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Programmed cell death is affected in the novel mouse mutant *Fused toes (Ft)*

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SUMMARY

We have identified a novel dominant mouse mutant that is characterised by fused toes on the fore limbs and a thymic hyperplasia, in heterozygous animals. Homozygosity of the mutation leads to malformation of the developing brain, lost of the genetic control of left-right asymmetry and to death around day 10 of development. Analysis of both limb development and induction of apoptosis in immature thymocytes in vitro suggest that programmed cell death is affected by the mutation. Since the mutation was caused via a transgene insertion we were able to map it to the D region on mouse chromosome 8. So far, no mutation that affects programmed cell death has been mapped to this chromosome. Thus, this mutation will allow the identification of a novel gene involved in programmed cell death during mammalian development.

Key words: insertional mutagenesis, limb development, apoptosis, situs inversus

INTRODUCTION

Recently limb development has become an attractive model for studying the molecular basis of complex processes such as morphogenesis. One reason is that mouse homologues of homeotic genes of *Drosophila*, the *Hox* genes, are expressed in a pattern that may define positional informations (Dollé et al., 1989; Izpisua-Belmonte et al., 1991). A number of years ago it was postulated that these type of genes were fundamental to establishing the overall structure of limbs (Wolpert, 1969; Summerbell and Lewis, 1975). However, limb morphogenesis requires more than pattern formation and it is very likely that several other essential genes do exist. Indeed, analysis of mouse limb mutants by means of the integration of a transgene, led to the identification of other genes involved in limb development. First, the formins were isolated and characterised by investigating the *limb deformity* (*ld*) mutation (Woychik et al., 1990; Maas et al., 1990). The *ld* gene generates several transcripts by alternative/differential splicing and therefore results in different proteins. The formins are considered to be putative transcription factors important for the establishment of the anteroposterior and dorsoventral axes (Trumpp et al., 1992). Second, the *Gli*3 gene was shown to be the gene mutated in the *add* and *Xt* mouse mutants (Pohl et al., 1990; Schimmang et al., 1992, 1994; Hoeven et al., 1993; Hui and Joyner, 1993). This gene product encodes a zinc finger protein which might be a transcription factor. The phenotype of the mutants suggests its role in the interaction of ectoderm with mesenchyme. Although all these genes are very interesting entry points to study the molecular basis of limb development, other important genes must exist because the number of mouse limb mutants is relatively high compared with the number of known genes (Lyon and Searle, 1989). Furthermore, particular aspects of morphogenesis, such as programmed cell death, have yet to be related to any known gene and mutation.

Apoptosis or programmed cell death is a well known phenomenon, which occurs in both development and the adult organism (for review, see, Kerr et al., 1972; Raff, 1992). During apoptosis cells undergo characteristic changes such as condensation of the nucleus, fragmentation of DNA into oligomeres of nucleosomes and degradation of proteins. Finally these cells are phagocytosed by surrounding cells, and thereby removed in a well organised process without affecting adjacent tissue structures.

During morphogenesis programmed cell death is essential for the modelling of different structures including limbs and digits (Saunders and Fallon, 1967). In the adult, programmed cell death is the mechanism by which hormone-dependent tissues such as the prostate gland regress after castration (Sanford et al., 1984). In addition, homeostasis and repertoire selection of T and B cells are regulated by programmed cell death (Goodnow et al., 1992; von Boehmer, 1992).

Since apoptosis is an active process within a cell, a specific gene programme has been postulated to be involved in apoptotic initiation and control (Wyllie et al., 1980). Recently, several genes have been described that seem to participate in apoptosis. First, expression of *bcl*-2 in B and T cells helps them escape from apoptosis (for reviews see Williams, 1991, and Veis et al., 1993). Recently, two *bcl*-2 related genes have been isolated, which can also influence programmed cell death

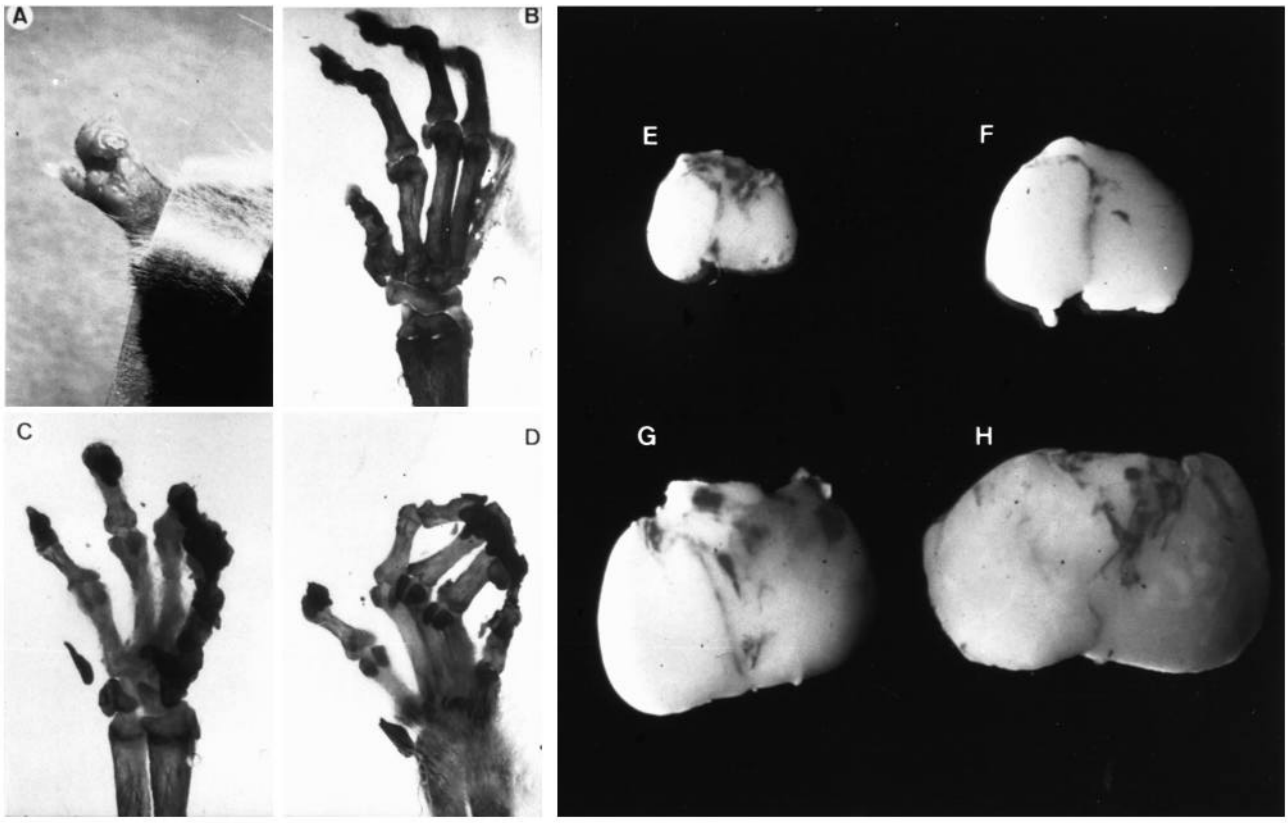

Fig. 1. The specific phenotypes of the *Ft*/+ mouse. (A-D) Fore limb alterations: A typical fore limb of a heterozygous *Ft*/+ mouse is illustrated in A. A skeletal stain of a wild-type right fore limb is shown (B) and compared to right fore limbs of *Ft*/+ mice (C,D). (E-H) Thymus alterations: A thymus from a 6-week old wild-type mouse is shown in E and can be compared with the thymus of a heterozygous *Ft*/+ mouse from the same litter at the same age (F). In addition, a thymus from a 4- and a 6-month old *Ft*/+ mouse is presented (G,H).

(Boise et al., 1993; Oltvai et al., 1993). Second, overexpression of p53 can induce apoptosis in myeloid cells (Yonish-Rouach et al., 1991). Similarly, programmed cell death is initiated by overexpression of c-*myc* or c-*fos* in fibroblasts (Evan et al., 1992; Smeyne et al., 1993). Third, expression of the Fas antigen can mediate apoptosis (Itoh et al., 1991). In this case, there is a mouse mutant (*lpr*) in which the Fas antigen is mutated and therefore not expressed in homozygous mice. These *lpr* mice develop a lymphadenopathy and suffer from an autoimmune disease, most likely as a consequence of a defect in the regulation of the peripheral T cell pool (Watanabe-Fukunaga et al., 1992). Finally, the orphan steroid receptor Nur 77 was found to be required for induction of apoptosis in T-cell hybridomas (Liu et al., 1994; Woronicz et al., 1994). However, in no case has a relation to programmed cell death, in the context of morphogenesis, been shown. In this study, we describe a mouse mutant in which programmed cell death is affected during limb development and in immature thymocytes.

MATERIAL AND METHODS

Mice

Mice have been bred in the animal house at the EMBL. The analyses of either hetero- or homozygous *Ft* mice were performed on a first generation backcross on C57BL/6 mice (the transgenic mouse was generated from F_2(C57BL/6 × SJL) mice).

Staining of cartilage and bone structures was done following standard procedures (Kimmel andTrammell, 1981).

Induction and elevation of apoptosis in thymocytes

Apoptosis was induced in immature thymocytes in vitro by addition of cortisone or crosslinking of TCR/CD3 complex via anti-CD3ε antibodies. Thymocytes undergoing apoptosis were quantitated by two independent methods: (i) detection of coordinate downregulation of the CD4 and CD8 coreceptors on immature CD4/8 double positive thymocytes (Swat et al., 1991) and (ii) detection of DNA fragmentation by flow cytometry (Nicoletti et al., 1991). Briefly, 2×10^6 freshly isolated thymocytes were cultered in 48-well microtiter plates (Costar, Asbach, FRG) in 0.5 ml serum-free medium (CG medium, Camon, Wiesbaden, FRG) with cortisone (Fluocinolone-acetonid, Sigma) at various concentrations and time intervals. Alternatively, thymocytes were incubated under the same conditions, in tissue plates precoated with graded amounts of purified anti-CD3ε Ab (clone 500A2).

In order to quantify apoptosis according to CD4/CD8 downregulation, 1×10^6 thymocytes were incubated for 30 minutes at 4°C with pretitered concentrations of phycoerythrin-conjugated anti-CD4 and FITC-conjugated anti-CD8 Ab (Becton and Dickinson, Heidelberg, FRG) diluted in PBS containing 5% FCS and 0.1% sodium azide. Cells were washed once in staining buffer and 20 000 cells/sample were analysed in a flow cytometer (FACScan, Becton and Dickinson). Dead cells were excluded from analysis by appropiate forward and side scatter gating. Two subsets of CD4/8 double-positive thymocytes could be distinguished according to the surface density of both receptors, $CD4^{low}/8^{low}$ and $CD4^{high}/8^{high}$. Percentage deletion was calculated according to the formula:

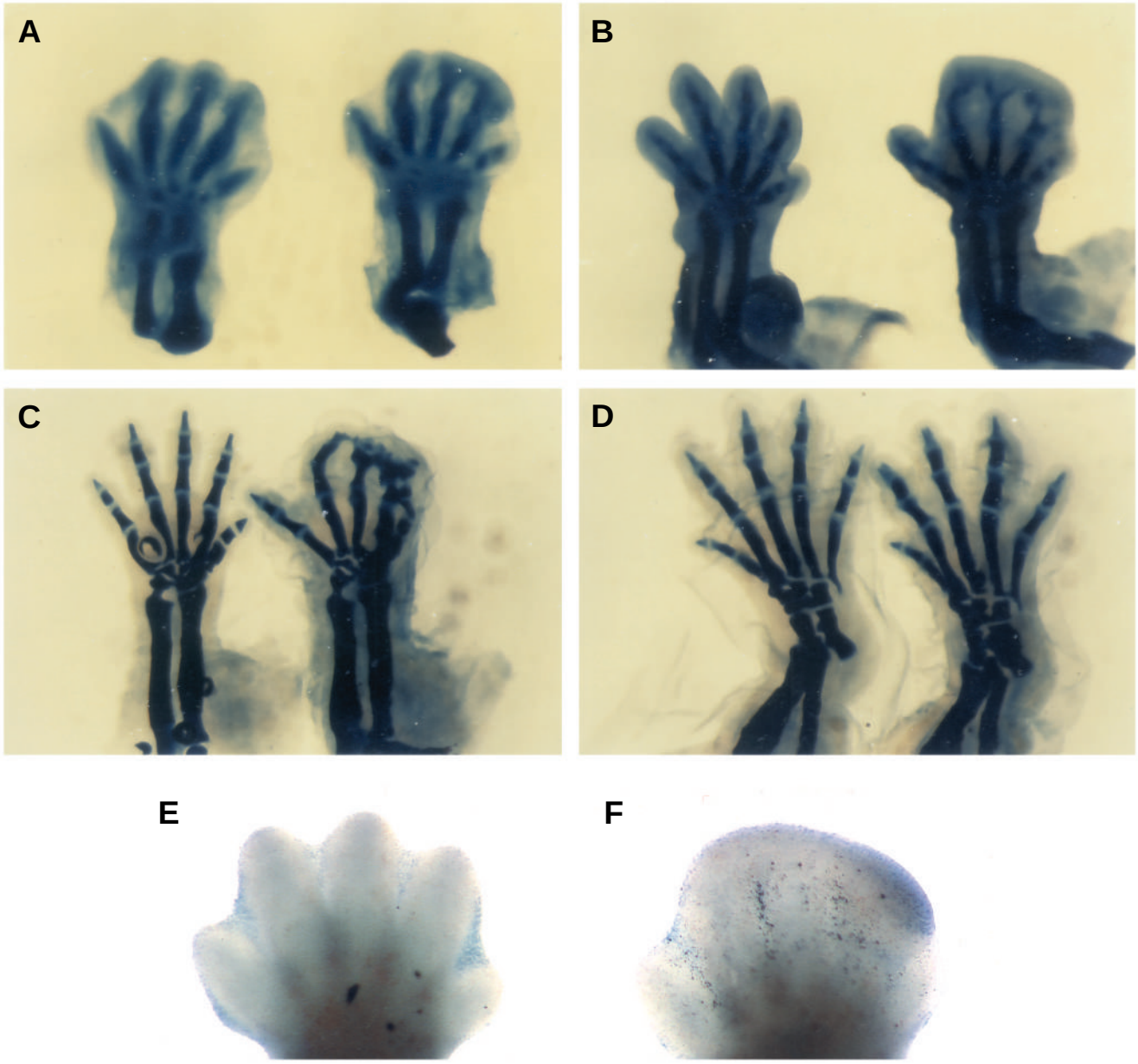

Fig. 2. The *Ft* mutant phenotypes in embryonic development. A-D are the cartilage stains of limbs from control littermates (left) and *Ft*/+ heterozygous embryos (right). Fore limbs of day 13.5, 14.5 and 15.5 embryos are shown in A, B and C, respectively. In D, hind limbs of 15.5-day old embryos are compared. A nile blue A staining of day 13.5 fore limbs from control littermates (E) and *Ft*/+ embryos (F) is also shown.

$$\%\ \text{deletion} = \frac{CD4^{low}/8^{low}}{CD4^{low}/8^{low} + CD4^{high}/8^{high}} \times 100\,.$$

In order to quantify by degradation of DNA, 1×10^6 thymocytes were incubated overnight at 4°C with 1.5 ml of a solution of propidium iodide (50 μg/ml in 0.1% sodium azide, 0.1% Triton X-100). 10 000 events/sample were measured by flow cytometry. Apoptotic nuclei were identified by a hypodiploid content of DNA and percentage deletion was calculated according to the ratio of hypodiploid to normodiploid nuclei (Nicoletti et al., 1991).

Cloning of the transgene integration site

Liver DNA, isolated from a heterozygous *Ft*/+ male, was partially digested with *Mbo*I and ligated into the *Bam*HI site of the Λ replacement vector DashII (Stratagene). 10^6 plaques were screened with a transgene-specific probe (human β-interferon promoter) and one positive clone was isolated. The insert was subsequently used to screen a C57BL/6 cosmid library to isolate the pre-integration site.

Mapping of the integration site

In situ hybridisation experiments were carried out using metaphase spreads from a WMP male mouse, in which all the autosomes, except 19, were in the form of metameric robertsonian translocations. For details of the procedure, see Mattei et al. (1985).

RESULTS

The phenotype of the *Ft* mutant

In the course of generating transgenic mice with the activated

human *Ha-ras* gene under the control of the human β-interferon promoter, one founder animal was detected that displayed an abnormality of both fore limbs (Fig. 1A). Breeding of this F_o mouse revealed a 100% correlation between the limb phenotype and the presence of the transgene. This linkage persisted over several generations (more than 12). Expression of the transgene was undetectable and there was never a sign of ras-induced tumor formation. Thus, the integration of the transgene has most probably induced a dominant mutation which affects the development of fore limbs.

Heterozygous mice of different generations and ages were inspected in more detail. First, bones of fore limbs were stained and their morphology was compared with fore limbs of wild-type mice. As exemplified in Fig. 1 we always found a bone fusion of the tip of digits no. 1-3 or of digits no. 1-4 in fore limbs of mutant mice. A fusion of digit no. 5 was never observed. Furthermore, we have never found a loss or gain of skeletal elements in the limbs. Based on this phenotype the mouse mutant was named *Ft* (*Fused toes*). Second, organs and skeletal elements were analysed by histopathology. All organs and the hind limbs were normal, except the thymus. As shown in Fig. 1E-H, the thymus of heterozygous *Ft* mice is enlarged early in life and, in contrast to wild-type mice, continues to grow. Histology and analyses of thymocytes revealed no gross alteration in either the thymus architecture or in the ratio of the different thymocyte subpopulations (data not shown). Thus, the *Ft* mutation is characterised by a fusion of fore limb digits and a thymic hyperplasia.

Programmed cell death is affected in fore limb and immature thymocytes

To investigate the reason for the fore limb digit fusion we analysed the morphological changes of limbs during development. Early limb development (day 9.5-12.5) looked normal, including the organisation of the apical ectodermal ridge. However, as outlined in Fig. 2, there is a clear effect visible in fore limbs of *Ft*/+ heterozygous mice by day 13.5. The interdigital cell death, which normally starts at about day 13, does not take place between digits no.1-4 (Fig. 2A). This phenomenon is even more pronounced at day 14.5 (Fig. 2B). As a consequence, the tips of the cartilage condensations in the digits become fused (Fig. 2C) leading to the phenotype visible in adult mice. Cell death seems to be normal between digit 4 and 5 in fore limbs and is also unaffected in hindlimbs (Fig. 2D). The absence of programmed cell death (PCD) in fore limbs of *Ft*/+ mice was confirmed by nile blue A staining. Accumulation of this dye is indicative for dying cells. Staining of day 13.5 *Ft*/+ mice fore limbs only resulted in blue stained cells between digits 4 and 5, but not between digits 1, 2, 3 and 4 (Fig. 2E).

The finding that PCD is influenced in fore limb development prompted us to investigate this process in thymocytes of mutant mice. PCD can be induced in immature thymocytes, in vitro and in vivo, by cortisone, radiation or crosslinking of the TCR/CD3 complex by antibodies (Cohen et al., 1992). We used all three methods to analyze the induction of apoptosis in immature thymocytes of *Ft*/+ heterozygous mice and age-matched control littermates. PCD was induced by graded concentrations of cortisone and was assessed by measuring the downregulation of the CD4/CD8 coreceptors on immature thymocytes after 6 hours in culture. As shown in Fig. 3A, treatment with cortisone led to a higher degree of deletion of thymocytes from wild-type mice as compared to *Ft*/+ mice at different concentrations of cortisone. When the kinetics of apoptosis induction by cortisone were compared between both groups of mice, induction of PCD was slower in thymocytes derived from *Ft*/+ mice than in their normal counterparts (Fig. 3B). Analogous results were obtained by induction of PCD via anti-CD3 antibodies and radiation (data not shown). The differences between *Ft*/+ and wild-type mice were highly reproducible and statistically significant, in all 18 pairs of mice (see legend, Fig. 3) and independent of the age of mice and methods of detection of apoptosis (coreceptor downregulation or DNA fragmentation, data not shown). Thus, freshly isolated immature thymocytes of *Ft*/+ mice apparently are more resistant to induction of PCD. Since both cortisone and TCR-mediated signalling are known to regulate thymocyte homeostasis in vivo via apoptosis (von Boehmer, 1992; Gonzalo et al., 1993), this lower susceptibility of thymocytes of *Ft*/+ mice might be causally related to development of thymic hyperplasia in this mutant strain. We have so far found no evidence for increased production of thymocytes to account for the hyperplasia.

Homozygous *Ft*/*Ft* mice die at midgestation

We were interested to know the phenotype of *Ft*/*Ft* homozygous animals. Therefore, heterozygous *Ft*/+ mice were mated and analysed for the presence and copy number of the transgene. However, all animals born from such a mating were either normal and nontransgenic or displayed the fore limb fusion, the thymus hyperplasia and had the same copy number

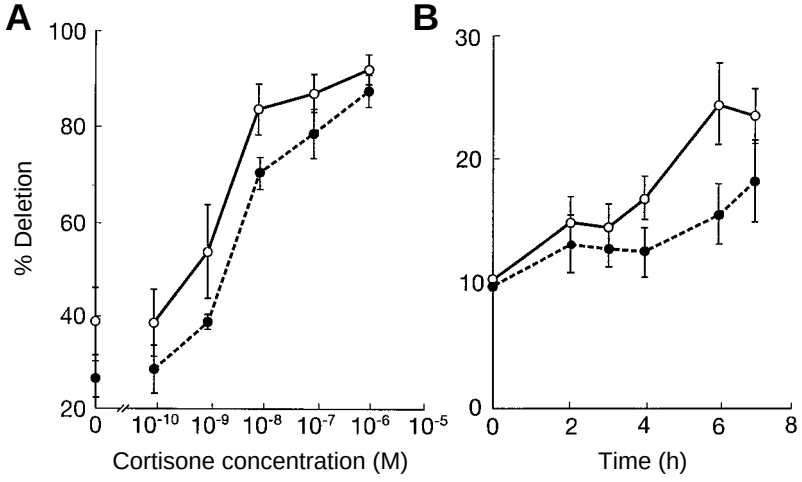

Fig. 3. Induction of apoptosis in immature thymocytes in vitro. (A) Whole thymocytes of wild-type (open circles) or *Ft*/+ mice (closed circles) of 2 months of age were treated with different concentrations of cortisone for 6 hours in vitro and induction of programmed cell death was quantified by analysing the deletion of double positive CD4/CD8 thymocytes (for details, see Materials and methods). Note the lower degree of cell death in *Ft*/+ mice ($P< 0.0025$). (B) Kinetics of cell death of the same groups of thymocytes are shown at a concentration of 10^{-10} M cortisone. Note the slower kinetics of apoptosis among thymocytes of *Ft*/+ mice ($P< 0.0026$). Each value represents the mean of four animals ± standard deviation. These results are representative of three independent experiments.

as heterozygous *Ft*/+ mice. This result suggested a prenathal lethality of homozygous *Ft*/*Ft* mice. Consequently, we examined embryos at different stages of development and correlated phenotype and genotype (genotype was confirmed by using a integration site-specific restriction fragment polymorphism, see below). Upon inspection of a total of 85 embryos from several dozen pregnant mice, we found 18 *Ft/Ft* embryos and observed that these homozygous mice were drastically altered in head structures at day 9.5. As shown in Fig. 4A, the telencephalon and mesencephalon are almost absent. Histological examination revealed that almost all cortical structures were reduced in these mice (data not shown). All other structures of these embryos looked relatively normal. In eight out of these eighteen *Ft/Ft* embryos the orientation of the tails were noted. In five cases the tails were found to be turned to the left (Fig. 4B) and only in the three others to the right. The latest stage where we could detect homozygous embryos was at day 10.5. Thus, homozygous *Ft*/*Ft* embryos must be dying at about day10. They have abnormal development of head structures and have lost their genetic control of the right-turning of the tail.

Cloning and mapping of the transgene integration site

The first step in identifying a mutated gene in *Ft* mice was the cloning of the transgene integration site. Therefore, a genomic DNA library generated from heterozygous *Ft*/+ mice was screened with transgene-specific probes. Only one lambda clone could be isolated carrying parts of the transgene and about 250 bp of flanking mouse DNA. Fortunately, this piece of mouse DNA was unique. Hybridisation of wild-type and heterozygous *Ft*/+ mouse DNA with this small flanking probe confirmed the cloning of the integration site. A restriction fragment polymorphism specific for the mutation was detectable (Fig. 5A). This probe was then used to screen for a wild-type cosmid that contained DNA spanning the preintegration site (not shown).

To map the integration site of the transgene one unique probe derived from the wild-type cosmid was used for in situ hybridisation on metaphase-spreads of mouse chromosomes. As demonstrated in Fig. 5B and C, specific signals were located in the D region of mouse chromosome 8.

DISCUSSION

Our analysis of *Ft* mice showed altered programmed cell death in fore limbs and thymus. Interdigital cell death in limb development is reduced between digits no. 1, 2, 3 and occasionally 4 but normal between digits 4 and 5. Hence, there is a polarity that is strongest in anterior structures in the fore limbs and not at all visible posteriorly. Since cell death in hind limbs is not obviously affected the changes in limb development can be considered as anteriorly restricted. In principle, hind limb structures are also susceptible to alterations since backcrossing for several generations of the *Ft* mice to the mouse strain C57BL/6 has led to fusion of toes no. 2 and 3 on hind limbs in about 15% of offspring (unpublished observation).

The finding of differences in the phenotypic changes in fore and hind limb is a common feature of most of the morphogenetic mutants described. For example, the mutant *add* affects primarly fore limbs and again only anteriorly (Pohl et al., 1990). Another mutant, called *legless*, produces strong changes on hind limb and mild alterations on fore limb (McNeish et al., 1988). One general reason for these differences might be the temporal difference of one day of fore and hind limb development. Another could be the specificity of gene expression.

Nevertheless, the only other structure in heterozygous *Ft* mice where apoptosis is affected is the thymus. As documented in Fig. 1, thymuses of *Ft* mice older than 5 months are enormously enlarged and contain up to 50 times more thymocytes than in age-matched control littermates. Despite massive thymic hyperplasia we have not detected abnormalities or signs of immunodeficiencies in these mice. Interestingly, the size of the peripheral T cell pool is not affected by this mutation (data not shown). A detailed analysis of thymopoiesis in *Ft*/+ mice (which will be the subject of a seperate report) showed that the efficacy of positive selection, i.e. the transition from double-positive to single-positive thymocytes is reduced and the emigration rate of thymocytes/day is increased only by a factor of 2. Thus, the rate of export of mature T cells in *Ft*/+ does not increase proportionally with the production of immature thymocytes. These findings may in part explain the normal cellularity of peripheral lymphoid organs. In adition, it is known from previous studies that the size of the peripheral T cell pool is autonomously regulated (Rocha et al., 1989).

We can only speculate about why no other structures are affected in the *Ft* mutant, for example in facial development, where cell death is an essential component in morphogenesis (Sulik et al., 1988). One explanation is the specificity of expression of the mutated gene. It could be that this gene is normally not expressed in unaffected structures.

Remarkably, homozygous *Ft*/*Ft* mice die around day 10 of embryonal development without giving any indication for a defect in cell death. They display gross alterations in head formation, in structures found to be altered in several dominant morphogenetic mouse mutant when crossed to homozygozity (Lyon and Searle, 1989). In addition, the homozygous *Ft* mice show a phenotype described as *situs inversus viscerum*, which is characterised by the absence of right-left asymmetry (Hummel and Chapman, 1959). The first sign for *situs inversus* is the absence of control of the tail location in 9.5-day old embryos. Wild-type embryos always have the tail turned to the right. The first mutation of this phenotype described has been mapped to mouse chromosome 12 (Hanzlik et al., 1990). Very recently, a second nonallelic *situs inversus* mutation was published, which maps to chromosome 4 (Yokoyama et al., 1993). *Ft* represents a third gene responsible for right-left assymetry, since it maps to mouse chromosome 8.

As our study suggests the *Ft* gene product might be involved in apoptosis during limb development and in immature thymocytes. This interaction could be direct or indirect. Therefore, we first tested genes suspected of participating directly in apoptosis, for changes in their expression profile. However, none of those analysed so far, e.g. c-*myc*, *bcl*-2, was found to be altered (data not shown). Also p53 seems not to be involved, since it is dispensible for mediating PCD after cortisone treatment of immature thymocytes as recently shown (Clarke et al., 1993; Lowe et al., 1993).

By in situ hybridisation to metaphase spreads of chromosomes, the *Ft* mutation has been mapped to the D region of chromosome 8. Although several genes and mutants are

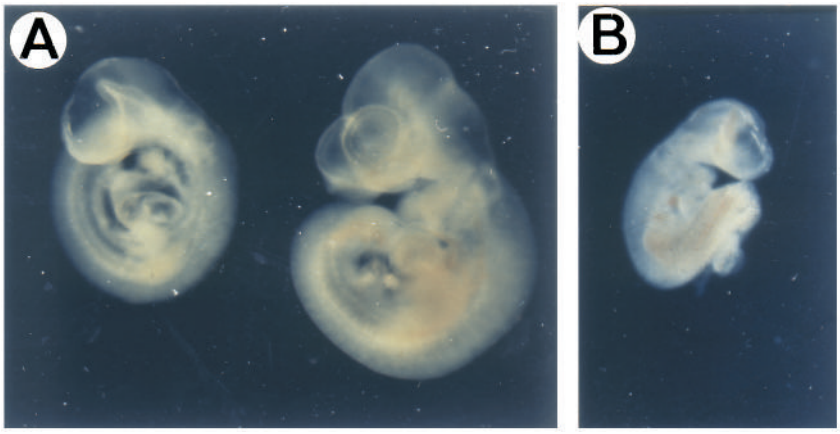

Fig. 4. The phenotypes of 9.5-day old homozygous *Ft*/*Ft* embryos are illustrated. A control littermate embryo (right in A) is presented. Note the different turns of the tails in A and B.

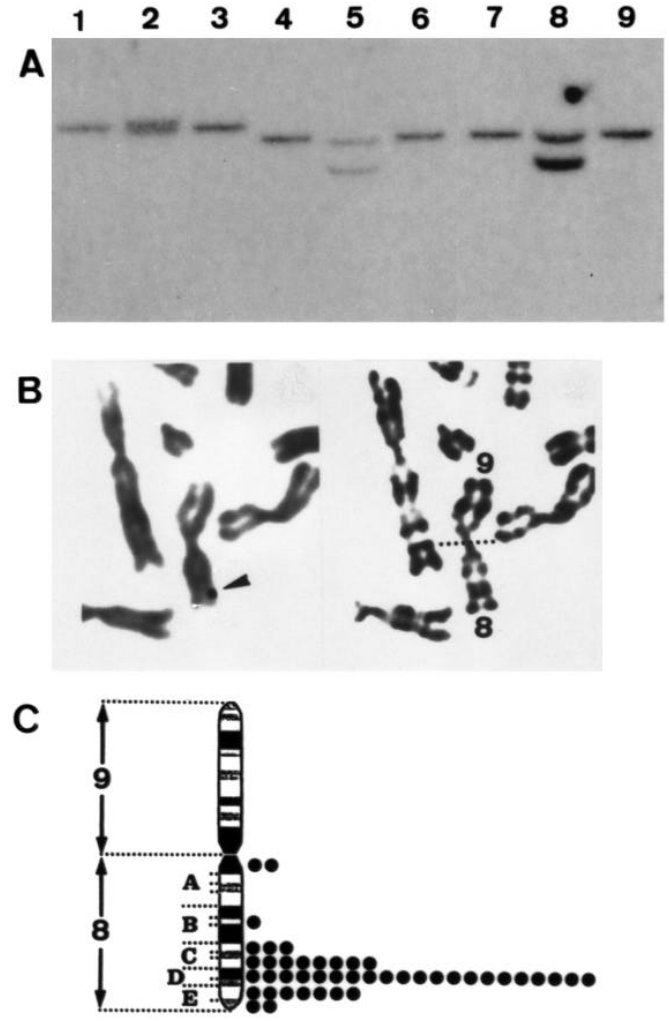

Fig. 5. Mapping of the *Ft* mutation. (A) A Southern analysis of genomic DNA isolated from either control littermate (lanes 1,4,7), *Ft*/+ mouse (lanes 2,5,8) or C57BL/6 (lanes 3,6,9) and digested with three different restriction enzymes. Genomic DNA, flanking the transgene integration site was used as probe. The *Ft* mutation was mapped by in situ hybridisation to metaphase spreads of WMP chromosomes as documented (left part of B) using the same DNA probe as in A. Chromosomes were subsequently identified by R-banding (right part of B). (C) Diagramme of WMP mouse Rb(8;9) chromosome, indicating the distribution of labelled sites on chromosome 8 (for details, see Materials and Methods).

mapped to this region, none of them has been described as involved in morphogenesis (Ceci and Lusis, 1992). We have nevertheless tested some for linkage to *Ft*. However, using pulse field gel electrophoresis with a resolution of about 1 Mb we have not found any evidence for the linkage of TAT, Zif 1 and 4 or uvomurolin, all of which map to the D region of chromosome 8 (unpublished data). Thus, the *Ft* mutation represents a new entry point to the molecular characterisation of chromosome 8 and reveals a novel mouse mutation.

We are very grateful to Thomas Pohl for construction of the *Ft*/+ genomic library, to Birgit Bosse for technical help, to Barbara Stace and Dave Stoddart for taking care of the mice at the EMBL and to Dimitri Komitowski for histopathological inspection of *Ft* mice. This work was supported by the Deutsche Forschungsgemeinschaft through SFB 271 and by the VW-Stiftung.

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