that enrichment for FLI binding sites had occurred in the selected pool of oligonucleotides. The DNA recovered after three rounds of selection was cloned into Bluescript for sequencing. The nucleotide sequences of the selected and cloned sites are presented in Table 1. The selected binding sites can be grouped in 3 different sets according to the number of GGA (or complementary TCC) identified. The largest set comprises 15 sequences, 13 of them showing a second GGA invariably located 3 nucleotides downstream of the central GGA. An identical pattern is observed within a set of selected sequences which show a third GGA located 3 nucleotides (or 2 in one case) upstream of the central GGA

core. A last set of 9 sequences reveals only one GGA motif which corresponds to the invariant core motif introduced in the pool of random oligonucleotides.

Selected DNA binding probes obtained by PCR amplification of the cloned inserts and end-labeled by kination were assayed in gel mobility-shift experiments with *in vitro*-translated FLI protein. DNA probes showing 2 or 3 GGA motifs were able to form stable protein-DNA complexes, whereas selected sequences having only a single GGA failed to form detectable complexes. A representative retardation gel using oligonucleotides 6 (3 GGA), 26 (2 GGA) and 15 (1 GGA) is shown in Figure 6. Probes 6 and 26 formed protein-DNA complexes, but differences could be observed in their relative affinities for the FLI protein. Oligonucleotide 15 failed to form a retarded complex. Quantitative gel retardation experiments were carried out in which the concentration of the FLI protein was held constant, while that of the DNA probe varied. The equilibrium dissociation constant (K_D) of FLI for a 3 GGA-binding site (oligonucleotide 14) was calculated to be $2x10^{-10}$ M. This value is within the same range as those observed for a truncated form of the ETS-1 protein containing the ETS binding domain (0.4 to $3.3x10^{-10}$ M for different binding sites; Nye *et al.*, 1992) and for the ETS-binding domain of ELK-1 ($0.85x10^{-10}$ M; Shore *et al.*, 1995).

Examination of the nucleotide frequencies detected strong preferences at several positions (Table 1). Downstream the central GGA, A was preferred at position 1 (frequency >0.65), and a clear consensus appeared at positions 3 to 6 (C, G, G, A, respectively, with frequencies >0.65). These positions correspond to the second GGA repeat spaced by 3 nucleotides from the central GGA. Upstream of the central core, preferences were less marked: C residues were favoured at positions -1 and -2 (frequencies of ~0.5), A and G being the preferred nucleotides at positions -3 and -5, respectively. The frequency of nucleotides therefore defines the consensus presented in Table 1. Sequence consensus have been reported for the ETS-1 protein (Fisher *et al.*, 1991; Nye *et al.*, 1992; Woods *et al.*, 1992). Whereas ETS-1 nucleotide preferences are clustered in a 9-bp region with a single GGA, FLI recognizes specific DNA sequences over a 15-bp region which shows GGA repeats. The FLI-preferred sequences are restricted to the same positions flanking the central GGA, but A residues are clearly favoured by FLI at position +1 (ETS-1 selected binding sites display either A or T residues at this position). One GGA repeat is clearly located at positions 4 to 6 and flanked by a C residue at position 3. The second repeat which appears in some selected sequences is located 2 or 3 nucleotides upstream of the central GGA. The presence of these GGA repeats may contribute to high affinity and specific binding of FLI, since the selected sequences having only one GGA do not form stable complexes when assayed by gel retardation. They most likely represent lower-affinity binding sequences selected during the process of selection/amplification which was performed in solution.

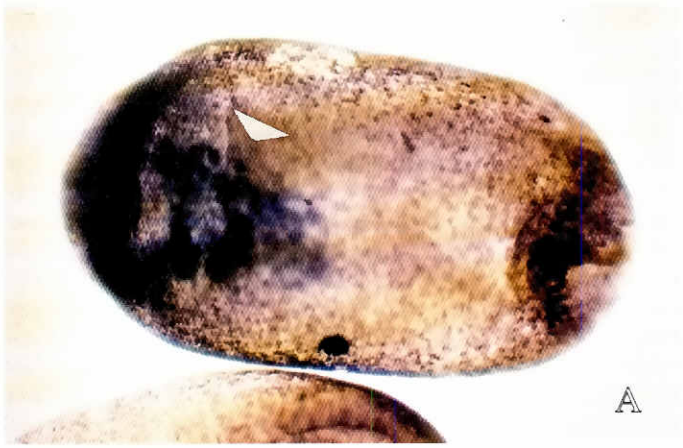
The binding preferences of FLI for sequences with repeated GGA motifs prompted us to screen a subset of eukaryotic promoter sequences derived from the EMBL Nucleotide Database for the occurrence of $GGAN_{2-3}GGAN_3GGA$ sequences. Such GGA repeats are found in the promoter region of a number of genes encoding adhesion molecules like α_v and β_3 integrins or constituents of the extra-cellular matrix like tenascin and vitronectin.

Discussion

We reported earlier that Xl-*Fli*, a gene coding for a transcription factor belonging to the *ets* family, is expressed in a restricted pattern in *Xenopus* embryos from early neurula up to at least stage 40/45 embryos (Meyer *et al.*, 1993). Using whole-mount *in situ* hybridization with digoxigenin-labeled probes, we show in this paper that in addition to the neural crest cells already characterized, other cell lineages contain Xl-*Fli* transcripts. At the sensitivity allowed by this technique, Xl-*Fli* expression is observed in cranial neural crest cells (CNC), angioblasts (Ag) and endothelial cells (EC), along the pronephric duct (PND) migration pathway and in the hypophysis (Ah).

The expression of Xl-*Fli* along the PND cells migration pathway can be detected as early as stage 17. This shows that the labeling most likely occurs at the level of the substratum on which the PND cells migrate, since at that stage, the pronephric duct rudiment is not yet individualized (Lynch and Fraser, 1990). This observation can be put together with earlier results of Cornish and Etkin (1993), who, using *borealis-laevis* chimera, showed that PND formation involved both cell migration from a

Fig. 3. Xl-*Fli* expression in the forming heart and vascular system. (A) *Ventral view of a stage 16/17 Xenopus embryo after whole-mount hybridization, showing a triangular shaped hybridization area (large arrowhead), in the presumptive cardiac region. Anterior is to the left, ventral to the front.* **(B-E)** *Sequence of 4 consecutive transverse sections (thickness ≈10 μm) of a stage 29/30 Xenopus embryo after whole-mount hybridization, showing in a caudo-rostral direction, the fusion of the two labeled procardiac tubes. Ventral is to the bottom. Bar, 50 μm.* **(F)** *Horizontal section of a stage 35/36 Xenopus embryo in the dorsal region after whole-mount hybridization, showing a characteristic labeling at the places where the plane of section encounters the intersomitic arteries (arrowheads). Ch, notochord; S, somites. Bar, 50 μm.* **(G)** *Transverse section of a stage 29/30 Xenopus embryo after whole-mount hybridization, showing a specific labeling at the level of vitelline veins (arrows). Ventral is to the left. En, endoderm. Bar, 50 μm.* **(H)** *Transverse section of a stage 34/35 Xenopus embryo, after whole-mount hybridization at the level of the hindbrain. The section has not been dewaxed prior to examination. Note the heavily stained cells sticking to the outer surface of the neural tube and which may correspond to angioblasts engaged in the vascularization of the neural tube (see text). Dorsal is to the top. RV, rhombencephalic ventricle; Rh, rhombencephalon. Bar, 10 μm.* **(I)** *Horizontal section through the dorsal region of a stage 33/34 embryo after whole-mount hybridization. Due to curvature of the embryo, oblique sections of the neural tube are observed at the level of the forebrain and hindbrain. Note the characteristic labeling in the dorsal region of the forebrain and in the ventral region of the hindbrain (full arrows), indicative of differentiating angioblasts sticking to the outer surface of the neural tube (see text). The hyaloid (retinal) artery, entering the eye by the choroid cleft, is clearly visualized in the space between the lens and the retina (large open arrows). In the left eye, it can be observed as a tunica, covering the posterior part of the lens and probably anastomosing with a circular vessel, the section of which is clearly observed on both sides of the lens (small open arrows). Also note the presence of a more diffuse labeling in the region anterior to the eye cup, and between the eye and the ear vesicle, corresponding to the streams of neural crest cells populating the mandibular, hyoid and branchial arches (arrowheads). Ch, notochord; E, eye; L, lens; P, prosencephalon; PV, prosencephalic ventricle; Rh, rhombencephalon; RV, rhombencephalic ventricle; OV, otic vesicle. Bar, 100 μm.*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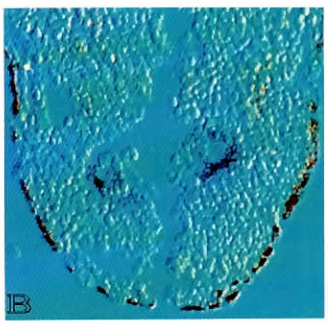
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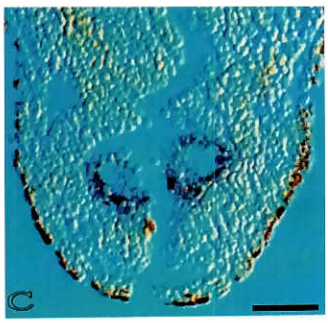
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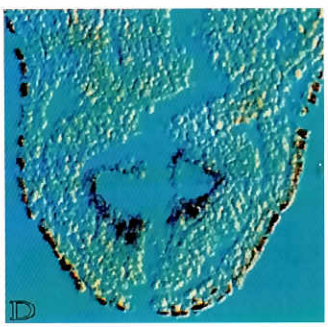
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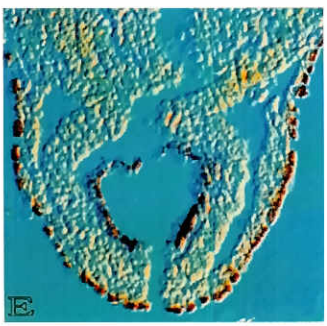
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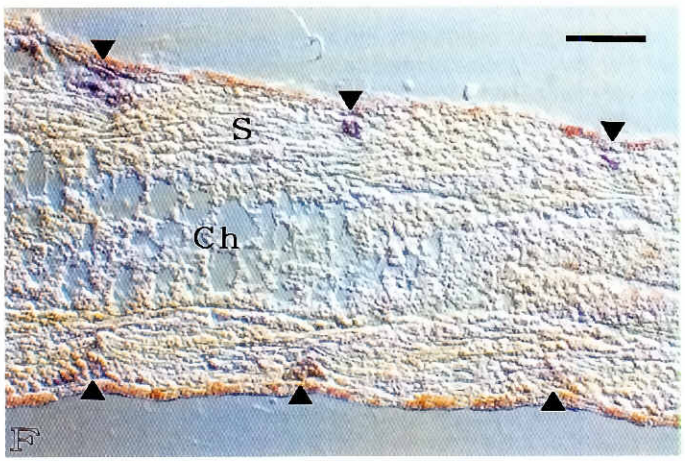
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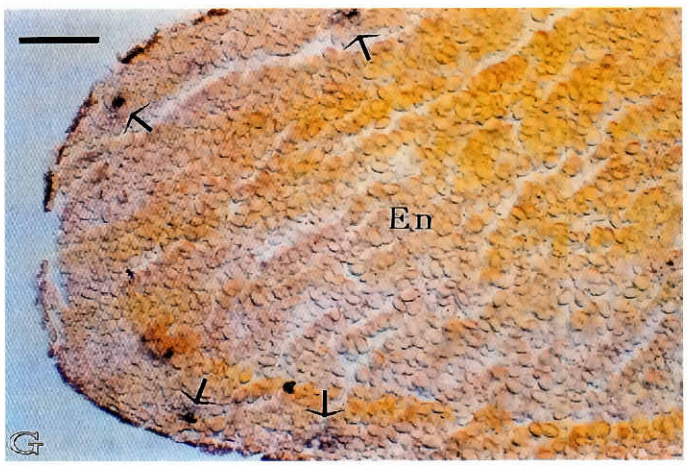
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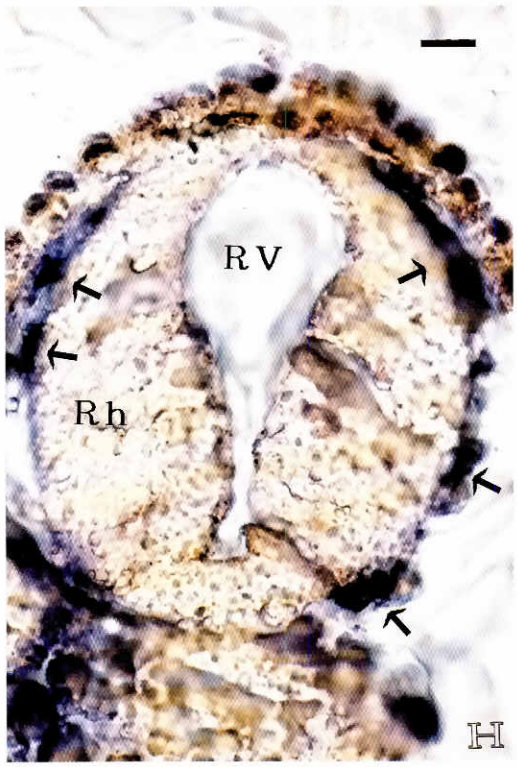
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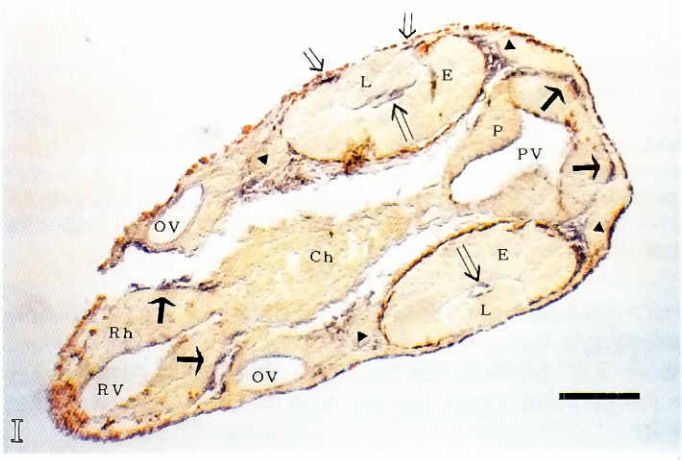
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TABLE 1

NUCLEOTIDE SEQUENCE OF THE SELECTED AND AMPLIFIED BINDING SITES

	Clone number	Selected sequences
Three sites	4.2	atgcGGAAGC**GGA**GCCGGAagta
	6	GGAAGT**GGA**ACCGGA
	14	TGGACC**GGA**ACCGGA
	18.3	GGAAGT**GGA**GCCGGA
	25	TGCTCC**GGA**AACGGA
	28	CGGTCC**GGA**AACGGA
Two sites	3.3	AGAACA**GGA**AACGGA
	4.1	TGCGGC**GGA**AACGGA
	7	GTCGCC**GGA**AGCGGA
	12.2	AAGACC**GGA**ACCGGA
	18.1	TGAACC**GGA**ACCGGA
	18.2	TTAACC**GGA**AACGGA
	26	AACACC**GGA**AACGGA
	27	TGCACC**GGA**AGCGGA
	29	CGCGCC**GGA**ACCGGA
	30	AGCCCC**GGA**ACCGGA
	31	CAGGCC**GGA**AGCGGA
	41	ATGGCC**GGA**AACGGA
	42	TGTACC**GGA**ACCGGA
	19.1	TTCCTC**GGA**TCGAGT
	20	TGCACC**GGA**TTTCCG
One site	3.1	TTTAAC**GGA**ATTATA
	3.2	CCTCTA**GGA**GATTGC
	12.1	TCGGCG**GGA**TTGTTT
	15	GGCAGT**GGA**ACATAA
	17	TAGCGA**GGA**TTCTGC
	19.2	AAAGAT**GGA**CTGCAC
	32	GCCATG**GGA**TATGTC
	38	AGTGGT**GGA**CAGCTC
	44	GGCAGT**GGA**ACATAA

Nucleotide preferences

	-6	-5	-4	-3	-2	-1	G	G	A	1	2	3	4	5	6
G	7	17	7	8	8	6	30	30		3	3	4	20	22	1
A	7	5	7	16	3	3			30	20	10	2	2	3	22
C	4	3	12	4	16	15				2	12	20	3	1	5
T	12	5	4	2	3	6				5	5	4	5	4	2

Consensus

•	G	•	A	C	C	G	G	A	A	•	C	G	G	A

The 30 sequences are ordered according to the number of GGA motifs (or complementary TCC, indicated by a dotted line). The central GGA present in the initial pool of random nucleotides is indicated in bold. Additional GGA motifs are underlined. Lowercase designation in the sequence of clone 4.2 corresponds to imposed flanking nucleotides common to all sequences. The frequency of each nucleotide flanking the GGA core motif is shown. Nucleotides with frequencies of ≥0.5 were used to generate the consensus.

PND rudiment and recruitment of posterior cells, probably belonging to the lateral plate mesoderm. In the light of the results above, it is very likely that the cells expressing XI-*Fli* transcripts in the peri-anal region, are the ones which will be recruited in PND.

The expression of the *Fli* gene in the erythropoietic cell lineages is consistent with a control of the erythroid transcription factor GATA-1 by myb-ets fusion proteins (Aurigemma *et al.*, 1992), ETS-1 ETS-2 and FLI polypeptides (Seth *et al.*, 1993). GATA-1 and GATA-2 were recently shown to define induction of hematopoietic mesoderm (Kelley *et al.*, 1994). Interestingly, the simultaneous occurrence of GATA and ETS target sequences in the promoter of glycoprotein IIB (Lemarchandel *et al.*, 1993) and cytosolic glutathione-peroxydase (Oprey *et al.*, 1994) was shown to be responsible for cell-type specific expression. Finally, GATA-4 was shown to be expressed in the presumptive cardiac ventral mesoderm, at the time that bilateral progenitors fuse and form the cardiac tube, and more specifically in the endocardium in later developmental stages (Kelley *et al.*, 1993). It could therefore be possible that XI-FLI, together with the GATA family of transcription factors are involved in the control of cell lineage-specific expression.

All the regions expressing the XI-*Fli* gene have in common the fact that they are sites of important cellular migrations. It is therefore very tempting to speculate that the XI-FLI protein could regulate the expression of adhesion molecules in migrating cells, in order to allow a decrease of adhesivity towards neighboring cells, as well as the recognition of guidance cues along the expected migration pathway. In support of this idea, it is noteworthy that CNC cells and PND cells, at least, have been shown to utilize partly overlapping guidance cues (Zackson and Steinberg, 1986; Thibaudeau *et al.*, 1993). The last authors reported that a glycosyl-phosphatidylinositol (GPI)-linked surface molecule, tentatively identified as alkaline phosphatase (AP), was associated to the migrating PND cells. The role of this endogenous alkaline phosphatase is still obscure, but it could work as a "receptor" for a constituent of the extracellular matrix (possibly in a phosphorylated state), or be involved in the cellular motility itself. It must also be stressed that AP is not the only GPI linked molecule which could be involved in the control of cellular adhesion, since a GPI-linked form of N-CAM was described (He *et al.*, 1986), as well as a truncated cadherin (Ranscht and Dours-Zimmermann, 1991; Vestal and Ranscht, 1992). More recently, a novel GPI-linked molecule was shown to be involved in epithelial cellular interactions (Rabino *et al.*, 1994). Although CNC and PND cells share partially overlapping sets of guidance cues, AP is not synthesized by CNC cells and therefore does not participate in the control of their migration. However, one could imagine that a common guidance cue is expressed at the level of the substratum where CNC and PND cells migrate (a constituent of the extracellular matrix for instance), which is recognized by cellular "receptors" specific either to CNC or PND cells (for discussion see Zackson and Steinberg, 1986). Strikingly, EC and CNC cells were reported to express common subsets of receptors to proteins of the extracellular matrix (Cheresh *et al.*, 1989; Delannet *et al.*, 1994). Among those, the $\alpha_V\beta_3$ integrin appears to play an important role both in the migration of neural crest cells (Delannet *et al.*, 1994) and in angiogenesis (Brooks *et al.*, 1994). A possible effect on the expression of receptors could be accompanied by a control of the expression of their ligands, like vitronectin for instance, which was suggested to play a crucial role in neural crest cell migration (Delannet *et al.*, 1994). The same $\alpha_V\beta_3$ integrin was found to be involved in endothelial cell migration on vitronectin (for review of the role of adhesion molecules in angio-

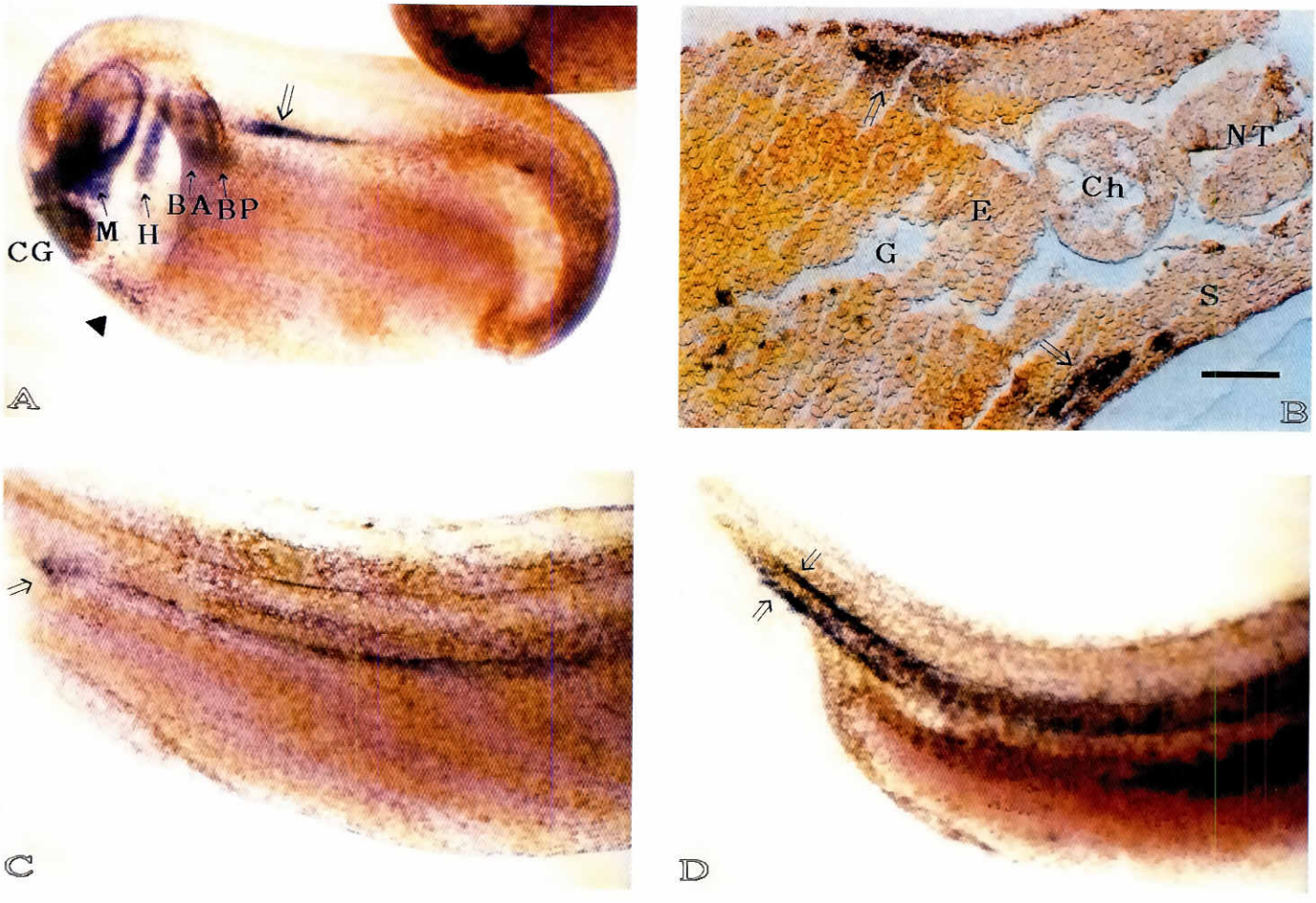

Fig. 4. Xl-*Fli* expression along the pronephric duct migration pathway. (A) *Whole-mount in situ hybridization on a stage 23 Xenopus embryo, cleared in BB/BA. Anterior is to the left, dorsal to the top. The labeling of the mandibular (M), hyoid (H), branchial anterior (BA) and branchial posterior (BP) arches is clearly visible. The labeling of the pronephric duct migration pathway (open arrow) at mid-flank of the embryo, is much shorter than in the stage 32/33 embryo shown in Fig. 1A. Also note the faint labeling left in the ventro-anterior region, in the presumptive heart area (arrowhead; see also Fig. 3A).* **(B)** *Transverse section of a stage 29/30 Xenopus embryo after whole-mount hybridization. The section is posterior to the liver region. Note the bilateral strong signal, localized subcutaneously at the border between the somite and the endoderm, and corresponding to the pronephric duct migration pathway (open arrows). Ventral is to the left. Ch, notochord; E, endoderm; G, gut; NT, neural tube; S, somite. Bar, 50 μm.* **(C)** *Whole-mount in situ hybridization on a stage 31/32 Xenopus embryo, showing the labeled Wolffian ducts merging into the cloaca (open arrow). Posterior is to the left.* **(D)** *Whole-mount in situ hybridization on a stage 35/36 Xenopus embryo, showing labeled blood vessels (open arrows) entering the tail-bud, posterior to the anal region. Posterior is to the left.*

genesis, see Bischof, 1995). It would not be surprising that these molecules also play an important role in the control of PND cell migration, as recent results showed that trunk neural crest cells give a contribution to the Wolffian duct (Collazo *et al.*, 1993).

It should be kept in mind that the regions expressing the Xl-*Fli* gene are also sites of transitions between epithelium and mesenchyme and vice-versa, since neural crest dispersion (Le Douarin, 1982), heart (Crossin and Hoffman, 1991) and kidney/pronephric duct (Ekblom, 1989; Rothenpieler and Dressler, 1993) formation involve such conversions. As the conversion between epithelium and mesenchyme was shown to involve deep modifications in the expression of adhesion molecules (Ekblom, 1989), Xl-*Fli* could play a role in these developmental processes.

In support of a role of the FLI protein in modulating cellular adhesivity, it must be emphasized that overexpression of the *Fli* gene during embryogenesis, upon microinjection of a synthetic transcript in the fertilized egg, is teratogenic and leads to malformations of the head and circulatory system (Remy *et al.*, unpublished results). High doses of the synthetic mRNA lead to important anterior truncations, most likely resulting from a perturbation of the inductive processes between embryonic layers leading to the progressive determination of the antero-posterior polarity (Ruiz i Altaba and Melton, 1990). Indeed, although the extent of mesoderm migration looks almost normal, overexpression of the *Fli* gene is accompanied by the appearance of round-shaped, isolated cells both at the site of injection and at the borders of the different embryonic layers. The altered morphology

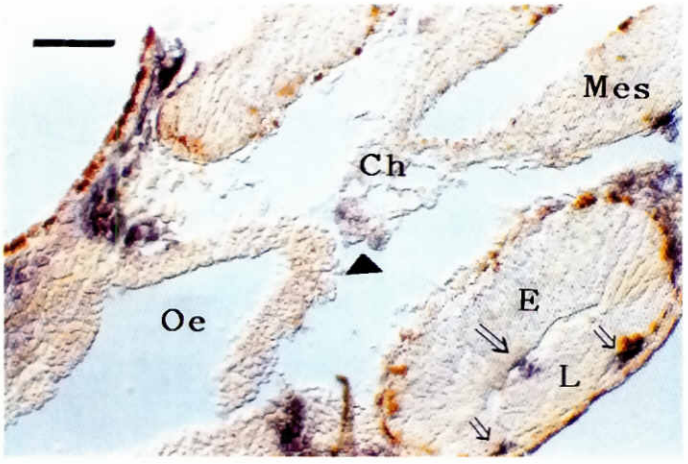

Fig. 5. Transverse section through the anterior part of a stage 29/30 embryo after whole-mount hybridization. *Note the group of cells hybridizing the probe, immobilized between the extreme tip of the notochord and the fingertip of the esophagus, most likely corresponding to the hypophysis (arrowhead). Hybridization can also be observed in the space between the lens and the retina, corresponding to the hyaloid (retinal) artery, cut at an angle (large open arrow) and at the periphery of the lens (small open arrow), probably corresponding to the section of a circular vessel. The trace of the mandibular arch is clearly visible on both sides of the esophagus. Also note the strongly positive isolated cell adhering to the outer surface of the midbrain and probably corresponding to a differentiating angioblast (see text). Ch, notochord; E, eye; L, lens; Mes, mesencephalon; Oe, esophagus. Bar, 50 μm.*

of these cells is strongly indicative of a modification in the cell-cell interactions.

Our results show that an *in vitro*-synthesized FLI protein recognizes efficiently regularly-spaced, direct repeats of a GGA motif. These results are clearly different from those of Zhang *et al.* (1993), who concluded that *Fli* recognize the consensus sequence CC/AGGAAGT. It must however be underlined that the experiments were carried out in a quite different way. Whereas we have been looking for the sequences preferred by FLI in a random library of oligonucleotides containing a central GGA motif, Zhang *et al.* (1993) measured the changes in the recognition of a target sequence, upon point mutation of residues flanking a single GGA motif, leaving no opportunity to detect a possible increase in the affinity upon repetition of the core motif at short distance. More than a definite consensus sequence containing a single core GGAA/T sequence, which is assumed to be recognized by transcription factors of the *ets* family, the FLI protein therefore appears to recognize the spacing of GGA core motifs. This could be determinant for the specificity of the interaction with the DNA, as was shown for other transcription factors (see Li *et al.*, 1993 and references therein). Of course, this does not preclude an effect of the residues flanking the GGA cores on the affinity of the complex, as suggested by the consensus sequence reported in Table 1. Another difference in the experimental protocols may be responsible for the discrepancy in the results with Zhang *et al.* (1993). Indeed, they conducted their bandshift experiments with a fusion protein synthesized in a bacterial system, whereas we have been using a synthetic protein translated *in vitro* in a rabbit reticulocyte system. The differences of the two systems in the ability to carry out post-translational modifications could also lead to variations in the sequences recognized.

In the light of the above results, it may not be fortuitous that the promoters of the integrin subunits α_V and β_3 do contain $GGAN_{2\text{-}3}GGAN_3GGA$ motifs, which are efficiently recognized *in vitro* by a synthetic FLI protein. These integrin subunits are expressed in migrating NC cells (Delannet *et al.*, 1994) as well as in cardiac endothelial cells (Brooks *et al.*, 1994). Furthermore, such consensus sequences are found in the promoter of vitronectin, a privileged ligand of the $\alpha_V\beta_3$ integrin, synthesized by CNC (Wayner *et al.*, 1991; Delannet *et al.*, 1994) and endothelial cells (Brooks *et al.*, 1994). The same is true for the tenascin promoter (Jones *et al.*, 1990) (tenascin is also known as cytotactin, J1 220/200 or hexabrachion), which is expressed in early endocardial cells and has been postulated to compete with fibronectin along cellular migration pathways (Tan *et al.*, 1987). Tenascin is also expressed in early cardiac endothelial cells and is down-regulated when they start to migrate to form the endocardial cushion tissue (Crossin and Hoffman, 1991).

Such a mechanism involving a regulation of cellular adhesivity, does not exclude an action at the level of proteases, more or less specific of the extra-cellular matrix, as was already proposed for another member of the *ets* family, the *ets*-1 gene (Wernert *et al.*, 1992b), suggesting a favouring role of the ETS-1 protein in cancer invasion (Vandenbunder *et al.*, 1994). The same *ets*-1 gene was shown to be expressed specifically in the endothelial cells of blood vessels, during both normal angiogenesis (Vandenbunder *et al.*, 1989) and tumor vascularization (Wernert *et al.*, 1992a). It may be significant that the *ets*-1 promoter also contains potential binding motifs for the FLI protein.

The *Fli* gene could therefore play a critical role in the control of important steps of embryonic development, like the patterning of the head skeleton, pronephric duct migration, cardiogenesis, vasculogenesis and/or angiogenesis. In support of this hypothesis, it is striking to observe that overexpression of Xl-*Fli* in the course of embryogenesis (Remy *et al.*, unpublished results) leads to developmental anomalies in the "compartments" where this gene is normally expressed. In addition to major defects in the antero-posterior polarity of the embryo, probably arising from perturbations in the inductive processes of gastrulation, anomalous vascularization is observed, leading to important hemangiomas, often accompanied by a blocking of red blood cell differentiation in the circulating blood, and a perturbation of the endothelial cell-lining of the heart cavity. Furthermore, malformation of the eyes is frequently encountered, which could reflect the involvement of neural crest cells in the formation of the head skeleton, and/or in inductive processes in eye development.

Materials and Methods

Digoxigenin-11-uridine-5'-triphosphate (DIG-11-UTP), anti-digoxigenin antibodies (anti-DIG-Fab fragment, conjugated to alkaline phosphatase) and blocking reagent were purchased from Boehringer Mannheim (Germany). Substrates for alkaline phosphatase were in the initial experiments bromo-chloro-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (Sigma, USA) and more recently BM-purple (Boehringer), which allows a better resolution with a comparable sensitivity.

Animals

Xenopus laevis were bought from the CNRS (France). *Xenopus* embryos were obtained as fully described elsewhere (Wolff *et al.*, 1991). Briefly, eggs were obtained from gonadotropin-injected females (HCG, Organon, France), fertilized *in vitro* and dejellied with cysteine. Embryos were allowed to develop at 21°C (Moon and Christian, 1989). They were staged according to Nieuwkoop and Faber (1967). Embryos were fixed in MEMFA (0.1 M MOPS pH 7.2, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde) and submitted to whole-mount *in situ* hybridization.

Digoxigenin-labeled RNA probes

RNA probes labeled with digoxigenin (Boehringer Mannheim, Germany) were obtained by *in vitro* transcription of a cloned Xl-*Fli* sequence (nucleotides 1 to 1372 of the Xl-*Fli* cDNA, cloned in Bluescript vector) under the control of T3- or T7-promoters for the antisense and sense probes and after *SmaI* or *ClaI* linearization, respectively. The manufacturer's instructions were followed. Before use, the RNA probes were shortened to a length of ≈0.5 kb upon incubation in 60 mM sodium carbonate, 40 mM sodium bicarbonate, pH 10.2 (Cox *et al.*, 1984).

Whole-mount hybridization

Whole-mount hybridizations were carried out essentially as described by Harland (1991), with a few minor modifications, as follows :

- the acetylation step was omitted, as it requires homogenization which may lead to mechanical damage to the embryos;
- blocking was achieved using the reagent supplied by Boehringer at 2% in hybridization buffer;
- the alkaline phosphatase-conjugated anti-digoxigenin antibodies were preadsorbed with *Xenopus laevis* "embryo powder", under the following conditions:

"embryo powder" was prepared by homogenization of stage 10-40 *Xenopus* embryos in a minimum volume of PBS (≈100 embryos in 200 µl of PBS buffer). Four volumes of ice-cold acetone were added to the suspension and the mixture was allowed to stand at 0°C for 30 min. After a 10 min centrifugation at 10000g, the supernatant was discarded and the precipitate was washed once more with cold acetone. After recovery, the precipitate was ground into a fine powder on a sheet of filter paper and air-dried.

3 mg of the "embryo powder" were resuspended in 0.5 ml of PBS buffer containing 2 mg/ml of bovine serumalbumin and 0.1% Triton X-100. After 30 min at 56°C, the suspension was cooled on ice and 5 µl of goat serum and 1 µl of anti-DIG antibody were added. The mixture was kept at 4°C for 2 h, on a rocking platform. After a 10 min centrifugation at 10000g, the supernatant was diluted 4-fold more and used in digoxigenin mapping.

- the best revelation was obtained using the new chromophoric substrate BM-purple (Boehringer), under the conditions recommended by the manufacturer. The staining was developed for 16 to 36 h (depending on embryo stages). The embryos were then rinsed in PBS buffer containing 0.1% Tween-20. They were post-fixed in MEMFA for 2 to 15 h, dehydrated by 4 washes with anhydrous methanol and cleared in benzylbenzoate/benzyl alcohol 2/1 v/v). After observation under the microscope, stained embryos were washed several times in 100% methanol, 100% butanol, incubated for 1 to 2 days in a Paraplast/butanol mixture (1/1 w/v) and finally embedded in paraplast. Embryos were then sectioned at a 10 µm thickness on a Jung 2055 Autocut microtome. Sections were mounted on gelatin-chromalun coated slides for examination.

FLI binding sites selection and amplification

The selection and amplification of FLI binding sites from a random pool of oligonucleotides were performed essentially according to the method of Pollock and Treisman (1990). The following oligonucleotides were used: random template R, 5'-GCTGCAGTCTAG AGCCTCATGC$(N)_6$GGA$(N)_6$AGTAGGCTAGAATTCGTATGTC-3'; primer A, 5'-GCTGCAGTCTAGAGCCTC-3'; primer B, 5'-GACATACGAATTCTAGCC-3'. The central 15 nucleotides of template R con

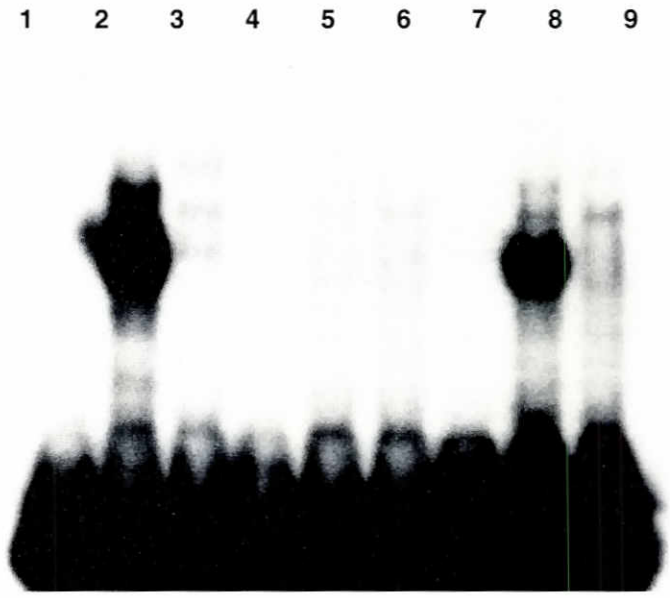

Fig. 6. Binding of FLI to selected sequences as assayed by gel retardation. *Three different oligonucleotides were used:* **(lanes 1-3)** *oligonucleotide 6 (3 GGA);* **(lanes 4-6)** *oligonucleotide 15 (1 GGA);* **(lanes 7-9)** *oligonucleotide 26 (1GGA). The sequences of the selected oligonucleotides are shown in Table 1. Lanes 1, 4 and 7, free probes. Lanes 2, 5 and 8, in vitro-translated FLI protein. Lanes 3, 6 and 9, unprogrammed reticulocyte lysate, used as a control.*

For the first round of selection, 1 µl (0.5 ng; 13 fmoles) of purified double-stranded probe was incubated for 10 min at room temperature with 2 µl of *in vitro* translated FLI protein (11 fmoles) and 1 µl (200 ng) poly(dI-dC) poly(dI-dC) in 20 µl of binding buffer E (20 mM HEPES pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 0.1% Nonidet NP40, 1 mg/ml BSA). 1 µl of FLI antiserum was added and the immune complexes were allowed to form on ice for 30 min. They were then incubated with 10 µl of protein A-Sepharose (equilibrated in buffer E) at 0°C for 1 h with gentle mixing. The immune complexes were washed with 250 µl of cold buffer E (without BSA) and the Sepharose beads were recovered by brief centrifugation. After a second washing/centrifugation step, the DNA was eluted from the beads at 45°C in 200 µl 50 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.5% SDS, 100 mM sodium acetate. The selected DNA was recovered by phenol/chloroform extraction and ethanol precipitation in the presence of 10 µg glycogen used as a carrier. Probe amplification was performed by PCR in a 10 µl-reaction mix containing 20 pmoles each primer A and B, 5 µCi α-[^{32}P]dCTP (3000 Ci/mmol), 20 µM dCTP, 50 µM each dATP, dTTP, dGTP, 1.5 mM $MgCl_2$ and 2.5 U *Taq* DNA polymerase (Eurobio) in the supplied PCR buffer. Amplification was carried out in 15 cycles of 1.5 min at 97°C, 2 min at 52°C, 1 min at 72°C. The amplified probe was purified on a 8% polyacrylamide gel. Two additional rounds of selection/amplification were performed in identical manner. The amplified products obtained after the second and third rounds were able to form protein-DNA complexes when analyzed in a gel retardation assay. To obtain sufficient quantities of DNA for cloning, the final product was reamplified and then cloned directionally between the *Xba* I and *Eco* RI sites of pBluescript KS$^+$ vector. The inserts were sequenced by the dideoxy sequencing method.

DNA binding reactions and gel mobility-shift assays

Selected DNA binding site probes for binding reactions were obtained by direct PCR amplification of the cloned inserts using primers A and B. The 59-bp probes were end-labeled using T4 polynucleotide kinase in the presence of γ^{32}[P]ATP. DNA binding reactions for gel retardation assays contained labeled probes (10 to 20 fmoles) and *in vitro*-translated FLI protein (~5 pmoles) in a final volume of 20 µl of binding buffer E (or alternatively 20 mM HEPES pH 7.9, 20% glycerol, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT). Poly(dI-dC).poly(dI-dC) was included as a non-specific competitor (1.5 µg). After incubation at room temperature for 20 min, DNA-protein complexes were resolved by electrophoresis on 6% non-denaturing polyacrylamide gels in 0.25xTBE at 10 V/cm. Gels were dried and DNA-protein complexes were visualized by autoradiography.

The equilibrium dissociation constant (K_D) for the DNA binding sites was obtained using gel mobility-shift assays in which the protein concentration was held constant (0.17×10^{-9} M) while the DNA concentration was varied ($0.035\text{-}0.28 \times 10^{-9}$ M). The amounts of unbound and bound probe were measured by image-plate analysis (Fuji BioImager). The data were graphically plotted according to Scatchard (ratio of bound and unbound versus bound). The equilibrium dissociation constant was calculated from the slope of a linear regression.

Protein and antibodies

FLI protein was produced by *in vitro* translation of a *Fli* synthetic mRNA using the Type II reticulocyte kit from Boehringer. *Fli* mRNA was obtained by *in vitro* transcription (SP6 Megascript kit, Ambion) of the Xl-*Fli* coding sequence cloned in the pSP64T transcription vector. Protein production was confirmed by SDS-polyacrylamide gel electrophoresis and autoradiography of ^{35}S-labeled proteins. The FLI-specific antiserum used for the selection/amplification procedure was obtained from rabbits injected with a glutathione S-transferase fusion protein containing amino acid residues 1 to 121 of the *Xenopus* FLI protein.

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