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Original Article

The avian *fli* gene is specifically expressed during embryogenesis in a subset of neural crest cells giving rise to mesenchyme

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ABSTRACT The ets-family of transcription factors is involved in the development of endothelial and hematopoietic cells. Among these genes, *fli* was shown to be responsible for erythroblastomas and Ewing's sarcomas. Its involvement in Ewing's sarcoma, a putative neurectodermal tumor, as well as the *in situ* hybridization studies performed in mice and *Xenopus* suggested a role in neural crest development. We cloned quail *fli* cDNA in order to analyze in more detail its expression in neural crest cells, which have been extensively studied in avian species. *Fli* gene maps on chicken chromosome 1 to band q31->q33. Two RNAs are transcribed, most likely arising from two different promoters. The analysis of its expression in neural crest cells reveals that it is expressed rather late, when the neural crest cells reach their target. Among the various lineages derived from the crest, it is restricted to the mesenchymal one. It is maintained at later stages in the cartilage of neural crest but also of mesodermal origin. In addition, *fli* is expressed in several mesoderm-derived cells: endothelial cells as well as intermediate and splanchnopleural mesoderm.

KEY WORDS: *chicken, quail, transcription factor, endothelial, neural crest, chromosomal localization, Ewing's sarcoma*

Introduction

The development of a complete organism from a single-cell zygote is a complex mechanism, requiring the temporal and cell-specific regulation of numerous genes. Proto-oncogenes encoding transcriptional effectors are likely to play a crucial role in embryogenesis, since they could be involved both in the control of cell proliferation and cell differentiation.

Among these proto-oncogenes, the *ets*-family (for a review, see Ghysdael and Boureux, 1997) could be of key importance. These genes are related to a retroviral oncogene (v-*ets*), expressed by the E-26 avian erythroblastosis virus as a fused gene with *gag* and *myb* (Leprince *et al.*, 1983; Nunn *et al.*, 1983). More than 30 related genes belonging to this family have been characterized in a wide range of species from the nematode (Beitel *et al.*, 1995) to human (Watson *et al.*, 1988a). The *ets* family is highly conserved, as shown for *ets-1* and *ets-2* genes in human and mouse (Watson *et al.*, 1988a), chicken (Boulukos *et al.*, 1988; Leprince *et al.*, 1988; Watson *et al.*, 1988b), *Xenopus laevis* (Stiegler *et al.*, 1990; Wolff *et al.*, 1990).

The corresponding proteins usually behave as transcriptional activators (for review, see Bosselut *et al.*, 1990; Gunther *et al.*, 1990; Ho *et al.*, 1990; Wasylyk *et al.*, 1993). However, several studies recently reported repressor activities for proteins belonging to the ets family: ets-1 (Prosser *et al.*, 1992; Wotton *et al.*, 1993), NET (Giovane *et al.*, 1994), ERF (Sgouras *et al.*, 1995). These proteins contain a well conserved DNA-binding domain, encompassing 85 amino acids, the ETS-domain. The latter was recently shown to adopt a helix-turn-helix conformation (Donaldson *et al.*, 1994; Liang *et al.*, 1994a,b). Transcription factors of the ets family were shown to bind regulatory sequences (EBS sites) containing a core GGAA/T motif (Karim *et al.*, 1990). Observations were more recently reported for *ets-1*, *ets-2* and *fli-1* binding to EBS sites, showing that orientation and spacing of the motifs could modulate the protein-DNA interaction (Hodge *et al.*, 1996; Venanzoni *et al.*, 1996).

Several studies have shown that members of the *ets* family play important roles both in oogenesis and embryogenesis. The maternal *ets*-2 transcript was shown to be required for *Xenopus* oocyte maturation (Chen *et al.*, 1990). *Ets-1* and *ets-2* genes were

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reported to be transcribed in different tissues and to be differentially regulated during mouse development (Kola *et al.*, 1993; Maroulakou *et al.*, 1994). *Ets-1* involvement in the control of chicken embryonic development was also reported (Vandenbunder *et al.*, 1989; Desbiens *et al.*, 1991; Pardanaud and Dieterlen-Lievre, 1993a,b; Quéva *et al.*, 1993). We could show that, in *Xenopus laevis*, the Xl-*fli* gene was specifically expressed in regions of the embryo affected by important cellular migrations, as well as in the migratory cells themselves: neural crest cells, pronephric duct cells and endothelial cells (Meyer *et al.*, 1993,1995). Similar observations were made for the *ets-1* and *ets-2* genes (Meyer *et al.*, 1997), as well as for the *erg* gene (Mager, unpublished results). The ectopic and over-expression of the Xl-*fli* gene during *X. laevis* embryogenesis was shown to be highly teratogenic. Anomalies affecting the antero-posterior polarity were observed (head truncations), together with developmental anomalies of the eyes and heart, and perturbations of erythrocytic differentiation (Remy *et al.*, 1996).

In this paper, we report the characterization of three different cDNA clones of the quail *fli* gene. Two of those correspond to cDNAs arising from different sites for initiation and termination of reverse transcription on the same mRNA template. The third one differs from those two at the 5' terminus, both in the 5'-UTR region and in the very beginning of the coding sequence. This 5'-UTR exhibits a significant homology with genomic regions located far upstream from the putative *fli*-promoters characterized in human, mouse and *Xenopus*. It is therefore very likely that the *fli* gene is transcribed from two different promoters at least. It must however be underlined that the 5'-sequence of this clone does not contain an initiator ATG in phase with the remaining part of the coding sequence common to the three cDNAs. The translation of mRNA most likely proceeds from the first downstream ATG.

We used a 1.5 kb PCR labeled cDNA probe in order to map the gene in avian species. Its localization on avian chromosome 1 reveals a conservation of its location relative to other genes between humans and avian species.

In situ hybridization using radioactive and digoxygenin-labeled probes was carried out to determine the expression pattern of the *fli* gene in the course of embryogenesis. The expression pattern confirms that *fli* is expressed in endothelial and neural crest cells as in other species (Meyer *et al.*, 1993,1995; Mélet *et al.*, 1996). In addition, we show that *fli* expression is restricted to mesectodermal neural crest cells from their arrival in the branchial arches to their differentiation into cartilage. It is never expressed in neurogenic and melanogenic neural crest cells.

Results

Quail fli-cDNAs

Three different clones of cDNA were sequenced (6.1, 10.1, 17.1). Two of them (10.1 and 17.1) clearly correspond to the same mRNA and originated from different sites for the initiation and termination of reverse transcription. Both contained an ATG, which corresponds to the open reading frame found in other species. The third one (6.1) differs from the two other clones in its 5'-terminal region, including the first 15 nucleotides of the open reading frame of clones (10.1) and (17.1). It must be stressed that no initiation ATG is found in this clone, upstream from the sequence common to all three clones. The translation therefore most likely proceeds from the first downstream ATG, leading to a shorter polypeptide, lacking the first 33 amino acids with respect to the putative translation products of the (10.1) and (17.1) clones. Unexpectedly, a search conducted in EMBL databank revealed a significant homology of this 5'-region of clone 6.1 (Fig. 1) with mouse, human and *Xenopus* genomic regions flanking the *fli* gene and thought to harbor the promoter (Barbeau *et al.*, 1996; our unpublished results). However, these homologous regions are far upstream (more than 1000bp) from the putative human and mouse transcription initiation sites. It is therefore very likely that the *fli* gene is transcribed from two different promoters at least.

The comparison of the 5'-UTR sequences among four different species [clone (10.1) or (17.1) of the quail, *Xenopus*, mouse and man] also reveals the existence of a short highly conserved motif immediately upstream of the initiator ATG, as illustrated in Figure 1, suggesting the existence of a conserved translational regulation. This sequence is however not found in the 5'-terminal sequence of clone (6.1).

Comparison of putative translation products in different species

Figure 2 shows an alignment of the putative FLI-polypeptide sequences of quail, *Xenopus* (Meyer *et al.*, 1993), mouse (Ben-David *et al.*, 1991) and man (Watson *et al.*, 1992). Although the homology between these different proteins decreases from man to *Xenopus*, the sequence is highly conserved between species, since the quail polypeptide exhibits 92, 90 and 85% identity with man, mouse and *Xenopus* counterparts respectively. These figures increase to 94, 93 and 92% if conservative replacements are taken into account.

An internal deletion of 22 amino acids is observed in the quail FLI polypeptide, with respect to the *Xenopus*, mouse and man homologs.

The putative quail polypeptide exhibits an ETS-domain, which is the most conserved region among the different species. In addition, multiple potential phosphorylation sites are conserved among the four sequences of Figure 2.

Chromosomal localization of Fli

A biotinylated probe was hybridized to chick early metaphase chromosome spreads. About 150 metaphases were analyzed giving 60-70% of signals on one or both chromatids on chromosome 1 to band q31->q33 (Fig. 3A and B). Very low non-specific background was observed. Due to the high stringency conditions used to prevent binding to related *ets*-family genes, signal was sometimes lost on one of the two chromatids.

Fli expression in the neural crest cells at early stages

The neural crest cells begin to migrate from the mesencephalic neural fold at stage HH9 and the migration wave progresses posteriorly. The neural folds prior to neural crest cell migration did not express *fli*, as verified at stages HH8, 11, 14-15 at different levels of the neural tube (Figs. 4,5 and 7). The neural crest cells, when they underwent their epithelial-mesenchymal transition and began to migrate, were neither labeled by the *fli* RNA probe. This was true either for the cephalic neural crest at stage HH10-11 (Fig. 4C,D), in the cervical region at stage HH12 and in the trunk at E3 (third day of embryonic development).

At stage HH14-15, the ectomesenchyme of the head was labeled by the *fli* probe as shown around the eye (Fig. 4A,B), in the

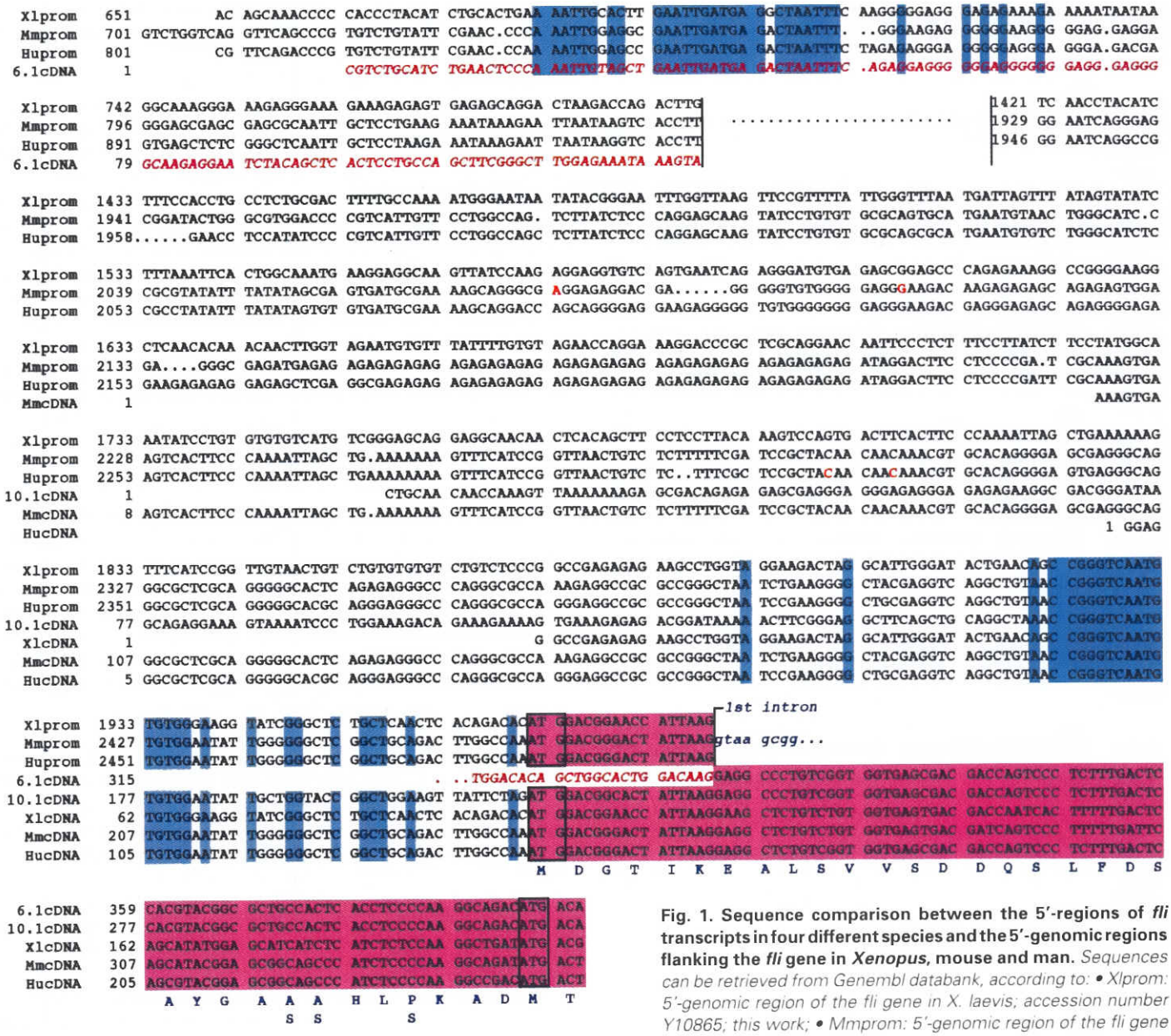

Fig. 1. Sequence comparison between the 5′-regions of *fli* transcripts in four different species and the 5′-genomic regions flanking the *fli* gene in *Xenopus*, mouse and man. *Sequences can be retrieved from Genembl databank, according to: • Xlprom: 5′-genomic region of the fli gene in X. laevis; accession number Y10865; this work; • Mmprom: 5′-genomic region of the fli gene in M. musculus; accession number L47615; Barbeau et al., (1996); • Huprom: 5′-genomic region of the fli gene in H. sapiens; accession number L47616; Barbeau et al., (1996); • 6.1cDNA: quail fli cDNA, clone 6.1; accession number Y14774; this work; • 10.1cDNA: quail fli cDNA, clone 10.1; accession number Y14773, this work; • XlcDNA: Xenopus fli cDNA; accession number X66979; Meyer et al., (1993); • MmcDNA: mouse fli cDNA; accession number X59421; Ben-David et al., (1991); • HucDNA: human fli cDNA; accession number X67001; Delattre et al., (1992). Sequences were aligned using the 'PILEUP' software from the GCG package. Genomic and cDNA sequences show a high degree of conservation in the coding region (highlighted in pink). This applies to clone 6.1, but only downstream from the glutamate residue in position 7 of the putative polypeptide (blue capital letters). Indeed, the 5′-region of clone 6.1, upstream from that residue (magenta capital italic letters), exhibits no convincing homology with the other sequences. Particularly, no initiator ATG can be found at the expected position. Therefore, translation should start at methionine 33 (boxed ATGs), conserved in all sequences. Strikingly, a significant conservation (shaded in cyan) is observed between the 5′-UTR of clone 6.1 and the three genomic sequences, more than 1kb upstream from the sites of transcription initiation described by Barbeau et al. (1996), in the mouse and human genes (red letters in the sequences). This would imply the existence of a second promoter, upstream from the one described by Barbeau et al. (1996). Sequence conservation is also observed in the 5′-UTR region (shaded in cyan), immediately upstream from the initiator ATG codons (boxed), most likely corresponding to translational regulatory regions.*

frontonasal process and the branchial arches (Figs. 4A,B and 5A,B). Premuscular condensations in the frontonasal process (Fig. 4A,B, arrows) and in the center of branchial arches at stage HH20 were not labeled. These mesenchymal cells are of mesodermal origin. To confirm unambiguously the neural crest origin of the *fli* expressing cells, two sets of experiments were carried out. First, double-labelings with HNK-1 monoclonal antibody (mAb) were performed. This antibody, although it does not label all the

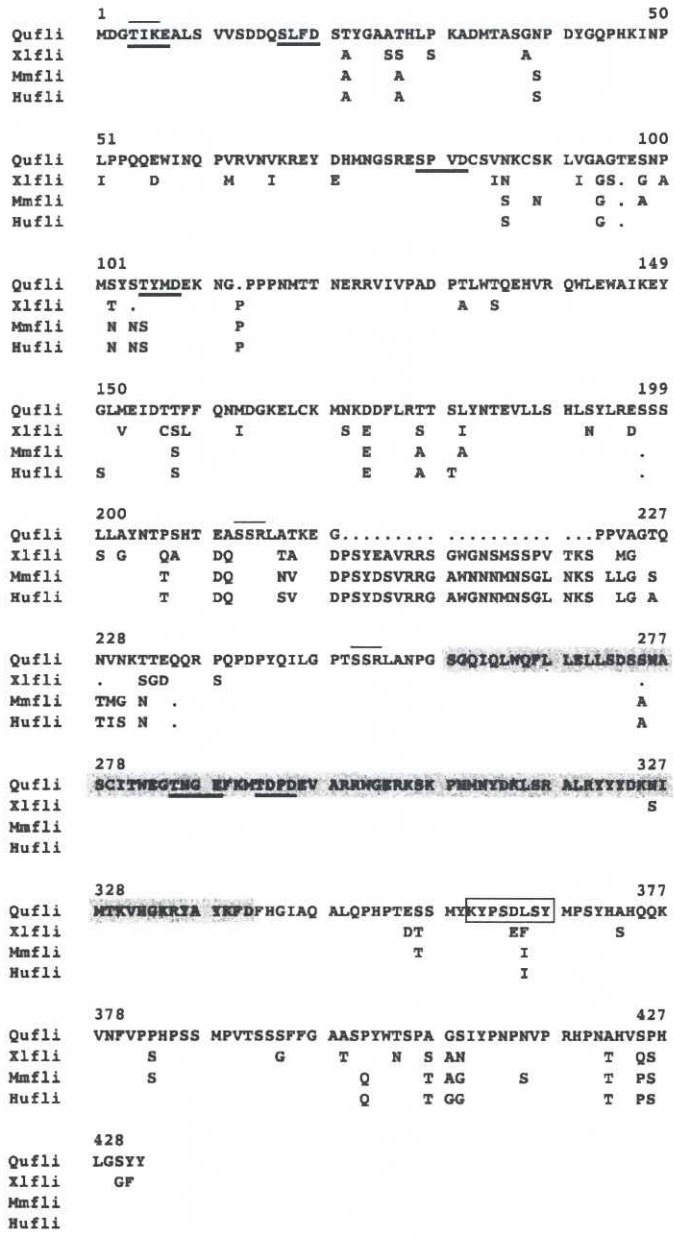

Fig. 2. Comparison of the putative translation products of *fli* cDNAs in four different species. *Only the amino acids differing from the quail sequence are shown. A high overall conservation is obvious, and is even more pronounced in the ETS- domain (shaded). Many potential phosphorylation sites can be detected: Ck2 (underlined in bold); PkC (black line on top of characters); tyrosine kinase (boxed).*

neural crest cells is, among the mesenchymal cells of the head, specific for the mesectoderm (Tucker *et al.*, 1988). Numerous cells were double-labeled, meaning that at least a subpopulation of crest cells expressed *fli* (Fig. 5A,B). However, some HNK-1-positive cells were not labeled. These cells corresponded to (i) the nerves and cranial ganglia (Fig. 5C,D, arrows), (ii) neural crest cells in the proximal part of the branchial arches (Fig. 5B, arrow). Neural crest cells became labeled as they reached the distal part of the arches. (Fig. 5B, arrowhead). At their last step of migration, it was demonstrated that they loose HNK-1 expression (Tucker *et al.*, 1984). However, they keep on expressing *fli* (asterisk).

We devised a second set of experiments to make sure that the mesenchymal cells of the branchial arches which were labeled were neural crest cells and not mesodermal cells. We replaced the neural fold of a chick at the level of rhombomeres 3 to 6 by their quail counterpart prior to migration. One day after grafting, quail cells were evidenced in branchial arches 2 and 3 using the QCPN mAb. These cells strictly corresponded to neural crest cells. The *fli* probe which hybridizes both to chick and quail RNAs was hybridized on adjacent sections. This confirmed that the zone which was labeled corresponded to the zone where neural crest cells were localized, at the periphery of the branchial arches (Fig. 6, arrowheads). The center of the branchial arch which contains mesodermal precursors of muscles was not labeled. In addition to neural crest cells, it has to be noted that endothelial cells of the aorta were labeled as well as the angioblasts which accumulated at the periphery of the neural tube prior to forming the brain vasculature (Fig. 6 arrow).

Fli expression in the derivatives of the neural crest

The examination of the early steps of neural crest cells settling strongly suggested a lineage specificity of *fli* expression. We therefore carefully analyzed the expression of *fli* in the derivatives of the neural crest. The neural crest is at the origin of a number of tissues and structures among which the peripheral nervous system, the melanocytes, some endocrine and paraendocrine cells and also the so-called mesectoderm which, in higher vertebrates, is restricted to the cephalic region of the neural axis (Le Douarin, 1982).

The neural crest cells of the trunk which do not yield mesectoderm were found not to express *fli*. This was true at early stages, at HH14-15 when these cells migrate in the somites or at E4 for melanoblasts migrating to the dermis. The crest derivatives in the nerves and the dorsal root ganglia in E3, 4, 6 chicks and E10 quails did not express *fli*. Neither did the parasympathetic ganglia in E4 and E6 chicks nor the crest cells of the adrenal medulla of E10 quails. The cells which migrate along the dorsal route give rise to the melanoblasts and melanocytes. These cells were observed in the E10 quail neck and were never labeled.

The vagal crest cells, which migrate from the region of somites 1 to 7 and which give rise to the enteric plexus (Le Douarin and Teillet, 1973), did not express *fli* either during their migration as shown at stage HH14-15 in Figure 6A,B (arrow) or after their settling in the gizzard and the intestine of E10 quails.

The mesectodermal subpopulation of the cephalic neural crest was the only one to be labeled. These cells give rise to the facial cartilages and bones, to the connective cells of the head and contribute to the glands of the neck (thymus, thyroid, parathyroids, ultimobranchial body, carotid body) (Le Lièvre and Le Douarin, 1975). The cells of the branchial arches were continuously labeled at stages HH14-15, at E3, 4 as well as their cartilaginous derivatives at E6. The glands of the neck were not *fli*-positive at E6.

Fli expression in mesodermal derivatives

At stage HH8, *fli* is expressed in isolated cells in the mesodermal layer. The MB1/QH1 epitope is an antigenic determinant common to endothelial and blood cells of the quail (Péault *et al.*,

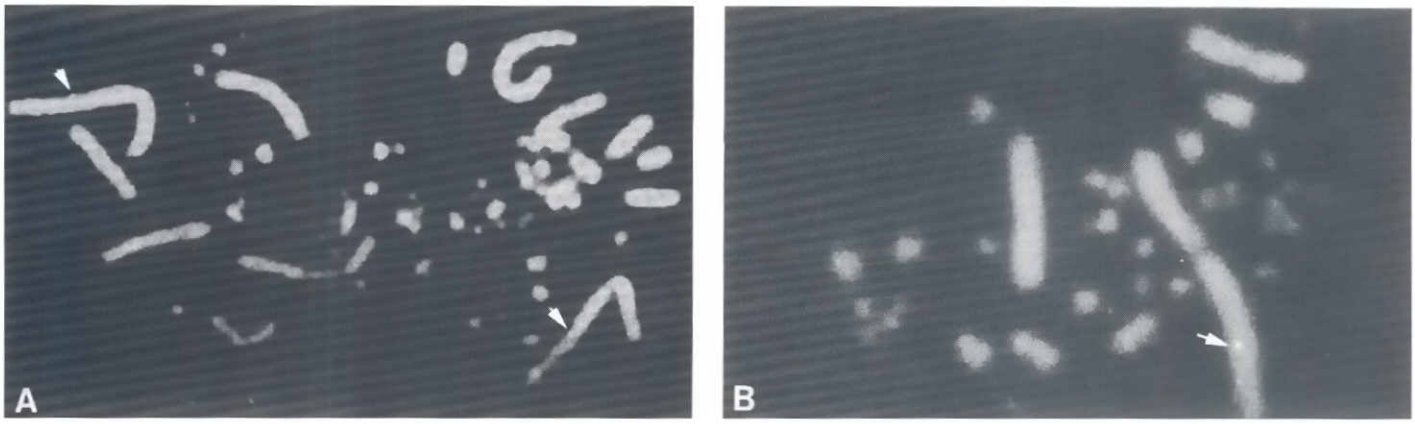

Fig. 3. Chromosomal localization. (A and B) *show metaphase spread (A) and partial metaphase spread (B) after hybridization with fli gene probe. Arrows indicate specific hybridization signals on chicken chromosome 1. The low resolution of the picture does not allow band recognition although the observation revealed hybridization on 1q31->q33.*

1983; Labastie *et al.*, 1986; Pardanaud *et al.*, 1987; Coffin and Poole, 1988; Poole and Coffin, 1988). The use of *fli* probe and of MB1/QH1 mAb on adjacent sections of quail origin showed that, at stage HH9, some *fli*-positive isolated cells were also MB1/QH1-positive (Fig. 7, arrow) although *fli*-positive cells were much more numerous than MB1/QH1 cells. This suggests that angioblasts express *fli* before MB1/QH1. At stage HH14, the somatopleure also contained isolated cells expressing *fli*. These cells are future vascular endothelial cells and originally come from the somites (Pardanaud *et al.*, 1989,1996; Pardanaud and Dieterlen-Lièvre, 1993a) (Fig. 8E,F). Conversely, the splanchnopleural mesoderm which generates its own endothelial cells was uniformly labeled at that stage (Fig. 8E,F). From stage HH11-12, *fli* labeled all vascular endothelia. This is shown for aortae (Fig. 8A,B), intersegmental arteries (Fig. 8F) and anterior cardinal vein (Fig. 8A,B). Unexpectedly, the amnion which does not contain blood islands was labeled as shown in Figure 8A and B. *Fli* expression in endothelial cells was maintained at least up to E10 in the quail.

The endocardium was labeled from its formation at stages HH11-12 (Fig. 8A,B) up to at least E6 (chick). Endocardial cells which underwent a conversion to mesenchyme and started their migration through the cardiac jelly to contribute to the septa and valves were also positive (Markwald *et al.*, 1975,1977; Manasek *et al.*, 1986) (Fig. 5E, arrow).

In the E4 aorta, the ventral aortic clusters, which are budding intraembryonic blood cells (Dieterlen-Lièvre and Martin, 1981), are labeled like the endothelium itself, as well as the cells which just bud from the cluster. The circulating blood cells were labeled only exceptionally (Fig. 8C,D).

The second early site of *fli* expression was the intermediate mesoderm from stage HH11 onward. The intermediate mesoderm extends from somite 5 to the cloaca and contains the 3 nephrogenic areas (pro-, meso-, metanephros). It expressed *fli* on its whole length at the investigated stages (HH11,12,14-15) (Fig. 9A,B).

Several mesenchymal cells of mesodermal origin were labeled. Although the somites themselves did not express *fli* (Fig. 9A,B), the sclerotome (Fig. 8A,B) when it segregated from the dermomyotome was labeled. Derived vertebral cartilage also expressed *fli* at E6. Neither the dermomyotome nor its dermal and muscular derivatives expressed the gene. All cartilage was labeled whether it was of neural crest origin as in the head, or of mesodermal origin as in vertebrae and in limbs. In E10 quails, the labeling became restricted to the perichondrium.

Discussion

In this study, we report the cloning, chromosomal localization and expression pattern of the avian homolog of *fli*, a member of the *ets* family of transcription factors.

Cloning and analysis of the cDNAs

cDNAs of the quail *fli* gene were cloned and sequenced. Sequence comparisons with other *fli* cDNAs bring evidence both for alternative splicing and existence of a second promoter. Indeed, the 5'-UTR region of clone (6.1) exhibits homology with genomic regions far upstream from the putative promoters characterized so far in man and mouse (Barbeau *et al.*, 1996).

Surprisingly, clone (6.1) does not contain any ATG upstream from the sequence common to all three *fli* cDNA clones. As the three coding phases in this 5'-region contain numerous stop codons, translation of the corresponding mRNA should therefore lead to a shorter polypeptide (399 amino acids; MW=45.3 kDa), lacking the first 33 amino acids with respect to the putative translation products of clones (10.1) and (17.1) (432 amino acids; 48.8 kDa).

Such a situation has already been reported for the ERG protein (Duterque-Coquillaud *et al.*, 1993), very similar to the FLI protein, where polypeptides down to 38 kDa are encoded by alternatively spliced transcripts. In support of this idea, this ATG is found in a rather good Kozak's context: GCAGAC**ATG**A, comparing with the GCCA/GCC**ATG**G consensus (Kozak, 1991). Quail FLI polypeptide also lacks 22 amino acids with respect to the proteins characterized so far in other species. This most likely arises from alternative splicing, as already observed for the closely related ERG proteins (Dhordain *et al.*, 1995; our unpublished results). It should be noted that the missing amino acids are rather conserved between FLI and ERG proteins and are flanking exons A81 and A72 of the latter , which have been shown to be alternatively spliced (Dhordain *et al.*, 1995).

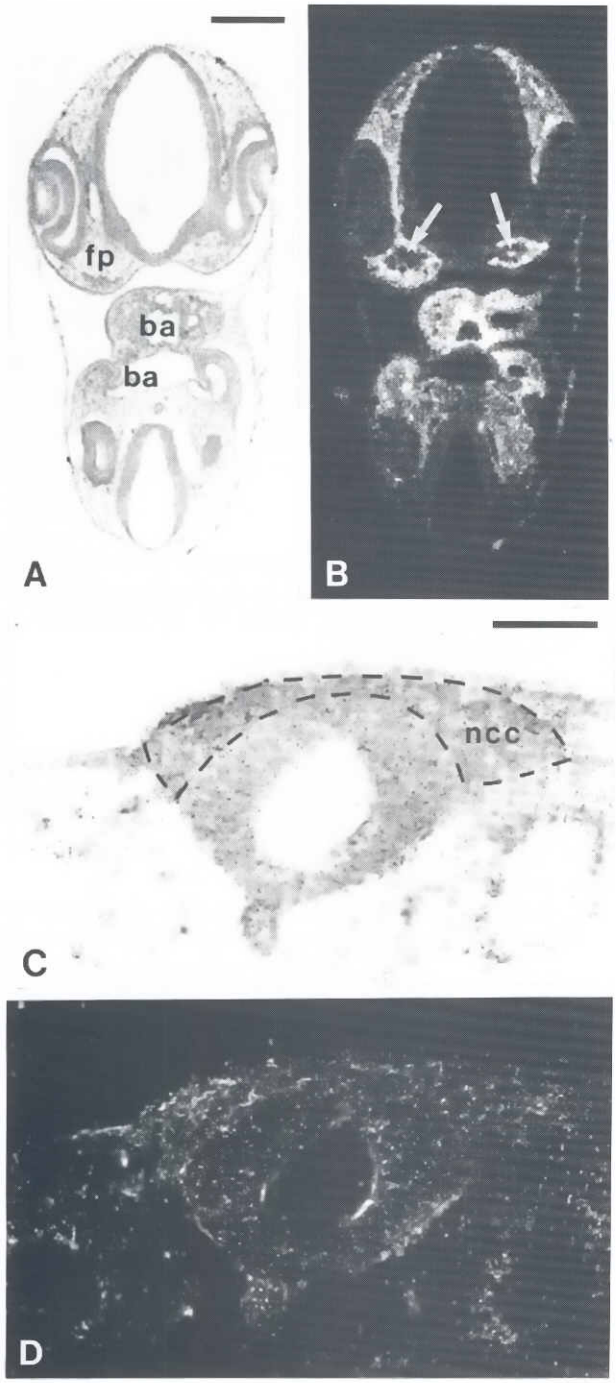

Fig. 4. Time course expression of *fli* in neural crest cells. (A and B) *Bright field and dark field photographs of a frontal section of the head of a stage HH14-15 quail embryo. The section shows the levels of the prosencephalon and the frontonasal process (fp) in its upper part, branchial arch 1 (ba) in the middle and rhombomeres at the level of the otic vesicle and branchial arch 2 in its lower part. The labeling is restricted to mesenchymal cells. At the level of the frontonasal process some mesenchymal cells of mesodermal origin are devoid of labeling (arrows). Note labeled cells in the amnios.* **(C and D)** *Bright field and dark field photographs of a transversal section of the rhombencephalon of a stage HH11 quail embryo. The neural crest cells (ncc) which migrate from the dorsal part of the neural tube are not labeled. Neither is the neural tube itself. Bar in A and B, 200 μm and 100 μm in C and D.*

The rather high conservation between species of the 5'-UTR sequence encountered in the (6.1) clone would plead for post-transcriptional control, as does the occurrence of highly conserved blocks in the 5'-UTRs of clones (10.1), (17.1) and human and mouse *fli* cDNAs. It would of course be very interesting to know whether these transcripts are tissue-specific. Experiments are in progress to isolate by RT/PCR the 5'-UTR of *Xenopus* and mouse cDNAs corresponding to clone (6.1).

The putative translation products of the *fli* cDNAs of course contain an ETS-type DNA-binding domain. Numerous putative phosphorylation sites are conserved in all the FLI-polypeptides known today. Among those, a tyrosine-kinase site located immediately downstream of the ETS-domain could be of importance for the biological activity of these proteins.

Chromosomal localization

The *fli* gene is mapped on human chromosome 11 q24 (Ouchida *et al.*, 1995). Our results confirm the conservation previously proposed between human chromosome 11 and chicken chromosome 1. The B-haemoglobin (HBB) and Progesterone receptor (PGR) genes were assigned to human chromosome 11 and chicken chromosome 1. This study contributes to the comparative gene maps among man and chicken. However, many more gene assignments need to be determined to precisely delineate the conserved chromosome segments.

Early expression in neural crest cells

The expression pattern of the mRNA from this gene was previously reported in mouse (Mélet *et al.*, 1996) and *X. laevis* (Meyer *et al.*, 1993,1995). In both species, its expression in hematopoietic and endothelial cells is clear and is confirmed in avian species in our study. Its expression in neural crest cells, although strongly suggested by the localization of the mRNA in the branchial arches, was never analyzed in details. In particular, nothing was known about the time course of its expression in the neural crest cells and the various lineages derived from these cells. We have used the avian model in which the neural crest cells have been extensively studied (Le Douarin, 1982) to study *fli* expression in these cells.

An early expression of *fli* in the intersomitic space raised the possibility that in the mouse *fli* was expressed early during neural crest cell migration (Mélet *et al.*, 1996). In *X. laevis*, *fli* is expressed in a position where early migrating crest cells should be located. Our study in avian species reveals that neither the neural fold from which the crest cells originate nor the early migrating cells are labeled. The observation of transversal sections co-labeled with HNK-1, an antibody specific for the neural crest cells in the branchial arches, indicates that neural crest cells begin to express *fli* only when they settle in the branchial arches. This expression pattern is different from that of the other members of the family. *ets-1* and ck-*erg*, are expressed in the cephalic and truncal neural crest cells as soon as they begin to migrate (Vandenbunder *et al.*, 1989; Maroulakou *et al.*, 1994; Dhordain *et al.*, 1995). A transient expression of *ets*-1 in the neural fold proper was noticed by Maroulakou *et al.* (1994) although this expression was not confirmed in *Xenopus* (Meyer *et al.*, 1997). Both ck-*erg* and *ets-1* continue to be produced, like *fli*, in the branchial arch-derived structures late in development. *Ets-2* expression in the branchial arches is also reported in *Xenopus* (Meyer *et al.*, 1997) and later in the neural crest derived cartilage in the head for the mouse (Maroulakou *et al.*, 1994).

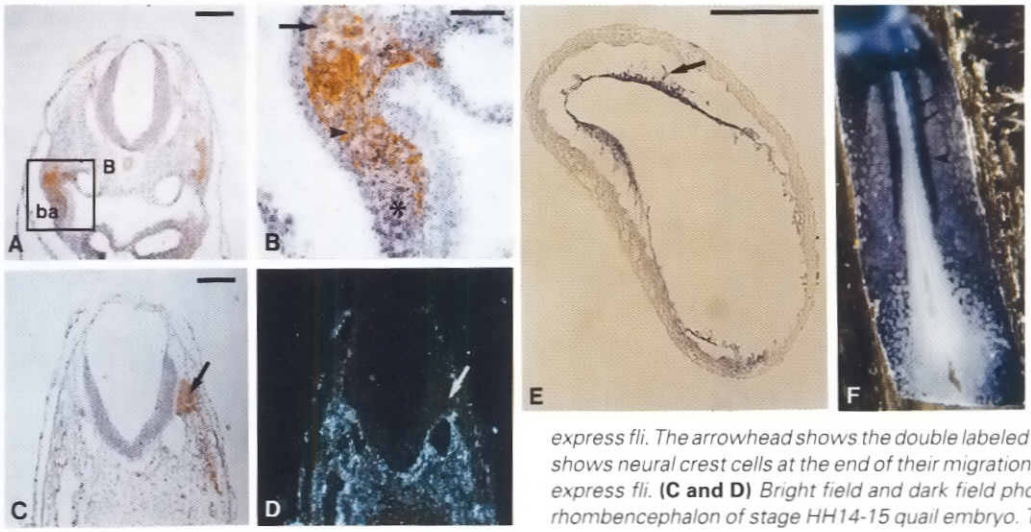

Fig. 5. Dynamic and lineage specific expression of *fli* in neural crest cells and in the heart. (A and B) *Transverse sections in the rhombencephalon of a stage HH14-15 quail embryo. The section was submitted to radioactive in situ hybridization with a fli probe (black dots) and immunocytochemistry with the HNK-1 antibody which recognizes a subset of neural crest cells (brown). The magnified section shows the neural crest cells at different positions during their migration toward the branchial arch (ba). The arrow shows neural crest cells which enter the arch stained with HNK-1 and which do not express fli. The arrowhead shows the double labeled neural crest cells in the arch. The asterisk shows neural crest cells at the end of their migration, which loose HNK-1 expression, but still express fli.* **(C and D)** *Bright field and dark field photographs of a transverse section of the rhombencephalon of stage HH14-15 quail embryo. The brown staining corresponding to the HNK-1 mAb location reveals the root of the trigeminal nerve (arrow). (D) These cells are not labeled (arrow). Also note labeled cells in the amnios.* **(E)** *Section through the heart after whole-mount in situ hybridization with a digoxigenin labeled antisense fli probe. Intense labeling can be observed in the endocardial cells (arrow), including those which start their conversion to mesenchyme.* **(F)** *Stage HH13 quail embryo after whole-mount in situ hybridization, showing a marked fli expression in the forming blood vessels of the embryo and extra-embryonic tissues. The endothelia of the two posterior cardinal veins and the intersomitic arteries are labeled. Bars, 100μm in A,C,D,E and 50 μm in B.*

The changes in the set of ets-family genes expressed in the neural crest cells reflect the main events of neural crest development: epithelio-mesenchymal transition, migration, settlement. These events can also be followed by changes in the adhesive properties of these cells (Bronner-Fraser, 1993 for review and references therein). For example, neural fold cells express c-cad6B (Nakagawa and Takeichi, 1995) and N-CAM (Thiery *et al.*, 1982) and lose these expressions as they begin to migrate. A subset of these cells then acquires the expression of c-cad7 (Nakagawa and Takeichi, 1995). This expression is lost as they reaggregate in the ganglia. As these cells settle in the branchial arches, they are in a fibronectin rich environment and express receptors for this molecule (Krotoski *et al.*, 1986). The members of the ets-family could play a role in regulating the expression of adhesion molecules in neural crest cells. *Fli* itself could regulate the adhesion molecules involved in the settling of neural crest cells.

Lineage specificity

We show that in the neural crest no other lineage than the mesectodermal is labeled. As a consequence, the expression is restricted to the neural crest of the head which is the only level which provides mesectoderm. This lineage restriction was not observed at early stages for *ets-1* since this gene is expressed in migrating neural crest cells in the trunk of the avian embryo (Vandenbunder *et al.*, 1989; Maroulakou *et al.*, 1994) and *Xenopus* (Meyer *et al.*, 1997). Moreover, *ets-1* and *ets-2* at later stages are expressed in neural derivatives of the neural crest (Meyer *et al.*, 1997).

Thus different members of the family are expressed at different times as well as in specific lineages in neural crest, suggesting that in this system these genes are not redundant but could play complementary roles.

Expression in endothelial cells

The involvement of ets family genes in the hemangioblastic system is well described. These genes are expressed in adulthood in various lineages of this system. The localization of *fli* transcripts suggests a participation of this gene in vasculogenesis and angiogenesis. It is expressed in the somatopleura when it is colonized by extrinsic progenitors for endothelial cells and in the splanchnopleura where endogenous angioblasts are generated (Pardanaud *et al.*, 1996). This expression pattern is very similar to those of c-*ets-1* (Kola

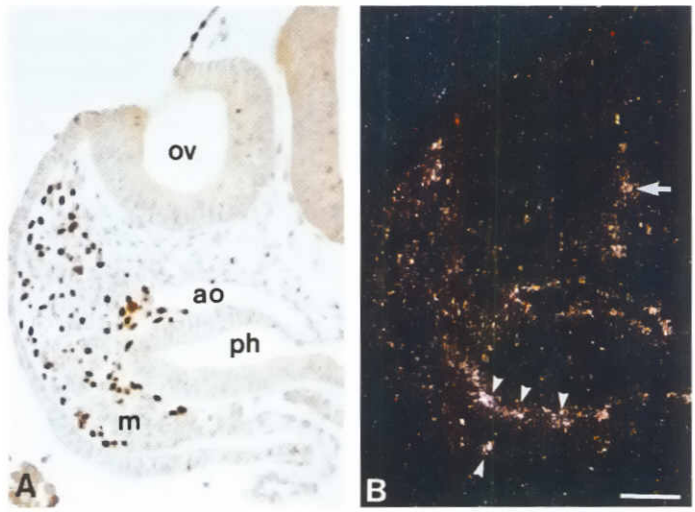

Fig. 6. *Fli* is expressed in the mesectoderm but not the mesoderm of the branchial arches. *Transverse section of a stage HH15 quail into chick chimera at the level of branchial arch 3. The neural fold of a chick has been replaced prior to crest migration by its quail counterpart at the level of rhombomeres 3 to 6. One day after grafting, quail cells, which correspond strictly to neural crest cells are evidenced using the QCPN mAb* **(A)**. *The center of branchial arch 3 is of mesodermal origin and thus it is not stained by QCPN (m). In* **(B)**, *an adjacent section shows that neural crest cells which are localized in the distal part of the branchial arches do express fli (arrowheads). The center of the arch, corresponding to the premuscular condensation of mesodermal origin is not labeled. Angioblasts at the periphery of the neural tube express fli as well as endothelia as in the aorta (ao). ov, otic vesicle; ph, pharynx. Bar, 200 μm.*

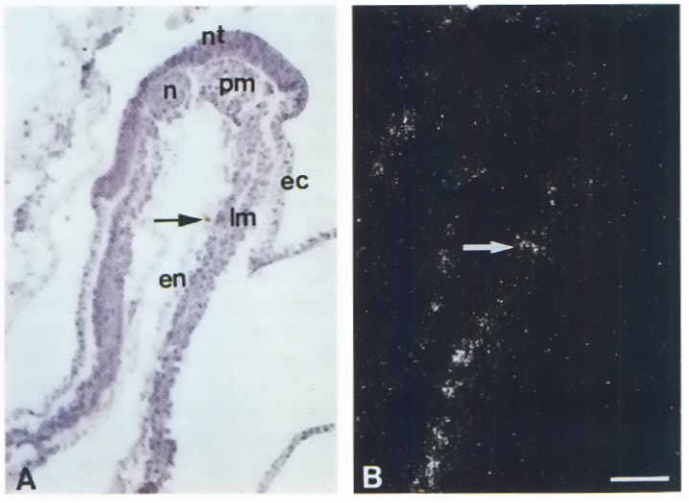

Fig. 7. *Fli* is expressed in a larger population than MB1/QH1. (A) *Transverse section at the level of the unsegmented mesoderm in a 7-somite stage quail embryo. Few MB1/QH1 cells are found between the lateral mesoderm and the endoderm (brown, arrow).* **(B)** *These cells also express fli.. Fli is however expressed in more cells than MB1/QH1. ec, ectoderm; en, endoderm; lm, lateral mesoderm; n, notochord; nt, neural tube; pm, paraxial mesoderm. Bar, 200 μm.*

et al., 1993; Pardanaud and Dieterlen-Lièvre, 1993b; Queva *et al.*, 1993; Maroulakou *et al.*, 1994) and ck-*erg* (Dhordain *et al.*, 1995). It is expressed later in many developing vascular structures including the heart, truncus arteriosus, and capillaries in various organs including the brain, the liver and the kidney. The expression of *ets-1* in endothelial cells is restricted to early development and is not linked to the maintenance of endothelial function. *Fli* is expressed at least up to E10 in endothelial cells in quail but at lower levels than in early development. The quail VEGF receptor RNAs, *Quek1* and *2* are expressed in endothelial cells in a similar pattern to *fli* although *Quek1* is expressed before the beginning of somitogenesis (Eichman *et al.*, 1993).

The amnion, which is avascular does express *fli*. Although we have no explanation for this expression, it has to be noted that other genes are also expressed in endothelial cells and amnion. It is the case for instance for endothelin-1 (Sunnergren *et al.*, 1990; Casey *et al.*, 1991) and tie-2 (Sato *et al.*, 1993).

Expression in other mesodermal cells

Our hybridizations also reveal that *fli*, like *ets-2* and *erg*, is expressed in all precartilaginous and cartilaginous condensations whatever their embryonic origin (Vandenbunder *et al.*, 1989; Maroulakou *et al.*, 1994; Dhordain *et al.*, 1995). Like ck-*erg* , *fli* is expressed early in the precursors of these cells in the sclerotome or in the neural crest cells. Unlike ck-*erg*, *fli* is not expressed in the dermomyotome nor in any muscle precursor in the cephalic mesoderm.

Fli is possibly expressed in the precursors of erythrocytes, as attested by its expression in the splanchnopleura and in aortic clusters at E3. It could in fact be expressed in the putative precursor of hematopoietic cells and angioblasts, the hemangioblast. *Fli* overexpression in *Xenopus* (Remy *et al.*, 1996) leads to an absence of circulating erythrocytes and their accumulation in ectopic positions. This suggests that *fli* could be involved in the release of hematopoietic precursors. Alternatively, *fli* could be involved in the differentiation of erythrocytes since it transactivates *gata-1*, which was shown to be involved in the terminal differentiation of erythrocytes (Seth *et al.*, 1993).

Finally, the wolfian duct is a mesodermal site of expression specific for *fli* and never positive for *erg* (Dhordain *et al.*, 1995). This *fli* expression was not reported in mouse (Mélet *et al.*, 1996) but was observed in *Xenopus* (Meyer *et al.*, 1995). The *ets-1* and *-2* genes are also expressed in the intermediate mesoderm (Vandenbunder *et al.*, 1989; Pardanaud and Dieterlen-Lièvre, 1993b; Meyer *et al.*, 1997).

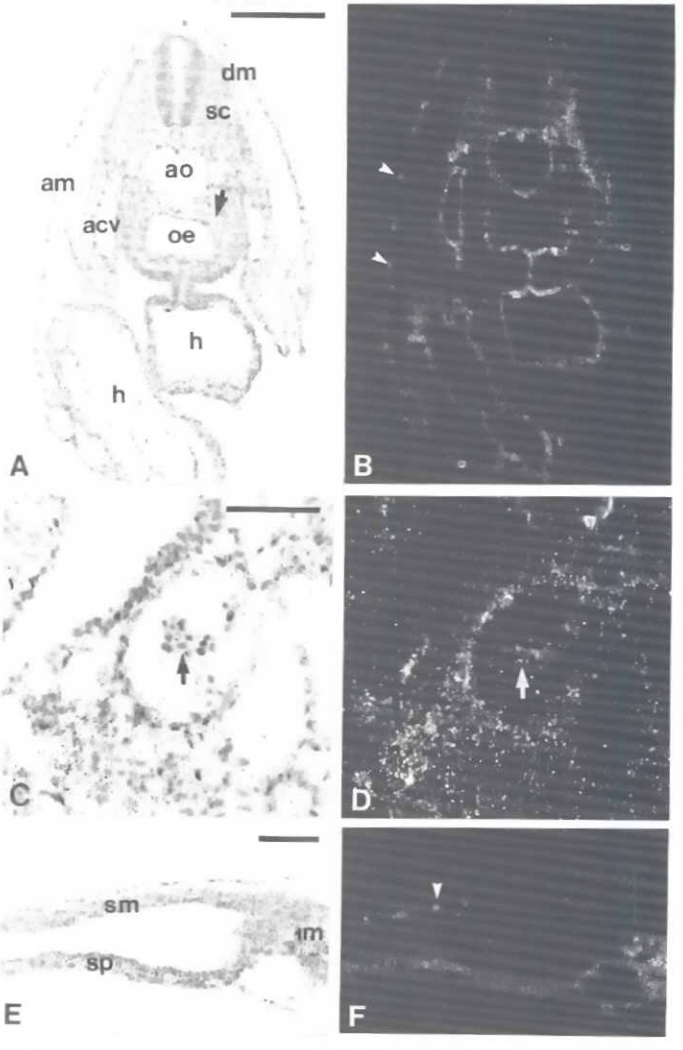

Fig. 8. *Fli* expression in the mesoderm. (A and B) *Bright field and dark field photographs of a transversal section of the cervical region of a stage HH14-15 quail embryo. Fli is expressed in endothelial cells in the heart (h), the anterior cardinal vein (acv) the aortae (ao). The labeling is also found in part of the amnion (arrowheads). The sclerotome (sc) express fli but not the dermomyotome (dm). The vagal neural crest cells which migrate to the oesophagus and down to the gut are not labeled (arrow).* **(C and D)** *Bright field and dark field photographs of a transverse section of aorta. The endothelium is labeled. Most of the circulating cells do not express fli except for one (arrow).* **(E and F)** *show the lateral and intermediate mesoderm of the trunk of a stage HH14 quail embryo (20 somites). Intermediate mesoderm (im) express fli as well as the splanchnopleura (sp). Isolated cells expressing fli are also found in somatopleura (sm). Bar, 200 μm in A,B,E,F and 50 μm in C, D.*

In human, *fli* is involved in different pathologies: erythroleukemias (Ben-David *et al.*, 1991), Ewing's sarcoma (Delattre *et al.*, 1992) and possibly lymphomas (Bergeron *et al.*, 1991,1992; Bhagirat *et al.*, 1995) and neuroblastomas (Burchill *et al.*, 1997). In these diseases, *fli* expression is impaired by either viral integration in the vicinity of the gene or by translocation events. In the case of Ewing's sarcoma, the cells originally affected by the mutation are not yet clearly defined. It was proposed from immunocytochemical and electron microscopy data that these cells have a neural crest origin (Navas-Palacios *et al.*, 1984; Llombart-Bosch *et al.*, 1987; Pinto *et al.*, 1989), since they share a number of characteristics with neural crest cells. *Fli* expression in these cells before differentiation pleads in favor of such a hypothesis. Alternatively, *fli* expression in mesenchymal cells of both neural crest and mesoderm origin suggests that Ewing's sarcoma could evolve from cells of both origins. The modification of *fli* expression in these cells could lead to a deregulation of target genes involved in proliferation.

The role of ets-family genes in neural crest development is still unclear but overexpression experiments in *Xenopus* suggest that the migration of neural crest cells is affected by the expression of this gene (Remy *et al.*, 1996).

Materials and Methods

Library screening and cDNA sequencing

A custom library (Invitrogen Corporation; San Diego; Ca) in pcDNAI (number of primary recombinants 1.7×10^6), constructed from 4-day quail embryo mRNA was screened with a *Xenopus fli* probe (nt 1-1370). Washes were carried out at moderate stringency (last washes at 0.5xSSC, 0.1% SDS, 50°C). Three different clones were selected on the basis of digestion by restriction enzymes. Sequencing was carried out directly in pcDNAI, on double-stranded DNA (Sanger *et al.*, 1977).

Chromosomal localization

Chromosome preparation

Primary fibroblast cell lines were isolated by trypsinization from 9-day chick embryos. Cell culturing and preparation of metaphase chromosomes spreads were done as previously described (Ladjali *et al.*, 1995). Cultures were synchronized with a double thymidine block during S phase in order to increase the yield of metaphase and early metaphase cells (Yunis, 1976; Viegas-Péquignot and Dutrillaux, 1978). R-bands were induced by the 5-bromo-2-deoxyuridine (BrdU; final concentration, 10 µg/ml) during the second half of the DNA synthesis phase. The chromosomes were classified according to Ladjali *et al.* (1993).

Probe labeling

The *fli* probe was prepared from a 1.5 kb quail cDNA cloned in pBluescript®II SK. Probe labeling was carried out using PCR in order to improve hybridization signals which are particularly difficult to observe with probes smaller than 3 kb (Richard *et al.*, 1994). The 50 µl of PCR reaction mixture contained 2 ng of the plasmid, 50 pmole for each primer T3 (5'-ATTAACCCTCACTAAAG-3') and T7 (3'-GATATCACTCAGCATAA-5'), 200 mM of each dNTP (dATP, dGTP, dCTP) and 200 mM of biotine-dUTP/dTTP (3/1) (biotine-11-dUTP, Sigma), and Taq DNA polymerase (Boehringer Mannheim). The amplification was performed by running 35 cycles (1 min at 94°C, 2 min at 48°C and 2 min at 72°C) in a DNA Thermal Cycler (Perkin Elmer Cetus).

In situ hybridization, probe detection and R-banding

The *in situ* hybridization was performed as previously described by Lemieux *et al.* (1992). Six ng of PCR-labeled fragment were used per slide (0.5 ng/µl) in the presence of quail competitor DNA. High stringency conditions were used to prevent binding to other ets-family genes. The biotinyled probe was detected with goat antibody at a final concentration of 10 µg/ml (Vector laboratories) followed by a fluorescein-labeled antigoat IgG at a final concentration of 5 µg/ml (Tebu Nordic, France). The slides were stained with propidium iodide at a final concentration of 1mg/ml. To reveal directly the R-banding pattern, the slides were mounted with -phenylenediamine (Lemieux *et al.*, 1992) .

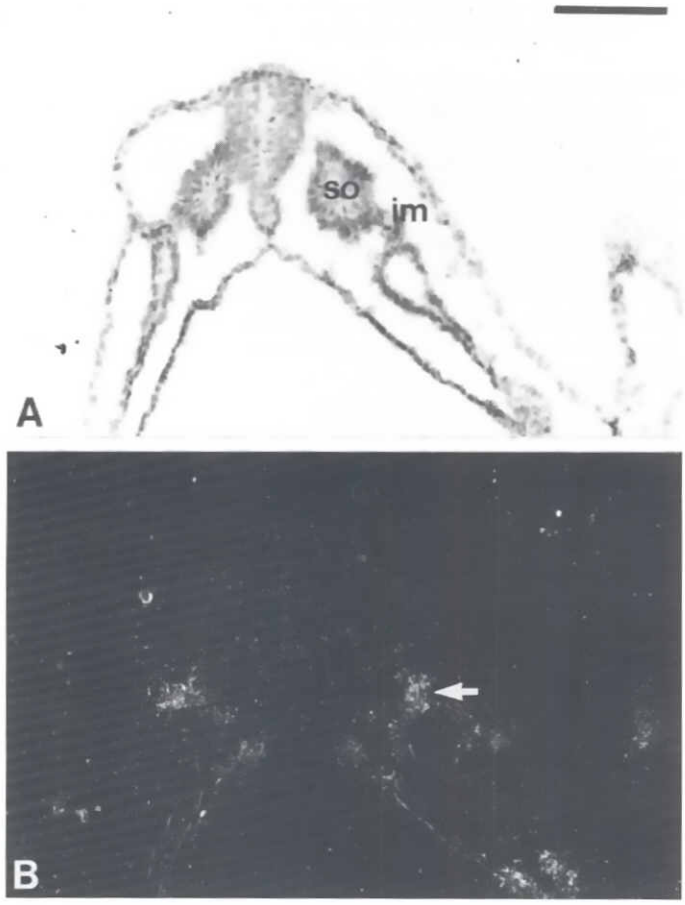

Fig. 9. *Fli* expression in the wolfian duct. (A and B) *Bright field and dark field photographs of a transversal section in the truncal region of a stage HH14-15 quail embryo. Fli is expressed in the intermediate mesoderm (im) (arrow) but not the somites (so). Bar, 200 µm.*

In situ hybridization

The presence of *fli* transcripts was investigated by using a 1.5 kb probe issued from the 5' side of clone 10.1, up to the *BamH1* site. *In situ* hybridizations on sections were carried out on stage 8 (4-somites) to stage 36 (Embryonic day 10: E10) quail and chick embryos, according to the developmental table of Hamburger and Hamilton (1951) (HH). Five to six µm paraffin sections were performed after fixation in Carnoy's fluid. They were treated as described in Eichmann *et al.* (1993). RNA probes were labeled by incorporation of ^{35}S-UTP (Amersham, 1000Ci/mmol) during synthesis (Promega, Riboprobe Gemini II) and used at a minimum concentration of 10^4 counts/min/µl. In some cases, the sections were stained with HNK-1 mAb before exposure to photographic emulsion, as described below. The time of exposure was of 15-21 days. The sections were counterstained with Gill's hematoxylin. Whole-mount *in situ* hybridizations on quail embryos, using digoxygenin-labeled probes, was carried out as described earlier for *Xenopus* embryos (Meyer *et al.*, 1995) with minor modifications:

- embryos were treated with 10 µg/ml proteinase K, at room temperature for a number of minutes equal to the stage number,

- pretreatment of the antibodies with embryo powder was omitted,
- no clearing of the embryos was carried out.

Immunocytochemistry

Sections were incubated with HNK-1 mAb (cell supernatant not diluted) 1 h in a humidified chamber at room temperature and, after washing in PBS, re-incubated for 1 h with horseradish peroxydase conjugated antibody against mouse IgG1 (Southern Biotechnology Associates). The reaction was developed in 250 µl PBS containing 20 mg diaminobenzidin and 100 µl H_2O_2 30%. Slides were then briefly rinsed in water and sections were stained with Gill's hematoxylin, ethanol dehydrated and mounted with Entellan (Merk). For MB1/QH1 immunocytochemistry, the same protocol was used except an overnight incubation at 4°C with the first antibody and the use of a horseradish peroxydase conjugated antibody against mouse IgM as a second antibody.

Quail/chick chimeras

Quail *(Coturnix coturnix japonica)* and chick *(Gallus gallus)* eggs from commercial sources were used. Microsurgery was performed on embryos at the 5-6-somite stage (about 30 h of incubation in a humidified atmosphere at 38°). A window was cut in the shell and India ink diluted 1:4 in PBS was injected into the sub-blastodermic cavity in order to make the embryonic structures more visible without using any "vital" stain. The vitelline membrane was windowed and the neural fold, i.e., one fourth of the depth of the neural tube, was removed at the level of rhombomeres 3 to 6 with a knife made by sharpening a steel needle on an Arkansas stone. The limits of the grafted tissues and of the sites of implantation were defined according to a map in which the anteroposterior limits of the rhombomeres were established (Grapin-Botton *et al.*, 1995). The rhombomere 3 to 6 neural fold was taken from a quail dissected in a dish and carried to the recipient egg using a micropipette controlled by buccal suction. Embryos were fixed in Carnoy's fixative one day after grafting, included in paraffin and sectioned on alternate slides. Quail cells were localized using the QCPN mAb and *fli* gene expression was analyzed on parallel slides.

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