

Overlapping roles and asymmetrical cross-regulation of the USF proteins in mice

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ABSTRACT USF1 and USF2 are ubiquitously expressed transcription factors implicated as antagonists of the c-Myc protooncprotein in the control of cellular proliferation. To determine the biological role of the USF proteins, mutant mice were generated by homologous recombination in embryonic stem cells. USF1-null mice were viable and fertile, with only slight behavioral abnormalities. However, these mice contained elevated levels of USF2, which may compensate for the absence of USF1. In contrast, USF2-null mice contained reduced levels of USF1 and displayed an obvious growth defect: they were 20–40% smaller at birth than their wild-type or heterozygous littermates and maintained a smaller size with proportionate features throughout postnatal development. Some of the USF-deficient mice, especially among the females, were prone to spontaneous epileptic seizures, suggesting that USF is important in normal brain function. Among the double mutants, an embryonic lethal phenotype was observed for mice that were homozygous for the *Usf2* mutation and either heterozygous or homozygous for the *Usf1* mutation, demonstrating that the USF proteins are essential in embryonic development.

USF was initially characterized as a transcription factor implicated in the regulation of the adenovirus major late promoter (1–4). In mammals, two different genes, *Usf1* and *Usf2*, are ubiquitously expressed (5, 6). The USF1 and USF2 polypeptides assemble in both homo- and heterodimers and interact specifically at DNA sites containing a CACGTG or CACATG core sequence (7, 8). Many of these USF binding sites are also potential targets for regulation by other transcription factors, including the various members of the Myc (9, 10) and TFE3 (11–13) families, which greatly complicates the identification of genes that are under USF control. Besides a common DNA-binding specificity, the Myc-related proteins also share with USF a strong structural similarity in their dimerization and DNA-binding domains, which contain a highly conserved basic helix–loop–helix leucine-zipper motif (14–16).

The relationship and extent of overlap between the functions of the *Usf1* and *Usf2* genes have been difficult to ascertain. The USF1 and USF2 polypeptides display a very high degree of homology in their C-terminal DNA-binding domains, with >70% identical residues (6). They also share a small but extremely conserved domain called USF-specific region, located just upstream of the basic region, that is implicated both in nuclear localization and in the specific activation of promoters containing an initiator element (17). USF1 and USF2 are otherwise quite different in their N-terminal regions, which include in each case at least one additional transcriptional activation domain (17, 18). Thus, although the different USF dimers display identical DNA-

binding properties, they may well control different sets of genes by establishing specific interactions with other transcription factors. The major form of USF present in most cell lines and tissues is the USF1·USF2 heterodimer. USF1 homodimers are less abundant and USF2 homodimers are usually quite scarce (7). There are also minor USF species that contain other products of the *Usf1* and *Usf2* genes generated by alternative splicing (5, 7, 19).

The important role of Myc in the control of cellular proliferation, differentiation, and apoptosis is well established (20, 21). Also, Myc overexpression is a common event in cancer progression that leads to uncontrolled growth (21). In contrast, the biological function of the USF proteins remains poorly understood. *In vitro* experiments suggest antagonistic roles of USF and Myc in growth control, because USF overexpression is sufficient to abolish Myc-mediated cellular transformation in primary rat embryo fibroblasts (22). Other properties of USF also point toward a role of this transcription factor in growth control. These include for instance the ability of both USF1 and USF2 to interact with proteins of the Fos family (23, 24) and to inhibit proliferation in several transformed cell lines (22). Also, recent findings in our laboratory indicate a cell cycle regulation of USF expression and activity (T. Lu and M.S., unpublished results) and a complete loss of USF function in several cancer cell lines (Y. Qyang, P. M. Ismail, X. Luo, T. Lu, and M.S., unpublished results).

To investigate the biological function of the USF proteins *in vivo*, we generated USF-deficient mice by using gene targeting in embryonic stem (ES) cells. Herein, we report the phenotype of individual and combined null-mutations in the *Usf1* and *Usf2* genes. These animals, as well as the cell lines that can be derived from them, should constitute a powerful model system to investigate the specialized and overlapping functions of the USF transcription factors and identify some of their specific target genes.

MATERIALS AND METHODS

Mutagenesis of the *Usf1* Gene in Murine ES Cells. A 129/SvEv mouse genomic library (Stratagene) was screened with a mouse *Usf1* cDNA clone. Among 11 positive λ -phage clones isolated, 3 were identified by PCR analysis as originating from the *Usf1* locus, whereas the others contained *Usf1* pseudogenes (25). The former were further characterized by restriction mapping and DNA sequencing to determine the genomic organization of the *Usf1* locus. A detailed description of the *Usf1* gene has been reported by others (19). A 3.5-kb *SacI*–*SpeI* 5' fragment and a 3.3-kb *XhoI*–*EcoRI* 3' fragment from the *Usf1* locus were used to construct a replacement gene targeting vector (Fig. 1A). A PGKneobpA resistance expression cassette (26) was inserted in the same orientation as the *Usf1* transcription between the two *Usf1* homologous regions.

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Abbreviations: MEF, mouse embryo fibroblast; ES cells, embryonic stem cells; EMSA, electrophoretic mobility shift assay.

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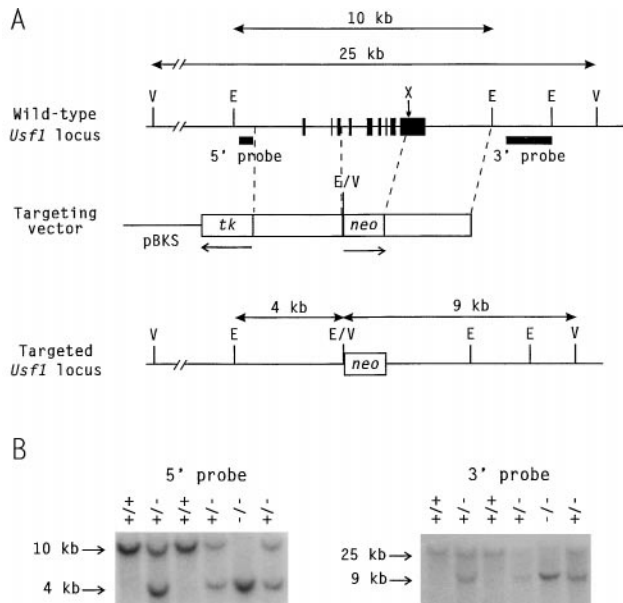


FIG. 1. Mutation of the *Usf1* locus. (A) Targeting strategy. (Top) Structure of the wild-type *Usf1* gene with solid boxes representing exons. The sizes of the restriction fragments detected by the indicated probes in wild-type DNA are shown above. E, *EcoRI*; V, *EcoRV*. (Middle) The gene-targeting vector. Open boxes, *Usf1* homologous regions; *neo*, the PGK-*neo* expression cassette that introduces novel *EcoRI* and *EcoRV* restriction sites; *tk*, the MC1-*tk* expression cassette used for negative selection. The arrows beneath *neo* and *tk* indicate the direction of transcription of each cassette. (Bottom) Structure of the targeted locus, with the sizes of the restriction fragments detected by the Southern probes shown above. (B) Southern blot showing genotypes of newborn mice from a heterozygote mating. *EcoRI*-digested tail DNA was hybridized with the 3' probe, and *EcoRV*-digested tail DNA was hybridized with the 5' probe. The wild-type and mutant bands are shown in each case. Lanes: +/+, wild type; +/-, heterozygous mutant; -/-, homozygous mutant.

An MC1tkpA herpes simplex virus thymidine kinase expression cassette (27) was added onto the long arm of homology to enrich for homologous recombinants by negative selection with 1-(2-deoxy-2-fluoro- β -D-arabinofuranosyl)-*S*-iodouracil. The targeting vector was linearized at a unique *NotI* site outside of the *Usf1* homology region. Fifty micrograms of linearized vector was electroporated into 2×10^7 AB-1 ES cells that were subsequently cultured in the presence of G418 and 1-(2-deoxy-2-fluoro- β -D-arabinofuranosyl)-*S*-iodouracil on mitotically inactivated STO fibroblasts (26, 28). Among 192 G418-resistant clones that were initially screened by *EcoRV* digestion and hybridization with a unique 3' probe external to the vector homology region, 64 were correctly targeted. Of those, 36 were expanded for further Southern blot analysis by *EcoRI* digestion and hybridization with a unique 5' probe and with a *neo* probe; 34 correctly targeted clones were identified.

Mutagenesis of the *Usf2* Gene in Murine ES Cells. The organization of the *Usf2* locus has been reported (29). A replacement gene targeting vector was constructed that contained a PGKneobpA cassette, a 2.3-kb *KpnI-NotI* 5' fragment, a 2.5-kb *EcoRV-KpnI* 3' fragment, and an MC1tkpA cassette downstream of the 3' fragment (Fig. 2A). This targeting vector (50 μ g) was linearized at a unique *KpnI* site outside of the homology region and electroporated into 2×10^7 ES cells. 1-(2-Deoxy-2-fluoro- β -D-arabinofuranosyl)-*S*-iodouracil-selected G418-resistant colonies were screened by *EcoRI* digestion and hybridization with a unique 5' probe external to the vector homology region, which identified 6 correctly targeted clones out of 364. These were expanded and further characterized by *EcoRI* digestion and hybridization

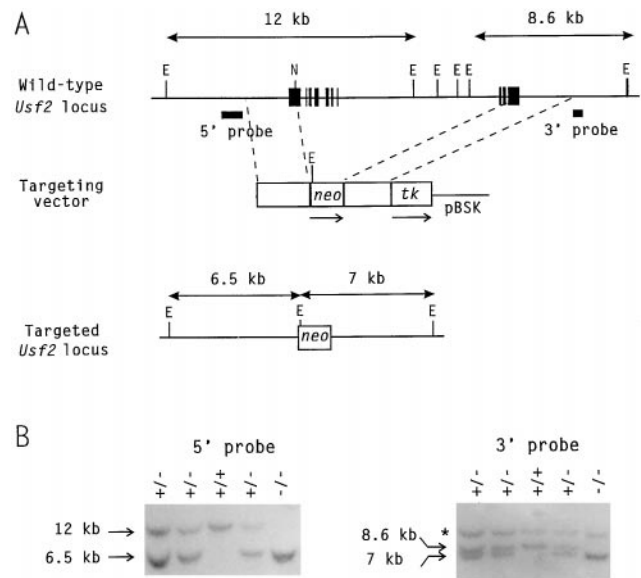


FIG. 2. Mutation of the *Usf2* locus. (A) Targeting strategy. Conventions are as indicated for Fig. 1. (B) Southern blot showing the genotypes of newborn mice. *EcoRI*-digested tail DNA was hybridized with the 5' and 3' probes. The wild-type and mutant bands are shown in each case. The asterisk indicates the migration of a band from a different locus that cross-reacted with the 3' probe.

with a unique 3' probe and a *neo* probe, which identified 5 correctly targeted clones.

Generation of Mutant Mice. Two of the *Usf1* mutant ES cell clones and four of the *Usf2* mutant ES cell clones were injected into C57BL/6J or C57BL/6J albino blastocysts, and the resulting chimeric embryos were transferred to the uterine horns of day 2.5 pseudopregnant foster mothers (30). Chimeras were identified among the resulting progeny by their ES-cell-derived agouti fur. Male chimeras were bred to C57BL/6 or C57BL/6 albino females to produce the first generation of heterozygous mutants. Both of the *Usf1* mutant clones and one of the *Usf2* mutant clones contributed to the germ line of chimeric mice.

For the *Usf1* mutation, the first generation heterozygotes were interbred to generate mice of all three genotypes. For the *Usf2* mutation, the low number of first generation heterozygotes was expanded by breeding these animals with C57BL/6 mice, and the second generation heterozygotes were used to generate mice of all three genotypes. USF2-null pups were very small and so were weaned 2–3 weeks later than their wild-type and heterozygous siblings. To generate the double mutants, *Usf1*(-/-) mice were first bred with *Usf2*(+/-) mice to generate animals that were heterozygous for both mutations. These double heterozygotes were interbred to generate mutants with all genotype combinations at both loci.

Analysis of USF Expression in Mutant Embryos. At 13.5 days, embryos generated by interbreeding either *Usf1*(+/-) or *Usf2*(+/-) mice were collected and used to establish mouse embryonic fibroblast (MEF) cell lines. For each MEF cell line, one plate of cells was used for preparation of genomic DNA and Southern blot analysis. Another plate was used for mini nuclear extract preparation as described by Schreiber *et al.* (31). The presence of different USF dimers in these extracts was identified by electrophoretic mobility shift assay (EMSA), using a radiolabeled 150-bp probe containing a USF-specific binding site. DNA-binding reactions (10 μ l) contained 100 mM Tris-HCl (pH 7.9), 60 mM KCl, 10 mM DTT, p[d(I-C)] (20 μ g/ml), 0.1% Triton X-100, and 1.5 μ l of nuclear extract (~3.4 μ g of protein). After a 20-min incubation at room temperature, the reaction mixtures were supplemented with 2 μ l of 30% glycerol and analyzed by electrophoresis as described (6).

For Western blot analysis, 16 μg of protein from each nuclear extract was resolved by SDS/PAGE on a 10% polyacrylamide gel and transferred to nitrocellulose. The blot was probed successively with USF2- and USF1-specific peptide antibodies (Santa Cruz Biotechnology). In each case, the horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies were detected by the ECL chemiluminescence method (Amersham).

RESULTS

Generation of *Usf1* and *Usf2* Mutant Alleles in the Mouse Germ Line. To mutate the *Usf1* gene in mouse ES cells, we generated a targeting vector that deleted about 3.4 kb of the *Usf1* locus, including most of the coding exons, by replacing them with a neomycin-resistance expression cassette (Fig. 1A). When the vector recombined with the endogenous gene, novel *EcoRI* and *EcoRV* sites were introduced (Fig. 1A). Correctly targeted clones could be detected by the presence of an additional 4-kb *EcoRI* band when hybridized with a 5' probe external to the vector homology region and an additional 9-kb *EcoRV* band when hybridized with a 3' probe external to the vector homology region (Fig. 1B). Correct targeting deleted the DNA region encoding amino acids 27–301 from the total 310 amino acids of USF1, which was predicted to functionally inactivate the locus. Two independent correctly targeted ES clones successfully contributed to the germ line of chimeric mice generated by blastocyst injection. The phenotypes of the *Usf1* mutants from these two clones in the C57BL/6 \times 129/SvEv hybrid genetic background were identical.

A similar strategy was used to generate a *Usf2* mutant allele in the mouse germ line. In this case, the targeting vector deleted about 11 kb of the *Usf2* locus, including most of the 5' untranslated region and all of the coding exons, thus creating a null allele. Recombination of the vector with the endogenous gene deleted a cluster of four *EcoRI* sites normally present in the seventh intron of the *Usf2* gene and introduced a novel site (Fig. 2A). In correctly targeted clones, hybridization with 5' or 3' probes external to the vector homology region detected novel 6.5-kb and 7-kb *EcoRI* bands, respectively (Fig. 2B).

Expression of the Different USF Dimers in Mutant Mice. The expression of USF in the different mutants was analyzed in embryos produced by interbreeding heterozygous mice (Fig. 3). Timed pregnancies were interrupted at day 13.5, and MEF cell lines were established from each embryo. Genomic DNA was prepared from each cell line for genotype determination, and nuclear extracts were used to examine by EMSA the absolute and relative levels of the USF DNA-binding complexes present in the different embryos (Fig. 3A and B).

As previously observed in many other cell types, the major USF DNA-binding species detected by EMSA in nuclear extracts from wild-type MEFs was the heterodimer containing full-length USF1 and USF2 polypeptides. The USF1 homodimers were present at a lower level and the USF2 homodimers were hardly detectable (Fig. 3A, lanes 1 and 6). As predicted for null mutants, nuclear extracts from MEFs homozygous for the *Usf1* mutation contained only USF2 homodimers (Fig. 3A, lanes 2 and 10). The abundance of these USF2 homodimers was similar to that of the heterodimers in wild-type MEFs, indicating an increase in USF2 polypeptides as a result of the *Usf1* mutation. Nuclear extracts from heterozygous embryos displayed a USF DNA-binding pattern that was intermediate between that observed in wild-type and homozygous embryos, suggesting a gene dosage effect. The abundance of USF polypeptides in the *Usf1* mutants was also examined by Western blotting using USF1- and USF2-specific antibodies (Fig. 3C). This analysis confirmed the complete absence of USF1 in *Usf1*($-/-$) embryos, whereas the abundance of USF1 in the heterozygotes was about half of that present in wild-type embryos. As predicted from the EMSA

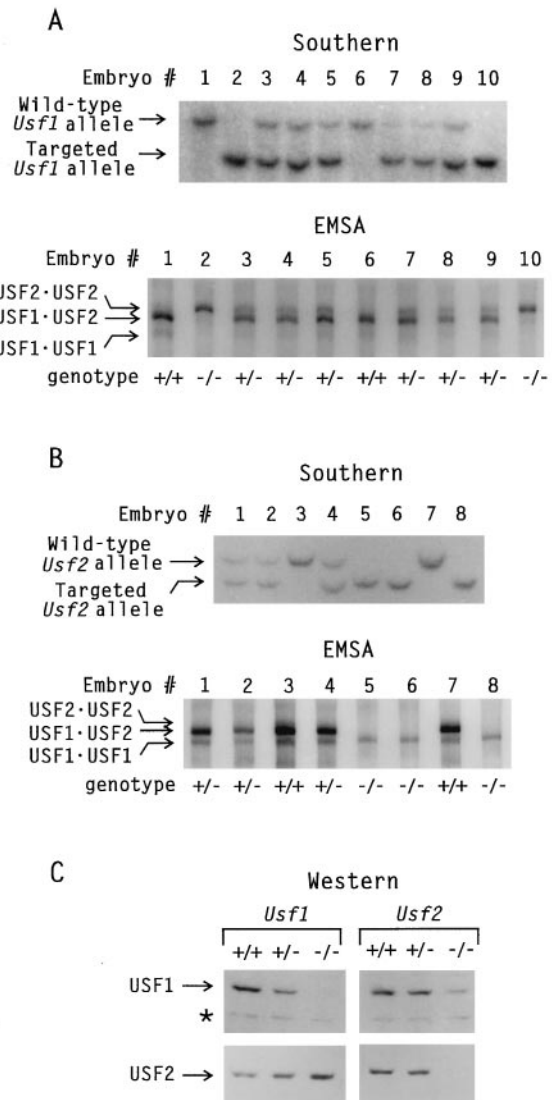


FIG. 3. Expression of USF in mutant embryos. (A) A pregnancy resulting from the mating of *Usf1*($+/-$) mice was interrupted at day 13.5 postconception, and MEF cell lines were established from each embryo. Genomic DNA was prepared from each cell line and used to establish the genotype of each embryo by Southern blotting. Mini nuclear extracts were prepared from each cell line and analyzed for USF DNA-binding species by EMSA, using a radiolabeled DNA fragment containing a USF-specific binding site under conditions that allow maximum resolution of the different USF dimers. Migration of protein–DNA complexes containing USF1 and USF2 polypeptides is indicated at left. (B) Same as in A but with embryos produced by *Usf2*($+/-$) mice. (C) Nuclear extracts from MEFs of the indicated genotypes were analyzed by Western blotting using successively USF2- and USF1-specific antibodies. The asterisk indicates the migration of a USF-unrelated protein that interacted with the USF1 antibodies and represents an internal marker for equal protein loading in the different lanes.

results, the abundance of the USF2 polypeptide increased stepwise as one or both copies of the *Usf1* gene were mutated. Quantitation revealed an increase in USF2 of 24% in *Usf1*($+/-$) embryos and 66% in *Usf1*($-/-$) embryos. From this result, we concluded that the phenotype of the *Usf1* mutants may be attenuated by the compensatory increase in USF2 if the two *Usf* genes performed partially redundant functions.

A similar experiment was carried out with MEFs derived from embryos produced by *Usf2* heterozygous mice (Fig. 3B). When analyzed by EMSA, *Usf2*($+/-$) MEFs displayed a USF

DNA-binding pattern very similar to that of the wild-type embryos. In contrast, *Usf2*(-/-) MEFs contained, as expected, USF1 homodimers exclusively (Fig. 3B, lanes 5, 6, and 8). However, the total level of DNA binding was in this case significantly reduced, indicating a decreased USF1 expression in these mutants. Indeed, the concentration of USF1 homodimers was no greater in the *Usf2*(-/-) embryos than it was in the corresponding heterozygous and wild-type embryos. Yet a significant increase in USF1 homodimers would have been expected if all of the USF1 polypeptides normally present as heterodimers were redistributed into homodimers in the absence of USF2. To exclude the possibility that additional USF1 polypeptides were present in *Usf2*(-/-) embryos in a form that was unable to bind DNA, further analysis was carried out by Western blotting. As shown in Fig. 3C, the complete absence of the USF2 polypeptide in *Usf2*(-/-) embryos was accompanied by a drastic decrease in the level of USF1, in perfect agreement with the EMSA results. Quantitation revealed that the level of USF1 in *Usf2*(-/-) embryos was 20% of the level present in wild-type embryos, indicating that the *Usf2* gene normally stimulates the expression of USF1.

From these analyses, we concluded that each *Usf* gene was capable of influencing the level of expression of the other gene. However, this cross-regulation was asymmetrical. USF2 expression decreased in a dose-dependent fashion in the presence of wild-type *Usf1* alleles, whereas USF1 expression increased considerably in the presence of the wild-type *Usf2* gene.

Phenotype of the *Usf1* Mutants. Mutant mice homozygous for the *Usf1* mutation were produced at the expected frequency from heterozygote matings. The pups were completely indistinguishable from their heterozygous and wild-type siblings. Anatomical and histological analyses in adults up to 3 months of age did not reveal any physical abnormality resulting from the *Usf1* mutation. USF1-null mice were also fertile and took good care of their young. However, some aspects of their behavior, especially among the females, were unusual, and they were also prone to spontaneous epileptic seizures.

First, we noticed that nearly all cages containing two or more USF1-null females had all or all but one animal lacking both whiskers and nasal fur. Whisker trimming and hair nibbling are known behavioral traits of mice, possibly related to social ranking, that are occasionally observed in animals sharing the same cage for extended periods of time (32, 33). In our own colony, this practice was observed in about 20% of all cages in which animals were maintained for several months and at the same frequency for the males and the females. In contrast, this practice was observed in more than 90% of the cages containing USF1-null females as young as 1.5 to 2 months of age.

Another characteristic of the USF1-null mice was their behavior under conditions of moderate stress. In particular, epileptic seizures were occasionally observed when the animals were handled just after awakening. The actual series of events observed during these seizures varied in different episodes. Most noticeable were cases where one or several of the following events were noted: presence of foamy saliva around the mouth, the animal squeaking before falling on their side, a brief period of intense convulsions, abnormal posturing (head bent backwards, curled up tail, folding back of limbs if picked up by the tail), complete immobility lasting up to 5 min,

and abundant urination. Progressive recovery required several minutes. Most of the USF1-null mice known to have had one or more seizures were female, although a few males were also affected. Similar behaviors were never observed in any of the wild-type or heterozygous control mice. From these observations, we concluded that the *Usf1* gene was important for normal behavior in response to stress.

Phenotype of the *Usf2* Mutants. USF2-null mice are viable. The numbers of wild-type, heterozygous, and homozygous pups born from heterozygote matings did not significantly differ from those expected for a Mendelian distribution (Table 1). However, the USF2-null mice displayed an obvious growth defect (Fig. 4). At birth, these animals could readily be distinguished from their siblings because they were noticeably smaller (Fig. 4A and B). They had in addition a distinct bend in the middle of the tail that disappeared within a few days (Fig. 4B). A significant percentage of USF2-null pups died shortly after birth (Table 1). It is unclear at this point whether this increased perinatal mortality rate had other causes besides the decreased ability of these animals to compete for their mother's milk. Indeed, many of them were still without milk several hours after birth. However, most of the USF2-null pups that survived this critical period (nearly 50%) subsequently developed in an apparently normal fashion.

At birth, the weight of the *Usf2* homozygotes was 20–40% less than the weight of their wild-type and heterozygous siblings. This size difference remained unchanged during postnatal development (Fig. 4A and C), and adult USF2-null mice displayed proportionate body features (Fig. 4D). About 10% of these animals had one or two spots of unpigmented fur on the belly. Fertility was also clearly affected by the *Usf2* mutation, because only one out of three males that mated at the age of 2.5 months with wild-type females eventually produced a litter. As for the *Usf1* mutants, occasional epileptic seizures were observed in some of the USF2-null females. Finally, although the cause of death is unclear at this point, USF2-null males had drastically decreased lifespans. Most of them died between the ages of 2.5 and 4.5 months, and no male so far has survived past 6 months of age. In contrast, all of the females, currently 4–10 months old, are still alive. From these results, we concluded that the *Usf2* gene was indispensable for normal embryonic development and also played an essential role in adult mice, with pleiotropic effects on at least brain function, fertility, and male lifespan.

Embryonic Lethality of the Double *Usf1/Usf2* Mutation. The viability of double *Usf1/Usf2* mutants was evaluated by using *Usf1*(+/-)/*Usf2*(+/-) mice. These double heterozygotes, which appeared normal, were obtained at the expected 50% frequency from crosses between *Usf1*(-/-) and *Usf2*(+/-) mice. The double heterozygotes were interbred to generate mice with all possible genotypes at the *Usf1* and *Usf2* loci. As illustrated in Table 2, their progeny was characterized by a complete absence of double-null mutants (0 instead of 18 expected for a Mendelian distribution). Also entirely lacking were *Usf1*(+/-)/*Usf2*(-/-) pups (0 instead of the expected 36). In addition, two genotypes were underrepresented, *Usf1*(-/-)/*Usf2*(+/-) and, as expected from the previous results, *Usf1*(+/-)/*Usf2*(-/-) (Table 2). From this analysis, we concluded that a minimum level of USF activity, whether contributed by products of the *Usf1* or *Usf2* genes, was

Table 1. Survival of *Usf2* homozygous mutants

Pups	Total no.	No. <i>Usf2</i> (+/+)	No. <i>Usf2</i> (+/-)	No. <i>Usf2</i> (-/-)
Born	354	91 (26%)	187 (53%)	76 (21%)
Died within 2 days	65	13	10	42
Survived past weaning	277	75	171	31

All pups from 43 litters produced by 14 breeding pairs of *Usf2* heterozygous mice were genotyped by tail DNA analysis. Of the 31 *Usf2*(-/-) mice that survived past weaning, 15 were male and 16 were female.

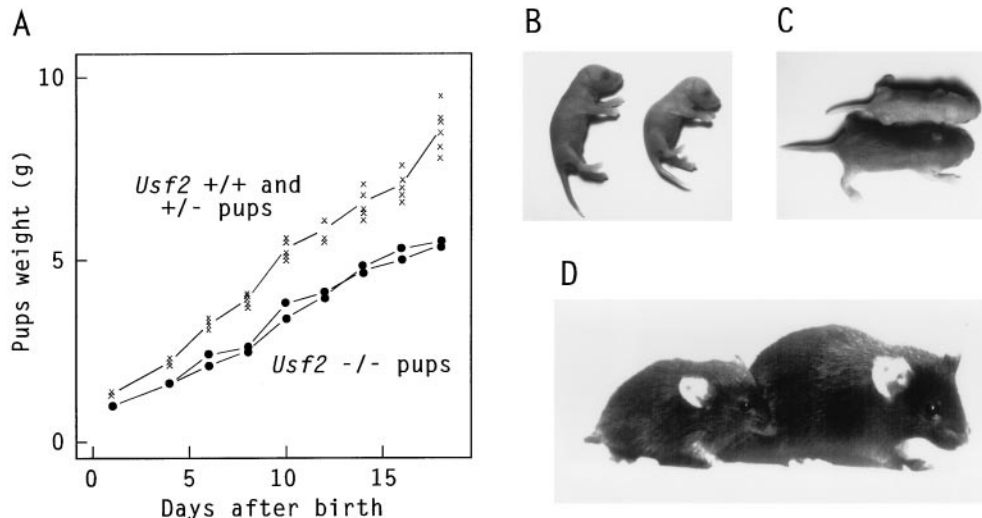


FIG. 4. Growth defect in *Usf2*($-/-$) mutant mice. (A) All pups in one litter produced by a *Usf2* heterozygous breeding pair were weighed at various times of postnatal development. Subsequent genotyping by tail DNA analysis revealed that the two smaller animals were homozygous mutants and that the wild-type and heterozygous pups all displayed similar weights. (B–D) Photographs of *Usf2*($-/-$) mutants next to one of their siblings at birth (B) and at 6 days (C) or 1 month of age (D). Note the smaller size and proportionate body features of the mutants and the characteristic kinky tail of the newborn USF2-null pup.

absolutely necessary for embryonic development. These results also indicate that the two *Usf* genes serve functions that are at least partially redundant.

DISCUSSION

We have analyzed the phenotypes resulting from mutations introduced either individually or in combination in the murine *Usf1* and *Usf2* loci. The targeting strategies used were expected to result in a null (*Usf2*) or essentially null mutation (*Usf1*). These predictions were confirmed by the complete absence of the corresponding USF polypeptides as observed both by EMSA and Western blotting.

The patterns of USF complexes detected by EMSA of nuclear extracts prepared from MEFs of different genotypes confirmed our interpretation of the wild-type pattern of USF–DNA complexes, with prominent bands corresponding to complexes containing USF1–USF2 heterodimers and USF1 homodimers and a minor band corresponding to the USF2 homodimers. These assignments were originally made by comparison with the patterns obtained by using recombinant USF polypeptides (6) and subsequently verified by antibody supershift assay. Because the USF1–USF2 heterodimers are normally the major USF species, their absence in both USF1- and USF2-null mice may be responsible for phenotypic traits that are common to both mutants. These include a propensity for spontaneous epileptic seizures, which is in both cases more common among the females and may reflect a decreased

ability of these animals to adapt to stressful situations. Preliminary examination of brain sections from several USF1-null mice did not reveal obvious histological abnormalities. More detailed analyses will thus be necessary to establish whether there is a structural basis for the observed neurologic defect.

Other aspects of the phenotype of USF-deficient mice were specific to the *Usf1* or *Usf2* mutants. For instance, the increased frequency of whisker-trimming behavior among USF1-null females was not observed in the *Usf2* mutants. More significantly, a distinct growth defect was found only in the USF2-null mice. Note that this particular phenotype is reminiscent of that of the insulin-like growth factor (IGF) II and, to a lesser extent, IGF-I null mice, which are also born smaller with an increased perinatal lethality (34, 35). On the other hand, a kinky tail similar of that of the USF2-null pups was one of the characteristics of the IGF-II receptor mutant embryos (36). It will therefore be of interest to investigate the status of the various components of the IGFs/IGF receptors pathway in the different USF-deficient mutant mice. Note also that in the case of the *Usf2* mutants, different abnormalities may be caused either by the absence of USF2 homodimers or by the general decrease in total USF activity, which is much more pronounced than in the *Usf1* mutants. To ascertain which of these differences is directly responsible for the observed phenotype, construction of *Usf2* mutants with enhanced USF1 expression may be necessary.

One clear difference between the *Usf1* and *Usf2* genes evidenced by the consequences of the individual mutations was

Table 2. Progeny of the double heterozygotes

Genotype	No. of pups	Observed frequency	Expected number	Expected frequency
<i>Usf1</i> (+/+)/ <i>Usf2</i> (+/+)	19	0.067	18	0.062
<i>Usf1</i> (+/+)/ <i>Usf2</i> (+/-)	61	0.214	36	0.125
<i>Usf1</i> (+/+)/ <i>Usf2</i> (-/-)	12	0.042	18	0.062
<i>Usf1</i> (+/-)/ <i>Usf2</i> (+/+)	47	0.164	36	0.125
<i>Usf1</i> (+/-)/ <i>Usf2</i> (+/-)	100	0.351	71	0.250
<i>Usf1</i> (+/-)/ <i>Usf2</i> (-/-)	0	0	36	0.125
<i>Usf1</i> (-/-)/ <i>Usf2</i> (+/+)	22	0.077	28	0.062
<i>Usf1</i> (-/-)/ <i>Usf2</i> (+/-)	24	0.084	36	0.125
<i>Usf1</i> (-/-)/ <i>Usf2</i> (-/-)	0	0	18	0.062

All 285 pups from 56 litters produced by 19 breeding pairs of *Usf1*(+/-)/*Usf2*(+/-) mice were genotyped by tail DNA analysis. There were no sex differences among the pups of the various genotypes.

the influence of each gene on the expression of the other. Indeed, USF1-null mice displayed an overall USF DNA-binding activity similar to that found in wild-type mice due to an enhanced USF2 expression and a drastic increase in the levels of USF2 homodimers. In contrast, USF2-null mice displayed a significant decrease in total DNA-binding activity and depressed USF1 expression. These results indicate that, in wild-type cells, the *Usf1* gene normally represses the level of the USF2 homodimers, whereas the *Usf2* gene increases expression of the USF1 polypeptides. The cross-regulation between the two *Usf* genes is thus clearly asymmetrical. This regulation may involve transcriptional mechanisms, as suggested by the presence of USF binding sites within the promoter of the *Usf2* gene (29), as well as posttranscriptional mechanisms requiring for instance the extremely conserved 5' and 3' untranslated regions of the *Usf1* and *Usf2* mRNAs (7, 19). Further analysis will be necessary to determine how each gene influences the expression of the other and whether the changes in USF expression observed in mutant embryos are reflected by similar changes in different adult tissues.

An embryonic-lethal phenotype was observed for the double *Usf1/Usf2* mutants, as well as for *Usf2*(-/-) mutants containing a single wild-type *Usf1* allele. Given that the latter probably contained even less USF1 activity than mice lacking only the *Usf2* gene, these results indicate that a minimum level of USF activity is absolutely required for embryonic development. Interestingly, a sea urchin transcription factor with strong homologies to the mammalian USF proteins has also been implicated in the regulation of gene expression during the development of *Lytechinus* embryos (37). In mice, this essential role of USF during embryonic development can be performed by the normal complement of USF species, which includes mostly heterodimers and USF1 homodimers. However, USF2 homodimers alone can serve the same function, as evidenced by the normal development of the USF1-null mice. Therefore, it is clear that the activities of the *Usf1* and *Usf2* genes are partially redundant. Because the phenotypes of the different mouse mutants also indicate specialized functions for the USF1 and USF2 proteins, it is most likely that these transcription factors regulate sets of genes that overlap but are not identical.

A major benefit provided by the various USF-deficient mice will be the availability of animal tissues and cell lines with different levels of expression of the USF1 and USF2 polypeptides. These should be particularly useful for investigating the role of USF in various phases of the cell cycle as well as in cellular immortalization and transformation. These cell lines and animal tissues should also allow unambiguous determination of genes that are specific targets of USF1, USF2, or both.

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