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Original Article

Overexpression of the *Xenopus* Xl-*fli* gene during early embryogenesis leads to anomalies in head and heart development and erythroid differentiation

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ABSTRACT The product of the Xl-*fli* gene, a *Xenopus laevis* transcription factor of the *ets* family, specifically expressed in several lineage of migratory cells during *Xenopus* development (Meyer *et al., Int. J. Dev. Biol. 39:* 909-919, 1995) was overproduced during *Xenopus* embryogenesis, upon microinjection of a synthetic transcript in the fertilized egg or in the early embryo. This results in anomalies of the antero-posterior and dorso-ventral polarities, and in tissue differentiation, particularly in the eye- and head cartilage development, as well as erythroid differentiation (absence of erythrocyte differentiation in the circulating blood, often accompanied by ectopic localization of mature erythrocytes, leading to important hemangiomas). Cytological examination reveals at gastrulation the existence of abnormal cells separating the different embryonic layers, suggesting modifications of the cellular adhesion properties. The possible involvement of the *fli* gene in controlling the dissemination of migratory cells is discussed.

KEYWORDS: *transcription factor, ets gene family, overexpression, ectopic expression, malformations*

Introduction

The *fli*-1 gene was first characterized in the mouse as being overexpressed upon integration of the Friend leukemia virus proviral DNA in the host cell DNA (Ben-David *et al.*, 1990; Ben-David and Bernstein, 1991) leading to erythroleukemia. More recently, the *fli* gene was associated to a human malignant disease, Ewing's sarcoma (EWS) (Delattre *et al.*, 1992; Sorensen *et al.*, 1993). In this disease, a translocation between chromosomes 11 and 22 leads in more than 80% of the patients to a hybrid protein containing the DNA-binding domain of the FLI protein and the N-terminal region (NTR) of the EWS protein, described as a RNA binding protein. This translocation therefore results in three consequences: i) placing the hybrid gene under the control of the EWS promoter, which appears to be quite efficient (Delattre *et al.*, 1992); ii) making the expression more ubiquitous (Delattre *et al.*, 1992); and iii) replacing the transactivation domain of the FLI protein by the EWS-NTR. Bailly *et al.* (1994) showed that the EWS-NTR indeed behaves as a very efficient transactivation domain. Interestingly, another hybrid protein composed of the EWS-NTR and the DNA-binding domain of the *erg* gene (Rao *et al.*, 1987; Reddy *et al.*, 1987; Reddy and Rao, 1991) has been characterized in Ewing's sarcoma (Zucman *et al.*, 1993). This might be particularly significant as the *erg* gene is very similar to the *fli* gene (Watson *et al.*, 1992; Meyer *et al.*, 1993). We cloned a Xl-*fli* cDNA from *Xenopus* neurula library and showed that the gene is expressed during embryogenesis in a restricted pattern evocative of neural crest cell distribution (Meyer *et al.*, 1993). Recent results of whole-mount *in situ* hybridization showed that *fli* transcripts are detected in different lineage of migratory cells (Meyer *et al.*, 1995). It was therefore of interest to overexpress this gene in early embryogenesis, looking for clues to the gene function. This paper reports a teratogenic effect of Xl-*fli* overexpression upon microinjection of a synthetic mRNA in the fertilized egg or in the early embryo. The effects observed can be interpreted in terms of interference with the cellular adhesion properties. The possible contribution of the *fli* gene to the control of cell migration is discussed.

Results

Injection of the synthetic fli mRNA results in accumulation of a functional translation product in the embryo

Xenopus embryos were injected with the synthetic mRNA from 45 min after fertilization up to the 8-cell stage and embry-

Abbreviations used in this paper: A-P, antero-posterior; D-V, dorso-ventral; ECM, extracellular matrix; EWS, Ewing's sarcoma; LTR, long terminal repeat; MSV, Moloney sarcoma virus; NC, neural crest; NTR, N-terminal region.

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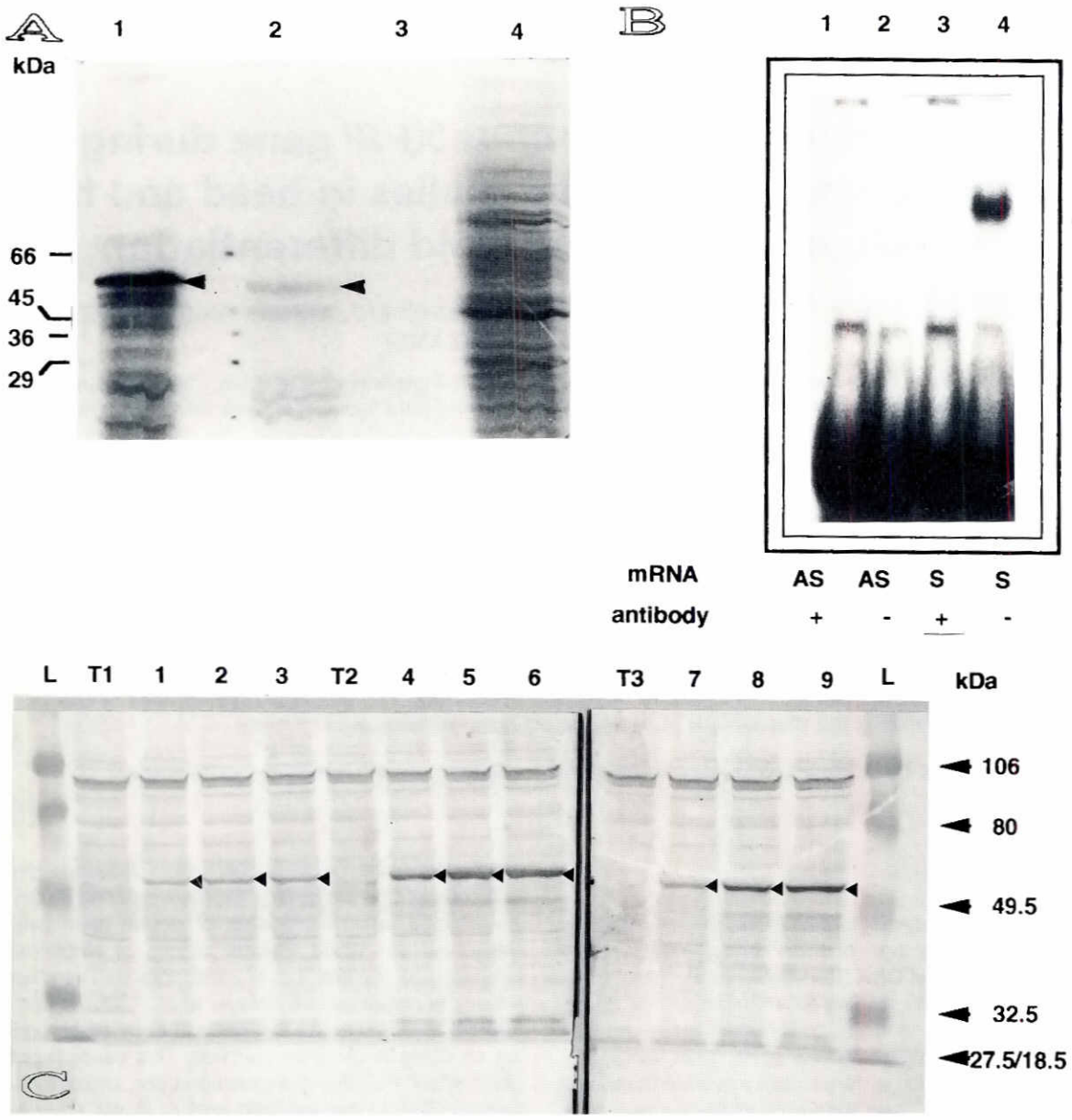

Fig. 1. Analysis of Xl-*fli* overexpression in injected embryos. (A) *Polyacrylamide gel electrophoresis of the ^{35}S-labeled translation products encoded by the Xl-fli synthetic transcript, in a rabbit reticulocyte system (lanes 1) and in a Xenopus oocyte system (lane 2, 3 and 4). Lane 1 shows the migration profile of the polypeptides recovered from the rabbit reticulocyte translation mixture. Lane 2 shows the polypeptides recovered from the oocyte extract upon immunoprecipitation with anti-FLI antibodies. In lane 3, the anti-FLI antibody was preincubated with the antigen prior to the immunoprecipitation reaction. Lane 4 shows the complete oocyte translation mixture, in the absence of immunoprecipitation.* **(B)** *Bandshifting activity of the overproduced FLI protein in extracts of Xenopus embryos at neurula stage towards the ETS-target sequence present in MSV-LTR. The embryos have been injected at the one-cell stage, either with the sense- (lanes 3 and 4) or antisense fli-synthetic transcript (lanes 1 and 2). The specificity of the complex formed with extracts of embryos injected with the sense transcript is assessed by the disappearance of the retarded band upon preincubation of the protein extract with an anti-FLI antibody (lane 3). No bandshifting activity is observed in control embryos, probably because endogenous FLI protein is present at a too low concentration. mRNA: S, injection with the sense transcript; AS, injection with the antisense transcript. antibody: (+), incubation in the presence of the anti-FLI antibody; (-), incubation in the absence of the antibody.* **(C)** *Immunocharacterization of the FLI protein overproduced in Xenopus embryos. Fertilized eggs were injected with increasing amounts of synthetic fli mRNA and allowed to develop for 2, 16 and 24 h. Embryos were then homogenized and the proteins were separated by SDS-PAGE and blotted to a nitrocellulose membrane. Proteins of control embryos of the same developmental stages were also analyzed. The Western blot was then probed with a rabbit polyclonal anti-FLI antibody. L, MW ladder; T1,T2,T3, control embryos analyzed at 2, 16 and 24 h, respectively. 1,2,3: proteins recovered 2 h after injection of the fertilized eggs with 0.55, 1.1 and 2.2 ng of mRNA, respectively. 4,5 and 6, proteins recovered after a 16 h incubation. 7,8 and 9, proteins recovered after a 24 h incubation. The bands corresponding to the Xl-fli protein present in the injected samples and absent from the control samples are indicated by an arrowhead. With the exception of a minor polypeptide of MW ≈30 kDa (probably corresponding to a degradation product), all other bands are non specific since they are observed both in the control and injected samples.*

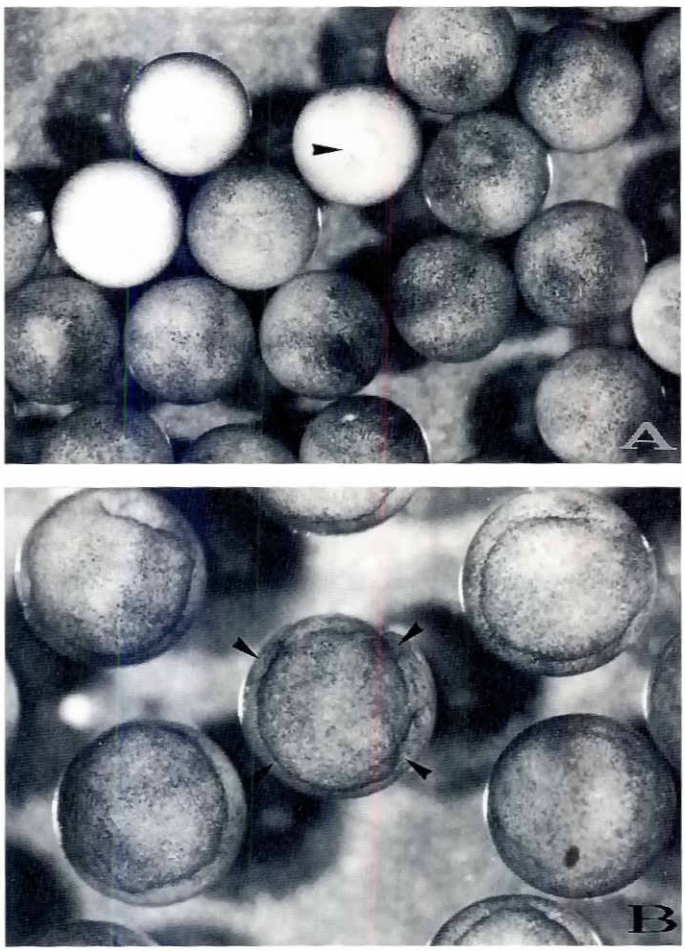

Fig. 2. Perturbation of gastrulation accompanying Xl-*fli* overexpression. (A) *Control Xenopus laevis embryos at the gastrula stage (st. 11.5/12). No superficial feature can be observed, except the blastoporal ring in the vegetal hemisphere.* **(B)** *Fertilized eggs injected with the sense fli synthetic transcript exhibit at the gastrula stage (st. 10.5/11) a distinctive cellular outgrowth (arrows) in the ventro-anterior region.*

onic development was allowed to proceed. Control injections were carried out using the antisense *in vitro* transcript. No effect was expected from an antisense injection, since: i) no maternal transcript of the *fli* gene could be detected (Meyer *et al.*, 1993); ii) the instability of injected RNAs most likely precludes any interference between the antisense synthetic RNA injected soon after fertilization and the zygotic transcripts, which only start to appear at the onset of neurulation (Meyer *et al.*, 1993); iii) mRNA silencing by the use of antisense transcripts in *Xenopus* embryos has proved of limited potential (Rebagliati and Melton, 1987; Colman, 1990). In agreement with these assumptions, antisense injected embryos presented a development identical to that of non-injected embryos (data not shown).

The ability of the synthetic transcript to encode a functional protein in the *Xenopus* system is illustrated in Figure 1. Panel A shows that the synthetic transcript is able to encode a major protein of molecular weight close to 51 kDa (to be compared with a theoretical MW of 51015 Da), both in an *in vitro* rabbit reticulocyte system (lane 1) and upon injection in *Xenopus* oocytes (lane 2). The lower labeling of the protein recovered by immunoprecipitation from oocyte extracts (lane 2), as compared to the one synthesized in the reticulocyte system (lane 1), is most likely due to the isotopic dilution of the ^{35}S labeled precursor by the oocyte pool of methionine. Both synthetic FLI proteins are able to give complexes with several ETS-target sequences like the one present in the LTR of Moloney sarcoma virus (MSV-LTR) (Gunther *et al.*, 1990) (data not shown). The disappearance of the bandshifted complex upon incubation with anti-FLI antibodies attested to the presence of the FLI polypeptide in the complex (data not shown). As this bandshift assay is very sensitive, it was used to check the persistence in later stages of embryogenesis of a functional FLI polypeptide translated from the synthetic transcript injected in early embryos. At neurula (stage 16/17), batches of 10 embryos were collected and homogenized in a lysis buffer (see Materials and Methods). The extracts were then tested for their ability to give a complex with the ETS-binding motif of the MSV-LTR (Gunther *et al.*, 1990). As shown in panel B of Figure 1 (lane 4), a marked complex band is observed in extracts from sense-injected embryos at the same level as the one obtained using an *in vitro* translated FLI protein (data not shown). The specificity of

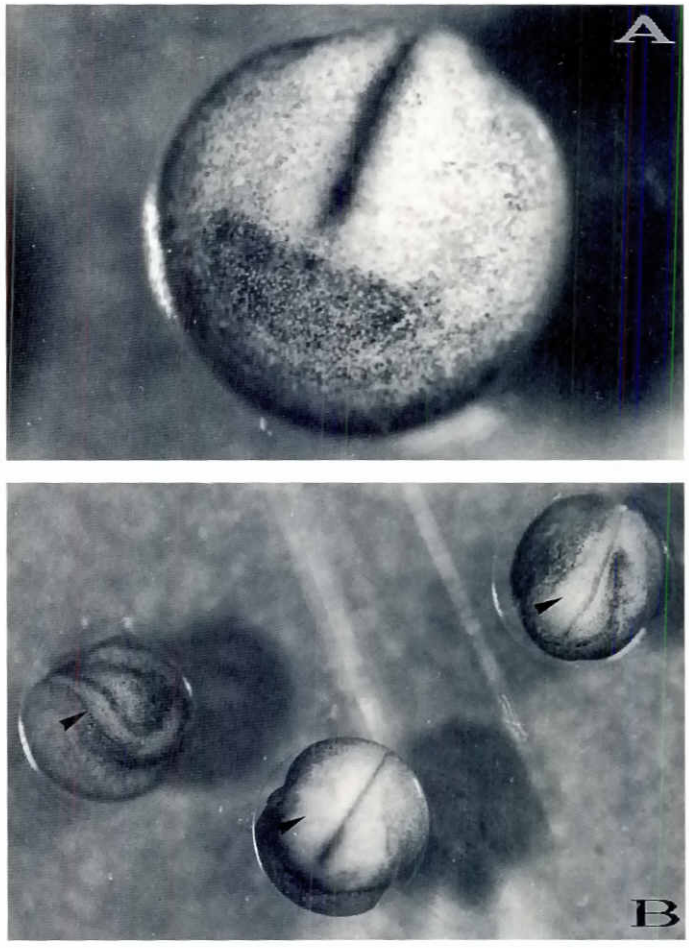

Fig. 3. Injected embryos exhibit hyperswollen neural folds. (A) *Control Xenopus laevis embryos at neurula stage (st. 18).* **(B)** *Fertilized eggs injected with the sense fli synthetic transcript exhibit at neurula stage (st. 17/18) a swelling of the border of the neural fold.*

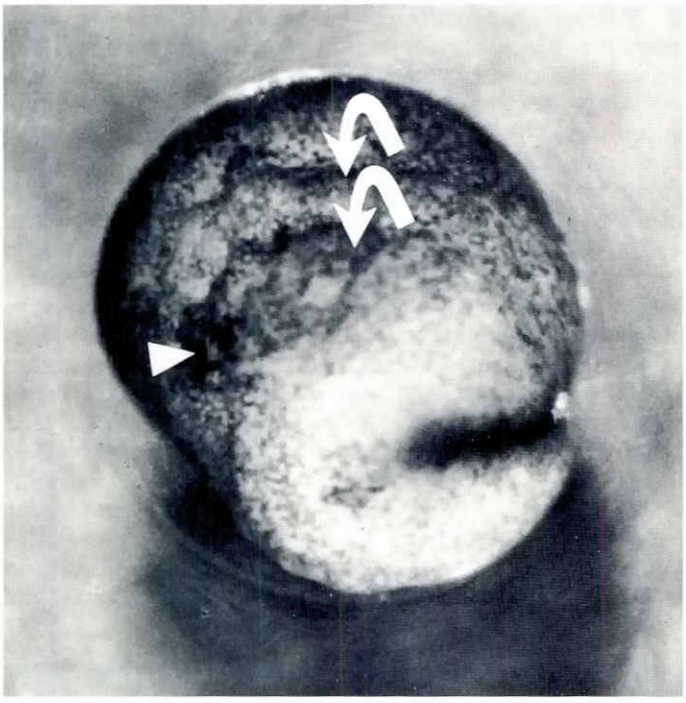

Fig. 4. Injected embryos show at neurula stage (st. 18/19) distinct streaks of cells extending from the dorsal to the ventral region, *evocative of an amplified and/or precocious streaming of neural crest cells towards branchial arches (curved arrows). Note the hyperpigmented region at the tip of the most anterior cell streak (arrowhead).*

the reaction is assessed both by the reversion of the complex upon incubation with an excess of unlabeled probe (data not shown) and by the suppression of the complex band upon incubation with anti-FLI antibodies (lane 3). Correlatively, no complex-forming protein is observed in extracts of antisense-injected embryos (lane 2), or in extracts of control neurula embryos (data not shown). The failure to detect a complex formation in the latter case is probably to be ascribed to a very low level of the endogenous FLI protein. The persistence of an overproduced FLI protein in injected embryos for at least 24 h is further illustrated in Figure 1C which shows the immunoprobing of a western blot carried out on extracts of embryos injected with increasing amounts of synthetic mRNA at 2, 16 and 24 h post-injection. Clearly, a polypeptide of the MW expected for the FLI protein is already present after 2 h, is accumulated at 16 h and is still present at high level after 24 h. Once more, the endogenous FLI protein cannot be detected in the extracts of control embryos.

Overexpression of the FLI protein has a marked teratogenic effect

Different amounts of synthetic transcripts ranging from 350 pg to 1.4 ng were injected in fertilized eggs or in the blastomeres of early embryos (up to the 8-cell stage).

Macroscopic examination

In agreement with the function of transcriptional effector assumed for the FLI protein, no obvious difference can be observed between sense/antisense-injected embryos and control embryos during the cleavage, when the zygotic transcription is silent. At gastrula stage, morphological differences can be observed in sense-injected embryos, whereas antisense-injected and control embryos remain indistinguishable. For the highest doses of the sense *fli* synthetic transcript (0.7 to 1.4 ng/egg), the injected embryos start to present a bulge on the ventral side, opposite to the blastoporal collar (Fig. 2B). During neurulation, the embryos appear almost normal, the only difference with the control (Fig. 3A) being a marked swelling of the neural folds, especially on the sides of the neural plate (Fig. 3B), a region which is known to give rise to the cranial neural crest (NC) cells (Sadaghiani and Thiébaud, 1987; Hopwood *et al.*, 1989; Turner and Weintraub, 1994). At late neurula stage, conspicuous subcutaneous streaks of cells moving from the dorsal region towards the branchial arches can be visualized (Fig. 4). Such a behavior could be expected if the neural crest cells proliferated abnormally and/or migrated precociously. Frequently, these cellular streaks exhibit a marked pigmentation at their tip, which could be related to the fact that NC-cells are the precursors of the melanocyte lin-

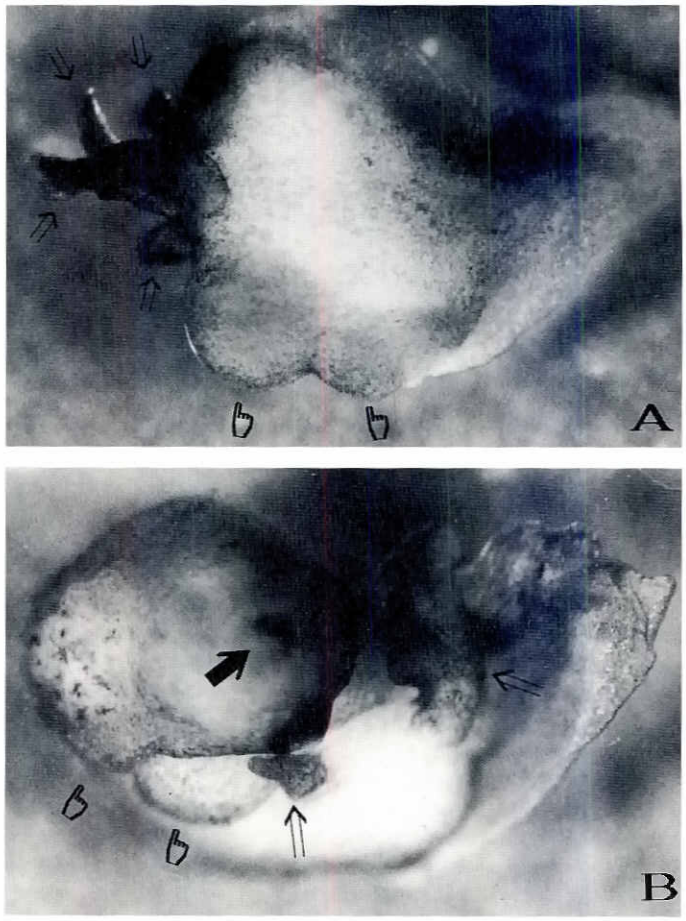

Fig. 5. Injected embryos exhibit blisters and cellular outgrowths at the level of ectoderm. (A) *Embryos injected with high amounts of the fli sense synthetic transcript (1 to 1.4 ng/egg) present head truncation phenotypes, similar to the one shown in this picture. Note also the ectodermal outgrowths in the most anterior region (arrows) and the important blisters in the ventral region (hand pointers).* **(B)** *A stage 33 injected embryo, showing a unique malformed eye (full arrow), ventro-anterior blisters (hand pointers), and ectodermal outgrowths (open arrows) which may look digitated like the most posterior one.*

TABLE 1

DEVELOPMENTAL ANOMALIES IN EMBRYOS INJECTED AT THE 8-CELL STAGE (500 PG OF XL-*FLI* SYNTHETIC MRNA), IN THE DORSAL OR VENTRAL BLASTOMERES

	dorso-animal blastomeres	ventro-animal blastomeres	normalized variable[a]	probability[b]
Nr. of batches	5(5,9,10,20,32)*	4(25,33,20,19)*		
Total nr. of injected embryos (N)	76	97		
% embryos surviving at stage 43	75%	66%	0.79	0.43
developmental anomalies of the eye	88%	52%	2.73	0.006
absence of erythrocytes in circulating blood	85%	56%	2.29	0.022
ectopic differentiation of erythrocytes	50%	38%	1.16	0.25
ectodermal cellular outgrowths	50%	80%	2.30	0.022
absence of beating heart	25%	2%	3.87	0.0001
fin anomalies	22%	n.o.	–	

*Numbers in brackets correspond to the number of embryos in the different batches. [a]The normalized variables were calculated according to: $(|P1-P2|)/\sqrt{(P1/N1+P2/N2)}$. [b]Probability that the two samples belong to the same population: a value above 0.1/0.2 indicates that the two mean values cannot be regarded as statistically different. n.o., not observed.

eage. Later on, the developing embryos are of much shorter length (4.9±0.2 mm for stage 33/34 control embryos compared to 3.7±0.5 mm for embryos of the same stages which have been injected with the sense *fli* synthetic transcript). They present more or less severe antero-posterior alterations:

At high doses of *fli* synthetic transcript (0.7 to 1.4 ng/egg), 1 to 2% of the injected embryos die precociously (mean value on three independent experiments). Of the remaining ones, 85 to 90% present major anomalies of the antero-posterior (A-P) and/or dorso-ventral (D-V) polarities (head truncation in Fig. 5A), whereas 10 to 15% show less severe developmental defaults (reduction or absence of cement gland: data not shown; abnormal or ectopic eyes, curved A-P axis, shorter body length: Fig. 5B). Simultaneously, most of the embryos (≈50% of the population) exhibit ectodermal blisters (Fig. 5C). In most cases, these blebs are heavily pigmented.

For these high doses of *fli* synthetic transcript, the lethality is rather high at later stages of development (50 to 60% around stage 30; ≈80% at stage 40; 95% at stage 46-47). At lower doses of *fli* synthetic transcript (0.35-0.5 ng/egg), the most severe A-P and D-V malformations almost disappear, leaving place to more subtle "phenotypes" (see Table 1):

- eye anomalies: cyclopy (Fig. 6A), or "vestigial" eye deeply buried into the head (Fig. 6B), supernumerary eye (Fig. 6C) most likely due to a duplication of the A-P axis in the most anterior region; heavily pigmented optic stalk (Fig. 6D). Altogether, these anomalies of the eye development affect roughly 40 to 50% of the embryos (extreme values ranging from 20 to 80% in 5 independent experiments). These anomalies appear significantly more frequent when the injection is carried out in the dorso-animal rather than in the ventro-animal blastomere, at the 8-cell stage (Table 1).
- anomalies of the erythroid differentiation: a failure of circulating red blood cells to differentiate had already been observed among

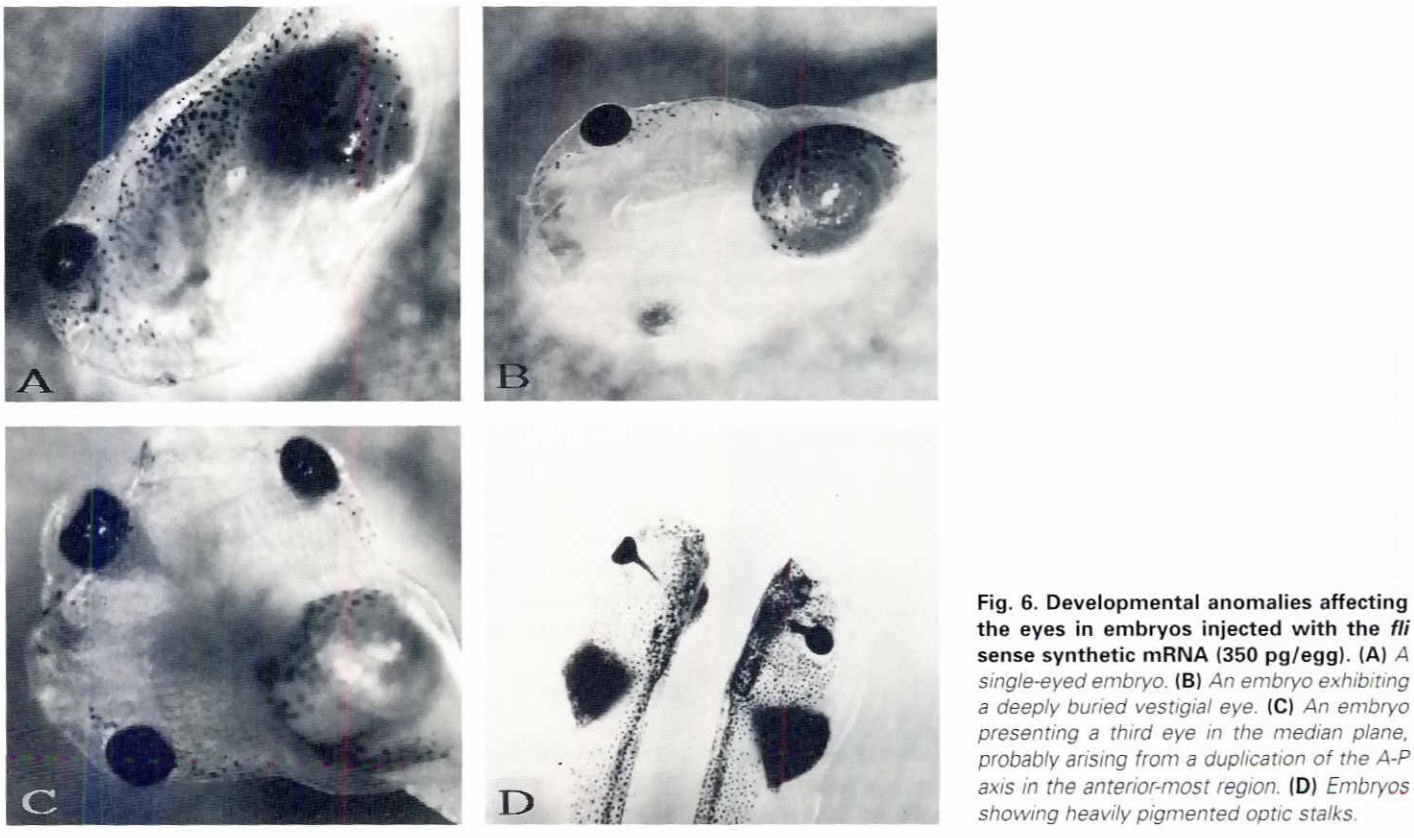

Fig. 6. Developmental anomalies affecting the eyes in embryos injected with the *fli* sense synthetic mRNA (350 pg/egg). (A) *A single-eyed embryo.* **(B)** *An embryo exhibiting a deeply buried vestigial eye.* **(C)** *An embryo presenting a third eye in the median plane, probably arising from a duplication of the A-P axis in the anterior-most region.* **(D)** *Embryos showing heavily pigmented optic stalks.*

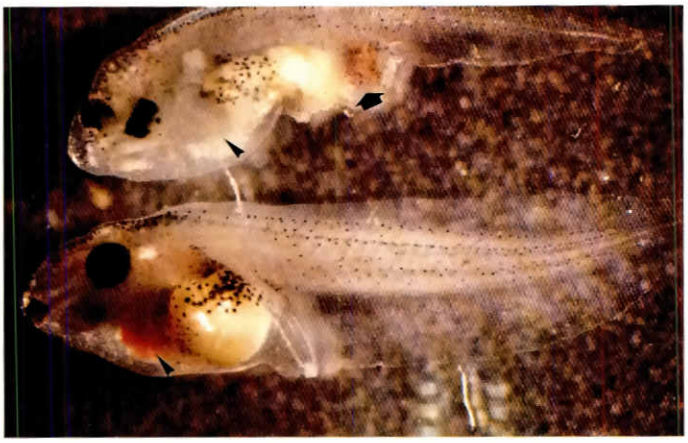

Fig. 7. Xl-*fli* overexpression leads to a blocking of erythropoiesis in circulating blood. *(Top) Stage 45 embryo injected with the sense fli synthetic transcript at the fertilized egg stage (700 pg/egg). Note the malformations affecting the eyes and guts, and the complete absence of red blood at the level of the heart, although the latter could clearly be seen beating under the microscope. (Bottom) Control embryo. Arrowhead, heart; large full arrow, reddish internal mass suggesting an ectopic accumulation of erythrocytes.*

the few embryos surviving at high doses of *fli* synthetic transcripts, later than stage 39/40 (Fig. 7). Although the heart was conspicuously beating, it was circulating colorless blood, revealing the absence of hemoglobin. However, in the specimen of Figure 7, a reddish mass could be distinguished caudally in the abdomen, suggesting ectopic erythroid differentiation. This observation was extended for the lowest doses of *fli* transcripts (350 pg/egg), where hemangiomas could be observed anywhere in 40 to 60% of the larvae, as illustrated in Figure 8. Table 1 shows that the blocking of erythroid differentiation is more frequent when the injection is carried out in the dorso-animal blastomeres. On the contrary, a much less significant difference is observed for ectopic erythroid differentiation.

Cytological examination

Araldite sections were stained and examined at different stages of embryonic development. At the end of blastulation and beginning of gastrulation, the cell layers in the non involuting dorsal marginal zone are clearly separated from each other (Fig. 9A). The same phenomenon is also observed in the ventral marginal zone, although to a lesser extent. Strikingly, round-shaped cells are deposited on the blastocoel floor. Furthermore, the endodermal mass shows islets of these abnormal round-shaped cells at a discrete location, which probably corresponds to the site of injection. These cells are clearly separated from each other and from the surrounding endodermal cells. Simultaneously, multiple "fractures" can be observed within the endodermal cell mass, which are not sectioning artefacts.

Fig. 8. Formation of a periocular hemangioma in a stage 45 *X. laevis* embryo injected with a *fli* sense synthetic mRNA *(350 pg/egg).*

Opposite to the blastoporal collar, at the level of the mesoderm free zone, a separation can be clearly detected between the inner and outer layers of ectoderm (see Fig. 9B, clearly showing a fracture at the level of the inner layer, as if the outer layer was expanding too fast). It should however be noticed that some extracellular fibrous material is left in the cavity, which could arise from a filling with extracellular matrix.

During gastrulation/neurulation, the injected embryos reveal marked anomalies. A striking difference with control embryos is observed at the level of the archenteron. Instead of being a vast cavity as in controls (Fig. 10A), it is reduced to a mere slit (Fig. 10B). Here too, the endodermal mass shows islets of round-shaped cells and evident signs of fractures (Fig. 10B). These disorganized cells also appear to creep between the different layers of ectoderm, mesoderm and archenteron roof, on the dorsal side of the embryo (see enlargement in Fig. 10C).

An example of eye malformation is shown on Figure 11A and B on parasagittal sections of a stage 45 embryo. On the one side, the eye appears almost normal, with the exception of a folded retina (short arrow), but on the other side, the eye cup did not invaginate. Simultaneously, the induction of the lens was perturbed resulting in a lens remaining attached to the ectoderm (full arrowhead). Also notice that the malformed eye cup is separated from the ectoderm by a thick layer of extra-cellular matrix (ECM; long arrows), infiltrated by mesenchymal cells flattened in shape, suggesting that they are actively migrating (see the two fold enlargement in the insert).

As already mentioned, even embryos which look almost normal as to their external morphology can present developmental alterations of the heart. This is also illustrated at the cytological level, as shown in Figure 13A and B, representing sections of the heart in control and *fli*-injected embryos at stage 45/46, respectively. Differences are clearly visible, affecting the cardiac jelly, which is thicker in the injected embryo, thereby reducing the volume of the heart cavity. Endothelial cells lining the ventricular cavity are much more numerous in the injected embryo and appear to pile up on the contrary to what is observed in the control embryo. As already observed at the level of the whole embryo, the heart cavity of the injected embryo does not contain any red blood cells, contrary to those clearly visible in the control. In a few cases (Table 1), no beating heart can be observed, essentially when the injection is carried out in the dorso-animal blastomeres at the 8-cell stage.

Discussion

The *fli* gene product, a transcription factor belonging to the *ets* family, was overexpressed in *Xenopus* fertilized eggs, yielding important anomalies in the embryo development. No effect of *fli*

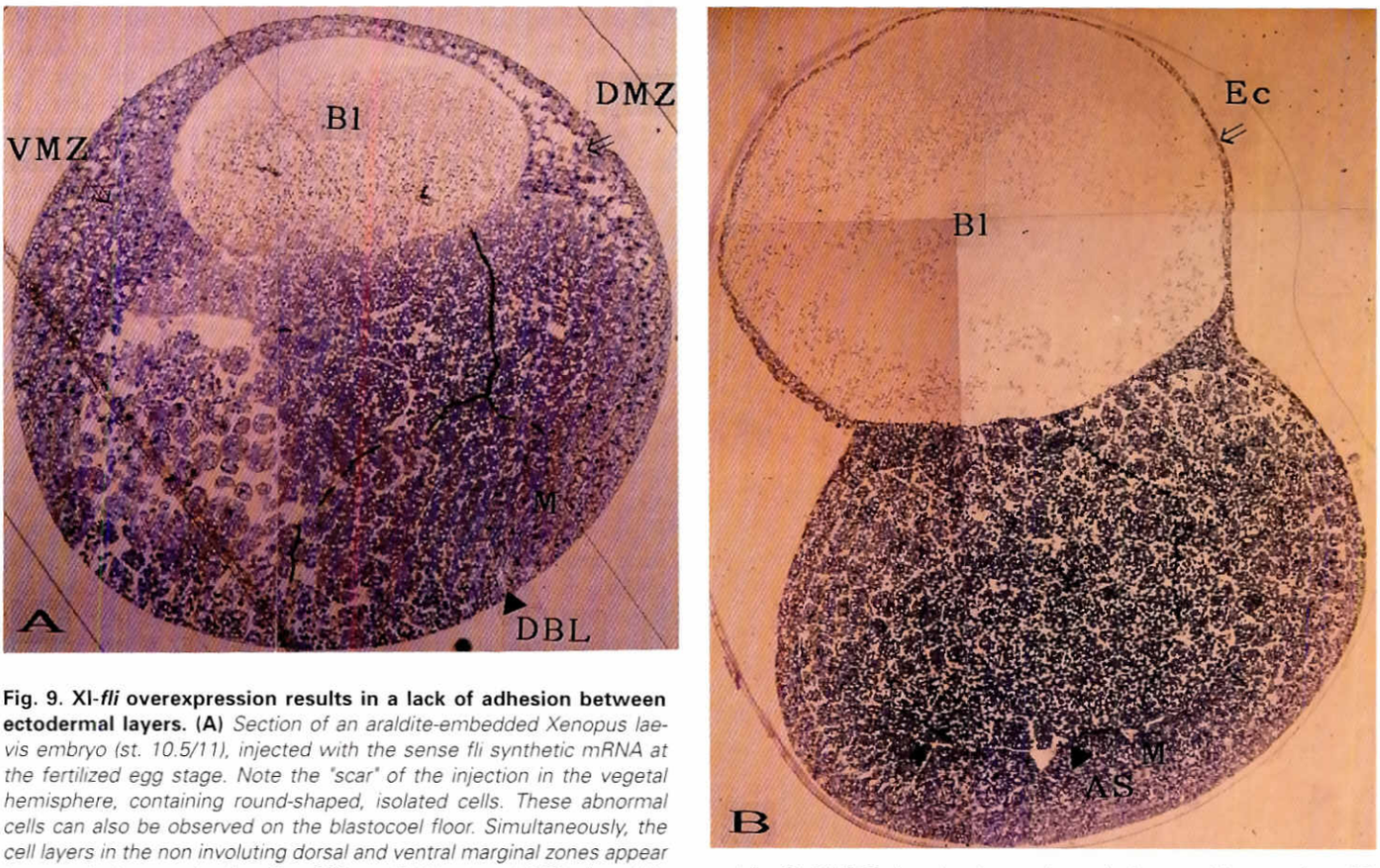

Fig. 9. Xl-*fli* overexpression results in a lack of adhesion between ectodermal layers. (A) *Section of an araldite-embedded Xenopus laevis embryo (st. 10.5/11), injected with the sense fli synthetic mRNA at the fertilized egg stage. Note the "scar" of the injection in the vegetal hemisphere, containing round-shaped, isolated cells. These abnormal cells can also be observed on the blastocoel floor. Simultaneously, the cell layers in the non involuting dorsal and ventral marginal zones appear to separate from each other. Bar, 200 μm. Bl, blastocoel; DBL, dorsal blastoporal lip; DMZ,VMZ, dorsal and ventral marginal zones; M, mesoderm.* **(B)** *Transverse section of an araldite-embedded gastrula embryo (st. 11/12) injected with the sense fli synthetic transcript at the fertilized egg stage, exhibiting a hyperswollen blastocoel. Note that the blastocoel appears limited by a single cell layer (the outer layer of epidermal ectoderm), the inner layer of the epidermal ectoderm looking broken at the level of the blastocoel floor. AS, archenteral slit; Bl, blastocoel; Ec, ectoderm; M, mesoderm; Y, yolk. Bar, 200 μm.*

synthetic mRNA injection was observed before the end of blastulation or the beginning of gastrulation, a period when zygotic transcription is highly effective and the exogenous FLI protein present at high level, as shown by the bandshifting activity persisting in neurula extracts, towards a viral target-sequence, and the immunological characterization of the FLI protein on western blots. The observation that the first effects of *fli* overexpression are detected at the transition from blastula to gastrula, is consistent with the function of transcription factor assumed for the FLI protein (for review, see Wasylyk *et al.*, 1993) as well as with earlier results showing that zygotic transcription massively occurs after the mid-blastula transition (MBT) (Newport and Kirschner, 1982a,b), although some zygotic transcription can occur prior to MBT (Kimelman *et al.*, 1987; Shiokawa *et al.*, 1989; Shiokawa, 1991).

Modification of the embryonic development is quite specific of the overexpression of the FLI protein as no effect is observed when an antisense *fli* transcript is injected into the fertilized egg. This absence of effect of the antisense transcript is not surprising as no maternal *fli* message could be detected in the embryo (Meyer *et al.*, 1993) and as the general unstability of mRNAs most likely precludes this transcript to persist up to the neurula stage, when the zygotic *fli* transcripts start to appear. Furthermore, the use of antisense transcripts in *Xenopus* embryos for selective mRNA silencing has proved of limited potential, because of the presence of unwinding activities (Rebagliati and Melton, 1987; Colman, 1990; Munir *et al.*, 1990).

The "phenotypes" observed in *fli*-injected embryos are more or less severe, ranging from limited malformations in the eyes, heart and/or absence of erythroid differentiation, to the complete absence of embryonic axes and the almost total absence of cellular differentiation, depending on the amount of synthetic transcript injected in the fertilized egg or early embryo. The heavy developmental anomalies are observed for amounts of *fli* mRNA ≥1 ng/egg and probably reflect important perturbations of the inductive processes which take place during gastrulation and lead to the progressive establishment of the A-P polarity. However, this perturbation of gastrulation does not merely reflect an arrest of mesoderm ingression, since its extent appears to be roughly the same as in control embryos. It is most likely due to a lack of adhesion between the different embryonic layers, interfering with induction processes. It could indeed reflect a role of the FLI protein in regulating the expression of adhesion molecules (belonging for instance to the cadherin and integrin families) and/or components of the extra-cellular matrix (ECM), as will be discussed. When lower amounts of synthetic transcripts

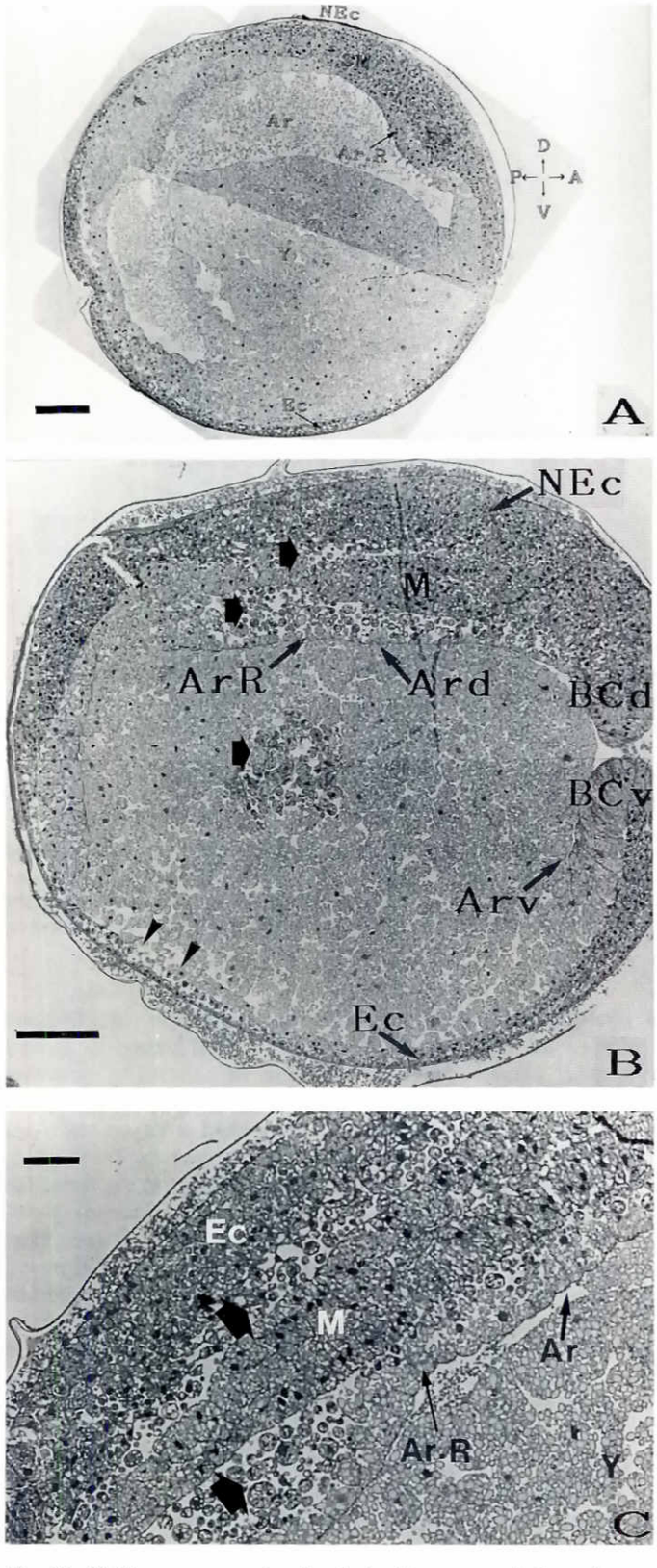

Fig. 10. Xl-*fli* overexpression leads to the accumulation of non-adherent cells at the borders of the different embryonic layers. (A) *Sagittal section of an araldite-embedded X. laevis embryo at neurula stage (control). The embryo exhibits a large archenteral cavity and in the dorsal region the three embryonic layers (neuroectoderm, mesoderm and endoderm) are in perfect contiguity. Anterior is to the right, dorsal to the top. Bar, 200 µm.* **(B)** *Section equivalent to (A) in a neurula embryo microinjected with a sense fli synthetic transcript (700 pg) at the fertilized egg stage. The archenteron is reduced to a mere slit. Although the mesoderm has actively migrated under the ectoderm, the borders between the three embryonic layers are now full of round-shaped isolated cells (thick arrows), probably present in an excess of ECM. Notice the "scar" of the injection site, exhibiting the same anomalous cells. Also remark the separation between the epithelial and sensorial layers of the ectoderm in the ventro-anterior region (arrowheads). Anterior is to the left, dorsal to the top. Bar, 200 µm.* **(C)** *Two-fold enlargement of the dorsal region of the section of (B) showing in more details the separation of the embryonic layers by anomalous cells (thick arrows). Bar, 100 µm. Ar: archenteron; Ar.d., archenteron (dorsal); Ar.R., archenteron roof; Ar.v., archenteron (ventral); Bcd, blastoporal collar (dorsal); Bcv, blastoporal collar (ventral); EA, eye anlage; Ec, ectoderm; M, mesoderm; Nec, neuroectoderm; SM, somitic mesoderm; Y, yolk.*

(≈0.35-0.5 ng/egg) are used, the heavy malformations regress and more subtle effects can be recognized (Table 1). However, even for a given amount of *fli* mRNA injected in the egg, the effects observed present a rather large variability, which is reflected by the dispersion of the frequencies of observation in several sets of independent experiments. This "phenotypic" variability is probably to be ascribed to a non homogeneous distribution of the *fli* synthetic mRNA among the different blastomeres, due to the viscosity of the cytoplasm in *Xenopus* eggs. Supporting this hypothesis is the observation that sections of *fli*-injected embryos reveal the "scar" of the injection in the vegetal hemisphere of the fertilized egg as islets of round-shaped cells separated from the surrounding endodermal cells. A difference in the amount of transcript remaining in the fertilized egg after injection may also contribute to this variability. Indeed, if the amount of synthetic transcript injected in the egg can be accurately controlled, the amount of *fli* mRNA leaking in the culture medium before wound healing might be somewhat different from egg to egg as a result of the depth of injection, more or less complete dejellyfication of the fertilized egg and other physical parameters, as elasticity of the egg surface or constraints during the transfer of the embryos from the injection plate to Petri dishes.

Even at high doses of *fli* mRNA, the first effects are not observed before the beginning of gastrulation and consist in the separation of cell layers in the non involuting marginal zone with a more pronounced effect in the dorsal region. This is accompanied by the presence of round-shaped cells on the blastoporal floor, as if these cells had modified adhesion properties. This observation is consistent with the appearance, in a large majority of treated embryos, of a hyper-swelling of the remaining blastocoel, which appears to be separated from the surrounding medium by a single cell layer, as if the adhesivity of the inner layer of the ectoderm towards the outer layer had been deeply altered. Strikingly, a recent report described identical "phenotypes" in embryos, whose cadherins had been depleted by an antisense approach (Heasman *et al.*, 1994).

The results above suggest that the function of the *fli* gene could be to control the cell adhesion system. The specific expression of the *fli* gene in neural crest cells at embryonic stages where these cells have already begun their migrations (Meyer *et al.*, 1993), would support this idea. Recent results

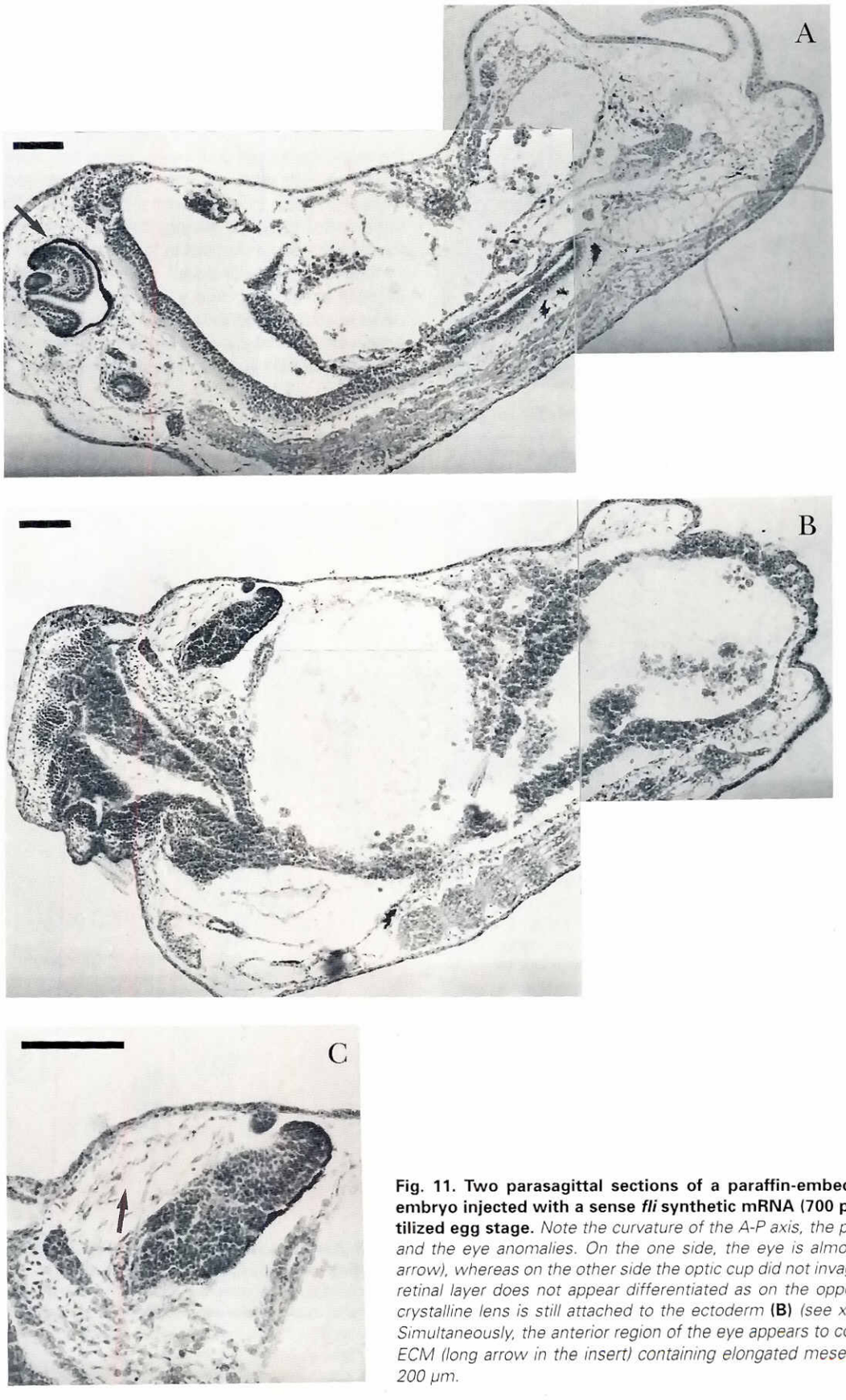

Fig. 11. Two parasagittal sections of a paraffin-embedded stage 37/38 embryo injected with a sense *fli* synthetic mRNA (700 pg/egg) at the fertilized egg stage. *Note the curvature of the A-P axis, the presence of blisters and the eye anomalies. On the one side, the eye is almost normal* **(A,** *long arrow), whereas on the other side the optic cup did not invaginate properly, the retinal layer does not appear differentiated as on the opposite side, and the crystalline lens is still attached to the ectoderm* **(B)** *(see x2 enlargement,* **C***). Simultaneously, the anterior region of the eye appears to contain an excess of ECM (long arrow in the insert) containing elongated mesenchymal cells. Bar, 200 μm.*

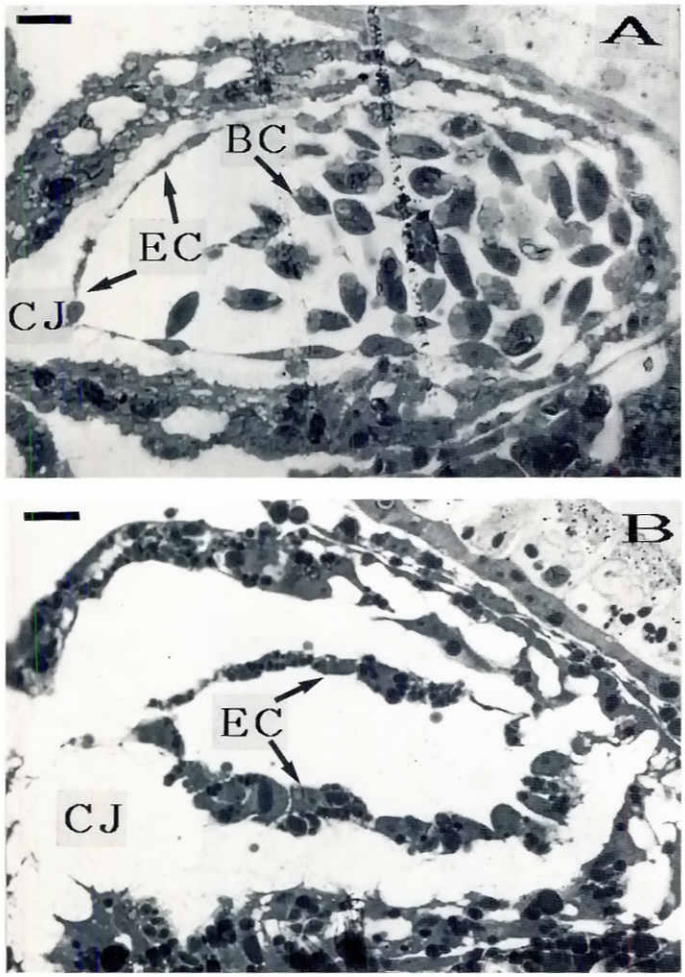

Fig. 12. Anomalies in heart development accompanying XI-*fli* overexpression. (A) *Sagittal section of an araldite-embedded stage 45 embryo (control). Notice the layer of endothelial cells (EC) lining the cardiac jelly (CJ) and the red blood cells (BC) filling the cardiac cavity.* **(B)** *Equivalent section in a stage 45 embryo injected with the fli sense synthetic mRNA (700 pg/egg) at the fertilized egg stage. Notice the thickening of the cardiac jelly (CJ) reducing the volume of the cardiac cavity and suggesting an increase in the secretion of a component of the ECM. The endothelial cells (EC) instead of forming a monolayer are now piling up as if they were proliferating abnormally or had modified adhesion properties. Bar, 30 µm.*

(Meyer *et al.*, 1995), showing that the *fli* gene is also expressed at sites where active cell migration does occur (cardiac endothelium, pronephric duct, hypophysis) are consistent with this idea. A role of the FLI protein in controlling the expression of adhesion molecules belonging to the integrin and cadherin families could not be exclusive of its participation in regulating the synthesis of specific constituents of the ECM. The presence of sequences recognized by the FLI protein in the promoter regions of the cytotactin and vitronectin genes, as well as their occurrence in the promoters of some integrins subunits (α_v and β_3 for instance; Meyer *et al.*, 1995) further support this hypothesis. An increase in the production of ECM due to *fli* overexpression could also be consistent with the presence of numerous blisters in the injected embryos. Further support would come from the observation of the remnants of a loose filling in the sections of Figure 9, the ECM being usually poorly conserved in standard fixatives, as well as from the observed accumulation of ECM in the heart (see below). Overexpression of the FLI protein could therefore deeply alter the migration of neural crest cells, resulting in anomalies of the head cartilages and eyes, since the neural crest cells are known to play a crucial role in these embryonic processes (Le Douarin, 1982). In agreement with this idea, Table 1 shows that the anomalies of eye development are significantly higher when the injection is carried out in the dorso-animal blastomeres.

Interestingly, lower doses of *fli* mRNA result in developmental defaults in the circulatory system, where the endogenous *fli* gene is also expressed. These defaults include an inhibition of erythroid differentiation, which is consistent with the association of erythroleukemia and *fli* overexpression in the mouse, upon

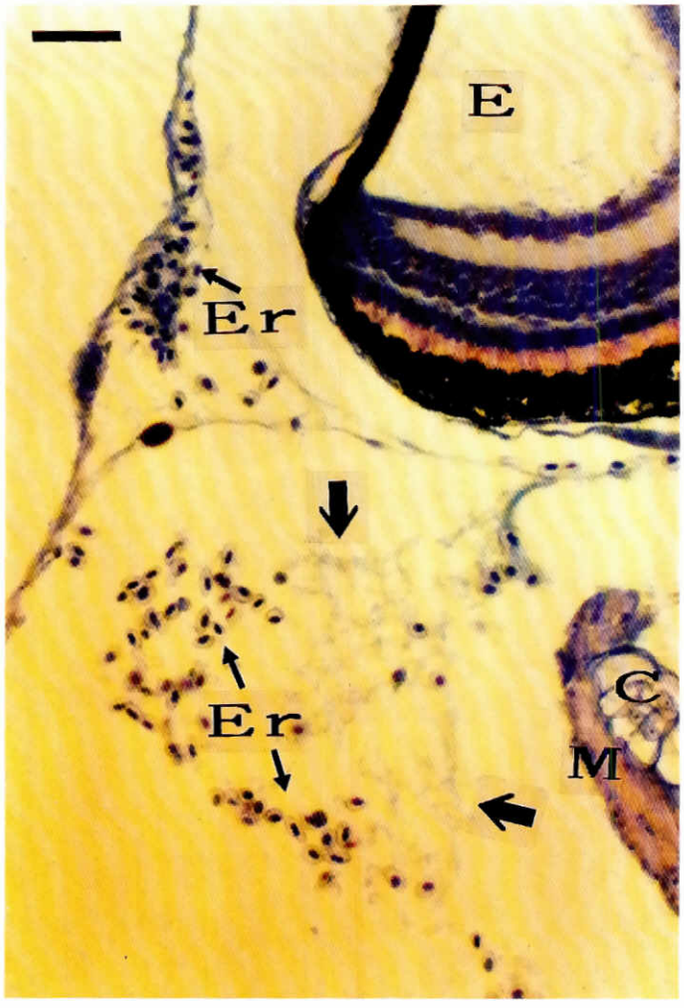

Fig. 13. Transverse section of a paraffin-embedded stage 45 embryo exhibiting a hemangioma in the periocular region (see Fig. 8), demonstrating the accumulation of erythrocytes (ellipsoidal nucleated cells) in avascular structures (thin arrows). *Also note the fibrous remnants of a filling material evocative of an excessive production of ECM (thick arrows). C, cartilage; E, eye; Er, erythrocytes; M, muscle. Bar, 20 µm.*

infection by the Friend leukemia virus. Table 1 shows that this anomaly is more frequent when the injection is carried out in the dorso-animal blastomere, which could reflect a role of both paraxial and ventral mesoderm in blood island formation (Kau and Turpen, 1983; Maéno *et al.*, 1985a,b). Strikingly, this absence of circulating red blood cells can be accompanied by the formation of hemangiomas which appear with a high frequency at various localizations in the larvae, in avascular structures, as shown in Figure 13. This shows that the differentiation of blood islands in vascular structures and circulating cells has been perturbed, misrouting information leading to ectopic differentiation of erythrocytes. Furthermore, the heart development itself is affected by *fli* overexpression. Table 1 shows that 25% of the embryos injected in the dorsal blastomeres do not show a beating heart, in agreement with the overexpression of *fli* in the known presumptive area for the heart. In many instances, the cardiac jelly is much more abundant than in control experiments and the endothelial cells (which normally express the *fli* gene; see Meyer *et al.*, 1995) are more numerous and appear to stack one upon the other instead of forming a cell monolayer. Once more, these observations could be indicative of perturbations affecting the synthesis of adhesion molecules and/or components of the ECM. Indeed, the cardiac jelly is synthesized by endothelial cells (Zhang *et al.*, 1995) and is constituted of sparse mesenchymal cells (derived from the endothelial cells) surrounded by an abundant ECM.

Of course, an effect of the FLI protein on the expression of cell adhesion molecules and/or components of the ECM could not be exclusive of other possible mechanisms allowing the dissemination of migratory cell populations. They could involve the up-regulation of genes encoding proteases, more or less specific for the ECM. It has for instance been shown that the *ets1* gene is expressed in mesencephalic neural crest cells (Vandenbunder *et al.*, 1989; Kola *et al.*, 1993) and stimulates the expression of metallo-proteases in fibroblasts of the stroma of various carcinoma (Wernert *et al.*, 1992), possibly facilitating the dispersion of metastases.

Experiments are in progress to assess the participation of the *fli* gene in these different possible mechanisms.

Materials and Methods

PCR

The coding sequence of the Xl-*fli* cDNA in λgt10 (Meyer *et al.*, 1993) was amplified using the following sense and antisense primers: 5'-GCTCATCTAGAATGGACGCAACCATTAAGGAA; 5'TTCTAAGCTTGTCGACCTAGTAAAAACCACCTAAGTG. The PCR product was purified by centrifugation on a miniprep spun column (Pharmacia), equilibrated in 50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 5 mM DTT, 50 μg/ml BSA. It was then treated with 5 units of T4 DNA polymerase (Eurogentec, Brussels, Belgium) in a total volume of 180 μl for 5 min at room temperature in the absence of dNTPs. After addition of 0.5 mM dNTPs, the incubation was resumed for 30 more min. The blunt-ended PCR product was then recovered by phenol/$CHCl_3$ and $CHCl_3$ extractions followed by ethanol precipitation.

Plasmids

The pSP64T vector (Krieg *et al.*, 1984) was chosen because of its ability to generate functional mRNAs by *in vitro* transcription. The plasmid was digested by BglII and blunt-ended upon incubation with 5 units of Klenow DNA polymerase in the presence of 200 μM dNTPs. The vector DNA was recovered by phenol/$CHCl_3$ and $CHCl_3$ extractions followed by ethanol precipitation. The insert was then ligated in the blunt-ended digested vector at different insert/vector ratios under standard conditions (Ausubel *et al.*, 1987). Recombinants in both sense and antisense orientations were selected. The sense recombinant used was fully sequenced.

In vitro transcription

The SP6 Megascript *in vitro* transcription kit from Ambion (Woodward, Texas, USA) was used under the conditions described by the manufacturer. The recombinant DNA was linearized by SmaI digestion. Capping was achieved upon addition of 4 mM m7G(5')ppp(5')G in the transcription mix. Under these conditions, the RNA yield ranges around 10 to 20 mg of RNA per mg of input DNA. The DNA matrix was destroyed upon incubation with RNAse-free DNAse (Boehringer, Mannheim, FRG). The RNA was phenol and $CHCl_3$ extracted, then recovered by ethanol precipitation. After drying, the RNA was dissolved in RNAse-free water at a concentration of ≈0.2 μg/μl.

Isolation and fertilization of Xenopus eggs

Wild-type *Xenopus* females were made to spawn upon treatment with 750 units of human chorionic gonadotropin injected in the dorsal lymph sac as described by Moon and Christian (1989). The eggs were then fertilized and dejellied according to the same authors.

Microinjection of the fertilized eggs

The dejellied fertilized eggs (starting from 45 min after fertilization) were microinjected with the *in vitro* transcribed RNA under the conditions described by Moon and Christian (1989). The apparatus used was the Nanoject automatic injector manufactured by Drummond Scientific company (Broomall, PA, USA). Each embryo was injected at the vegetal pole with 0.35 to 1.4 ng of synthetic RNA, in a volume of 4.6 to 23 nl of injection buffer (10 mM Tris-HCl pH 7.4, 88 mM NaCl, 1 mM KCl).

Antibodies

Polyclonal antibodies specific for different regions of the *Xenopus* FLI protein were obtained upon immunization of rabbits with fusion proteins between the desired FLI domains and the glutathion S-transferase. The fusion proteins were obtained using the pGEX2T expression vector and partially purified by affinity chromatography on glutathion-Sepharose columns. The antibodies were then checked by ELISA. For the bandshift experiments, an antibody against the N-terminal region (amino-acids 1 to 121) was used.

In vitro translation of the synthetic mRNA

In vitro translations of the synthetic mRNA used in microinjections were done using the Type II reticulocyte kit from Boehringer Mannheim (Germany) under the conditions recommended by the manufacturer. The translation products were analyzed by SDS-electrophoresis on a 10% polyacrylamide gel.

Bandshift experiments

Injected embryos were recovered at neurula stage and homogenized as described by Hinkley *et al.* (1992). The "biological" activity of the FLI protein formed upon translation of the synthetic mRNA was assessed by its bandshifting activity towards a target sequence contained in the LTR of the Moloney sarcoma virus. The reaction mix contained 20 mM Hepes buffer pH 7.9, 50 mM KCl, 10 mM DTT, 1 mM EDTA, 5 to 10 μl of embryo extracts (or 1 μl of an *in vitro* translation mixture), 1 to 1.5 μg of poly(dI-dC) and a large excess of probe labeled by 5'-phosphorylation. After addition of the probe, the reaction mixture was incubated for 20 min at 0°C and the complexes were resolved by electrophoresis on a 6% polyacrylamide gel in 0.25xTBE buffer at room temperature. The specificity of the interaction was controlled by displacement by an excess of non-labeled probe as well as by suppression of the bandshift upon incubation of the complex in the presence of anti-FLI antibodies.

Embryo embedding, sectioning and staining

Embryos were fixed overnight in 4% paraformaldehyde and dehydrated through an increasing ethanol series (70%, 95% and 100%), treated twice with propylene oxide at room temperature for 10 min. They were then submitted to an incubation in a 50% propylene oxide-50% araldite resin for at least 2 h, (under an incandescence bulb, so as to maintain a convenient fluidity of the resin), followed by 2 one-hour treatments in araldite, before being incubated in fresh resin at 70°C, up to complete hardening of the resin (usually 48 h). The embedded embryos were then sectioned using a Reichert OMU-2 ultramicrotome equipped with a diamond knife at a 1 to 1.5 mm thickness. The sections were then processed with sodium methoxide for 1 min at room temperature to get rid of the resin, washed twice rapidly with a 50% methanol-50% benzene mixture and twice with acetone. After rehydration, the sections were stained with 0.5% toluidine blue in 5% sodium borate solution, or Mayer's hemalung (Merck, Darmstadt, Germany), diluted at 50% in water. Alternatively, the toluidine blue staining can be achieved without elimination of the resin by sodium methoxide simply by covering the sections with the dye solution and letting stand on a heating plate for 1 to 2 min at 65°C.

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