Phenotypic and Molecular Analysis of a Transgenic Insertional Allele of the Mouse Fused Locus

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ABSTRACT

Spontaneous mutations at the mouse Fused (Fu) locus cause dominant skeletal and neurological defects and recessive lethal embryonic defects including neuroectodermal abnormalities and axial duplications. Here, we describe a new allele at the Fu locus caused by a transgenic insertional mutation, He46. Embryos homozygous for the He46 insertion die at day 9–10 post coitum and display phenotypic defects similar to those associated with Fu alleles. The He46 locus was cloned and shown to contain a 20-kb deletion at the site of transgene insertion with no other detectable rearrangements. Genomic probes from the He46 locus were mapped to a genetic locus closely linked to the Fu locus and were hybridized to a YAC contig covering the Fu critical region. Compound heterozygotes between Fu and He46 were inviable and displayed abnormalities at the same stage of embryogenesis as do homozygotes for either of the two mutations, demonstrating that these two recessive lethal mutations belong to the same complementation group. A genomic probe from the wild-type He46 locus detected a transcript that is disrupted by the transgenic insertion, representing a candidate for the wild-type allele of Fused.

The Fused locus of the mouse has been a subject of interest for many years because of its apparent role in a variety of developmental processes, as well as its unusual genetic properties. Fused (Fu), the original spontaneous mutation reported in 1937, is a dominant mutation that causes neural tube duplications resulting in tail bifurcations, asymmetrical fusion of vertebrae leading to kinking and shortening of the tail and fusion of ribs, as well as deafness, waltzing behavior, and urogenital abnormalities (Reed 1937; Gluecksohn-Waelsch 1954; Theiler and Gluecksohn-Waelsch 1956; Deol 1966). The Fu mutation displays variable expressivity and incomplete penetrance, which is lower when the mutation is maternally inherited, suggesting that the locus is subject to a form of maternal imprinting (Reed 1937; Dunn and Gluecksohn-Waelsch 1954; Cattanach 1986; Ruvinsky and Agulnik 1990). In addition, the Fu mutation can be passed for several generations in an intact state, in which no phenotype is evident, but from which the mutant phenotype can reappear at a very low frequency in subsequent generations (Belavaev et al. 1981).

Two more severe alleles at the Fu locus, Kinky (FuK) (Caspari and David 1940) and Knobby (FuKb) (Jacobs-Cohen et al. 1984) exist as well. Heterozygotes for either of these semi-dominant alleles display skeletal abnormalities similar to those of Fu/ Fu heterozygotes, but homozygotes for FuK or FuKb die at ~8–9 and 9–10 days post coitum (dpc), respectively (Gluecksohn-Schoenheimer 1949; Jacobs-Cohen et al. 1984). Among the defects reported in these homozygous embryos (as well as in occasional Fu/Fu embryos) is the partial or complete duplication of axial structures, suggesting that the Fu gene may play a role in primary embryonic induction (Gluecksohn-Schoenheimer 1949; Theiler and Gluecksohn-Waelsch 1956; Jacobs-Cohen et al. 1984). Genetic studies have suggested that all three mutant alleles of Fu carry gain-of-function mutations (Greenspan and O'Brien 1986), because mice heterozygous for a large deletion including the Fu locus do not display tail defects (Lyon and Bechtol, 1977). While genetic and physical mapping studies have limited the FuKb locus to a 2-cM region of chromosome 17 (Rossi et al. 1994), the gene has not been identified.

The analysis of insertional mutations in the mouse caused by the integration of transgene DNA has often been pursued as an approach to identify novel genes involved in mouse embryogenesis (Gridley 1991). This approach has led to the cloning of the genes corresponding to several previously identified mutations, including limb deformity (Maas et al. 1990; Woychik et al. 1990), microphthalmia (Hodgkinson et al. 1993), and

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pigmy (Xiang et al. 1990), as well as to the identification of other new genes of developmental importance, such as nodal (Zhou et al. 1993; Conlon et al. 1994). We previously described two insertional mutations associated with embryonic lethality at the compaction and gastrulation stages (Lee et al. 1992; Cheng and Costantini 1995), and here we describe a third insertional mutation that arose in the transgenic line He-46 (Costantini et al. 1989). While He-46 heterozygotes were apparently normal, homozygous mice could not be produced, and embryological studies showed that they died at day 9.5–10.5 dpc, displaying multiple abnormalities, many of which resembled those in homozygotes for Fu<sup>x</sup> or Fu<sup>xk</sup>. Cloning of the He-46 insertion locus allowed us to genetically map the He-46 locus on chromosome 17 and to show that He-46 is tightly linked to Fu<sup>xk</sup>. Complementation testing revealed that He-46 and Fu<sup>xk</sup> are allelic, as compound heterozygotes were inviable and displayed abnormalities at the same stage of embryogenesis as do homozygotes for either of the two mutations. A transcript detected by Northern blot analyses, which is encoded at the wild-type He-46 locus and disrupted by the transgenic insertion, represents a candidate for the wild-type allele of the Fu gene.

**MATERIALS AND METHODS**

**Transgenic and mutant animals:** The He-46 transgenic line was originally derived by microinjection of an 8.1 kb HindIII fragment of the human e-globin gene into a zygote of strain (CBA/J X C57BL/6J)F<sub>2</sub>, and the line was maintained by breeding to (CBA/J X C57BL/6J)F<sub>1</sub> animals and by intercrosses among transgenic heterozygotes. Therefore, the He-46 mutation was carried on a mixed CBA/J and C57BL/6J background. The BTBR Fu<sup>x</sup>+/– mutant mice were obtained from Dr. William Dove (University of Wisconsin) and were maintained by intercrossing or backcrossing to BTBR.

**Histological analysis:** Embryos were dissected from pregnant females as described (Hogan et al. 1986), fixed overnight in Bouin’s solution or 10% formalin, and stored at 70% ethanol at 4°. The embryos were dehydrated through an ethanol series, cleared in toluene, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin.

**Identification of homozygous He-46 embryos:** Embryos homozygous for the He-46 mutation were identified by one of several methods. For embryos at E9.5 or later, DNA isolated from the embryo and/or yolk sac was analyzed by Southern blot hybridization with a probe that distinguished the wild-type from the mutant He-46 locus (Figure 4), or the yolk sac was analyzed by PCR, as described below. For embryos at E7.5–E8.5, the ectoplacental cone (EPC) was removed using embryonic lethality at the compaction and gastrulation stages (Lee et al. 1992; Cheng and Costantini 1995). Probe F detected a 7.0 kb fragment in genomic lambda library to identify clones representing the wild-type preinsertion locus. Clones isolated with probe A did not overlap with clones isolated with probe F, so a third probe isolated from one of these new lambda clones (probe D, Figure 4c) was used to isolate additional clones, which completed the contig. Restriction maps of the wild-type and mutant chromosomes (Figure 4) were derived from restriction maps of lambda clones, in addition to genomic Southern hybridizations of DNA from transgenic and wild-type mice, using probes from this locus. Cloning, DNA preparation, radiolabeling, and hybridization were performed by standard procedures (Sambrook et al. 1989).

**Northern blot hybridization:** Total RNA was isolated from wild-type ES cells [line CCE (Robertson et al. 1986)] and ES cells homozygous for the He-46 transgene [line 1.1.2 (S-W. Cheng and F. Costantini, unpublished data)] by the method of Gomzcynski and Sacchi (1987). The RNA was electrophoresed on formaldehyde-agarose gels, blotted onto GeneScreen-plus filters (NEN Research Products), and hybridized with probe F (Figure 4b), which had been 32P-labeled by random priming (Boehringer Mannheim). Hybridization and washing conditions were as described (Lee et al. 1992), except that the successive washes were in buffers containing 4X SSC, 1X SSC and 0.1X SSC. Autoradiography of the filter was rehybridized with a probe for mouse β-actin mRNA (the HindIII-KpnI fragment of pTRI-β-Actin-Mouse, Ambion, Inc.) and reexposed to X-ray film.

**Genetic mapping:** In situ hybridization to metaphase chromosome spreads derived from heterozygous He-46 mice was performed as described (Lee et al. 1990). Genetic mapping of the He-46 locus was first performed by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × Mus spretus)F<sub>1</sub> X C57BL/6J] mice as described (Copeland and Jenkins 1991). Probe E detected a 7.0 kb fragment in BamHI-digested C57BL/6J DNA and a 8.5 kb fragment was detected in BamHI-digested M. spretus DNA. The presence or absence of the 8.5 kb spretus-specific BamHI fragment was followed in a total of 193 backcross mice. Recombination distances were calculated as described (Green 1981) using the computer program SPRETUS MADNESS. This analysis placed the He-46 locus 1.29 ± 0.91 cm distal to D17Loh66d.
and 0.63 ± 0.62 cm proximal to Pim 1 (N. A. Jenkins and N. G. Copeland, personal communication). The chromosomal position of the He46 locus was further refined by analyzing DNAs from animals carrying recombinant chromosomes in the T to H2 region of chromosome 12, recovered from a balanced-lethal cross between two M. musculus strains, A/J.Twa and BTBR (King et al. 1989). Probe D hybridized to a 2.1-kb band in BTBR DNA and to a 2.25-kb band in A/J.Twa DNA digested with EcoRI. To place the He46 locus between two of the six genetic markers typed in these animals, restriction fragment length polymorphism (RFLP) analysis was initially performed on two animals carrying recombinant chromosomes in each of five recombination intervals. These results placed the He46 locus between D17L, k5M and yf and 35 additional animals carrying recombinant chromosomes in this interval were subsequently analyzed.

**Compensation tests**: PCR was performed in 20 μl of 50 mM KCl/10 mM Tris-HCl pH 8.3/1.5 mM MgCl2/0.5 units Taq polymerase/1 μl of each 5B12 primer/0.50 μM of each EP primer/125 mM dNTP at cycling conditions of 1 cycle at 90 sec at 94°, followed by 35 cycles of 30 sec at 94°, 30 sec at 60°, 90 sec at 72°. The FbH12-linked (CA), primers were 5B12F (5'-ACTCCAGCTACAGAGCATCG-3') and 5B12R (5'-TGATAGTGTACAGGATGTGCAG-3'), which amplify a fragment of the mutant c-globin transgene. PCR products were analyzed on 10% native polyacrylamide gels.

Embryo DNA was prepared from ~5 mg (wet wt) of yolk sac that was digested in 50 mM KCl/10 mM Tris-HCl pH 8.3/1.5 mM MgCl2 supplemented with 0.05% sodium dodecyl sulfate (SDS) and 50 μg/ml proteinase K at 55° for 1 hr. The resulting mixture was heated at 95° for 10 min to inactivate the proteinase K, chilled on ice for 15 min and briefly centrifuged to precipitate the SDS.

Adult DNA was prepared from 1-cm pieces of tail that were digested in a solution containing 100 mM NaCl, 100 mM EDTA, 0.5% SDS, 10 mM Tris-HCl pH 8.0, and 3.2 mg/ml proteinase K at 55° for 2 hr with occasional vortexing. The resulting solution was extracted with phenol/chloroform (4:1) and precipitated in 2 vol ethanol. The DNA was dissolved in 200 μl of H2O, of which 0.2 μl was used for PCR.

**Mapping He46 in YAC panel**: C19.B11 (YAC1), B16.C1 (YAC2), C65.G6 (YAC3), C22.E8 (YAC4) and FDE.H5 (YAC5) have been described previously (Rossi et al. 1994) using the names in brackets. C101.H7 and C101.H1 were referred to in Rossi et al. (1992) as YAC3 and YAC4, respectively. C143.G5, FBP.G7 and FBN.11 were obtained by screening the Princeton and MIT YAC libraries (Green and Olson, 1990; Rossi et al. 1992; Kusumi et al. 1993) with primers derived from the sequence of the left end of C19.B11. The end was recovered by vectorette PCR (Riley et al. 1990) and sequenced directly (Sanger et al. 1977). The primers were 5'-TTCTGGAACTCTGGCTGCT-3' and 5'-TCTAGTTCAGCAACCTG-3'. Yeast DNA and mouse genomic DNA were prepared as previously described (Rossi et al. 1992). Ten micrograms of DNA was digested with EcoRI, separated on a 1% agarose gel, transferred to Genescreen, hybridized to probe E (Figure 4c) and labeled by oligo-labeling (Southern 1975; Feinberg and Vogelstein 1984). The filter was washed at 65° in 0.1x SSC, 0.05% SDS and exposed to X-ray film.

**RESULTS**

Embryonic defects and lethality in He46 homozygotes: The He46 transgenic line was produced by the microinjection of a human c-globin gene into fertilized mouse eggs. Southern and dot blot analyses showed that heterozygotes carried 70–80 copies of the 8-kb transgene in a single tandem array, and no expression of the globin transgene could be detected at the RNA level in heterozygous embryos (data not shown). Heterozygotes were normal and fertile, and animals of both sexes transmitted the transgene in Mendelian proportions. However, attempts to produce adult mice homozygous for the transgenic insert were unsuccessful (Costantini et al. 1989). To determine the stage of development at which homozygotes were dying, heterozygous He46 mice were intercrossed, and the females were sacrificed at various stages of gestation. The embryos were recovered and examined, and their genotypes at the insertion locus were determined by either Southern blotting (e.g., Figure 4f). PCR (e.g., Figure 4g) or in situ hybridization analyses (data not shown).

At 7.5 dpc (E7.5), He46 homozygotes could not be distinguished morphologically from their wild-type or heterozygous littermates (Figure 1, a and b), suggesting that the He46 mutation does not interfere with preimplantation development or early gastrulation. At E8.5, however, most mutant homozygotes appeared somewhat retarded in development, resembling normal embryos at E8.0 (Figure 1, c and d). By E9.5, all the mutant homozygotes were severely abnormal, although the extent of development and the nature of the defects observed varied considerably from embryo to embryo, even within the same litter. E10.5 homozygotes displayed little additional growth or development, and by E12.5 virtually all homozygotes had been resorbed.

Several examples of E9.5 He46 homozygous embryos are shown in Figure 2 and histological sections are shown in Figure 3. Compared to normal E9.5 embryos (Figure 2, a and d), the most advanced subset of mutant homozygotes (e.g., Figure 2, b–d left) were only slightly retarded in overall development. They displayed 12–18 somites, compared to 21–25 in wild-type littermates, and had completed or nearly completed the process of turning. In these embryos, the most severe abnormalities were restricted to the cranial region, where the head folds had usually failed to close (Figures 2, c and d and 3c), and the forebrain was absent or severely underdeveloped (Figures 2b and 3b). In the trunk, the most common abnormality in these embryos was a kinky neural tube (Figure 3c). The heart appeared normal in most cases, but occasionally the pericardium was abnormally expanded (Figure 2d, left). Another subset of E9.5 homozygotes was more severely retarded, with head folds resembling those of a normal E8.5 embryo, only 4–8 somites, and turning only beginning (Figure 2, e and f). Other defects frequently observed included cardia bifida (failure of the two cardiac primordia to fuse, e.g., Figures 2f and 3d) or an abnormal accumulation of cellular debris in the amniotic cavity (not shown).

The most unusual abnormalities observed in He46 ho-
FIGURE 1.—Histological sections of wild-type and He46 homozygous embryos at E7.5 and E8.5. (a) Wild-type E7.5 embryo. C, chorion; AL, allantois; AM, amnion; EE, embryonic ectoderm. Bar, 0.2 mm (a–d at same magnification). (b) He46/He46 littermate of the embryo shown in a. The posterior amniotic fold (AF) has not yet fused with the anterior amniotic fluid, but the embryo appears normal. EPC, ectoplacental cone. (c) Sagittal section of a wild-type E8.5 embryo. H, heart; HF, head folds; NE, neuroectoderm; PS, primitive streak mesoderm; YS, yolk sac. (d) Sagittal section of He46/He46 embryo at E8.5, which is smaller and less developed than its littermate in c.
mozygotes were the apparent duplication of parts of the embryo and the presence of embryonic structures (either duplicated structures or parts of a single embryo) outside the amnion and yolk sac. One homozygous mutant conceptus contained two allantoises connected to the placenta, one originating from an abnormal structure (apparently a secondary embryonic axis) at right angles to the main axis and the second originating from an extra structure near the head folds of the primary embryo. The posterior end of the primary embryo protruded through the visceral yolk sac into the yolk cavity (Figure 2h). Several homozygous embryos contained ex-
Figure 3.—Histological sections of wild-type (a) and homozygous Hc46 embryos (b–h) at E9.5. (a) Transverse section through the head of normal embryo shown in Figure 2a, showing hindbrain (HB), maxillary component of first branchial arch (BA), optic vesicle (OV), telencephalon (T) and tail bud (TB). Bar, 0.5 mm (all panels are at the same magnification). (b) Transverse section through the head of the abnormal embryo shown in Figure 2b, displaying severe truncation and malformation of the telencephalon. (c) Frontal section of abnormal embryo shown in Figure 2d, showing open head folds (HF) and kinked neural tube (NT). S, somites; H, heart. (d) Transverse section of Hc46/Hc46 embryo shown in Figure 2f, displaying two unfused heart primordia. (e–h) Four frontal sections through an Hc46/Hc46 embryo with duplicated embryonic formations (bold arrows) rostral to the head folds (HF) of the primary embryo. One structure reminiscent of a tail bud in cross section can be seen outside the yolk sac in f but can be seen in g and h (arrows) to join to an abnormal mass of tissue closer to the head folds and within the yolk sac. The caudal end of the primary embryo (C) is at bottom, as seen in g and h. A second abnormal structure also protruding through the yolk sac is seen at the upper left in e (arrow at left).

In summary, Hc46 homozygotes at E9.5 displayed a spectrum of abnormal development, ranging from mild growth retardation with defects limited to the cranial region to more severe retardation, to unusual malformations and partial duplications.

Molecular cloning of the Hc46 locus and detection of a transcript disrupted by the transgene insertion: To clone the mutant and corresponding wild-type loci, a genomic lambda library was prepared from heterozygous Hc46 mouse DNA and screened with a probe for the human $\alpha$-globin transgene. Several clones containing both mouse DNA and transgene DNA were identified (Figure 4d). Single copy probes isolated from the mouse DNA segments of these clones (probes A and E, Figure 4c) hybridized to bands of different sizes in wild-type DNA vs. homozygous Hc46 DNA (Figure 4f), indicating that these clones represented the left and right transgene DNA/mouse DNA junctions.

Probes from the mouse DNA portions of the junction clones were next used to screen a C57BL/6J genomic DNA library, and a series of overlapping wild-type clones, spanning the preinsertion locus, was isolated by genomic walking. Comparison of the wild-type (Figure 4b) and mutant (Figure 4e) genomic DNA restriction maps suggested that a 20-kb deletion had occurred at the site of transgene integration, which was confirmed by the inability of probes B and D to hybridize to DNA from homozygous Hc46 embryos (Figure 4f). Other
Transgenic Insertional Fused Allele

Figure 4.—Structure of the wild-type and mutant He46 loci, and Southern blot and PCR analyses. (a) Scale in kilobase pairs. (b) Restriction map of the wild-type mouse genome at the He46 locus. B, BamHI; E, EcoRI; H, HincII; K, KpnI; N, NolI; S, SadI; X, XbaI. The positions of PCR primers P3 and P4 are indicated in the inset, where the vertical bar indicates the right boundary of the deletion. (c) Single copy probes used for cloning and analysis of the He46 locus. Probe A, 2.8-kb SadI/EcoRI fragment; probe B, 0.7-kb Tth1111/KpnI fragment; probe D, 0.25-kb EcoRI/SalI fragment (SalI site in lambda vector); probe E, 2.7-kb EcoRI/SalI fragment (SalI site in lambda vector); probe F, 1.1-kb KpnI/EcoRI fragment. (d) Restriction maps of two lambda clones containing the left and right junctions between the human ε-globin transgene array (thick lines) and the flanking mouse DNA (thin lines), aligned with the genomic restriction maps. The positions of PCR primers P1 and P3 are indicated in the inset. (e) Restriction map of the mouse genome surrounding the tandem array of ε-globin transgenes (arrowheads) in the mutant He46 genome. The dotted line indicates the extent of mouse DNA deleted at this locus. (f) Southern blot analyses of wild-type (w), heterozygous (h) and homozygous He46 (H) DNAs with the indicated probes and restriction enzymes. Probes A and E, which are near the two junctions, detect bands of different sizes in wild-type and homozygous DNAs, and both bands in heterozygous DNAs. Probes B and D, which are deleted in the He46 genome, detect a band only in wild-type and heterozygous DNAs. The positions of molecular size markers (in kb) are shown next to each panel. (g) Identification of wild-type (w), heterozygous (h) and homozygous He46 (H) embryos by PCR analysis. DNA samples from E9.5 embryos were amplified using a mixture of primers P1, P3 and P4. Primer P1 is derived from the ε-globin transgene, primer P3 from the flanking mouse DNA, and primer P4 from the mouse DNA within the region deleted in the He46 allele. The 327-bp band derives from the wild-type allele (primers P3 and P4) and is seen in wild-type and heterozygous DNA samples. The 550-bp band derives from the mutant allele (primers P1 and P3) and is seen in heterozygous and homozygous He46 samples. Lane m, molecular size markers; 506- and 298-bp bands are indicated at left.
than the deletion, the He46 and wild-type genomes flanking the insertion site appeared to be colinear, based on restriction mapping of the lambda clones and genomic Southern analysis.

In an attempt to identify a gene encoded at this locus, we tested several single copy genomic probes for their ability to hybridize to Northern blots containing total RNA from mouse embryos or embryonic stem cells. As shown in Figure 5, probe F, from within the deleted region, hybridized to a 3.9-kb transcript in wild-type embryonic stem (ES) cells, while an ES cell line derived from homozygous He46 mutant embryos (S.-W. CHENG, J. J. LEE and F. COSTANTINI, unpublished data) lacked the 3.9-kb transcript, as expected. The 3.9-kb RNA was also present in RNA from wild-type whole embryos at E9.5–E16.5 (data not shown). Thus, we have detected a transcript encoded at the He46 locus, representing a gene whose disruption may be responsible for the mutant phenotype.

**Genetic mapping of the He46 locus:** The He46 insertion locus was first assigned to chromosome 17 by in situ hybridization to metaphase chromosome spreads (RADICE et al. 1991) prepared from a heterozygous He46 mouse, using a probe for the human e-globin transgene (data not shown). To place the He46 locus on the mouse genetic map, probe E (Figure 4c) was mapped in a M. musculus/M. spretus interspecific backcross (COPELAND and JENKINS 1991). While this localized the transgene insertion between the markers Pim1 and D17Leth66D (Figure 6), it could not provide a precise location in this interval because of recombination suppression, apparently due to an inversion between M. musculus and M. spretus in a region of chromosome 17 proximal to D17Leth66D (HIMMELBAUER and SILVER 1993). The He46 locus was then mapped in a panel of recombinant mice derived from a cross between the two M. musculus strains, A/J.TWS and BTBR (KING et al. 1989). Probe D (Figure 4c), which detected a RFLP...
between these two strains, was used to analyze DNAs from 37 mice previously shown to have recombination events in the interval between D17Leh54 and if, markers that lie within the D17Leh66D-Pim1 interval (Figure 6). We found eight recombination events between He46 and if, and 29 between D17Leh54 and He46, placing the He46 locus within this interval, ~1 cM proximal to if.

Since the Fu locus is known to map in this region and given the phenotypic similarities between homozygous He46 embryos and those homozygous for Fu alleles (see Discussion), we next asked whether the He46 locus resided within a series of yeast artificial chromosome clones that represented a partial physical map of the 2-cM interval covering the Fu locus. As shown in Figure 7, probe E hybridized to a subset of overlapping YAC clones from the Fu locus. This analysis placed the He46 locus in between two genetic markers (the proximal end of YAC C19B11 and Hba-ps4, a pseudogene of α-globin) that failed to recombine with FuK' in a 983-animal interspecific backcross (Rossi et al. 1994).

The He46 and FuK' mutations fail to complement: The genetic and physical mapping data suggested that the He46 insertional mutation and the three Fu alleles might affect the same gene. If so, a compound heterozygote carrying an He46 allele and a lethal Fu allele (FuK or FuK') should be inviable, i.e., the mutations should fail to complement. For this purpose, heterozygous He46 males were mated with heterozygous FuK'/BTBR females, and the offspring were analyzed for inheritance of the He46 mutation, using the e-globin transgene as a diagnostic marker, and for inheritance of the tail kink phenotype (Figure 8). Among 69 live born progeny analyzed, there were 21 wild type, 31 He46/+, and 17 Fu"/+; but no He46/FuK' progeny, strongly suggesting that such compound heterozygotes are inviable (p < 0.0001). Thus, based on its failure to complement the prenatal lethality of FuK', He46 is allelic to FuK'.

When 17 embryos from such a cross were recovered at E9.5 and E11.5 and genotyped by PCR analysis of
yolk sac or embryo tissue, a total of four He46/FuKi embryos were definitively identified (e.g., Figure 8, embryo lane), three at E9.5 and one at E11.5 (another two E9.5 embryos undergoing resorption appeared to be He46/FuKi, although their genotypes were uncertain because of infiltration with maternal cells). Two of the E9.5 He46/FuKi embryos were clearly abnormal, exhibiting phenotypic defects characteristic of FuK'/FuK' and He46/He46 embryos. One of these was slightly retarded, with a flattened head and an expanded pericardium, and the other was severely retarded, with poorly formed head folds, a large globular mass attached to the head folds, and an abnormal allantois (Figure 2j). One E9.5 and one E11.5 He46/FuKi embryo appeared normal, indicating that some He46/FuKi embryos develop to a later stage than He46/He46 homozygotes (but not necessarily later than FuK'/FuK' homozygotes on the BTBR background, a fraction of which appear normal as late as E11.5) (unpublished data).

**DISCUSSION**

In this paper, we describe a transgenic insertional mutation, He46, which represents a new allele at the Fused locus of the mouse. The He46 mutation was first identified based on its recessive lethal phenotype. Embryological analyses showed that the homozygous embryos died at E9-E10, displaying a wide spectrum of abnormalities, many of which were similar to those previously described for one or more alleles of Fu. The insertional nature of the mutation allowed the He46 locus to be cloned, and comparison of the wild-type and transgenic loci showed that insertion of the transgene had been accompanied by the deletion of 20 kb of chromosomal DNA, without any other detectable rearrangements. A genomic probe from the locus hybridized to a transcript present in wild-type embryos and ES cells, but not in homozygous He46 ES cells, thus identifying a gene disrupted by the transgene insertion/deletion. We found that the He46 locus mapped to chromosome 17, within a YAC contig covering the FuK critical region. Finally, we showed that He46/FuK compound heterozygotes were inviable, demonstrating that these two recessive lethal mutations belong to the same complementation group.

Homozygous He46 embryos examined at E9.5, a stage when all were clearly abnormal, displayed a spectrum of abnormalities affecting the neural ectoderm, heart and extraembryonic membranes, as well as a wide range of developmental retardation. The extent to which this variation is due to modifying genetic loci cannot yet be evaluated, as the embryos studied had a mixed genetic background. Based on published descriptions of Fu, FuK' and FuK homozygous embryos at E9-E10 (Dunn and Caspari 1945; Theiler and Gluecksohn-Waelsch 1956; Jacobs-Cohen et al. 1984), the similarities to He46 homozygous embryos are striking. Many of the abnormal features observed among He46 homozygotes have also been reported in homozygotes for one or more Fu alleles, particularly FuK (e.g., Figure 2i). These include the following: incomplete closure or malformation of the head folds (observed in Fu, FuK' and FuK) crooked neural tube (Fu and FuK), cardiac bafida (FuK), enlarged heart (FuK), duplications of the allantois (FuK), partial duplications of the embryonic axis (Fu and FuK), unorganized embryonic tissue at the cephalic end of the embryo (FuK), embryonic tissue found outside of the amnion and yolk sac (FuK), and necrotic cells in the amniotic cavity (FuK and FuK). There were also significant differences between He46 and other Fu alleles: for example, FuK/FuK embryos arrest and die at an earlier stage (by E8.5), rarely developing a neural tube, heart or somites, and they display ectodermal swellings and outgrowths at E7.5-E8.5 (Jacobs-Cohen et al. 1984), which were not observed in the limited sample of He46 homozygotes examined at these stages. Fu/Fu homozygous embryos, most of which are viable, displayed frequent duplications and bifurcations at the posterior...
end of the neural tube (Theiler and Gluecksohn-Waelsch 1956). Overall, however, the phenotypic similarities add weight to the genetic evidence that *He* 46 is an allele of the *Fu* locus.

It has been proposed (Greenspan and O'Brien 1986) that *Fu, Fu* sub* and Fu* sub* are gain-of-function rather than loss-of-function alleles, based primarily on their genetic nonequivalence to a chromosome 17 deletion in the partial *t* haplotype *t* sub* sub , which removes *Fu* (Lyon and Bechtol 1977; Himmelbauer et al. 1994). Heterozygous inheritance of this deletion, which by definition represents a loss-of-function allele, results in no dominant phenotypic defects. Additional evidence that the dominant tail phenotype of the *Fu* sub* sub mutation cannot be attributed to a loss-of-function of the normal allele was provided by the observation that *Fu* sub* /++ tertiary trisomic mice maintained the kinky tail phenotype (Ruvinsky et al. 1991). More likely, the mutant *Fu* alleles induce the dominant developmental defects as a result of inappropriate patterns of expression during embryogenesis or by producing proteins whose function is modified by the mutation.

In contrast, the *He* 46 allele genetically resembles the *Fu* sub* sub deletion in that both are recessive embryonic lethals and neither has any dominant effects (Lyon and Bechtol 1977). While the effects of the *He* 46 insertion/deletion on the structure and expression of the *Fu* gene remain to be defined, the physical nature of this genetic lesion (and the identification of a transcription unit disrupted by the insertion/deletion) appears consistent with the hypothesis that *He* 46 represents a loss-of-function allele of *Fu*. The phenotypic similarities between *He* 46 and *Fu* sub* sub homozygous embryos, and the noncomplementation of *He* 46 and *Fu* sub* sub, suggest that the *Fu* sub* sub mutation interferes with the normal embryonic function of the gene, causing the recessive lethal effects, while at the same time creating a new and inappropriate function, leading to the dominant effects in *Fu* sub* heterozygotes. The viability of *Fu/Fu* animals, on the other hand, suggests that the normal function of *Fu* is not disrupted in this allele. Indeed, animals that are *Fu/+* are viable, indicating that one copy of the *Fu* allele can provide sufficient gene product for normal gene function during embryogenesis.

This work provides another example of the ability of random insertional mutations in the mouse to provide molecular access to developmentally important genetic loci (Gridley 1991). Analysis of the normal developmental functions of the *Fused* gene should be very interesting, given the pleiotropic effects of mutant alleles. The duplications of axial and other embryonic structures observed in embryos homozygous for several of the alleles suggest that the normal gene may play a role in primary embryonic induction. Studies of chimeric mice produced using embryonic stem cells homozygous for the *He* 46 allele suggest that the normal gene also plays an important role in craniofacial development (unpublished data), which is consistent with the cranial malformation seen in the most advanced *He* 46 homozygous embryos. The mechanisms of parental imprinting and heritable inactivation of the spontaneous *Fu* alleles should also be amenable to molecular analysis once the gene is identified. The studies reported here have led to the detection of a 3.9-kb transcript encoded at the *He* 46 locus, representing a strong candidate for the wild-type *Fused* gene. Characterization of this gene and its expression patterns in *He* 46, *Fu, Fu* sub* and *Fu* sub* mice should provide further insight into the molecular basis of the developmental defects associated with these alleles.

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