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Expression of the zinc finger gene *Gli*3 is affected in the morphogenetic mouse mutant *extra-toes* (*Xt*)

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Summary

Genetic analysis and homology between the phenotypic alterations of the human *Greig Cephalopolysyndactyly Syndrome* (*GCPS*) and the mouse mutant *extra-toes* (*Xt*) have suggested a dominant mutation in the same gene of both species. Recently, the *GLI*3 gene, a member of the *Krüppel*-related zinc finger genes, has been proposed as a candidate gene for *GCPS*. We examined the expression of the mouse *Gli*3 gene in both *Xt* mutant animals and during normal mouse development. Northern and RNAase protection analysis of embryos revealed that *Gli*3 expression was reduced about 50% in heterozygous *Xt*/+ mice and completely absent in homozygous *Xt*/*Xt* mice. In addition, in situ analysis of wild-type mice documented *Gli*3 expression in the developing limb and brain, structures affected in *Xt* mutant mice. This pattern suggests an important function of the *Gli*3 gene during morphogenesis.

Key words: *Gli*3 zinc finger gene, *extra-toes* (*Xt*), limb development, brain development, *Greig Cephalopolysyndactyly Syndrome* (*GCPS*).

Introduction

Recent advances in the molecular and genetic analysis of mouse development have made it possible to address the molecular basis of processes involved in pattern formation and morphogenesis (for reviews see Rossant and Joyner, 1989; Gridley, 1991; Gossler and Balling, 1992; Rossant and Hopkins, 1992). This has been illustrated by the recent characterization of mouse mutants, which has led to the identification of several important genes (Epstein et al., 1991; Hill et al., 1991; Suter et al., 1992; Thomas et al., 1991; for review see Reith and Bernstein, 1991). In this context, the developing limb has attracted much attention as a model system since many mutations exist affecting its proper development. Among them the mutant *extra-toes* (*Xt*) has been described as a member of a group of mutant loci affecting the limb skeletal pattern (Hinchcliffe and Johnson, 1980). The phenotype of *Xt* mice is characterized by the formation of extra digits (polydactyly) on the preaxial (anterior) site of the limb (Johnson, 1967). This polydactyly is possibly the result of an enlarged apical ectodermal ridge (AER), which presumably induces some of the underlying mesenchyme to form additional skeletal elements. In homozygous *Xt*/*Xt* mice, more proximal parts of the limb skeleton are also affected. Animals die in utero, or at birth, with a range of abnormalities including malformations of the brain, central nervous system and sense organs.

As a result of morphological studies and comparative gene mapping studies, *Xt* has been proposed to be an animal model for the human *Greig Cephalopolysyndactyly Syndrome* (*GCPS*) (Winter and Huson, 1988), an autosomal mutation affecting limb and craniofacial development (Greig, 1926). Recently the zinc finger gene *GLI*3 has been proposed as a candidate gene for *GCPS* since it was shown to be interrupted by translocations in Greig syndrome families (Vortkamp et al., 1991). *GLI*3 has been isolated and described as a member of a gene family that is related to the *Drosophila* gap gene *Krüppel* (Ruppert et al., 1988, 1990). This family has been proposed to play a role in embryonic development and tissue-specific differentiation (Ruppert et al., 1988).

We describe the analysis of *Gli*3 gene expression in wild-type and mutant *Xt* mice and during normal development. Our results strongly support the suggestion that interference with *Gli*3 gene function during embryogenesis results in the *Xt* phenotype.

Materials and methods

Mice

Mice carrying the *Xt* mutation in a (C3Hx101) F_2 background were obtained from the MRC Radiobiology Unit, Harwell, England and Thomas Franz, Universitätskrankenhaus Eppendorf, Hamburg, Germany. The middle of the day when vaginal plug

formation was found is defined as gestational day 0.5. Embryos were isolated at gestational day 9, 12.5, 13.5 and 17. Embryos at gestational day 17 were macroscopically identified as wild-type, heterozygous *Xt*/+ and homozygous *Xt*/*Xt* embryos as described (Johnson, 1967).

Isolation and analysis of RNA and DNA

DNA and RNA from embryos or organs of adult mice were prepared and analysed using standard protocols. Analysis of polyadenylated mRNA on northern blots was performed under normal stringency, using a cDNA of the human *GLI*3 gene (Ruppert et al., 1990) and an actin probe (Spiegelman et al., 1983). For Southern blot and RNAase protection analysis part of a mouse cDNA encoding *Gli*3 was used. This partial cDNA was isolated from an 8.5 day fetal mouse library using a segment of the human *GLI*3 cDNA as a probe (Vortkamp et al., 1992). The isolated *Gli*3 cDNA contains a stretch of sequence that is highly conserved (85% identity on the nucleotide level) to a 5′ part of the human *GLI*3 cDNA (nucleotides 26-415). The antisense probe used for RNAase protection analysis has a length of 239 basepairs and the protected fragment corresponds to nucleotides 415 to 201 of the human *GLI*3 cDNA. For analysis of the integrity and amount of RNA, an antisense actin probe was used as a control. Relative levels of *Gli*3 expression detected on northern blots and in RNAase protection assays were determined by laser scanning.

In situ hybridization

In situ hybridizations were performed as described earlier omitting the prehybridization step (Dollé and Duboule, 1989). The *Gli*3 RNA probe was identical to the one used for RNAase protection analysis (see above).

Results

Analysis of the Gli*3 gene in wild-type and* Xt *mutant mice*

At day 17 post-coitum (p.c.) the *Xt* mutant phenotype is fully visible. Therefore, a transcript crucial for this mutation should be detectable before or at this time in wild-type mice. To examine *Gli*3 expression, we first used a human cDNA probe encoding the entire open-reading frame of the *GLI*3 protein (Ruppert et al., 1990). On northern blots containing RNA isolated from a day 17 p.c. wild-type embryo, a single RNA species of about 8 kb was detected (Fig. 1). This RNA comigrated with the *GLI*3 mRNA detected in the human embryonal carcinoma cell line NTera-2 and was also detected when a mouse cDNA encoding part of the *Gli*3 gene was used (see below). The same analysis was carried out on mRNAs isolated from heterozygous *Xt*/+ and homozygous *Xt*/*Xt* littermates. Integrity and amount of mRNAs used were controlled by hybridizing the identical northern blot with an actin gene probe. Interestingly, our results showed a reduced amount of the 8 kb mRNA in *Xt*/+ heterozygotes and non-detectable levels of the transcript in *Xt*/*Xt* homozygous animals (Fig. 1).

To verify that we had detected a *Gli*3-specific transcript, we performed our further analysis with a mouse cDNA encoding part of the *Gli*3 gene. This cDNA shares a high degree of homology with a 5′ part of the human *GLI*3 cDNA (for details, see Materials and methods). Hybridization of the *Gli*3 cDNA probe to Southern blots resulted in the detection of single restriction fragments of genomic mouse DNA (Fig. 2A). Therefore, this probe is specific for

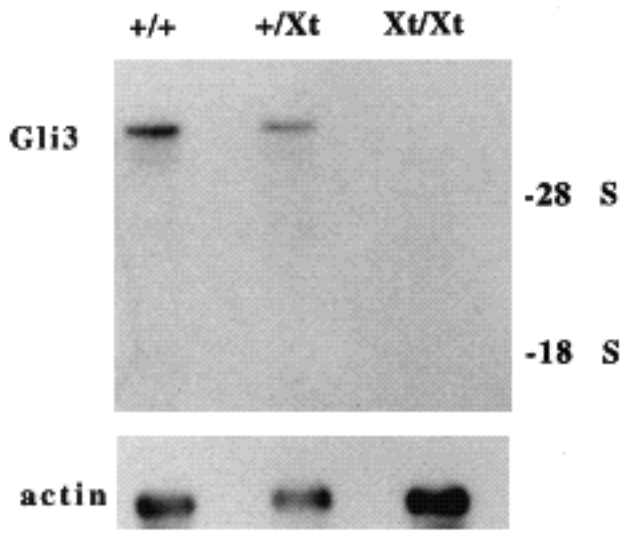

Fig. 1. Expression of *Gli*3 in wild-type and *Xt* mutant embryos of gestational day 17. 2 μg of poly(A)$^+$ RNA isolated from embryos of different genotypes (as indicated) was hybridized with a cDNA probe for the human *GLI*3 and actin on northern blots. The position of the 18S and 28S ribosomal RNAs are marked.

*Gli*3 and does not cross-hybridize with other members of the *GLI-Krüppel* gene family. Southern blot analysis of genomic DNA from *Xt*/+ heterozygous suggested hemizygosity for the *Gli3* gene and was confirmed as a deletion due to the absence of hybridization with genomic DNA from *Xt*/*Xt* homozygous mice (Fig. 2A).

In order to assay the effect of the deletion at the *Gli*3 locus on the expression of the gene, we performed RNAase protection analysis using the partial cDNA of the mouse *Gli*3 gene (see Materials and methods). Again RNAs of each sample were assayed for actin in a parallel RNAase protection analysis (data not shown). A probe derived from the *Gli*3 cDNA protected a 214 nucleotide fragment in both wild-type and *Xt*/+ heterozygous RNAs (Fig. 2B). In the RNA from *Xt*/+ heterozygous littermates, a reduced amount of the *Gli*3-specific fragment was observed. Quantification of this specific fragment in relation to the actin-specific signal revealed in *Xt*/+ heterozygotes a significant reduction of about 50% with respect to the wild-type level. In RNA isolated from *Xt*/*Xt* homozygous littermates, no protection of the Gli3-specific probe was detected (Fig. 2B).

To obtain further insight in the expression pattern of *Gli*3, we examined further developmental stages and adult organs of wild-type mice by northern blot and RNAase protection analysis. A specifically protected *Gli*3 fragment was found in embryonic stem cells and day 9 p.c. embryos (Fig. 3). In adult mouse tissues, RNAase protection analysis revealed the presence of *Gli*3 mRNA in all RNAs examined. The highest levels of *Gli*3 mRNAs were found in brain. Lower amounts of *Gli*3 mRNAs were observed in testis, kidney, muscle, salivary gland, thymus, spleen and lung. Trace amounts of a *Gli*3-specific transcript were found in liver. In embryos of day 9-14 p.c. and in brain, gonads, kidney, muscle, thymus and spleen, the presence of a *Gli*3 mRNA of about 8 kb in size was confirmed by northern blot analysis (data not shown).

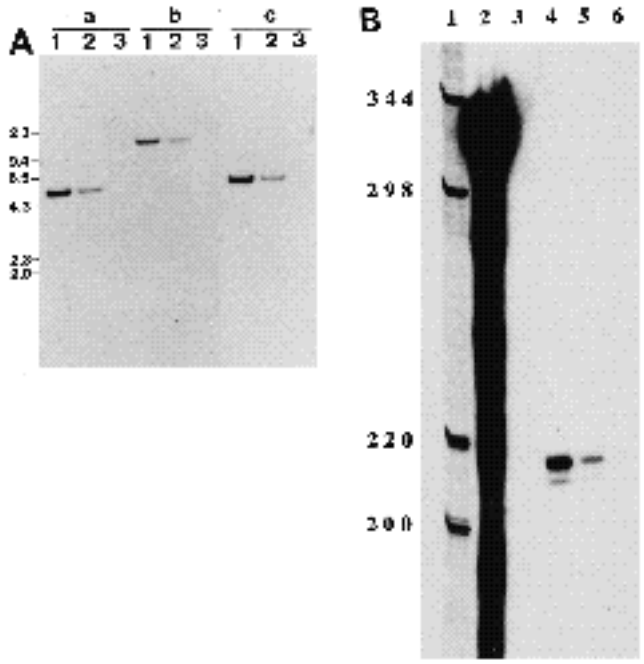

Fig. 2. The *Gli*3 gene and its expression in wild-type and *Xt* mutant mice. (A) Southern blot analysis of genomic DNA from wild-type (1), *Xt*/+ heterozygous (2) and *Xt*/*Xt* homozygous (3) mice digested with the enzymes *Bam*HI (a), *Bgl*I (b) or *Eco*RI (c) and probed with a mouse *Gli*3 cDNA probe. To ensure equal amounts of the individual samples, the same blot was rehybridized with several other probes (data not shown). (B) Total RNA (20 μg) of embryos at day 17 p.c. or tRNA were hybridized with a mouse *Gli*3 antisense riboprobe in a RNAase protection analysis. lane 1, DNA length marker; lane 2, riboprobe; lane 3, tRNA; lane 4, wild-type embryo; lane 5, *Xt*/+ heterozygous; lane 6, *Xt*/*Xt* homozygous.

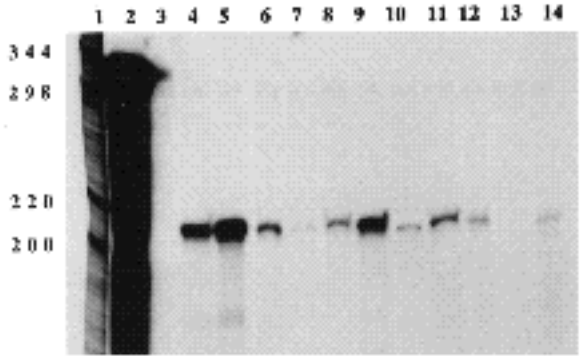

Fig. 3. Expression of *Gli*3 in development and tissues of adult mice. A RNAase protection analysis was performed using a *Gli*3 antisense riboprobe. Either 20 μg of total RNA was used (lane 4, embryonic stem cells; lane 5, embryo at gestational day 9) or 40 μg of total RNA in the case of adult tissues (lanes 6 to 14, corresponding to gonads, kidney, muscle, brain, salivary gland, thymus, spleen, liver and lung). Lane 1, DNA length marker; lane 2, riboprobe; lane 3, tRNA.

In situ hybridization analysis of Gli*3 expression*

The spatial expression pattern of *Gli*3 was studied by in situ RNA analysis between day 10 and 16 p.c. The two

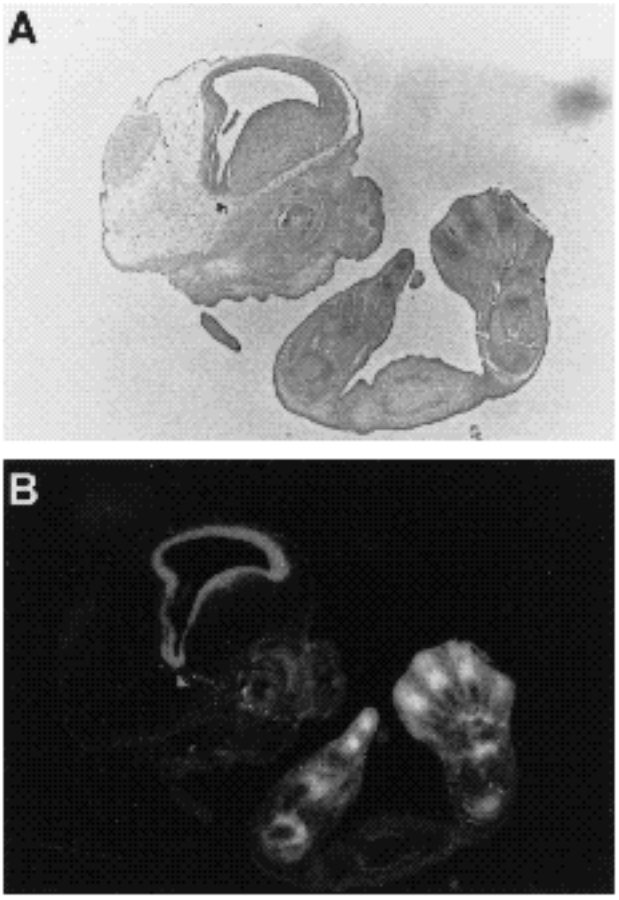

Fig. 4. In situ analysis of *Gli*3 expression in a day 12.5 p.c. embryo. Bright-field (A) and dark-field (B) view of a parasagittal section through head, forelimb and hindlimb, which has been hybridized with a *Gli*3-specific antisense riboprobe.

major sites where *Gli*3-specific transcripts were found were the developing brain and limbs as documented in Fig. 4 by a section through a day 12.5 p.c. embryo. A detailed analysis of this expression was performed on sagittal and transverse sections of wild-type embryos at day 13.5 p.c. In transverse sections, at the level of the forelimbs, *Gli*3 expression was localized in the presumptive digits and the future humeral head (Fig. 5A). Analysis of serial sections through forelimb and hindlimb showed that all parts of the developing limb skeleton expressed *Gli*3 (data not shown). An examination at the cellular level revealed that *Gli*3-specific transcripts were highly concentrated in the perichondrium and almost entirely excluded from the cartilage cells (Fig. 5B,C).

The other site of high *Gli*3 expression was the developing brain. *Gli*3 was expressed in the neuroepithelium of the inner walls of the forebrain, midbrain and hindbrain. In the telencephalon, the expression of *Gli*3 extended ventrally to the corpus striatum (Fig. 6B-E) and very anteriorly to the area of the developing olfactory lobes (Fig. 6F). The corpus striatum is contiguos with the diencephalon (Fig. 6E) from which part of it is thought to originate (Price et al., 1991). In the diencephalon, *Gli*3 transcripts were found in the dorsolateral wall, forming the different parts of the thalamus

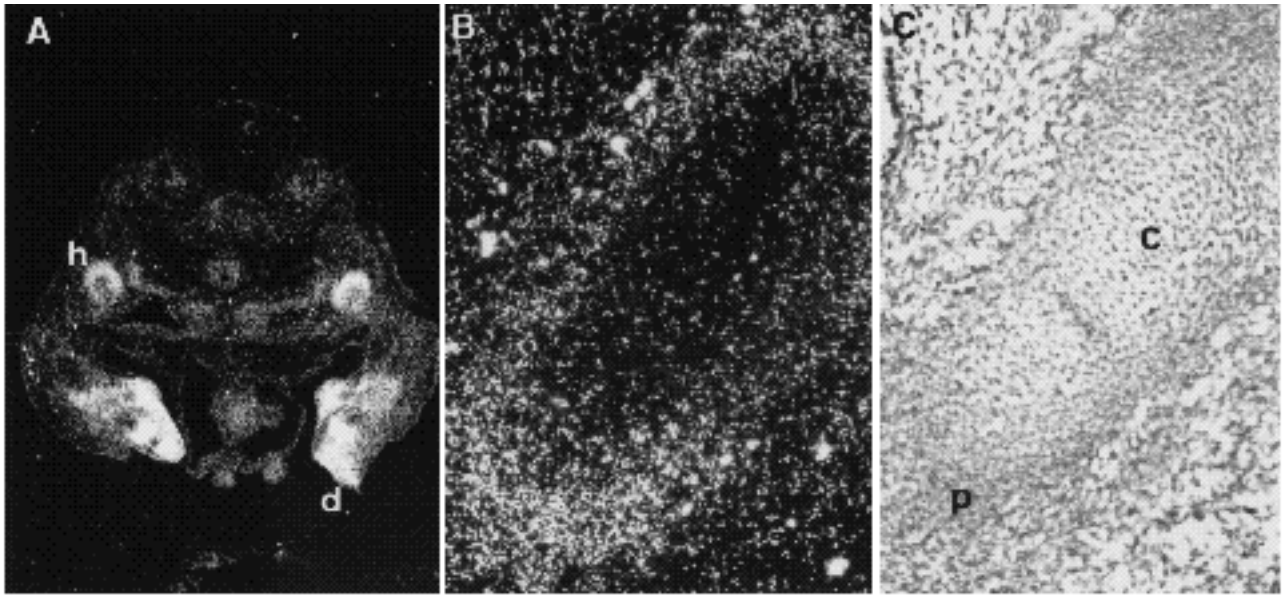

Fig. 5. Expression of *Gli*3 in the developing limb skeleton of a day 13.5 p.c. embryo. (A) Dark-field view of a transverse section at the level of the forelimbs showing expression of *Gli*3 in the humeral head and at the digits as observed by in situ hybridization. Dark-field (B) and bright-field (C; magnification 440×) view of a sagittal section through a developing metatarsal bone of the hindlimb. Abbreviations are d, digits; h, humeral head; p, perichondrium; c, forming cartilage.

(Fig. 6A-E) as well as in the ventral floor termed the infundibulum (Fig. 6F). Rathke's pocket, the future anterior part of the pituitary gland, was also positive for *Gli*3 RNA (data not shown). Expression of *Gli*3 was also seen in the more anterior part of the hypothalamus, such as in the region of the optic chiasma. A high level of transcripts was detected in the roof of the mesencephalon (Fig. 6B-D), extending to the metencephalic areas containing the cerebellum and the pons (Fig. 6B,C,E). The hindbrain was positive from the roof fold (posterior choroid plexus) onwards which marks the beginning of the myelencephalon (Fig. 6B,C,F).

Discussion

The Gli*3 gene is mutated in* Xt *mice*

Several lines of evidence indicate that the *Gli*3 gene plays a causative role for the *Xt* phenotype. The present study complements recent studies of the *Xt* locus, which show that the *Gli*3 gene carries a deletion in its 5′ coding region (Vortkamp et al., 1992). A mapping analysis of this deletion makes it very likely to be identical to the deletion of at least 80 kb of DNA found before in *Xt* mice (Pohl et al., 1990 and unpublished data). To gain more insight into the organization of the mouse *Gli*3 gene of which so far only partial cDNAs are available, we are currently attempting to obtain a full-length cDNA and genomic DNA. Our present analysis shows that the consequence of the *Xt* mutation is the absence of *Gli*3 expression. Therefore, the deletion found in the *Gli*3 gene is apparently not restricted to a small part of its coding region, but includes further 5′ located sequences. These data suggest that *Xt* is an allele of *Gli*3 and that the *Xt* phenotype results from the absence of *Gli*3 expression due to a deletion of its promoter and 5′ coding region. This is underlined by the observation that the temporal and spatial expression pattern of this gene correlates well with the *Xt* phenotype. The highest level of *Gli*3 expression was found in the limb and brain, two of the most severely affected structures in *Xt*/*Xt* homozygous mice. In addition, preliminary analysis revealed several other sites such as the spinal cord and vertebrae where *Gli*3 expression was detected and which are also affected in the *Xt* mutant. The mutation of the *Gli*3 gene as the direct cause for *Xt* is also supported by the analysis of its human homologue *GLI*3. In man, translocations of the *GLI*3 gene are observed in patients carrying *GCPS*, a syndrome affecting limb and craniofacial development (Vortkamp et al., 1991).

Another potential allele of *Gli*3 is the recessive mutation *add* (*anterior digit pattern deformity*, Pohl et al., 1990). It will be of high interest to analyse *Gli*3 expression in these mice. To provide final evidence for the *Gli*3 gene being responsible for the *Xt* and *add* phenotype, we are presently attempting to perform transgenic rescue experiments.

The Gli*3 gene in morphogenesis*

The limb skeletal elements of the *Xt* mutant have aberrant shapes; they may be fused, reduced or elongated. Most remarkably, the appearance of supernumerary skeletal elements are seen on the anterior side of both handplate and footplate leading to the formation of extra digits (Johnson, 1967). Consequently, growth and patterning of the limb skeleton are affected. Limb bones are formed by endochondral ossification involving the formation of a cartilaginous intermediate (Hinchcliffe and Johnson, 1980). The developing cartilage is surrounded by the perichondrium, a mesenchymal layer of cells, which probably has a role in the shaping of the forming bone (Bélanger, 1977). The *Gli*3

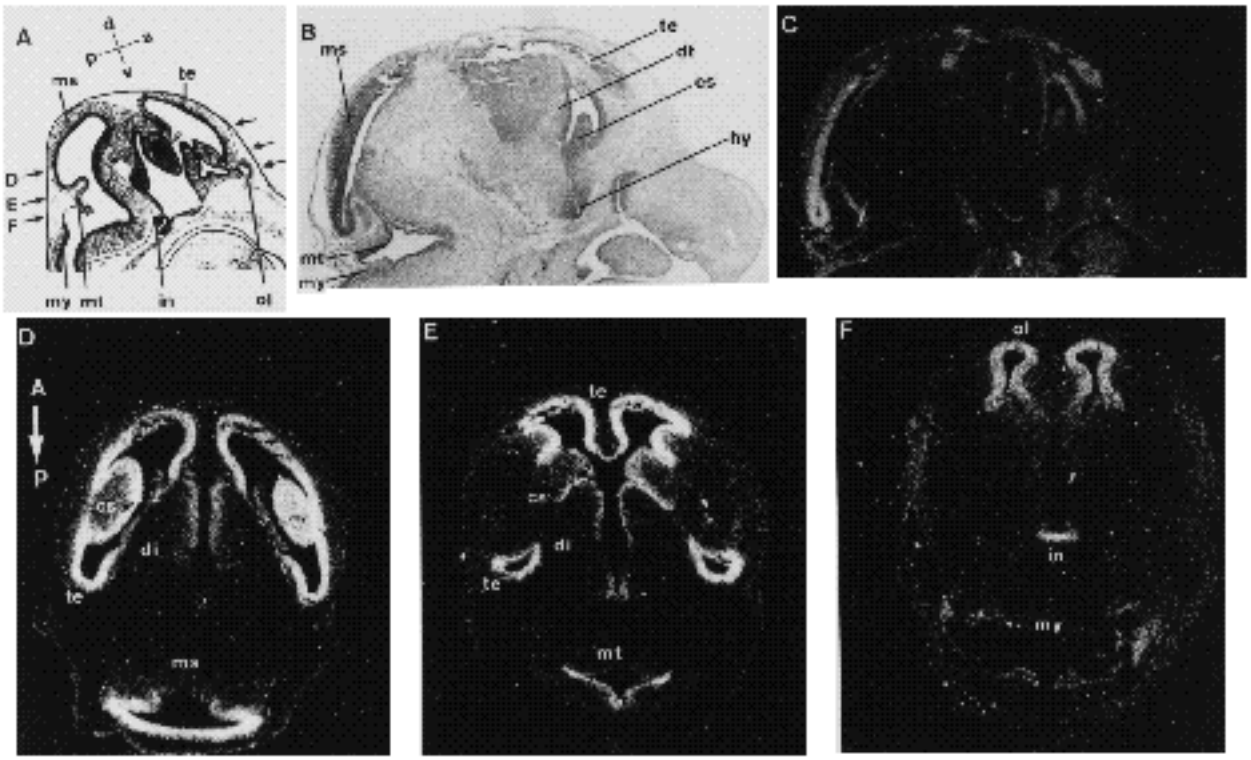

Fig. 6. Spatial expression of *Gli*3 in the embryonic brain. (A) Schematic diagram of a mid-sagittal section through the embryonic head at at around day 13.5 of development. The anterior-posterior (a,p) and dorsal-ventral (d,v) axes are indicated. Arrowheads point out the various planes of section in D-F. Bright-field (B) and dark-field (C) view of an in situ analysis using a *Gli*3-specific antisense riboprobe. The section is more parasagittal than the diagram in A. (D-F) Serial transverse sections through the fetal head as outlined in A. The anterior-posterior (a,p) axis is indicated in D. Abbreviations are cs, corpus striatum; di, diencephalon; dt, dorsal thalamus; ht, hypothalamus; in, infundibulum; ms, mesencephalon; mt, metencephalon; my, myelencephalon; ol, olfactory lobes; te, telencephalon.

gene is highly expressed in the perichondrium. Therefore, mutations of this gene may affect the normal process of cartilage formation and result in an abnormal skeletal pattern within the limb.

Reduction of *Gli*3 gene expression in *Xt*/+ heterozygotes causes haploinsufficiency, as suggested earlier (Pohl et al., 1990). The same explanation has recently been proposed for the semidominant phenotype caused by mutations in *Pax* genes (Hill and van Heyningen, 1992). *Gli*3 expression is decreased in heterozygous *Xt*/+ mice and absent in homozygous *Xt*/*Xt* animals, which inversely correlates with the number of digits formed (Johnson, 1967). Since so far there is no indication for a polarized *Gli*3 expression along the anterior-posterior axis during development (Schimmang and Rüther, unpublished data), it seems that the cells on the anterior side of the limb are more affected by a reduction of gene dosage. Alternatively, interference with *Gli*3 may initially result in an increased size of the limb bud and AER, which would induce the formation of additional digits.

In embryonic brain, *Gli*3 expression is widespread in the future cortex. Again, this correlates well with the *Xt* phenotype. *Xt*/*Xt* homozygotes have brain malformations, which have been explained by neural overgrowth during a delayed closure of the neural tube or thereafter (Johnson, 1967). In the newborn animal, this seems to impair the proper formation of the cerebral hemispheres, which are decreased in size and partially covered by an unusual bulging of the dorsolateral mesencephalic walls. The telencephalon of *Xt*/*Xt* mice lacks olfactory lobes and the diencephalon lacks a pineal gland, which is derived from the anterior part of the thalamic roof. More ventrally, the infundibulum and the optic chiasma are ill-defined or absent. The cerebellum remains undifferentiated and the myelencephalon, like the whole brain, is twisted about its major axis. As in the case of the limbs, a lack of *Gli*3 expression correlates with the absence, reduction or overgrowth of a structure.

The *Gli*3 gene may also have a function earlier during embryogenesis since it was found to be expressed in embryonic stem cells. Remarkably, we detected expression of *Gli*3 in the embryo at day 9 p.c., the stage when the first detectable phenotypic abnormalities in *Xt*/*Xt* homozygous animals are visible (Johnson, 1967).

The relatively high levels of *Gli*3 in adult brain suggests a function of *Gli*3 in differentiated cells of the nervous system. Furthermore, *Gli*3 is apparently present in a broad variety of other tissues as is also observed for its human counterpart *GLI*3 (Ruppert et al., 1988), although expression of *GLI*3 has not yet been described in human brain. In mice, no abnormalities have been found upon histological examination of gonads, heart, kidney, liver, lung, spleen, thymus, stomach and adrenal gland in *Xt*/*Xt* homozygous viscera (Johnson, 1967). However, a more detailed analysis may reveal further roles for this gene during organogenesis and cell differentiation.

Gli*3 as a member of the* GLI-Krüppel *zinc-finger gene family*

*Gli*3 is a member of the *GLI-Krüppel* gene family, for which further genes have been implicated in developmental processes in other species (Ruiz i Altaba et al., 1987; Orenic et al., 1990; Schuh et al., 1986). Like several other vertebrate zinc finger genes, the *GLI* genes have been found to be related to *Drosophila* genes such as *Krüppel* and *cubi - tus interruptus Dominant* (Ruppert et al., 1988, 1990). However, in contrast to their *Drosophila* counterparts, there is a lack of direct genetic evidence for the involvement of vertebrate zinc finger genes in embryonic pattern formation or cellular differentiation (for a review, see El-Baradi and Pieler, 1991). Apart from the human *WT1* gene, involved in kidney development (for a review, see Haber and Buckler, 1992), *Gli*3 is one of the first examples of a mammalian zinc finger gene for which direct genetic evidence of its involvement in embryonic development exists. The analysis of the expression of the *Gli*3 gene and the *Xt* phenotype in early embryonic development and the isolation of *Gli*3 target genes will elucidate its potential regulatory function during pattern formation and morphogenesis.

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