A murine homolog of the yeast RNA1 gene is required for postimplantation development

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A gene has been characterized that is required for postimplantation mouse development. The gene, designated *fug1*, was disrupted in embryonic stem cells by the U3Neo gene trap retrovirus, and the disrupted allele was introduced into the germ line. Homozygous mutant embryos arrest at the egg cylinder stage at about embryonic day 6 and are mostly resorbed by day 8.5. The appearance of the proamniotic cavity is delayed, and epiblast cells that surround the cavity are disorganized. *fug1* transcripts are undetectable at E6 but are induced throughout the embryo after E6.5. The gene is expressed at low levels in all adult tissues examined, maps to chromosome 15, and is conserved among mammals. The cDNA sequence encodes a protein of 589 amino acids, the first 400 of which are 38% identical to the *Saccharomyces cerevisiae* RNA1 gene. Regions of greatest similarity include a long acidic domain and 11 leucine-rich motifs, thought to mediate high affinity protein-protein interactions. These similarities suggest that Fug1 may be required for developmental changes in RNA processing or chromatin structure prior to gastrulation.

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Genes linked to recessive mutations that affect mammalian development have been difficult to isolate. Genetic screens are expensive and yield relatively few mutants. Recessive phenotypes can be identified only after backcrossing mutagenized animals, and developmental phenotypes, particularly those that result in early embryonic death, are usually apparent only after sacrificing animals during pregnancy. Consequently, most of the ~1300 genetic loci that have been described in mice (Green 1989) are associated with easily identified phenotypes affecting postnatal life. Even fewer genes that affect early embryonic development have been cloned (Gridley et al. 1990; Herrmann et al. 1990; Lee et al. 1992; Michaud et al. 1993; Zhou et al. 1993).

To circumvent some of the problems associated with conventional mouse genetics, gene trap vectors have been developed to disrupt genes expressed in embryonic stem (ES) cells (Gossler et al. 1989; Friedrich and Soriano 1991; von Melchner et al. 1992). The disrupted genes are introduced into the germ line and mice are bred to ho-

mozygosity to assess gene functions. For example, we have developed retrovirus vectors that contain coding sequences for a selectable marker inserted into the U3 region of a defective Moloney murine leukemia virus (MLV) (von Melchner and Ruley 1989; Reddy et al. 1991; Chang et al. 1993). Provirus integration positions the U3 gene only 30 nucleotides from the flanking cellular DNA and selection for U3 gene expression generates clones in which the virus has integrated in or near 5' exons of expressed cellular genes.

Several features of gene trap mutagenesis are particularly useful with regard to analyzing gene functions in mice. First, the mutation frequency following gene trap selection approaches 100%. Thus, the transcribed sequences disrupted in cells recovered after gene trap selection have properties of genes transcribed by RNA polymerase II. They encode spliced transcripts, typically hybridize to single copy cellular DNA, and are expressed in cells prior to selection (Friedrich and Soriano 1991; Skarnes et al. 1992; von Melchner et al. 1992). Moreover, several integration sites have disrupted previously characterized genes (von Melchner et al. 1992; Pruitt 1992). This greatly reduces the number of animals needed to generate mutants, and the affected genes can be charac-

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terized even when the mutations cause no obvious phenotypes. Second, mutations in genes required for embryonic development are readily identified by the failure to recover offspring that are homozygous for the provirus insert. This has been observed with >40% of the disrupted genes analyzed (Friedrich and Soriano 1991; Skarnes et al. 1992; von Melchner et al. 1992). Third, evidence from our laboratory suggests that most genes, including weakly expressed genes, can be targeted by gene trap mutagenesis (Chang et al. 1993). Finally, collections of ES cell clones can be screened in vitro for interesting mutations before being introduced into the germ line. For example, it is possible to screen for mutations involving developmentally regulated genes or genes with known sequence (Gossler et al. 1989; Reddy et al. 1992; von Melchner et al. 1992).

In this paper we have characterized a gene whose disruption by the U3Neo gene trap vector generates an embryonic lethal mutation. Mice homozygous for the disrupted allele arrest at the time in development at which the gene is normally induced (ca. embryonic day 6.5). The gene, designated *fug1* (failure to undergo gastrulation) is similar to the *RNA1* genes of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*.

Results

Mice homozygous for the 1B6 provirus die in utero

The 1B6-neo ES cell clone was isolated by infecting D3 ES cells with the U3Neo gene trap retrovirus and selecting in G418, as described previously (von Melchner et al. 1992). Ribonuclease protection and Northern blot analysis indicated that the U3Neo gene was expressed from a promoter in the flanking cellular DNA. Sequences 5' of the site of the proviral integration (isolated by inverse PCR) hybridized to single-copy cellular DNA and provided a probe that distinguished between the disrupted and normal alleles (not shown). While heterozygous mice carrying the 1B6 provirus appeared normal, none of the 34 offspring initially derived from crossing 1B6 heterozygotes was homozygous for the integrated provirus (von Melchner et al. 1992). This suggested that the gene disrupted in 1B6 mice was required for embryonic development.

Heterozygous mice derived after four or more backcrosses into a C57Bl/6 background were subsequently mated, but none of the 53 progeny was homozygous for the mutant allele (data not shown). Embryos were subsequently dissected at 13, 11, and 8.5 days postcoitum and genotyped by Southern blotting. Of the 55 conceptuses, 14 were resorbed, and no homozygous mutants

were observed among the remaining 41 embryos (not shown). This suggests that mice homozygous for the 1B6 provirus are lost in utero before day 8.5 but after implantation. In all, 84 heterozygotes and 44 wild-type homozygotes have been obtained from crosses between 1B6 heterozygotes, similar to the ratio expected for a recessive lethal mutation. Further analysis suggested that homozygous embryos arrested in development prior to gastrulation (see below); consequently, the disrupted gene was designated *fug1*.

Analysis of the gene disrupted by the 1B6 provirus

Sequences upstream of U3 gene trap proviruses cloned by inverse PCR frequently hybridize to transcripts expressed by the disrupted cellular genes. However, 5' sequences flanking the 1B6 integration site failed to hybridize to cellular transcripts (von Melchner et al. 1992). We therefore isolated sequences downstream of the virus by anchored PCR (Frohman et al. 1988). The cloned PCR product (designated 1B6-13) contained genomic sequences 3' to the provirus and were used to isolate cDNA clones of the *fug1* gene. Comparisons between the *fug1* cDNA and genomic sequences revealed that integration occurred in a 48-nucleotide exon (Fig. 1) only 16 nucleotides downstream from an intron/exon boundary. This accounts for the failure of the 5'-flanking sequence to detect cellular transcripts by Northern blot hybridization. *fug1* transcripts are differentially spliced, as only one of three cDNAs spanning the integration site contained the 48-nucleotide exon. By Southern analysis, the 1B6 provirus appears to have integrated normally, without causing obvious rearrangements, duplications, or deletions of host DNA (data not shown).

Twelve independent cDNAs derived from transcripts of the disrupted gene have been characterized. The 1B6-6 cDNA was isolated from a PCC3 embryonic carcinoma cell cDNA library (Stratagene) by using the 1B6-13 probe. The 1B6-6 cDNA was then used to isolate four other cDNAs from the same library. Finally, an almost full-length cDNA (1B6-1) was used to probe an 8.5-day mouse embryonic cDNA library, resulting in 69 positive plaques (of 1.2×10^6 plaques), of which 7 were characterized. As estimated by their relative abundance in cDNA libraries, *fug1* transcripts comprise ~0.006% of the total mRNA in 8.5 day embryos.

Although the *fug1* cDNA sequence (Fig. 2) was similar in size to the transcript (3 kb) as measured on Northern blots (see below), primer extension analysis using D3 ES cellular RNA revealed that native transcripts extended an additional 101 nucleotides upstream of the 5' end of

Figure 1. Integration site of the 1B6 provirus. Genomic sequences surrounding the 1B6 provirus were used to isolate cDNAs of the disrupted gene. Comparison of the cDNA and genomic sequences reveals a 48-nucleotide exon into which the provirus inserted. The site of provirus integration and splice acceptor and donor sites are indicated.

at the 3' end of the 1B6-14b clone, and a B1 repeat is present in the 3'-untranslated region. The cDNA sequence has been confirmed in at least two independent clones except for 92 nucleotides at the 3' end.

The 1B6 cDNA sequence contains an open reading frame (ORF) of 589 amino acids and includes a highly acidic region composed of 27 glutamic acids and 11 aspartic

Figure 2. Sequence of *fug1* cDNA. The nucleotide sequence and deduced amino acid sequence for the longest open reading frame (ORF) are shown. The integration site for the 1B6 provirus is shown (*). An alternatively spliced 48-nucleotide exon and polyadenylation signal are underlined. A B1 repeat encompasses nucleotides 2490–2637.

acids, interrupted by only 2 glycines and 1 glutamine (Fig. 2). Analysis of the predicted translation product by the MacVector (IBI) and Prosite (Intelligenetics) programs did not reveal known protein sequence domains indicative of possible function. However, the FastDB (Intelligenetics) program revealed a striking similarity between the 1B6 ORF and the RNA1 protein of *S. cerevisiae*. The aligned protein sequences show 38% amino acid identity and 47% similarity over the entire length (407 amino acids) of RNA1. Subsequently, the sequence of the *S. pombe* RNA1 gene was reported (Melchior et al. 1993). As shown in Figure 3, Fug1 is nearly as similar to the two yeast proteins as they are to each another.

RNA1 is a member of a larger group of proteins that possess leucine-rich motifs (LRMs) (Schneider and Schweiger 1992; Melchior et al. 1993). LRMs are thought to mediate protein-protein interactions, such as the tight binding of the ribonuclease/angiogenin inhibitor to angiogenin. The 11 LRM repeats and a long acidic domain comprise the regions of greatest similarity (Fig. 3). Two amino acids mutated in the temperature-sensitive lethal *rna1-1* allele and required for the mutant phenotype (Traglia et al. 1989) are also present in Fug1. However, the carboxy-terminal regions are not conserved, as Fug1 is 182 amino acids longer than RNA1 of *S. cerevisiae*. Whereas the significance of the extra sequence is unknown, the carboxy-terminal region of RNA1 beyond the acidic domain can be deleted without affecting function (Traglia et al. 1989).

The Fug1 gene is activated prior to gastrulation and is widely expressed in adult tissues

The expression of *fug1* was assessed by Northern blot analysis of total cellular RNAs using the 1B6-1 cDNA as a probe. All adult tissues examined (Fig. 4), as well as several established fibroblast cell lines (data not shown),

expressed similar levels of a 3-kb transcript. The autoradiograms of the same Northern blot, probed with either 1B6-1 or glyceraldehyde 3-phosphate dehydrogenase (GAP), indicated that the ratio of *fug1* to GAP transcripts in the uninfected D3 cells was more than twofold greater than in 1B6 cells (Fig. 4). This would be expected if proviral integration greatly reduced the expression from the affected allele.

Whole-mount in situ hybridization was performed on normal mouse embryos between 6 and 9.5 days of embryonic development (Fig. 5; data not shown). Embryos were dissected out of the yolk sac, and the ectoplacental cone was removed. The embryos were also sectioned to provide a more detailed analysis of expression (Fig. 6). *fug1* was ubiquitously expressed in 8.5- and 9.5-day embryos as observed both in whole embryos and in sections (data not shown). In sections of 7.5-day embryos (Fig. 6), modest expression was seen throughout the embryo, in both embryonic and extraembryonic tissues. However, expression was highest in the embryonic ectoderm (Fig. 6C) and lowest in the most posterior part of the primitive streak. This is the part of the streak that contributes to the extraembryonic mesoderm (Lawson and Pederson 1992). In short, highest levels were observed in the cells that form the embryo proper. Ectoderm was also preferentially stained with probes for the constitutive GAP gene (Fig. 6E), which may reflect the higher cell density. However, unlike *fug1*, GAP expression was always uniform within the ectoderm. *fug1* expression was reproducibly low (in three experiments) in 6.5-day embryos (Fig. 5; data not shown), even after 24 hr of alkaline phosphatase reaction. Some 6.5-day embryos exhibited faint staining above background while others were indistinguishable from controls. Importantly, 6-day embryos show no staining (Fig. 5). These results suggest that *fug1* expression is regulated during embryogenesis, with levels increasing dramatically just prior to gastrulation.

Figure 4. Northern analysis of *fug1* transcripts from different tissues. Unselected (20 μ g) RNA from the indicated mouse tissue or ES cell line was first hybridized to a 32 P-labeled 1B6-1 cDNA probe and then stripped and hybridized to a GAP probe, as indicated. The migration of the 28S and 18S RNAs are indicated. The 1B6-1 and GAP blots were exposed for 4 days and 8 hr with enhancing screens, respectively.

Effects of virus integration on *fug1* gene expression

The 48-nucleotide exon into which the 1B6 provirus integrated is alternatively spliced, as it was present in only one of three cDNAs. To measure the relative abundance of each transcript, primers on either side of the exon were used to amplify reverse transcription products obtained with RNA from different tissues and cell lines. In all tissues examined, transcripts containing (*fug1* B) and lacking (*fug1* A) the 48-nucleotide exon were present at a ratio of $\sim 1:3$ (data not shown). The *fug1* B transcript was not detected in two rat cell lines (RAT1 and REF52), while it was present in two mouse cell lines (10T1/2 and D3) (data not shown). Together, these results suggest that alternative splicing has little significance with regard to gene regulation.

It was important to understand the extent to which the 1B6 provirus interfered with *fug1* expression. While *fug1*-U3Neo fusion transcripts terminate almost exclusively in the 5' long terminal repeat (LTR) (von Melchner et al. 1992), it was possible that some *fug1* A transcripts could be expressed by splicing around the provirus. We tested whether *fug1* sequences downstream of the provirus were expressed in presumptive 1B6 homozygotes by in situ hybridization. If expression of sequences 3' of the integration site was abolished, then homozygous embryos should not hybridize to the 1B6-1 cDNA probe. As summarized in Table 1 and illustrated in Figure 5, approximately one-fourth of the 7.5- and 8.5-day embryos derived from inbreeding 1B6 mice were negative for *fug1* expression. These embryos were smaller and less developed than normal and thus appeared to be homozygous mutants. These results suggest that the 1B6 provirus induced a null mutation. However, as *fug1* transcripts are undetectable before E6.5 (Fig. 5), the lack of *fug1* expression could result from failure of homozygous embryos to develop beyond the point at which the gene is expressed.

Phenotype of embryos homozygous for the 1B6 provirus

Embryos presumably homozygous for the 1B6 provirus were identified based on the occurrence of abnormal embryos ($\sim$ one-fourth of all embryos) in crosses between 1B6 heterozygotes but not in crosses in which one or both of the parents were wild type (Table 1). The failure of these abnormal embryos to express *fug1* suggested further that they represent 1B6 homozygotes. At E7.5 these embryos were one-fourth to one-third normal size (Figs. 5 and 7) and lacked a discernible exocoelom and ectoplacental cavity. Additionally, the ectoplacental cone did not appear to be vascularized as it is in normal embryos (data not shown). The absence of vascularization could be due to a halt in development of the embryo prior to the initiation of the uterine reaction. Alternatively, it could be a consequence of the rejection of the embryo by the uterus. Abnormal embryos were also seen at E6.5. These embryos were half the size of their normal siblings and were similar in appearance to E7.5 mutants, albeit 25–50% smaller (Fig. 7). Only two abnormal embryos were observed at E8.5. One was recovered (Table 1) and appeared similar in size to the E7.5 mutants (data not shown). In summary, homozygous mutants arrest in development around E6, continue to grow until E7–E8.5, and most are resorbed by E8.5 (Table 1).

Sections of mutant embryos at E6.5, E7.5, and E8.5 revealed two embryonic cell layers, presumably representing the primitive endoderm and ectoderm or epiblast (Fig. 7; data not shown). The mutant embryos contained what appeared to be a small proamniotic cavity that was sometimes observable at E7.5 (Fig. 7) and was relatively large in one mutant at E8.5 (data not shown). However, while the primitive ectoderm forms an organized columnar epithelium surrounding the cavity in normal embryos, there was little or no organization of the ectoderm in mutant embryos (Fig. 7). The embryonic cells also appeared less cohesive, as compared with the tight epithelium of normal embryos. The mutants lacked an obvious mesoderm layer, even at E8.5. However, mesoderm cells could be present, but disorganized. In summary, *fug1* appears to be required for the organization of the primitive ectoderm around the proamniotic cavity.

fug1 is conserved among mammals

The phylogenetic conservation of *fug1* was assessed by probing DNAs from different species ("zoo blot") with the 1B6-1 cDNA. As shown in Figure 8, hybridization was only observed in mammals, suggesting that *fug1* sequences are not highly conserved. Reducing the stringency of hybridization did not reveal any cross-reactive bands in zebrafish, *Drosophila*, or any additional bands in yeast DNA (data not shown). Finally, *fug1* appears to be present as a single-copy gene in mice, as only a single hybridizing band was detected by the 1B6-1 probe following digestion with either *Sall* or *SmaI* (data not shown).

Figure 5. *fug1* is expressed in 7.5-day embryos but not in 6-day or mutant embryos. (A) 7.25-Day embryos derived from inbreeding 1B6 heterozygotes. (M) Presumptive mutant embryos; (W) normal embryos. All were hybridized in situ with an antisense 1B6-1 probe. Color reactions proceeded overnight. (B) 7.5-Day embryos hybridized either with the 1B6-1 sense (S) or antisense (AS) probes and reactions were developed for 4 hr. 6.5-Day (C,E) or 6-day embryos (D) hybridized with a 1B6-1 antisense (C,D) or sense (E) probe. For C–E, color reaction proceeded overnight. The embryos in C, D, and E were enlarged threefold relative to the embryos in A and B.

Fug1 maps to chromosome 15

The mouse chromosomal location of *Fug1* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J) × *Mus spretus*] *F1* × C57BL/6J mice. This interspecific backcross mapping panel has been typed for >1300 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins 1991). C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a mouse cDNA *fug1* probe. A 6.8-kb *M. spretus* *HincII* RFLP (see Materials and methods) was used to follow the segregation of the *fug1* locus in backcross mice. The mapping results indicated that *Fug1* is located in the distal region of mouse chromosome 15 linked to thyroglobulin (*Tgn*), simian sarcoma oncogene (*Sis*) and glycerol phosphate dehydrogenase 1 (*Gdcl*). Although 144 mice were analyzed for every marker, up to 171 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are centromere– *Tgn*–12/147–*Fug1*–0/157–*Sis*–23/171–*Gdcl*. The recombination frequencies [expressed as genetic distances in centiMorgans (cM) ± the standard error] are– *TgM*–8.2 ± 2.3 [*Fug1*, *Sis*] 13.5 ± 2.6–*Gdcl*. No recombinants were detected between *Fug1* and *Sis* in 157 animals typed in common suggesting that the two loci

are within 1.9 cM of each other (upper 95% confidence limit).

We have compared our interspecific map of chromosome 15 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (compiled by M.T. Davisson, T.H. Roderick, A.L. Hillyard, and D.P. Doolittle and provided from GBASE, a computerized data base maintained at The Jackson Laboratory, Bar Harbor, ME). *fug1* mapped in a region of the composite map that contains one mouse mutation, blind (*Bld*) (Watson 1968). Heterozygotes for *Bld* are blind, and homozygotes die by day 8 of embryonic development (Vankin and Caspari 1979). Presumptive *Bld* homozygotes lose contact with uterine tissue and are retarded in growth as early as E6. By E7.5, the mutants formed amniotic folds and limited mesoderm, which we have not observed in *fug1* homozygotes. Due to a similar map position and similar timing of embryonic death, *Bld* may be an allele of *fug1*. Unfortunately, we have been unable to test this possibility because *Bld* mice are no longer available (M. Watson, pers. comm.).

Discussion

Gene trap mutagenesis provides an effective means to study gene functions in mice. In the present study, we have characterized a gene that was disrupted in ES cells by the U3Neo gene trap retrovirus and subsequently introduced into the germ line. The disrupted gene, designated *fug1*, appears to be a mammalian homolog of the yeast *RNA1* gene. Homozygous mutants arrest at the egg

Figure 6. *fug1* expression in gastrulation stage embryos. Embryos (around E7.5) were hybridized in situ with either with a 1B6-1 anti-sense (A, C, D) or sense (B) probe or an antisense GAP probe (E) and then sectioned. Color reactions went for 4 hr (E), 6 hr (A, C, D), and overnight (B). (ec) Ectoplacental cavity; (ex) exocoelom; (ac) amniotic cavity; (al) allantois; (ect) ectoderm; (mes) mesoderm; (ve) visceral endoderm.

cylinder stage of embryonic development, just prior to the time at which the gene is normally induced. Following gastrulation, *fug1* is expressed in all embryonic and adult tissues examined.

Activation of U3Neo gene expression

Earlier Northern blot and ribonuclease protection experiments indicated that gene trap selection generates proviruses that have inserted in or near 5' exons of expressed cellular genes (von Melchner et al. 1992). U3 fusion transcripts were expressed from flanking cellular promoters and contained an average of 2–300 nucle-

otides of cellular RNA. In the present study, we show that this mechanism was responsible for the activation of U3 gene expression in the 1B6 clone. U3Neo transcripts were expressed as a result of integration into a 5' exon of the *fug1* gene and contained ~225 nucleotides of appended cellular RNA. As has been observed previously (von Melchner and Ruley 1989; von Melchner et al. 1992), the target gene was expressed in ES cells prior to virus integration and was not activated as a result of integration. Integration occurred only 16 nucleotides from an intron/exon junction, accounting for the failure of 5'-flanking sequences to hybridize to cellular transcripts (von Melchner et al. 1992). Finally, *fug1* tran-

Table 1. Developmental arrest and lack of target gene expression in embryos homozygous for the 1B6 integration

Cross	Age	Normal	Abnormal	Resorbed
Het × Het	E8	34	2 (0/1)	15
Het × Het	E7	90 (21/21)	17 (0/5)	11
Het × Het	E6	32	13	0
Het × WT	E8	7	0	0
Het × WT	E7	19	0	0
Het × WT	E6	9	0	0
WT × WT	E8	62	0	3
WT × WT	E7	110 (36/36)	0	5
WT × WT	E6	32	0	2

Summarized are the phenotypes of embryos recovered from crossing mice heterozygous for the 1B6 provirus after 6, 7, or 8 days p.c. Embryos designated E8, E7, and E6 represent embryos staged between 6–6.9, 7–7.9, and 8–8.5 days of embryonic development (Theiler 1989). Embryos were isolated by dissection and classified as normal or abnormal. Abnormal embryos were approximately one-third normal size. Numbers in parentheses indicate the number of stained and unstained embryos as determined by in situ hybridization, probing with cDNA sequences 3' of the site of provirus integration (1B6-1).

scripts are not highly expressed (<0.01% total mRNA), as estimated either by Northern blot hybridization or by their representation in cDNA libraries. Thus, even weak promoters are capable of activating U3 gene expression, as suggested previously (Chang et al. 1993).

*Recessive lethal phenotype resulting from loss of *fug1* expression*

Several observations suggest that insertional disruption of the *fug1* gene is responsible for the recessive lethal phenotype observed in 1B6 homozygotes. First, the 1B6 provirus was backcrossed into C57Bl/6 mice for five generations and was invariably linked to embryonic death among the 128 inbred progeny genotyped. Consequently, any second site mutation causing this phenotype would have to be closely linked to the provirus. Second, the provirus appears to abolish *fug1* gene expression, as fusion transcripts terminate in the 5' LTR, and we were unable to detect *fug1* transcripts 3' of the provirus in homozygous mutant embryos. Third, developmental arrest occurs around the time at which the *fug1* gene is normally induced, around E6.5. Finally, embryonic death was probably not caused by expression of the *neo* transgene, as high levels of neomycin phosphotransferase have been constitutively expressed throughout development without detrimental effects (Friedrich and Soriano 1991; M. Roshon and H.E. Ruley, unpubl.).

**fug1* is required for postimplantation development*

Little is known about the genes required for the differentiation of the primitive ectoderm and endoderm, the formation of the proamniotic cavity, and the organization of the ectoderm into an organized epithelium. Al-

though a number of mutations have been described that result in embryonic death at or before gastrulation (Green 1989), only a few of the responsible genes have been cloned (Herrmann et al. 1990; Lee et al. 1992; Zhou et al. 1993).

fug1 is required for the organized development of the egg cylinder stage embryo and subsequent gastrulation. Embryos homozygous for the disrupted *fug1* allele form a proamniotic cavity, but only after a considerable delay. Ectodermal cells surrounding the cavity are disorganized, even in mutants found at E8.5. The ectodermal cells may be impaired in their ability to organize into an epithelium; alternatively, cell-cell contacts may form but are not maintained.

Several observations suggest that *fug1* is not simply required for cell viability. Defects are not observed until E6, after maternal RNAs are typically degraded (Sawicki et al. 1981) and after embryos require at least some de novo gene expression (Johnson 1981). Moreover, the mutant embryos continue to grow for several days after abnormalities are first observed, and the cells remain intact as judged by microscopic examination of tissue sections. Nevertheless, we cannot exclude the possibility that maternal or early embryonic products can maintain viability until E8.5.

**Fug1* is similar to the yeast *RNA1* protein*

Fug1 is related to the *RNA1* proteins of *S. cerevisiae* and *S. pombe*. The regions of greatest similarity include a large acidic domain and 11 LRMs, thought to mediate high affinity protein-protein interactions (Schneider and Schweiger 1992). *RNA1* is essential for yeast cell viability. Both loss-of-function and temperature-conditional mutations cause pleiotropic defects in the synthesis, processing, and nuclear transport of precursors for all three classes of RNA (Hopper et al. 1978; Atkinson et al. 1985; Forrester et al. 1992). Remarkably, *RNA1* protein appears to be localized to the cytoplasm (Hopper et al. 1990), and like *RNA1*, *Fug1* possesses no obvious nuclear localization signal.

Some of the effects of *RNA1* on RNA metabolism are nearly identical to those caused by *PRP20* (Forrester et al. 1992), suggesting that the two genes may function in the same pathway. Consistent with this notion, *SRN1*, a negative regulator of carbon catabolite repression, suppresses both *RNA1* and some *PRP* mutations (Pearson et al. 1982; Tung et al. 1992). Because *PRP20* encodes a nuclear protein (Fleischmann et al. 1991), it may be a target of a cytoplasmic activity controlled by *RNA1*.

RNA1 appears to affect RNA metabolism indirectly, as it is excluded from the nucleus and (like *fug1*) is expressed at low levels (Traglia et al. 1989). Moreover, splicing defects seen in vivo are not observed with mutant cell extracts in vitro (Atkinson et al. 1985). How does *RNA1* exert such pleiotropic effects on RNA metabolism? One possibility is that *RNA1* may influence chromatin structure, as has been suggested of *PRP20* (Forrester et al. 1992).

At this point we do not know whether biochemical

Figure 7. Sections of normal and abnormal 6.5- and 7.5-day embryos. Embryos were derived from inbreeding 1B6 heterozygotes and serially sectioned. Representative sections of normal 6.5- and 7.5-day embryos are shown in A and C, respectively, and representative sections of mutant 6.5- and 7.5-day siblings are shown in B and D, respectively. Photomicrograph of mutant embryo in D is enlarged three times that of the normal littermate (C). (epc) Ectoplacental cone; (pa) proamniotic cavity; (ect) primitive ectoderm; (ve) visceral endoderm; (rm) Reichert's membrane; (ec) ectoplacental cavity; (ex) exocoelom; (ac) amniotic cavity; (mes) mesoderm; (ect) ectoderm; (ve) visceral endoderm.

functions of RNA1 are conserved by Fug1, as similar proteins (e.g., Ras) may function quite differently in mammalian cells and yeast (Lowy and Willumsen 1993). The additional 182-amino-acid residues at the carboxyl terminus of Fug1 may also indicate functional differences. Future studies will therefore address a number of questions, including the following: (1) What are the biochemical functions of RNA1 and Fug1, and are these conserved? (2) Can protein partners thought to interact

with LRMs of Fug1 and RNA1 be identified and are they related? (3) How does the activation of *fug1* expression between E6.5 and E7 influence development? Specifically, is *fug1* required for widespread changes in chromatin structure and gene expression (Cedar and Razin 1990; Kafri et al. 1992) that occur during this period? The identification of a mammalian RNA1 homolog and its insertional disruption in mice will assist efforts to answer these questions.

Figure 8. *fugu* is conserved in mammals. Southern analysis of DNA from different organisms: 10 μ g, unless otherwise indicated, of either the indicated organism or cell line was digested with *Eco*RI and hybridized to 32 P-labeled probes derived from the entire 1B6-1 cDNA sequence. *Drosophila* DNA is from *Drosophila melanogaster*, Fish is from zebrafish, and yeast is from *S. cerevisiae*. The CV-1 cell line was used for monkey DNA, CCL64 for mink, CHTG49 for hamster, and D3 for mouse. Mink DNA (2 μ g) was underloaded relative to other mammalian DNAs. The blot was hybridized at 42°C overnight as described in Materials and methods and washed two times each in 2 $\times$, 1 $\times$, and 0.2 $\times$ SSC for 20 min each at 68°C. Size markers, in kb, are indicated.

Materials and methods

Nucleic acid blot hybridization

DNA and RNA were isolated from cultured cells or tail biopsies as described previously (Laird et al. 1991; Titus 1991). Southern and Northern blots (Sambrook et al. 1989) were transferred to Hybond N+ nylon membranes (Amersham) and hybridized to [32 P]dCTP-labeled probes (Feinberg and Volgelstein 1984).

Primer extension, RT-PCR, and 5' RACE

Primer extension analysis of *fugu* transcripts was performed as described (Sambrook et al. 1989), using 10^6 cpm of 5' end-labeled primer, annealing overnight at 30.5°C.

RT-PCR was performed as described (Innis et al. 1990), using primers EX-1: 5'-ACGCGGACTCTCAAGCAGTGA-3' and EX-2: 5'-TTGCCCTCCAATCGTAGAGCTT-3' as flanking primers.

5' RACE (Frohman et al. 1988) was performed using three nested primers complementary to the U3Neo-coding strand and an anchor/adaptor primer combination. Specific primers were as follows: NeoA, 5'-attgtctgtgtgccagtcata; NeoB, 5'-CGAAT-

AGCCTCTCCACCCAA; NeoC, 5'-CCATCTTGTTCATCA-TGCCAAACGATCC; anchor, 5'-ATTGAATTCTCTAGAAC-GCGTCTCGAG(T)₂₀i and adaptor, 5'-ATTGAATTCTCTA-GAACGCGTCTCGAG. Total cellular RNA (10 μ g) was reverse transcribed for 90 min at 45°C in 25 μ l of reaction mix containing 1 $\times$ PCR buffer (Perkin Elmer), 200 μ M DNTPs (Perkin-Elmer), 8 mM dithiothreitol, 100 nM NeoA primer, 30 units of RNasin (Promega) and 200 units of superscript reverse transcriptase (GIBCO-BRL). DNA/RNA hybrids were treated (30 min at 37°C) with RNase A (10 μ g, Boehringer/Mannheim) and RNase H (2.5 U, GIBCO-BRL), and excess primer was removed by centrifugation through a Qiagen spin column according to the manufacturer's instructions. Poly(A)⁺ tracts were added to the first-strand products by using terminal transferase in a 20- μ l reaction (15 min, 37°C) containing 1 $\times$ terminal transferase buffer (GIBCO-BRL), 160 μ M dATP and 15 units of terminal transferase (GIBCO-BRL). The tailed first strand product (15 μ l) was subjected to 10 cycles of PCR (1 min at 90°C; 2 min at 55°C; 3 min at 72°C) in 100 μ l of 1 $\times$ PCR buffer (Perkin-Elmer) and 0.5 units of *Taq* polymerase (Perkin-Elmer). Aliquots (10 μ l) of the first PCR product were amplified for 45 cycles using 1 mM Neo C primer, and 1 mM adaptor primer as before.

DNA sequencing

Plasmid DNA (5 μ g) was used in each sequencing reaction as described (Hsiao 1991), with the following modifications: (1) a 1:8 dilution of the G-mix was used; (2) termination reaction used 1.0 μ l of termination and 1.5 μ l of extension mix; (3) labeling was for 4 min at room temperature; and (4) termination was for 5 min at 37°C. All reactions, after labeling, were incubated in 96-well plates (conical bottom), and were initiated and terminated by centrifugation. Initial sequences were obtained by using T3 and T7 primers and later extended by using custom 17- to 18-nucleotide primers (Oligos, Etc.).

Two microliters of each sequencing reaction was loaded onto a Betagen AutoTrans apparatus. The gels [6% (19:1 acryl./bis) Sequagel (EC-833 from National Diagnostics)], were prerun at 825 constant volts for 30–60 min. Free nucleotides were electrophoresed into a small strip of nylon membrane, and the membrane was changed. Gels were run at 825 constant volts and transferred onto 60 cm of membrane at a 1.5 web speed and a 450-min web time.

Whole-mount in situ hybridization

Detailed methods for whole-mount in situ hybridization were based on procedures provided by Jill McMahon (Roche Institute, Nutley) and have been described elsewhere (Sasaki and Hogan 1993). Fixed and detergent-permeabilized embryos were hybridized to single-stranded RNA probes synthesized in vitro using UTP labeled with the plant steroid digoxigenin (DIG-11-UTP). The DIG-labeled RNA was localized using a conjugate of anti-digoxigenin Fab antibody and calf intestinal alkaline phosphatase. The enzyme activity of the reporter was detected by a color reaction with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT), resulting in the formation of a water-insoluble purple precipitate. The yield of labeled probe was assessed before use by gel electrophoresis and by testing dilutions spotted onto a nylon filter for color reaction. Embryos were timed from the day of plugging (noon = E0.5).

Interspecific backcross mapping

Interspecific backcross progeny were generated by mating (C57BL/6J $\times$ M. spretus)F₁ females and C57BL/6J males as de-

scribed (Copeland and Jenkins 1991). A total of 205 N₂ mice were used to map the *fug1* locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described (Jenkins et al. 1982). All blots were prepared with Zetabind nylon membrane (AMF-Cuno). The probe (310-bp 1B6-6 cDNA) was labeled with [α^{32}]dCTP using a nick translation labeling kit (Boehringer Mannheim); washing was done to a final stringency of $2.0\times$ SSCP, 0.1% SDS at 65°C. A major fragment of 7.5 kb was detected in *HincII*-digested C57BL/6J DNA, and a major fragment of 6.8 kb was detected in *HincII*-digested *M. spretus* DNA. The presence or absence of the 6.8-kb *M. spretus*-specific *HincII* fragment was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to *Fug1*, including *Tgn*, *Sis*, and *Gcdl*, has been reported previously (Brannan et al. 1992). Recombination distances were calculated as described (Green 1981) using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombinational events required to explain the allele distribution patterns.

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