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A retroviral gene trap insertion into the histone 3.3A gene causes partial neonatal lethality, stunted growth, neuromuscular deficits and male sub-fertility in transgenic mice

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Spermatogenesis is a complex developmental process involving cell division and differentiation. Approximately half of all sterile males have defects in spermatogenesis or sperm function. An insight into the molecular control points regulating this process might help in treating male infertility. Gene trapping in embryonic stem cells and the generation of transgenic mice represents one route to identify genes expressed during spermatogenesis. The trapped gene is tagged with a *lacZ* reporter gene so that the expression pattern of the gene can be visualized by staining for β-galactosidase activity. We have screened transgenic mouse lines for expression of trapped genes in the gonads. One such trap event was shown to be in the replacement histone 3.3A gene (*H3.3A*). This gene was expressed ubiquitously during embryonic development until 13.5 days post-coitum and in the adult heart, kidney, brain, testes and ovaries. This mutation resulted in postnatal death of 50% of homozygous mutants. Surviving mutants displayed reduced growth rates when competing with wild-type siblings for food. Mutant mice also had a neuromuscular deficit and males displayed reduced copulatory activity. When copulations did occur, these resulted in very few pregnancies, suggesting that mutations in the *H3.3A* gene may contribute to some cases of impaired fertility in man.

INTRODUCTION

Spermatogenesis is the complex developmental process by which diploid spermatogonial cells in the testis give rise to haploid spermatozoa. While the morphological changes that occur during this process are well documented, far less is known about the molecular control points. The WHO estimate that 11.2% of cases of male infertility are caused by defective spermatogenesis (1). This represents ~500 000 males in North America alone. The identification of genes involved in spermatogenesis will increase our understanding of this elaborate developmental process and provide a greater insight into clinical syndromes like infertility and testicular cancer, possibly allowing therapeutic intervention. In addition, an understanding of the control points in spermatogenesis may provide potential new targets for fertility regulation.

To identify genes that may regulate spermatogenesis, we have screened transgenic mice carrying retroviral gene trap insertions for those that show gonadal expression of the trapped gene. The retroviral gene trap contains a promoterless *lacZ* reporter gene which can be used to profile the spatial and temporal expression pattern of the trapped gene (2). In addition, the generation of a fused RNA transcript between the trapped gene and vector sequences facilitates cloning of the trapped gene (3). One mouse line with *lacZ* expression in the gonads was cloned and the trapped gene identified as the replacement histone 3.3A gene (*H3.3A*).

The histones are a family of basic proteins that organize the structure of DNA within the cell nucleus. The core histones (H2A, H2B, H3 and H4) pack the DNA into nucleosomes which are arranged into a higher level of chromatin structure by binding to the H1 linker histone (for a review see ref. 4). In mammals, each histone class except H4 is subdivided into several subtypes or variants which show tissue-specific patterns of expression (5,6). This is particularly evident during spermatogenesis, when chromatin undergoes substantial reorganization and substitution of histones by stage-specific subtypes (7). Indeed, a number of testis-specific histones have been identified, including TH2A, TH2B (8), TH3 (9) and H1t (10).

The majority of histone genes are expressed during DNA replication. There is, however, a subset of replication-independent histones or replacement histones where expression is not coupled to the cell cycle. This group includes the histone subtypes H1°, H2A.X, H2A.Z and H3.3. In contrast

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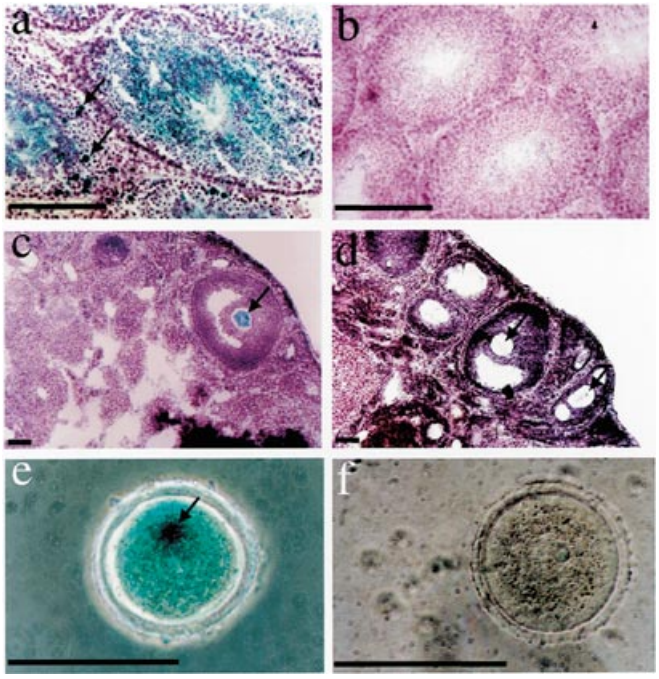

Figure 1. β-Gal activity in the gonads of mice from the 14E7 gene trap line. Cryosections of testes (**a** and **b**), ovaries (**c** and **d**) and oocytes (**e** and **f**) stained for β-gal activity and counterstained with haematoxylin and eosin. Samples from 14E7 heterozygotes (a, c and e) are compared with wild-type controls (b, d and f). All scale bars, 100 μm.

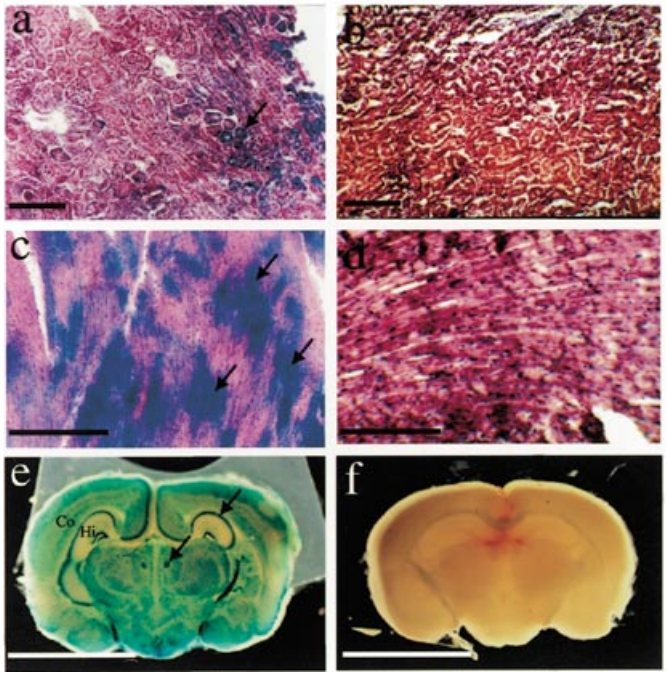

Figure 2. β-Gal activity in non-gonadal tissues. (**a**) Transgenic kidney, proximal convoluted tubule is arrowed; (**b**) wild-type kidney; (**c**) transgenic heart; (**d**) wild-type heart; (**e**) transgenic brain, cell bodies of hippocampus and fasciculus retroflexus are arrowed; (**f**) wild-type brain. (a–d) Cryosections; (e and f) 2 mm brain sections. Scale bars: (a–d) 100 μm; (e and f) 0.5 cm.

to mammalian replication-dependent histone genes, which are organized into two clusters in the genome, replacement histone genes are solitary, encode polyadenylated mRNA, have long untranslated regions (UTRs) and some, including H3.3, also contain introns (for a review see ref. 7). The function of the replacement histones is still largely unknown, although they have been repeatedly described in connection with differentiated and non-proliferating cells (11–15).

Two *H3.3* variants exist among vertebrates; *H3.3A* and *H3.3B*. These genes encode identical proteins that differ from the H3.1 and H3.2 proteins at amino acids 89, 90 and 96 (16). The two *H3.3* genes differ extensively in their 5′- and 3′-UTRs, have different genomic structures and have been shown to be differentially expressed during differentiation processes (15,17). Gene trapping the *H3.3A* gene has allowed us to assess the function of this gene during development and in the adult. This disruption results in some neonatal mortality while surviving mutants display stunted growth, neuromuscular deficits and sub-fertility in adult males.

RESULTS

Expression pattern of the trapped gene

Screening of 14 gene trap strains of mice identified a line with *lacZ* reporter gene expression in the testis and ovary (GTE.14E7). In the adult testis, staining occurred within seminiferous tubules and the most intense staining appeared to be associated with maturing germ cells rather than spermatogonia (Fig. 1a). Staining was particularly intense in what appeared to be primary spermatocytes (Fig. 1a, arrowed) and was also observed in haploid spermatids and the tails of spermatozoa. Staining in Sertoli cells was restricted to the cytoplasm of residual bodies removed from developing spermatids. No staining was observed in Leydig cells. To investigate the temporal expression pattern of the trapped gene, testes were sectioned and stained for β-galactosidase (β-gal) activity during the first spermatogenic wave at puberty. Testes from 14E7 heterozygotes showed faint punctate staining at 5 days post-partum indicative of low levels of expression prior to the initiation of spermatogenesis (data not shown). As the first cycle of spermatogenesis progressed, the intensity of staining increased, consistent with expression in spermatocytes. An identical staining pattern was observed in homozygous mice. In the ovary, β-gal activity was confined to developing eggs (Fig. 1c, arrowed); staining was found throughout the egg cytoplasm as well as in an intense blue spot close to the germinal vesicle (Fig. 1e).

In the adult kidney, staining was restricted to some proximal convoluted tubules (Fig. 2a, arrowed), although staining was not observed in all tubules. No staining was observed in the renal corpuscles. Patchy staining was also found in the atria and ventricles of the heart (Fig. 2c, arrowed). The patchy staining in the kidney and the heart probably represents a fixation artifact. The adult brain showed widespread staining, with strongest expression in cell bodies, including the olfactory bulbs, striatum, hippocampus (Fig. 2e, arrowed) and fasciculus retroflexus (Fig. 2e, arrowed), lateral hypothalamus, cortex and cerebellum.

During embryonic development, β-gal activity was found at 7.5 days post-coitum (d.p.c.), when gene expression was associated

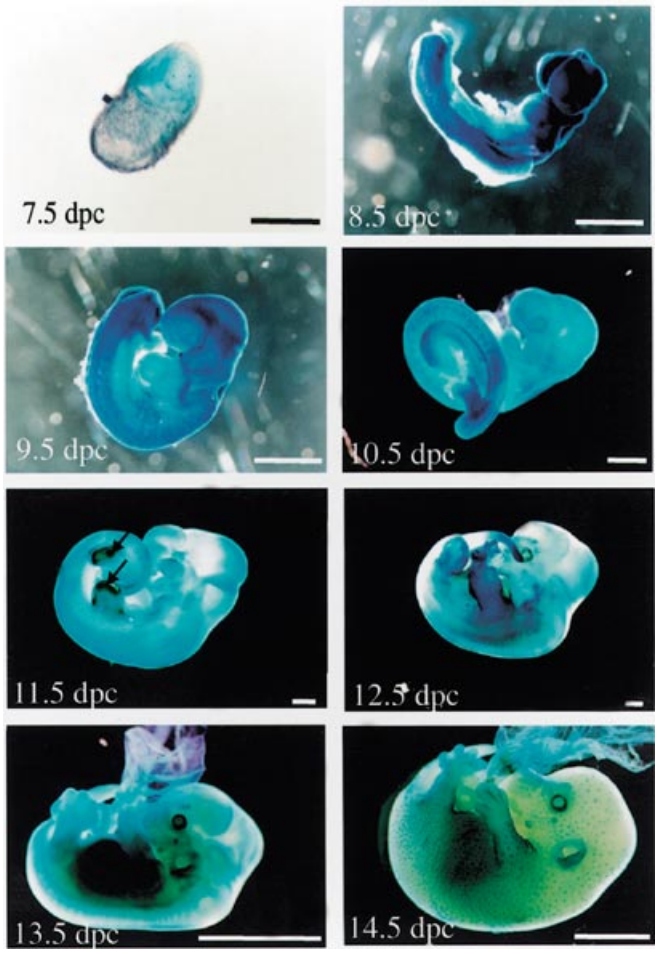

Figure 3. β-Gal activity in mid-gestation embryos. Scale bars: 7.5 d.p.c., 150 μm; 8.5 d.p.c., 0.5 mm; 9.5–12.5 d.p.c., 1 mm; 13.5 and 14.5 d.p.c., 0.5 cm.

with the developing embryo but not the extra-embryonic tissues (Fig. 3). Widespread embryonic expression was seen from 8.5 to 10.5 d.p.c. but at later stages of development *lacZ* expression became more restricted (Fig. 3). Between 11.5 and 12.5 d.p.c., intense staining was detected on the apical surface of developing limbs (Fig. 3, arrowed), while at 14.5 d.p.c. staining was concentrated in the developing hair follicles and the outer ear.

Identification of the trapped gene

Northern analysis revealed a transcript of ~4.1 kb when hybridized to a *neo*-specific probe (data not shown). This transcript is ~200 bp larger than the expected retroviral gene trap transcript, indicating a fusion transcript between retroviral *lacZ*/*neo* sequences and the trapped gene.

Inverse PCR from testis cDNA identified 36 bp of fused 5′-sequence. The cloned cDNA flank was found to have 100% homology to the *Mus musculus* histone variant gene *H3.3A*. Comparison with published mouse *H3.3A* cDNA sequence (18) and human genomic sequences (GenBank accession no. M77656) indicated that the gene trap vector had integrated at position 751 of the 1537 bp first intron upstream of the ATG translation start.

To confirm that the cloned *H3.3A* sequence was the true flanking sequence, primers specific for the *H3.3A* 5′-UTR were used with *lacZ* primers to amplify cDNA from transgenic testis RNA. All PCR products were of the expected sizes for a fused transcript from the *H3.3A* gene (data not shown). In addition, an intron 1-specific probe hybridized to a single 1.5 kb *Stu*I band using wild-type DNA and to an additional 8 kb band in DNA from heterozygotes (Fig. 4A). In homozygote mutants, only the larger band was detected, confirming trapping of the *H3.3A* gene (Fig. 4A).

Retroviral insertion into *H3.3A* creates a hypomorphic mutation

Expression of the *H3.3A* gene during mid-gestation development was analysed by northern blotting. A 1.3 kb transcript was detected from 11.5 to 16.5 d.p.c. (Fig. 4B), which corresponds with the embryonic β-gal staining pattern observed for the trapped gene (Fig. 3). To determine whether insertion of the gene trap vector had generated an *H3.3A* null allele, northern analysis was performed on RNA isolated from 16.5 d.p.c. mutant embryos (Fig. 4C). The *H3.3A* probe detected a 1.3 kb transcript in wild-type embryos but no transcript was detected in homozygous mutant embryos (Fig. 4C, $n = 9$). However, the more sensitive RT–PCR using an exon 1 primer specific for the *H3.3A* gene and an exon 4 primer revealed some wild-type *H3.3A* transcript (Fig. 4D). Preliminary experiments to determine the level of *H3.3A* transcripts in mutant mice by semi-quantitative RT–PCR indicate a 4–7 times reduction compared with normal levels (data not shown). To eliminate artifactual amplification of *H3.3A* pseudogenes in this assay, samples were treated with DNase and shown not to support a PCR reaction in the absence of reverse transcription. These data indicate that the retroviral insertion has created a hypomorphic mutation with reduced levels of *H3.3A* mRNA below the limits of detection by northern blotting.

Disruption of *H3.3A* causes some neonatal lethality

H3.3A mutants were born in approximately Mendelian ratios (+/+, 23; +/–, 38; –/–, 20). Birth weights of homozygous mutant pups were not significantly different from litter mates [+/+ and +/–, 1.51 ± 0.07 g ($n = 61$); –/–, 1.44 ± 0.06 g ($n = 18$)] and could not be distinguished from them. However, 50% (10/20) of the mutants died within 24 h, which was statistically significant ($P < 0.005$).

Homozygous mutant pups show reduced growth rates in competition with littermates

Surviving homozygous mutant pups showed significantly reduced growth rates ($P < 0.0001$) from as young as 1 day postpartum. The differences in weight between mutant and non-mutant siblings increased during the first month after birth (Fig. 5A). As non-mutants approached adult body weight the mutants continued to grow, reducing the difference in body weight (Fig. 5B). However, at 4 months old, homozygous mutants were still significantly smaller (+/+, 30.41 ± 3.39 g versus –/–, 24.75 ± 1.15 g; $P = 0.016$). Culling of non-mutants between 3 and 10 days after birth resulted in normal growth of mutant pups (Fig. 5A), suggesting that the reduced growth rates were due to competition with non-mutant littermates.

The surviving mutants began to lose weight around weaning (~18 days after birth). Initially this weight loss appeared to be

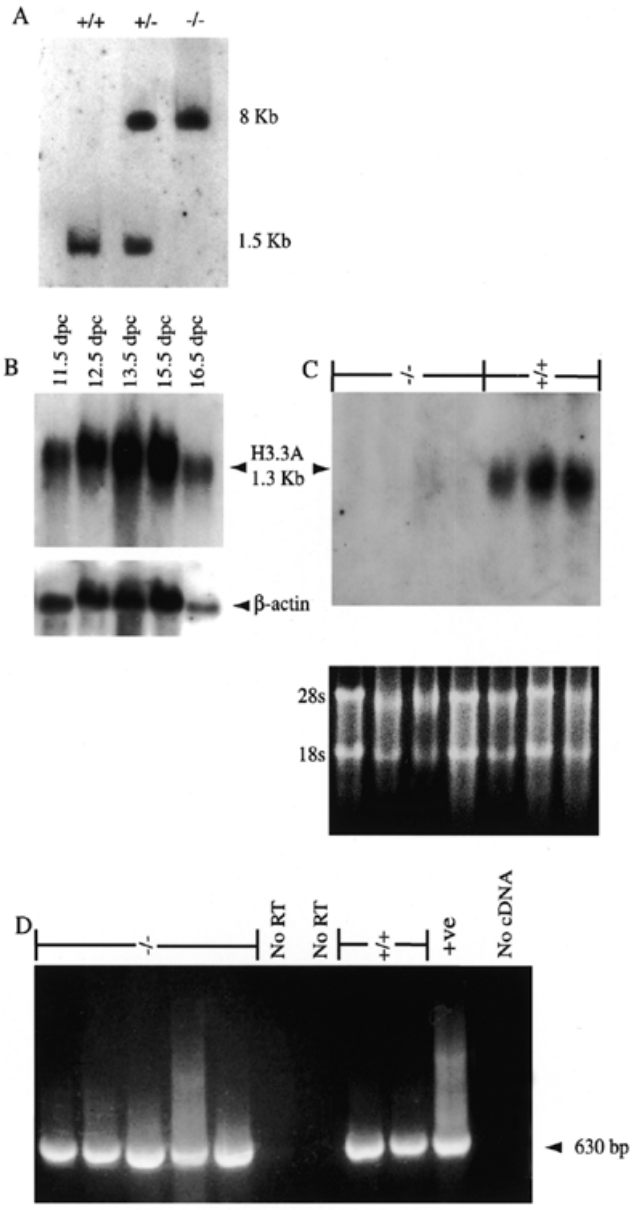

Figure 4. Molecular characterization of the *H3.3A* gene trap. (**A**) Southern analysis of the gene trap insertion into the *H3.3A* gene. Hybridization of an *H3.3A* probe to wild-type (+/+) DNA detected a single 1.5 kb band. DNA from a heterozygote (+/–) displayed this wild-type band in addition to an 8 kb band. Only the 8 kb fragment was detected in homozygous mutants (–/–). All DNA samples were digested with *Stu*I. (**B**) *H3.3A* gene expression in mid-gestation embryos. RNA from wild-type embryos was probed with an *H3.3A*-specific probe and then stripped and re-probed with β-actin to show similar RNA loadings per lane. (**C**) Northern analysis of *H3.3A* transcripts in 16.5 d.p.c. embryos. +/+, wild-type; –/–, homozygous mutant. (**D**) Detection of *H3.3A* transcripts in homozygous mutant embryos by RT–PCR. Primers in exons 1 and 4 were used to amplify a 630 bp product from RNA isolated from 16.5 d.p.c. embryos. No amplification was seen without a reverse transcription step or if no cDNA was included in the reaction. +/+, wild-type; –/–, homozygous mutant.

terminal but it was found that if pups were given food on the floor of the cage from day 18, the weight loss was minimized and did not result in death. Wet food was therefore provided until mutants were ~2 months old. At this age mutants were able to maintain their weight on the standard diet in a food hopper.

Mutant mice show impaired tasks involving neuromuscular activity

The ability of the mice to hang from a wire grid was assessed. By 19 days after birth, control mice (wild-type or heterozygotes) were able to hang suspended from the grid for an average of 31.5 ± 1.7 s (n = 6) and this increased to the maximum recorded time of 60 s by day 21 (Fig. 5C). In contrast, age- and weight-matched mutants performed poorly at this task (6.7 ± 1.0 s at 19 days and 11.0 ± 1.7 s at 21 days; n = 6). Although the ability of the mutant mice to hold onto the grid increased up to 35 days old (after this time no significant improvement was seen), on average they could only grip the grid for a maximum of 45.1 ± 5.15 s.

To investigate whether the impaired cage grip could result from a neuromuscular deficit, 10 control mice (five heterozygotes and five wild-type) and five age- and weight-matched mutants (6 weeks old) were put through a standardized behavioural screen [SHIRPA test (19)]. This revealed significant differences (P = 0.01–0.005) between mutants and control mice in tests that evaluated neuromuscular functions (grip strength, wire manoeuvre and muscle tone). Unfortunately these tests do not differentiate between the neurological and muscular components of the phenotype. Comparison of the weight and gross morphology of the flexor digitorum from five homozygous mutants and six wild-type (129 Sv/Ev) controls detected no differences between the two groups.

Mutant mice show reduced fertility

Since expression of the *H3.3A* gene trap was initially identified in the gonads, matings were set up to assess fertility of both male and female homozygous mutants. Four of six homozygous mutant females were fertile; the other two did not breed despite being housed with a total of four wild-type males over a 4 month period. The proportion of infertile females is significantly different from wild-type 129 Sv/Ev females (69/71 matings produced offspring; P = 0.028). However, given the small number of mutant females examined, further studies are required. Ovarian morphology of the two infertile females appeared normal.

Over an 8 week period, four wild-type males plugged a total of 65 females, of which 48 became pregnant (Table 1). In contrast, six homozygous mutant males set up under identical conditions plugged only 16 females (5/6 males produced at least one plug), only one of which became pregnant and gave birth to four heterozygous pups. The number of copulations per male, as well as the number of pregnancies per copulatory plug, was significantly lower for mutants than for non-mutants (P < 0.0001) (Table 1). Analysis of the male reproductive tract revealed no obvious differences in the testis (n = 6), epididymis (n = 5) or vas deferens (n = 4) and sperm numbers appeared normal.

DISCUSSION

We have characterized a retroviral gene trap line initially identified as showing expression of the trapped gene in the gonads. The retroviral gene trap vector had inserted into the first intron of the *H3.3A* gene. Previous reports have noted the presence of a 71 bp region within this intron with 90% identity between human and chicken sequences (20). This region is just

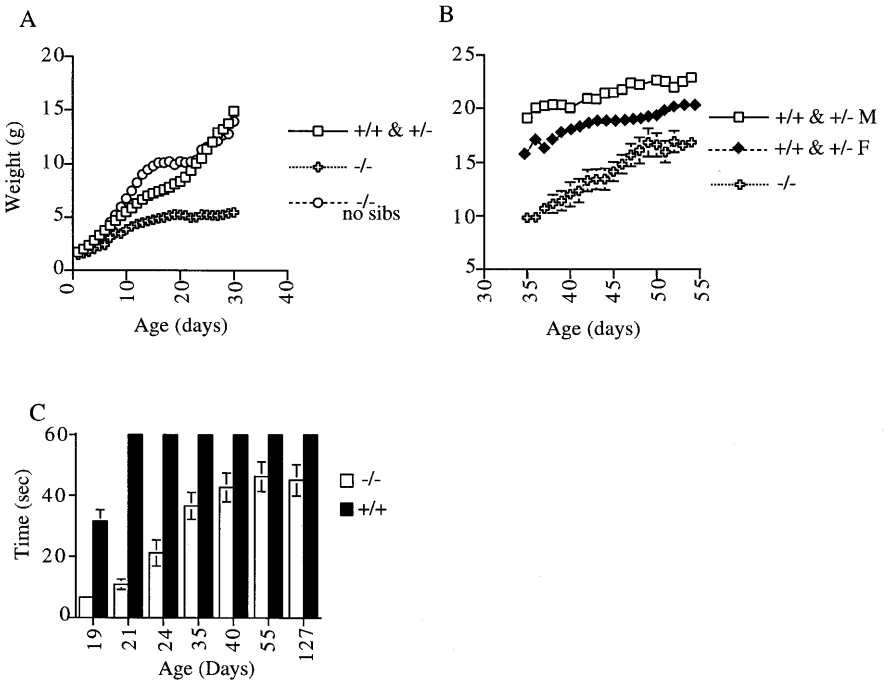

Figure 5. Growth and agility of homozygous mutants. (**A**) Weight gain of *H3.3A* mutants (plus symbols, *n* = 7) and non-mutant litter mates (squares, *n* = 30) without culling of non-mutants. Homozygous mutant pups grow normally after culling non-mutant siblings (circles, *n* = 4). (**B**) Difference in weight gain becomes less after 35 days post-partum (*n* = 5 for all groups). M, males; F, females. (**C**) Ability of mice to hang from a wire grid. Age- and weight-matched mice were placed onto a wire grid which was then inverted. Mice were timed for 60 s or until they fell off. Open bars, mutants (*n* = 6); closed bars, wild-type littermates (*n* = 6). The size of the mutants had no effect on cage grip times. The possibility of a learning effect was ruled out by repeating the experiment with separate groups of pups tested at 19 and 55 days old. Results from this group were similar to those shown above. +/+, wild-type; +/–, heterozygote; –/–, homozygous mutant.

Table 1. Analysis of male fertility in mice homozygous for gene trap integration into the *H3.3A* gene

	Wild-type (%)	Homozygous mutant (%)	*P* value
Females plugged	65/95 (68)	16/160 (10)	<0.0001
Matings resulting in pregnancy	48/65 (74)	1/16 (6)	<0.0001

Male and female mice were caged together and females checked daily for the presence of a copulatory plug in the vagina. Plugged females were removed and housed in isolation for 21 days to determine whether pregnant.

upstream of and includes the first of three thymidine tracts (21) which act as RNA polymerase II transcription termination sites in the human gene (20). Outside this region, the human and chicken sequences show no significant similarity. Comparison between the human and mouse intron 1 sequences revealed similar thymidine tracts ~200 bp upstream of the retroviral insertion site, although 9 bp of sequence around the second thymidine tract are complementary, rather than identical, to the corresponding region in the human intron. It is not yet known whether these sequences have a regulatory function in the mouse or whether insertion of the retrovirus will affect any function.

The *H3.3A* gene is expressed as a single 1.3 kb transcript in many different tissues (15,17,22). There are, however, differences in the level of expression between tissues and different stages of development (15,22). The widespread embryonic expression of *H3.3A* (17,22) is reflected by the *lacZ* expression in heterozygous embryos. Thus, *lacZ* expression resulting from insertion of the retroviral gene trap vector into intron 1 of the *H3.3A* gene appears to follow the expression pattern of the endogenous gene.

The absence of detectable *H3.3A* mRNA by northern blot analysis of homozygous mutant embryos (16.5 d.p.c.) demonstrates a reduction in *H3.3A* transcripts, although detection of transcript by RT–PCR indicated that the mutation is not an absolute null but represents a hypomorphic mutation. These results, together with the absence of a detectable phenotype at birth, suggest that wild-type levels of H3.3A are not essential for embryonic development. Survival of 50% of homozygous mutants after birth suggests that the residual level of *H3.3A* transcript might allow survival of these mice. Alternatively, the presence of multiple copies of functional *H3* genes, including *H3.3A* pseudogenes and the *H3.3B* gene, which encodes an identical protein, might allow some functional redundancy. Compensation in the histone family of genes has been shown *in vitro*. Up to half the histone genes located within the two major histone clusters have been deleted in a chicken B cell line (DT40). These deletions resulted in an up-regulation of other histone sub-types, thus resulting in no detectable change in total levels of H3 transcript (23). Although extrapolation to an *in vivo* system is difficult, disruption of the murine H1° gene by homologous recombination failed to reveal any abnormal phenotypes (24). Similar results have also been reported in other organisms such as *Tetrahymena* and *Xenopus* (reviewed in ref. 25). It is not clear, however, why some of the mutants die while others manage to survive, unless the level of functional compensation is variable

between individuals, perhaps because of penetrance differences. It is unlikely to result from modifier gene effects since the mice are maintained on an inbred genetic background.

The cause of the neonatal lethality has not yet been determined. No gross abnormalities were detected by histological examination of the major organs shortly after birth. However, it was not possible to determine whether the pups analysed would have survived or died. It is possible that the tissues examined were taken from those homozygous mutants that would have survived and may therefore not be expected to display an abnormal phenotype.

The observed neuromuscular phenotype may explain the terminal weight loss that coincides with weaning. At weaning, pups are too small to be able to feed on solid food without grasping the food hopper with their front feet. As mice continue to grow, their need to grip the food hopper during feeding declines and their ability to grip a wire grid improves. Together these factors could explain why mutants do not require special feeding as adults. Interestingly, rippling muscle disease, an autosomal dominant disorder characterized by electrically silent percussion-induced muscular contractions, maps to the same location on human chromosome 1 (q41–42) as *H3.3A* (26). Thus, *H3.3A* represents a candidate gene for this syndrome.

The neuromuscular disorder may also contribute to the reduced fertility of these animals by compromising their copulatory performance. However, the infrequency of mating is not the sole cause of male sub-fertility, as the average number of pregnancies per copulatory plug is also significantly reduced. The male reproductive tract appears histologically normal. It is possible that sperm are not correctly capacitated in the female reproductive tract, thus resulting in reduced motility. These data are the first to show that the mammalian H3.3A protein has a role in gamete function and fertility. It is possible that mutations within this gene may also contribute to some cases of human male infertility.

MATERIALS AND METHODS

Generation of transgenic mouse lines

The ROSAβgeo retroviral gene trap vector was kindly provided by P. Soriano (Fred Hutchinson Cancer Research Center, Seattle, WA) and contains the splice acceptor sequence from the adenovirus major late transcript adjacent to the selectable marker and an in-frame fusion between the *lacZ* and neomycin resistance genes (*neo*) (2). CCE-1B embryonic stem (ES) cells, grown on mitotically inactivated STO feeders, were infected with retroviral supernatant at a low multiplicity and gene trap events selected in G418 (200 μg/ml; Gibco BRL, Paisley, UK). Mice carrying the gene trap mutation were generated by injection of ES cell clones into host blastocysts and crossing of germline chimeras with 129 Sv/Ev mice.

Staining for trapped gene expression

Tissues were mounted in OCT (Tissue-Tek; Miles Scientific, Elkhart, IN) and snap frozen in liquid nitrogen. Cryostat sections of 15 μm were cut, attached to slides and fixed for 5 min in 0.4% paraformaldehyde/phosphate-buffered saline (PBS), washed three times in PBS, pH 7.4, and stained overnight in PBS containing 2 mM $MgCl_2$, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 0.4 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). After staining, sections were fixed in 4% paraformaldehyde and counterstained with haematoxylin and eosin. Brains were cut into 2 mm slices before whole mount staining.

Timed matings were set up between transgenic males and wild-type females with the plug day defined as 0.5 d.p.c. Isolated embryos (3.5–12.5 d.p.c.) were fixed in 0.4% paraformaldehyde/PBS for 5–15 min and washed thoroughly in PBS before staining.

Northern blotting and RT–PCR

Total RNA was isolated from tissues using TRIzol reagent (Gibco BRL). Aliquots of 20 μg of RNA were size fractionated through a denaturing 1% agarose–2.2 M formaldehyde gel and transferred to Hybond N membrane (Amersham Pharmacia, Little Chalfont, UK) by capillary action. Hybridization was performed overnight with ^{32}P-radiolabelled probes and filters were washed at a final stringency of 0.1× SSC, 0.1% SDS at 65°C. cDNA was synthesized from total RNA by incubation with random hexanucleotide primers and M-MuLV reverse transcriptase (Boehringer Mannheim, Lewes, UK) according to the manufacturer's protocol. Standard PCR reactions contained 1× PARR buffer (Cambio, Cambridge, UK), 1.5 mM dNTP, 200 μM each primer, 1 U *Taq* polymerase (Stratagene, Amsterdam, The Netherlands). Wild-type *H3.3A* transcripts were detected using an exon 1-specific primer (accgcctctcgcttgccgag) which does not hybridize to the *H3.3B* gene, and a return primer located in exon 4 (ggaaacgccaatacctgattctg).

Cloning of the trapped gene by inverse PCR

Cloning of flanking exon sequence was performed as described previously (3). After nested PCR using primers designed to *lacZ* (VC7, ccagggttttcccgtca; VC8, gccatgtcacagatcatc; LZ144, ccgtctgaatttgacctgagcgcat) amplification of flanking sequence was confirmed by Southern analysis using an internal *lacZ* oligonucleotide probe (VC9, cgaagagtttgtcctcaacc). The amplification product was cloned into pCR2.1 (Invitrogen, Groningen, The Netherlands) and sequenced. This sequence was used to search the GenBank/EMBL database for homology to published sequences using the BLASTN program.

Southern analysis to confirm the disruption event

An aliquot of 10 μg of genomic DNA was digested with *Stu*I and hybridized with a probe specific for intron 1 of the *H3.3A* gene 5′ of the insertion site. Membranes were washed at a final stringency of 0.1× SSC, 0.1% SDS at 68°C for 10 min.

Sequencing intron 1 from the murine *H3.3A* gene

H3.3A intron 1 sequence flanking the retroviral insertion was amplified by PCR using primers located within the intron (5′, H3.3A, accgcctctcgcttgccgag; 3′, H3.3E2F2, cagtcttgcgagcggcttttgta) and retroviral LTR-specific primers (5′, RLTR3, cttgtggtctcgctgttcctt; 3′, 337, gctagcttgccaaacctacaggt). These products were cloned into pCR2.1 and sequenced. Sequence data were deposited with GenBank (accession no. AJ237671).

Genotyping mice by PCR

Genomic DNA was prepared from tail biopsies. Initially PCR primers designed to the *neo* gene sequence (323, gctagcttgccaaacctacaggt; 784, gggtggagagcgtattcggctat) were used to identify heterozygote animals (cycle conditions: 95°C for 5 min denaturation step followed by 35 cycles of 93°C for 30 s, 60°C for 30 s, 70°C for 1 min). Once genomic flanks had been cloned, primers 5′ and 3′ to the vector insertion (H3.3A5′IR, cgtggcaggggatggggtattct; H3.3AE2F2, cagtcttgcgagcggctttgta) along with an LTR-specific primer (337, gctagcttgccaaacctacaggt) were used to amplify wild-type and mutated alleles. PCR results were initially confirmed by Southern blotting.

Histological analysis

Neonatal and adult tissues were examined for histological defects. Tissues were fixed in 4% formalin, dehydrated through a series of alcohols, wax embedded and 7 μm sections cut and stained with haematoxylin and eosin.

Behavioural testing

Mice (19–127 days post-partum) were placed on a wire grid consisting of 5 mm parallel bars 1 cm apart which was then slowly inverted. The grid was held ~20 cm from the bench top to discourage the mouse from jumping off. The time until the mouse fell off was measured up to a maximum of 60 s. The test was repeated six times per mouse with 30 s rest between each test. SHIRPA testing (SmithKline Beecham Pharmaceuticals, Harwell MRC Mouse Genome Centre, Imperial College School of Medicine and Royal London Hospital Phenotype Assessment) was also performed (19).

Fertility testing

Males (six mutant and four wild-type) were housed in individual cages. Three wild-type 129 Sv/Ev females were placed into each cage and checked for a vaginal plug daily. Plugged females were removed and stored for 21 days to check for pregnancy. The fertility of females was examined by housing each mutant female with one wild-type 129 Sv/Ev male. Males were changed after 3 weeks if females did not become pregnant.

Statistical analysis

Fisher's exact test was used to analyse deviation from Mendelian ratios, postnatal survival, frequency of matings/pregnancies and the SHIRPA testing. The Mann–Whitney *U*-test was used to compare averages of results obtained in the analysis of pup, testis and muscle weights.

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