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Genetics

Provirus integration into a gene encoding a ubiquitin-conjugating enzyme results in a placental defect and embryonic lethality

(insertional mutagenesis/retroviruses/protein degradation)

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ABSTRACT **Ubiquitin-conjugating enzymes (E2 or Ubc) constitute a family of conserved proteins that play a key role in ubiquitin-dependent degradation of proteins in eukaryotes. We describe here a transgenic mouse strain where retrovirus integration into an *Ubc* gene, designated *UbcM4*, results in a recessive-lethal mutation. *UbcM4* is the mouse homologue of the previously described human *UbcH7* that is involved in the *in vitro* ubiquitination of several proteins including the tumor suppressor protein p53. The provirus is located in the first intron of the gene. When both alleles are mutated the level of steady-state mRNA is reduced by about 70%. About a third of homozygous mutant embryos die around day 11.5 of gestation. Embryos that survive that stage are growth retarded and die perinatally. The lethal phenotype is most likely caused by impairment of placenta development as this is the only organ that consistently showed pathological defects. The placental labyrinth is drastically reduced in size and vascularization is disturbed. The *UbcM4* mouse mutant represents the first example in mammals of a mutation in a gene involved in ubiquitin conjugation. Its recessive-lethal phenotype demonstrates that the ubiquitin system plays an essential role during mouse development.**

Protein degradation is an important process by which cells control the activity of normal proteins and avoid the potentially toxic effects of aberrant proteins. A major pathway for selective breakdown of proteins in eukaryotes is the ubiquitin/proteasome system in which proteins are conjugated to the polypeptide ubiquitin and, thus tagged, are degraded by the proteasome complex (1–3). Substrates of this pathway include cyclins, cyclin-dependent kinase inhibitors, transcription factors, the tumor suppressor protein p53, and a variety of oncoproteins (2). Very recent evidence suggests that ubiquitination of membrane-bound receptors might also serve as a signal for degradation via the lysosomal pathway (4). Although ubiquitin conjugation is mainly thought to tag proteins for destruction, several lines of evidence suggest that it might also be involved in other cellular functions. This is indicated, for example, by the presence of stable ubiquitinated proteins, such as histones (5), and by the existence of enzymes that remove ubiquitin from conjugates and therefore make this modification reversible (6). Reversible ubiquitination might have a role in modulating the function of target proteins similar to protein phosphorylation.

Attachment of ubiquitin to cellular proteins is carried out by the sequential action of three classes of enzymes, ubiquitin-activating enzymes (referred to as E1 or Uba), ubiquitin-conjugating enzymes (E2 or Ubc), and ubiquitin-protein ligases (E3 or Ubr) (7). In an initial activating step the E1 enzyme forms a thiolester bond with the C terminus of ubiquitin, which is then transferred to a specific cysteine residue of the E2 enzyme. Although E2 enzymes can directly donate ubiquitin to proteins, E3 enzymes are typically required for recognition of specific proteolytic substrates and at least in one case have been shown to be directly involved in the final transfer of ubiquitin to a substrate protein (8). E2 enzymes are encoded by a multigene family and several members of this family have been isolated from various organisms including plants. The function of E2 enzymes has been studied most extensively in yeast by using a genetic approach. Mutations in individual genes for different yeast E2 enzymes revealed that these proteins participate in a wide variety of essential cellular functions (1). In *Drosophila* an E2 enzyme has been shown to be involved in nervous system development (9, 10). Based on these results it is most likely that in higher organisms, including man, mutations in the genes coding for the different enzymes of the ubiquitin-conjugating system will contribute to abnormal development and disease. Indirect evidence for such a relationship has accumulated over the past years (11).

No mouse mutants with spontaneous or experimentally induced alterations have been previously described that help to clarify the role of any of the *E2* genes during differentiation and development. In the present report we describe a transgenic mouse mutant where integration of a retrovirus into an *E2* gene, designated *UbcM4*, results in a recessive-lethal phenotype. Homozygous embryos die *in utero* most likely as a result of impaired placenta development.

MATERIALS AND METHODS

Derivation of A6 Mouse Strain. Cells of the embryonal stem (ES) cell line J1 (12) were infected by growing on a monolayer of x-ray irradiated Psi-2 cells producing mp10 virus (13) in ES cell culture medium containing 8 μg/ml polybrene. After coculture for 48 h, ES cells were trypsinized and selected in medium containing G418 (125 μg/ml of active component) for 10 days. G418-resistant colonies were picked and expanded. Cell lines were analyzed by Southern blot analysis for the presence of proviral DNA. ES cell lines with single copy proviral genome were injected into BALB/c or C57BL/6 blastocysts, and injected embryos were transferred into uteri of pseudopregnant F1(C57BL/6J × CBA) foster mothers as described (12). Of nine independent ES cell lines injected, six contributed to the germ line of the mouse. Two of the six transgenic lines, A6 and A8, resulted in recessive defects in the homozygotes. The inbred A6 mouse strain was obtained by breeding a male chimera with a female 129/Sv mouse. The 129/Sv strain was also used for all subsequent breedings in our colony. The genotyping was initially done by quantitative Southern blotting of tail DNA. After the provirus flanking probe (5′ fA6 in Fig. 2) was obtained all genotypes were confirmed by qualitative Southern blots and/or by PCR analysis (see below). Embryos were obtained

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Abbreviations: E2 or Ubc, ubiquitin-conjugating enzyme; ES, embryonal stem; LTR, long terminal repeat; Mo-MuLV, Moloney murine leukemia virus; RACE, rapid amplification of cDNA ends.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. X97042).

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from timed matings; the day of plug detection was counted as day 0.5 of gestation.

Molecular Cloning. The proviral integration site, subsequently referred to as A6 locus, was cloned using standard methods (14). Initially a cellular DNA fragment flanking the 5′ end of the mp10 provirus was cloned by inverse PCR (15). This fragment, designated 5′ fA6 (see Fig. 2), was subcloned in the Bluescript vector (Stratagene). To obtain phage λ clones representing the A6 locus from wild-type mice, a 129Sv mouse genomic library in λFIXII (Stratagene) was screened with a radiolabeled probe derived from fragment 5′ fA6. Positive plaques were purified by further rounds of screening. For fine mapping and structural analyses, subfragments from the λ clones were subcloned according to standard procedure. The physical map of the A6 locus shown in Fig. 2 was obtained from Southern blot analyses of wild-type and mutant genomic DNA as well as from restriction analyses of the λ and plasmid clones.

Genotype Analysis. For genotyping, DNA was isolated from the yolk sac of dissected embryos or from the terminal third of the tail of adult animals and analyzed by PCR using three different primers (A, B, and C) as shown schematically in Fig. 3. The sequence of primer A was 5′-TGGCTCGAACTCTG-GTGGGT-3′ and primer B was 5′-GCTCTCTTCTACA-GAACAGG-3′. Both sequences were derived from a genomic subclone representing the preintegration site of the provirus. Primer C (5′-CAGGGTCATTTTCAGGTCCT-3′) was derived from the published sequence of the Moloney murine leukemia virus (Mo-MuLV) LTR (positions 8148–8167 in ref. 16). The reaction was carried out in a thermocycler (Perkin–Elmer) for 35 cycles consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. PCR products were visualized after separation by agarose gel electrophoresis in the presence of ethidium bromide under UV light.

Isolation and Sequencing of cDNA Clones. A mouse brain cDNA library (CLONTECH) was screened with the genomic probe pA62-2 (see Fig. 2). Positive plaques were purified and the DNA inserts were recloned in the Bluescript vector (Stratagene). The 5′ end of the *UbcM4* transcripts in brain and placenta was amplified by using a kit for rapid amplification of cDNA ends (5′ RACE) as described in the instruction manual (Life Technologies, Gaithersburg, MD). The nucleotide sequence was determined by the dideoxy method, using fluorescence-labeled dideoxy terminators (17). Subsequent separation and analysis were performed on a model 373A DNA sequencer (Applied Biosystems).

RNA Preparation and Analysis. Total cellular RNA was isolated by the guanidinium isothiocyanate procedure, fractionated (15 μg per lane) by electrophoresis in 1% agarose gels containing formaldehyde, and transferred by capillary blotting onto nylon membranes (14). After staining with methylene blue, rRNA bands were used to confirm the integrity and amount of RNA in each lane. Filters were hybridized for 18–20 h at 65°C in a standard hybridization solution without formamide (14).

^{32}P-radiolabeled probes were prepared by the random-priming procedure (18). Final filter washes were done at 55°C with a solution of 0.1× standard saline citrate (SSC; 1× SSC is 0.15 M NaCl/0.015 M sodium citrate) and 0.1% SDS.

Histology. Embryos and placentas were removed and fixed by immersion in 4% paraformaldehyde. Tissue was embedded in paraffin and sections of 5 micrometer thickness taken. Laminin immunoreactivity was detected using a rabbit antilaminin antiserum (Boehringer Mannheim), biotinylated secondary antibody, and the avidin-biotin-peroxidase complex (Boehringer Mannheim) according to the manufacturer's protocol. Peroxidase was visualized using diaminobenzidine/hydrogenperoxid.

RESULTS

Provirus Integration at the A6 Locus Results in a Recessive-Lethal Phenotype. The transgenic mouse strain A6 was generated by infection of preimplantation mouse embryos with the recombinant retrovirus mp10 (13). This virus is a replication-defective derivative of the Mo-MuLV and carries a neomycin resistance gene under the transcriptional control of the Mo-MuLV LTR. The A6 mouse strain carries one copy of the provirus in its germ line. Animals heterozygous for provirus integration at the A6 locus did not display any overt abnormalities during development and postnatal life. However, crosses between heterozygous parents failed to produce homozygous mutant offspring at weaning, indicative of lethality at an earlier age (Table 1). Embryos were isolated at different stages of gestation and genotyped by PCR analysis of yolk sac DNA as described below. Homozygous A6 embryos were recovered in the expected proportions between days 10.5 and 19.5 of gestation (counting the day of vaginal plug as day 0.5). However, the proportion of normally developed homozygous embryos decreased with age, accompanied by an increasing number of embryos that were dead, or alive but with clear signs of growth retardation (Table 1). On day 10.5 of gestation, genotyped homozygotes were morphologically indistinguishable from wild-type and heterozygous embryos. Beyond that stage of development, live homozygotes could be identified by a reduction of body size, which became more pronounced toward term. Roughly 30% of the homozygotes, however, were found dead between day 10.5 of gestation and term and showed developmental features (e.g., in the footplate characteristic of day 11.5 or 12.5 of gestation). As none of the remaining 70% homozygotes was found alive in born litters, it is assumed that they die perinatally.

Homozygous A6 Embryos Display Abnormalities in the Placenta. The prenatal development of A6 homozygotes was examined histologically on days 10.5–19.5 of gestation. Genotyped homozygotes did not show any malformation of vital organs. However, in two of eight homozygous placentas examined for laminin immunoreactivity on day 11.5 of gestation, the chorionic plate appeared thinner and mesenchymal invasion of the trophoblast was considerably reduced (Fig. 1 *a* and *b*). This suggests that in some A6 homozygotes the establishment of maternal–fetal exchange is impaired and may cause their death in midgestation. Homozygotes that survived beyond day 11.5 of gestation could be identified phenotypically late in gestation because they were smaller than the heterozygous or wild-type littermates. The histological examination of these homozygotes did not reveal malformations of any vital organ. Frequently, the placentas of homozygotes at late stages of gestation appeared pale and smaller than normal. Histological examination revealed that the labyrinth of the mutant placentas was reduced in size (Fig. 1 *c* and *d*). The distribution of mesenchyme and the fetal capillaries in the labyrinth was investigated immunohistochemically using antibodies to laminin, which stains the basal lamina of fetal capillaries in the labyrinth. In wild-type embryos, the fetal blood vessels were evenly distributed throughout the labyrinth and had a rather

Table 1. Genotype of offspring and embryos derived from parents heterozygous at the A6 locus

Age	Wild type, wt/wt	Heterozygous, A6/wt	Homozygous, A6/A6
E10.5	21	44	20
E11.5	27	35	25 (0/5)
E12.5	27	37	12 (3/1)
E13.5	15	21	17 (7/2)
E14.5	12	18	8 (2/3)
E15.5	32	65	22 (3/13)
E16.5	14	28	10 (1/5)
E17.5	24	51	20 (6/9)
E18.5	21	28	19 (4/6)
E19.5	12	28	14 (5/7)
Weaning	90	131	0

Total numbers of embryos (dead or alive) are given in each lane. Numbers in brackets indicate homozygous embryos that were significantly reduced in size (>30%) or dead, respectively. E10.5 to E19.5 represent day 10.5 to 19.5 of gestation.

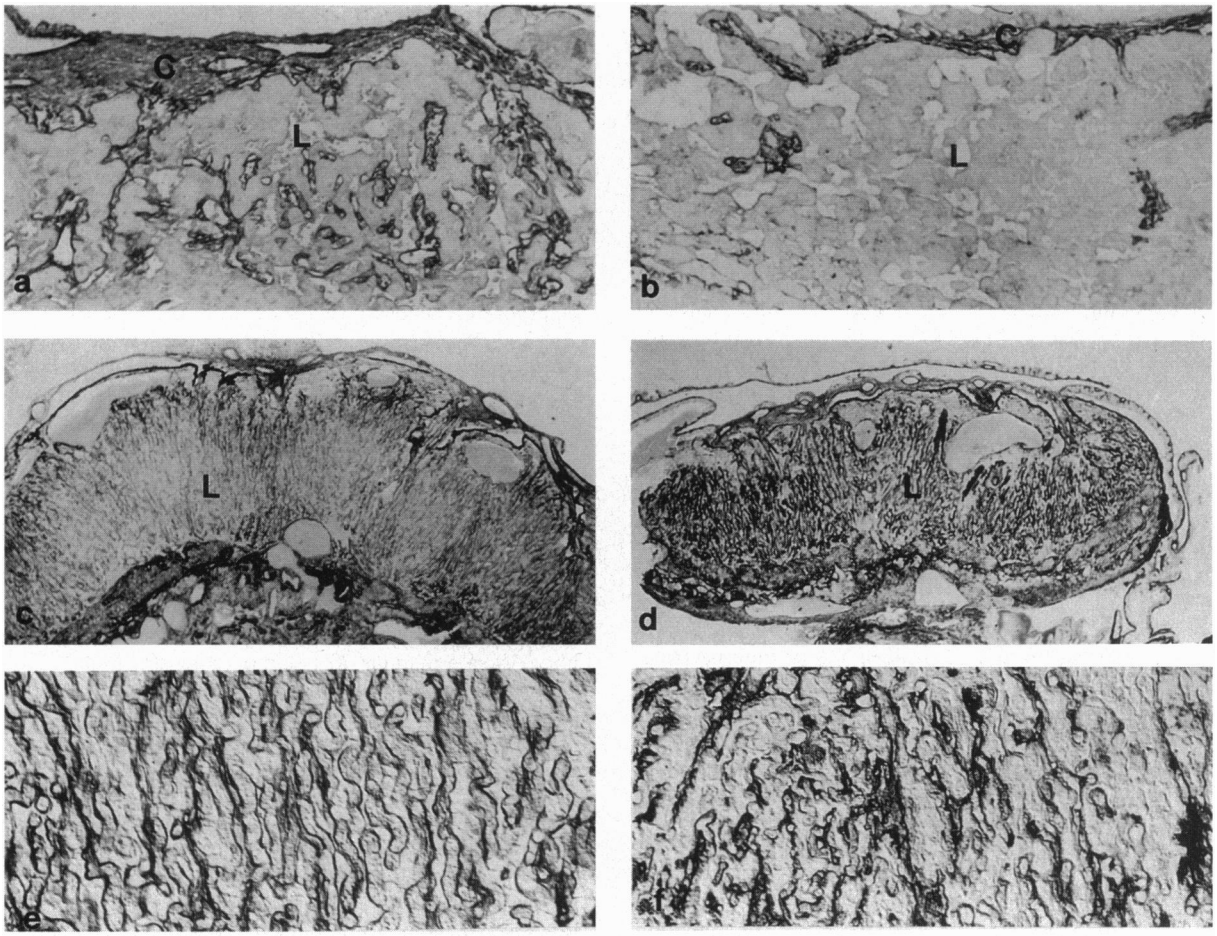

FIG. 1. Immunohistochemical localization of laminin in cross sections of placentas on day 11.5 (*a* and *b*) and day 18.5 (*c–f*) of gestation. Paraffin sections were taken through similar planes of wild-type (*a*, *c*, and *e*) and A6 homozygous mutant (*b*, *d*, and *f*) placentas. The laminin immunoreactivity (black) within the labyrinth (L) marks the fetal mesenchyme and the basal lamina of the fetal blood vessels. (*a*) On day 11.5 of gestation, fetal mesenchyme invades the trophoblast and establishes a widespread system of fetal blood vessels in the labyrinth (L). (*b*) In some A6 homozygous mutants, the chorionic plate is much thinner and fetal blood vessels in the labyrinth (L) are scarce. Although this particular homozygote was alive and appeared phenotypically normal, such defective mesenchymal invasion of the labyrinth may account for the 30% of homozygous dying immediately after day 11.5 of gestation. Most A6 homozygous placentas examined on day 11.5 of gestation showed a normal mesenchymal invasion with respect to the pattern of laminin immunoreactivity. (*c* and *d*) Late in gestation, the mutant placenta (*d*) is smaller in cross section than the wild-type placenta. (*e* and *f*) At higher magnification, the fetal blood vessels of the labyrinth in wild type (*e*) appear orderly spaced between maternal blood spaces, while in the mutant placenta the fetal blood vessels show frequent branching, variations in caliber, and a denser laminin immunoreactivity. (*a* and *b*, ×130; *c* and *d*, ×32; *e* and *f*, ×230.)

uniform caliber (Fig. 1 *c* and *e*). The fetal blood vessels in mutant placentas, however, showed more branching and a considerable variation in caliber, which may cause an impairment of maternal fetal exchange of gases and nutrients, and hence decrease the normal growth rate of the fetus in late gestation (Fig. 1 *d* and *f*).

The A6 Locus Encodes a Ubiquitin-Conjugating Enzyme. To analyze the molecular nature of the mutation at the A6 locus a 430-bp DNA fragment flanking the 5′ LTR of the provirus was cloned by inverse PCR. When used as a probe on Southern blots the flanking fragment, designated 5′ fA6 (Fig. 2), was shown to hybridize to a single band in genomic DNA from wild-type animals. In heterozygous animals, this probe detected an additional band due to the insertion of the provirus (data not shown). Additional flanking sequences were isolated by screening a phage λ library of genomic mouse DNA with probe 5′ fA6. Three overlapping λ clones, designated λA61, λA62, and λA64 (Fig. 2) were purified and their DNA characterized by restriction mapping. A subfragment of λA61 representing the preintegration site was completely sequenced. Based on this information oligonucleotides were designed that in combination with an oligonucleotide from the 5′ LTR allowed genotyping of A6 mice by PCR analysis as shown in Fig. 3. Assignment of genotypes of all embryos and offspring from A6 mouse intercrosses was based on this PCR assay.

To examine whether the provirus was inserted in the proximity of a gene, the λ clones were dissected into subfragments that were then used as hybridization probes on Southern blots of genomic DNAs from different species. A 1.8-kb fragment, designated pA62-2 in Fig. 2, and found to be nonrepetitive and evolutionarily conserved, hybridized to several RNA tran-

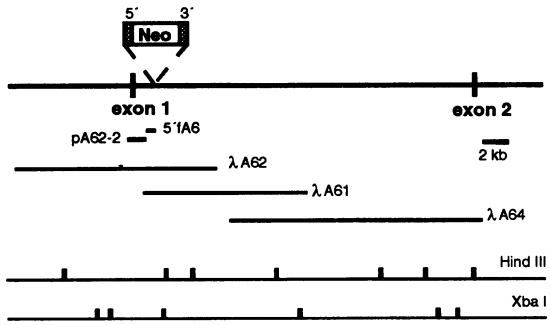

FIG. 2. Schematic representation of the A6 locus. The first two exons at the 5′ end of the *UbcM4* gene and the integration site of the provirus containing a neomycin resistance gene are shown. The position of phage λ and plasmid clones mentioned in *Results* and restriction sites for *Hin*dIII and *Xba*I are indicated.

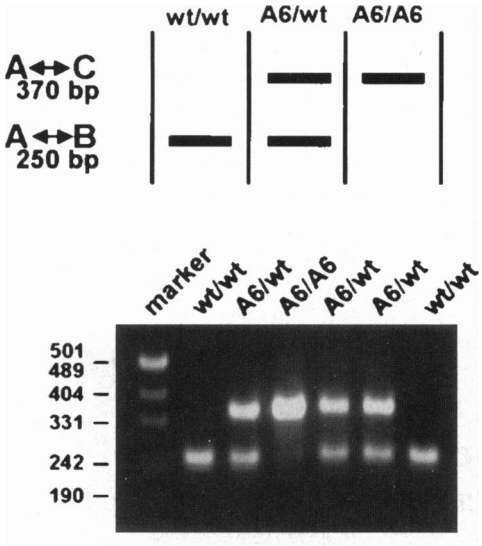

FIG. 3. Genotype analysis of A6 mice by PCR. The schematic drawing shows the position of the primers and the expected lengths of the fragments. The gel at the bottom shows the results of a genotype analysis from yolk sac DNA of day 12.5 embryos from heterozygous A6 parents. (For details, see *Materials and Methods*.)

scripts when used as a probe on Northern blots containing poly(A)$^+$ RNA from day 15.5 placentas and embryos and from adult brain. To further characterize these transcripts, a brain λgt10 cDNA library was screened with this fragment, resulting in the isolation of several cDNA clones. The sequence of the longest cDNA clone is shown in Fig. 4. By 5′ RACE analysis using different oligonucleotides from the initially isolated cDNA it was confirmed that the 5′ end shown in Fig. 4 represents the 5′ most start site of the transcript. However, by the same method and, independently, by primer extension analysis, other possible start sites were found within the first 20 bp, indicating heterogeneity at the 5′ end.

The sequence shown in Fig. 4 has a major open reading frame of 462 bp beginning with a potential ATG initiation codon at nucleotide 1 and ending with a TAA terminator codon at position 463, coding for a protein with a calculated mass of about 17 kDa. Two other possible initiation codons are present at positions 22 and 55; however, the ATG codon at position 1 conforms best to the consensus for translation start signals (19). The sequence 3′ to the TAA stop codon at position 463 contains several stop codons in all three reading frames and therefore is most likely part of the 3′-untranslated region.

The 17-kDa protein encoded by the open reading frame belongs to the family of ubiquitin-conjugating enzymes, Ubc, and its amino acid sequence shows 100% identity to the recently described human *UbcH7*, which *in vitro* can ubiquitinate several proteins including the tumor-suppressor protein p53 (20). As the *Ubc* gene described here is the fourth published family member from mouse we will refer to it as *UbcM4* (for *UbcM1*, *-2*, and *-3*; see ref. 21).

The Provirus Is Inserted in the First Intron of the *UbcM4* Gene. To map the proviral integration site relative to the transcriptional unit of the cellular gene, a partial exon/intron structure of the *UbcM4* gene was determined. For this purpose restriction digests of the above described genomic clones λA61, λA62, and λA64 (compare Fig. 2) were hybridized with oligonucleotides derived from the cDNA sequence to identify fragments containing exon sequences. These fragments were subcloned, partially sequenced, and exon–intron junctions identified by comparison of genomic and cDNA sequences. By this approach exons 1 and 2 were mapped that together represent 110 bp from the 5′ end of the cDNA. These two exons are separated by 26 kb of intervening sequences (Fig. 2). Further restriction mapping showed that the provirus had integrated into the first intron about 2 kb downstream of the first exon–intron boundary in the same transcriptional orientation as the *UbcM4* gene. The results are summarized schematically in Fig. 2.

Provirus Affects Expression of the *UbcM4* Gene. When used as a probe on Northern blots with total RNA from different tissues of adult mice, the *UbcM4* cDNA hybridized with at least four transcripts that were estimated to be 0.9, 1.8, 2.6, and 2.9 kb long (Fig. 5). The gene is expressed at about the same levels in all organs tested. The 2.9-kb transcript formed the most prominent band in all tissues except testes, where the 0.9-kb transcript was most abundant. The relationship between the

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-31                                                                ATTTTGGGAAAGCAGCAGCACCAGATCCAAG
  1 ATGGCGGCCAGCAGGAGGCTGATGAAGGAGCTTGAAGAGATCCGCAAATGTGGAATGAAAAACTTCCGTAACATCCAGGTTGATGAAGCT
    M  A  A  S  R  R  L  M  K  E  L  E  E  I  R  K  C  G  M  K  N  F  R  N  I  Q  V  D  E  A    30
 91 AATTTATTGACTTGGCAAGGGCTTATTGTTCCTGACAACCCTCCATATGATAAGGGAGCCTTCAGAATTGAAATCAACTTTCCAGCAGAG
    N  L  L  T  W  Q  G  L  I  V  P  D  N  P  P  Y  D  K  G  A  F  R  I  E  I  N  F  P  A  E    60
181 TATCCATTCAAACCACCCAAGATCACATTTAAAACAAAGATCTACCACCCTAACATCGATGAGAAGGGGCAGGTCTGTCTGCCAGTCATT
    Y  P  F  K  P  P  K  I  T  F  K  T  K  I  Y  H  P  N  I  D  E  K  G  Q  V  C  L  P  V  I    90
271 AGTGCTGAAAACTGGAAGCCAGCCACCAAGACTGACCAAGTAATCCAGTCCCTCATAGCACTGGTGAATGACCCCCAGCCTGAGCACCCA
    S  A  E  N  W  K  P  A  T  K  T  D  Q  V  I  Q  S  L  I  A  L  V  N  D  P  Q  P  E  H  P   120
361 CTCCGGGCTGACCTAGCTGAAGAATACTCTAAGGACCGTAAAAAATTCTGTAAGAATGCTGAAGAGTTTACAAAGAAATATGGGGAAAAG
    L  R  A  D  L  A  E  E  Y  S  K  D  R  K  K  F  C  K  N  A  E  E  F  T  K  K  Y  G  E  K   150
451 CGACCTGTGGACTAAAATCTGCCATGAGTGATTCCAGCAAGTTTGAGCAGAGACCCCGAGCAGTGCATTCAGACACCCCGCAAAGCAGGA
    R  P  V  D  *                                                                               154
541 CTCTGTGGAAATTGACACGTGCCACCAACTGGCGTCCGCTTGTGGCAGTTACTAACTTTCTACAGTTTTCTTAATCAAAAGTGGTCTAGG
631 TAACCTGTAAAGAAGGATTAAAAAATTCAGATGTTCTAGTTCTGCTTGCTTTGTTTTAAAAATCACTGCTTTAGTCTACTTCAAAAGAAT
721 GGCATTTCTTTTCTTGCCCAAATTTACTCAAAGTTGCAGTTAGCCCAGAAGGTTTAAAGAAAAAGGTTGTTGTGGTTTCTTCCCCCTTCC
811 CCTCTCCCCACTTTTCGATTCCCACTTCCACAGACCAAGGCCCCAGCTCCCCACAGAGGTGGCCGCAGCACTGACAACGTTTCCTGTGCC
901 AACACTGCCTGTGGGTAATGAGTAGCTCACGCACCCCATTCCAGCTTAGTGATGTCTTCAAAAAATTATAAAACAGTATCAATGCCCTGT
991 CCCAAAGGAACATCCATGTATTCAAAGAGAAGCCGTCTTTACGTAAAATTTTAGCAAAGGGTTTCCACAATAATAATAGACCCGACAGAC
1081 AGACCCCCATGGTCCCTATCTGTAGTCAGACTGTGCTGGCTGGGTCTCTGCAGGGAACCTGTTATAGCAGGTTTGTGGTGACCTTGGAGG
1171 GAGGGTCTGCCAGCAGAGAATACAGTGCGCTTGTGTGATGGACTTCTCACAGTCAGCAGCCCTTCCTGAAGCTGTGGTAGACCTGGTCAT
1261 CACAGACCAAAAAAAAAAAAAA
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FIG. 4. Nucleotide sequence of *UbcM4* cDNA and deduced amino acid sequence of the protein. Numbers at the left refer to nucleotide positions, and those at the right refer to amino acid positions. The poly(A) tract at the 3′ end is also present in the genomic DNA and, therefore, does not represent the poly(A) tail of the mRNA.

four transcripts is not completely clear. However, preliminary evidence suggests that they all code for the same protein and only vary in the length of the 3′ untranslated region (data not shown). The 3′ untranslated region of the cDNA shown in Fig. 4 is therefore incomplete in respect to the longer transcripts. The gene is transcribed early in development as shown by the presence of all four transcripts in embryos and placentas of different developmental stages (Fig. 6). Taken together, these results suggest that the *UbcM4* gene is ubiquitously expressed throughout embryonic development and in adult animals.

To test whether proviral insertion affected the level of transcription of the *UbcM4* gene, total RNAs from embryos and placentas of different developmental stages from litters of heterozygous A6 parents were analyzed on Northern blots. In each case yolk sac DNA was used for genotype analysis. As an example the results are shown for day 15.5 embryos and placentas from the same litter (Fig. 7). Compared with their wild-type littermates, the level of all four transcripts is significantly reduced in heterozygous and even more so in homozygous A6 embryos and placentas. To quantitate the reduction in expression autoradiograms were scanned with a densitometer and the amount of RNA in each lane was normalized by rehybridizing the blots with a glyceraldehyde-3-phosphate dehydrogenase probe. This analysis showed the level of transcripts to be reduced by 30 ± 5% in heterozygous and 70 ± 8% in homozygous as compared with wild-type embryos and placentas. Roughly the same results were obtained at all developmental stages studied in more detail, such as day 11.5, 12.5, 15.5, and 17.5 of gestation. In summary, the results clearly indicate that provirus integration affects expression of the *UbcM4* gene by reducing the amount of steady-state mRNA in A6 embryos and placentas.

DISCUSSION

The A6 mouse mutant described in this report represents an example in mammals for mutation of a gene involved in ubiquitin conjugation. The recessive-lethal phenotype of the A6 mutant demonstrates that the ubiquitin system plays an essential role during mouse development, most likely by mediating programmed degradation of proteins such as transcription factors or receptors. A characteristic feature of the A6 mutant is the observation that the retrovirus integration into the *UbcM4* gene did not result in a complete transcriptional block but in a reduction in the level of steady-state mRNA by about 70%. Obviously, the level of *UbcM4* expression is very crucial for its proper function and reduction below a certain threshold is sufficient to cause a recessive-lethal phenotype. It has to be emphasized, however, that the reduced expression was only shown at the RNA level as no suitable antibodies are available at the present time to study protein levels.

Mutations of *Ubc* genes have so far been studied only in yeast and *Drosophila*. Genetic analysis of the ubiquitin-conjugation system in yeast showed that the E2 enzymes participate in

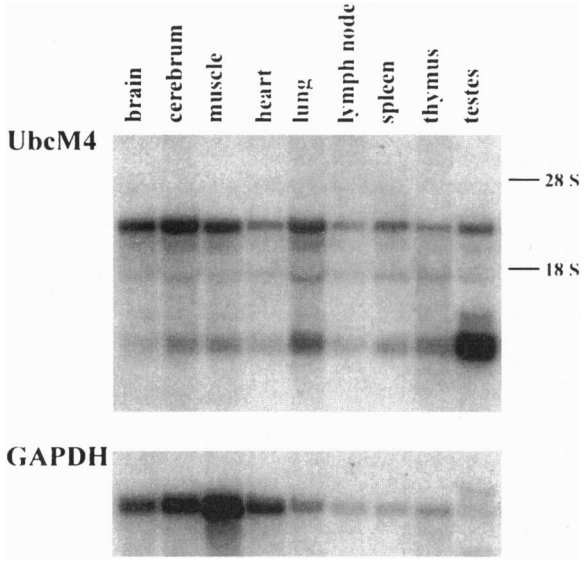

FIG. 5. Expression of the *UbcM4* gene in different organs of adult mice. For the Northern blot analysis of total RNA the *UbcM4* cDNA shown in Fig. 4 was used as a probe. The positions of 28S and 18S rRNAs are indicated. The blot was subsequently rehybridized with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

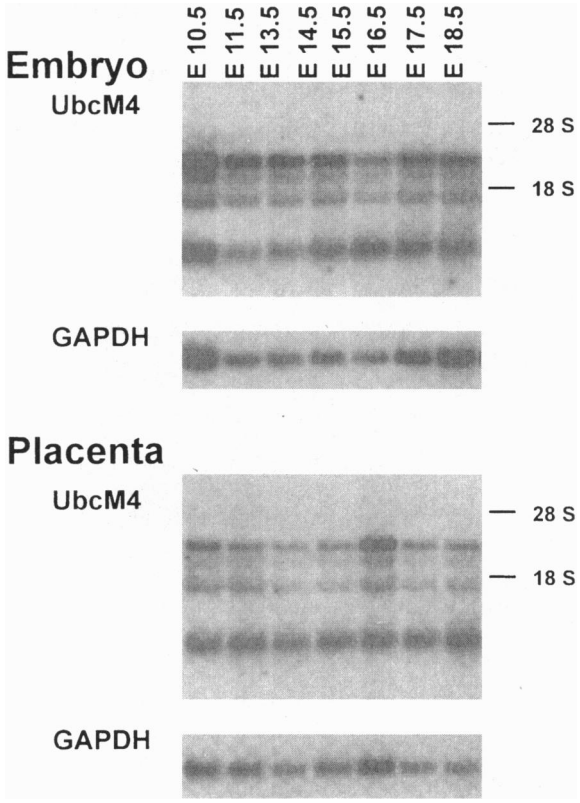

FIG. 6. Expression of the *UbcM4* gene in embryos and placentas at different developmental stages. E10.5–E18.5 represent days 10.5–18.5 of gestation. For details, see the legend to Fig. 5.

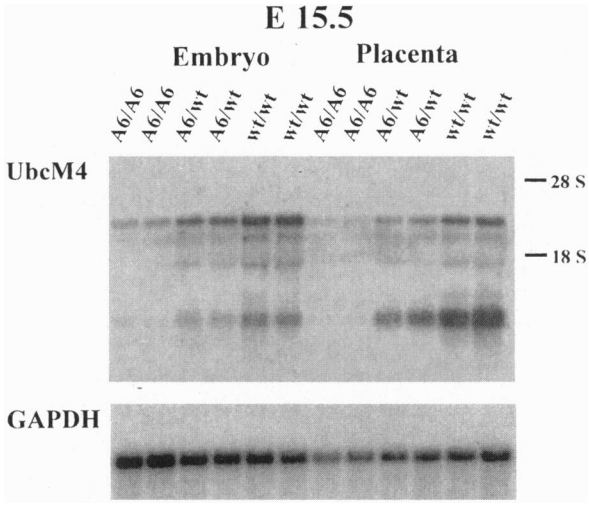

FIG. 7. Expression of the *UbcM4* gene in A6 wild-type and mutant embryos and placentas. RNAs from embryos and their corresponding placentas at day 15.5 of gestation (E15.5) from the same litter of heterozygous A6 parents were analyzed as described in the legend to Fig. 4. The genotypes of the A6 wild-type (wt/wt), heterozygous (A6/wt), and homozygous (A6/A6) embryos were determined by PCR analysis of yolk sac cDNA.

essential cellular functions such as DNA repair, cell-cycle progression, organelle biosynthesis, heat shock resistance, and cadmium tolerance (1, 22). In *Drosophila*, mutation of the *bendless* gene, which encodes an E2 enzyme, affects development of the nervous system (9, 10). In this case a missense mutation in the active site region of an E2 enzyme that is predominantly expressed in the neural system gives rise to the altered phenotype.

We speculate that the lethal phenotype of homozygous A6 mutants is caused by impairment of the placenta, as this is the only organ that consistently showed histological abnormalities in its structure. However, at the present time we cannot exclude subtle alterations within the embryo itself that might contribute to its death. The placenta is essential for embryonic survival beyond day 11.5 of gestation as it forms vascular connections necessary for maternal-fetal exchange of gases, nutrients, and waste products (23). The A6 mutation seems to affect primarily the fetal mesenchyme in the labyrinth layer, as the latter is narrower than in wild-type placentas and characterized by frequent branching and disorganized arrangements of fetal blood vessels. Thus, the transport of nutrients to the embryo may be limited in the damaged labyrinth region of the homozygous mutants. The lethal phenotype of mouse mutants with targeted disruption of the genes for the trophoblast-specific transcription factor Mash-2 and the hepatocyte growth factor/scatter factor has also been attributed to defects in the labyrinth layer (24–26).

Homozygous A6 embryos die at two different stages during development: around day 11.5 of gestation and just before or around birth. This variation in the time of death is not due to differences in the genetic background as the A6 mice were always kept in the same inbred background. The fact that death of A6 homozygotes was first observed around day 11.5 of gestation is in agreement with a placental defect, as mouse mutants with complete abrogation of placental function also die around that time (27–29). Homozygous A6 embryos that live beyond day 11.5 or 12.5 of gestation are growth retarded. We speculate that these embryos form a placenta with sufficient function to allow survival but not normal growth and development. It is, however, unclear why these embryos die perinatally.

The *UbcM4* gene product described in this paper belongs to a family of closely related proteins that participate in ubiquitination and thereby target proteins for degradation (1–3). *UbcM4* consists essentially only of the highly conserved E2 core domain; there are no sequences extending beyond the N terminus of the core, as has been described for the three other known mouse Ubcs (1, 21). The amino acid sequence of *UbcM4* is 100% identical with the recently published human *UbcH7* enzyme. *UbcH7* was shown to be involved in the *in vitro* ubiquitination of p53, non-"N-end rule" substrates, the precursor of NF-κB and c-Fos (20, 30–32). The A6 mutant can serve as a model system to analyze if any of these *in vitro* substrates are also ubiquitinated *in vivo*. In the case of p53, we have so far no evidence that this protein is accumulated in homozygous A6 placentas due to less efficient ubiquitin-mediated proteolysis (A.G. and K.H., unpublished results).

The placenta-specific effect of the mutation cannot be explained by a tissue-specific expression pattern of the *UbcM4* gene or a cell-specific effect of the retrovirus-induced mutation. According to our results, *UbcM4* is expressed ubiquitiously and its transcription is reduced not only in mutant placentas but also in mutant embryos. As the specificity of protein ubiquitination is determined most likely by differential interaction between E2 and E3 enzymes (see, for example, ref. 20), one might speculate that a placenta-specific E3 enzyme in cooperation with the ubiquitously expressed *UbcM4* is necessary for the ubiquitin-mediated proteolysis of as yet unknown proteins required for placenta development.

Several retrovirus-induced mutations in mice occurring either spontaneously or through experimental manipulation have been described previously (33, 34). In at least two cases provirus insertion disrupts expression of cellular genes in a tissue-specific manner (35, 36). A distinct feature of the A6 mutant is the fact that provirus integration results only in a partial inactivation of the *UbcM4* gene: in all tissues of homozygous embryos studied so far the level of steady-state mRNA is reduced by about 70% when analyzed on Northern blots. The mechanism by which the provirus partially inactivates the gene is unknown. One can speculate that integration into the first intron disrupts some regulatory element or that the neomycin resistance gene in the provirus acts as a transcriptional silencer on the *UbcM4* gene as has been shown previously (37). To test these hypotheses, experiments are underway to define the cis-acting elements of the *UbcM4* gene and to delete the neomycin resistance gene from the provirus. The results will contribute to our understanding of how retroviral insertion disrupts mammalian gene expression.

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