A novel transgenic line of mice exhibiting autosomal recessive male-specific lethality and non-alcoholic fatty liver disease

Vincent E. Sollars, Benjamin J. McEntee, Julie B. Engiles, Jay L. Rothstein and Arthur M. Buchberg*

Kimmel Cancer Center, Thomas Jefferson University, 233 South 10th Street, Philadelphia, PA 19107, USA

Received July 2, 2002; Revised and Accepted August 16, 2002 DDBJ/EMBL/GenBank accession nos. XM_129372, XP_129372

We have isolated a Meis1a transgenic mouse line exhibiting recessive male-specific lethality and non-alcoholic fatty liver disease (NAFLD), which coincides with pubescence and is androgen-dependent. The phenotype is due to disruption of an endogenous locus, since other Meis1a transgenic lines do not exhibit these phenotypes. Necropsy analysis revealed hepatic microvesicular steatosis in pubescent male homozygous mice, which is absent in transgenic females. The transgene insertion site was localized to chromosome 1 and further refined by cloning the flanking regions. Sequence analysis shows that the integration site disrupts a putative metallo-β-lactamase gene with a 21.3 kb deletion encompassing exons 5–7.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) consists of a broad spectrum of diseases, all of which are associated with the accumulation of lipid-containing vesicles in hepatocytes (1). If this condition is associated with parenchymal inflammation, it is categorized as a subtype of NAFLD first recognized by Ludwig and colleagues (2) and termed non-alcoholic steatohepatitis (NASH). NAFLD has a risk of becoming aggressive if it is found to include hepatocyte ballooning and necrosis, with or without fibrosis, Mallory bodies or cirrhosis (1–7). The major endogenous risk factors thought to be associated with this disease are obesity, female gender, hyperlipidemia and diabetes (1–3,5). However, an investigation has revealed that these risk factors are not always associated with the disease, and consequently other factors must also play a role (8).

Although NAFLD can be a non-progressive disease, some cases of NAFLD degenerate into NASH and become associated with cirrhosis of the liver and death. Additionally, it has been reported that the degeneration of NAFLD into irreversible liver damage can occur fairly rapidly (8). The prevalence of NAFLD is high, with an incidence of 10–24% of the general population in different countries. In obese individuals, the incidence is even greater, with a prevalence of 57.5–74.0% (9,10). As the trend of obesity increases in the modern world, NAFLD has the potential to become one of the most common forms of liver disease.

A unique phenotype was revealed when examining Meis1 transgenic mouse lines generated in our laboratory. Myeloid ecotropic viral integrarion site 1 (Meis1), a member of the three-amino-acid loop extension protein (TALE) homeo-domain-containing gene family, was first identified in our laboratory as a common site of viral integration in murine myeloid tumors (11). Overexpression of Meis1 and Homeobox A9 (Hoxa9) in murine bone marrow cells results in acute, aggressive, polyclonal leukemia resembling acute myeloid leukemia in humans (12). Meis1 plays a functional role in the translocation of Pbx proteins, another class of TALE homeodomain proteins, into the nucleus (13), and also has additional unknown functions.

Analysis of Meis1 transgenic lines showed that the Tg2770 line exhibited pronounced death of males around puberty, at 3–8 weeks. Further studies determined that the premature morbidity was associated with males homozygous for the transgene. Since the male lethality is only observed in the Tg2770 line and not with other Meis1 transgenic lines, it is most plausible that the lethality is due to an insertional mutation rather than the expression of the transgene. The unusual phenotype of this transgenic line and the possible implications of our data for understanding gender-specific gene expression prompted further analysis.

This paper presents data generated from our analysis of the Tg2770 transgenic mouse line. Tg2770 was examined at both the pathological and molecular levels to determine the cause of the defect. The results include analysis of several organ systems...
Pathology of the Tg2770 line

When heterozygous mice for the transgene were intercrossed to generate homozygotes, we observed peripubescent lethality of homozygous Tg2770 males (but not females). The homozygous males had a mean time of death of 39.5 days post partum (p.p.) (Fig. 1B); heterozygous males and both homozygous and heterozygous females were unaffected. The lethality in these males was rapid, occurring within 12–24 hours of the first signs of distress. Inheritance of the peripubescent lethality suggests that a single autosomal recessive locus is responsible for the phenotype. Furthermore, lethality is not background-dependent, because the phenotype is still present after the Tg2770 transgene was backcrossed onto the Mus castaneus, C57BL/6J or BXH2 inbred strains (Fig. 1B and data not shown). Southern blot analysis indicated that the copy number of the transgene was 1–2 (data not shown), and there was a 100% concordance between homozygous males and lethality.

In addition to the peripubescent lethality, the homozygous males were smaller than their littermates. Just prior to morbidity the homozygous male Tg2770 mice exhibited scruffy fur, extreme lethargy, respiratory distress, pinkish/purplish skin color and prominent blood vasculature of the extremities.

Necropsy of moribund male homozygous Tg2770 mice revealed a slightly enlarged, salmon-colored liver with visible small, pale granules dispersed throughout the organ. No defects in the heart, lung, kidney and brain were found upon gross examination. Hematoxylin and eosin (H&E) sections of the livers of these mice revealed extreme accumulation of small vesicles (Fig. 2A), which upon specific staining with osmium tetroxide (OsO4) were shown to be composed of lipids (Fig. 2C). There is no indication of inflammatory cells, biliary stasis, bile duct proliferation or cellular necrosis accompanying the steatosis of the liver. Thus, the transgene integration in the homozygous condition produces microvesicular steatosis of the liver in male mice. Necropsy also revealed that the seminal vesicles and surrounding fat pads were underdeveloped in these mice, although the testes appeared to be unaffected and functional, since male homozygous mice that survived long enough to breed were fertile.

Androgen sensitivity of the phenotype

Since the phenotype of male lethality was strikingly coincident with puberty, it seemed likely that androgen signaling could be necessary for the lethality. To test this hypothesis, we used the ArTfm mutation, which is an X-linked mutation. The ArTfm allele results from a single base deletion in exon 1 of the androgen receptor. This results in an almost complete absence of androgen receptor transcripts, and hemizygous males are insensitive to androgens. Thus, hemizygous males are genetically male, but phenotypically female (15). We used an outcross to introduce the ArTfm allele into the Tg2770 line, followed by a backcross to the original Tg2770 line to generate homozygous Tg2770 mice (Fig. 3).

Tg2770 homozygous mice also hemizygous for ArTfm exhibited rescue of the male-specific lethality (Fig. 3A). All 12 of the Tg2770/Tg2770, ArTfm/Y progeny survived at

as well as sequencing studies of the integration site of the Meis1 transgene. The Tg2770 transgenic line provides a novel model for NAFLD, as well as male-specific lethality.

RESULTS

Derivation of the Tg2770 transgenic line

In our efforts to determine the role of Meis1 in leukemogenesis, Meis1 transgenic mice were generated. The transgenic construct was made by placing a Meis1a (the most common transcript) cDNA under the control of an immunoglobulin heavy-chain enhancer and promoter region (14) (Fig. 1A).

Multiple founders that contained the transgene were generated, which upon further analysis showed transgene mRNA expression in three of these lines (data not shown). The Tg2770 line was generated on a pure FVB/NJ inbred background and is one of the lines that was found to exhibit transgene mRNA expression.

Figure 1. The Meis1a transgene and Tg2770 male-specific lethality. (A) The Meis1a open reading frame (ORF) was inserted into the pGem-7Zf– vector from Promega (Madison, WI) containing additional immunoglobulin heavy-chain promoter/enhancer element sequences (14) and SV40 transcription stop sequences. The construct was placed in the MCS of the vector for DNA production, then excised by restriction enzyme digestion and purified for microinjection to generate transgenic mice. (B) Kaplan–Meier plot of the lethality data in the Tg2770 strain. The solid black line represents data for the Tg2770 mutation on the original FVB background, while the gray line shows the results of a single outcross of Tg2770 heterozygotes to Mus castaneus followed by a backcross to Tg2770 to generate homozygous males. Heterozygous males and heterozygous or homozygous females exhibited no premature morbidity. A total of 118 homozygous males were scored on the FVB background, resulting in a mean lifetime of 39.5±14.5 days. A total of 24 homozygous males were scored on the M. castaneus background, resulting in a mean lifetime of 40.5±8.3 days. There is no significant difference in the mean lifetime on either background.
least 200 days, in contrast to the 14 \( Tg2770/Tg2770, +/Y \) progeny, which succumbed to peripubescent lethality (Fig. 3B). The death of homozygous male mice that do not contain the \( Ar^{Tjm} \) mutation adds evidence to the supposition that the lethality is not background-dependent, since the \( Ar^{Tjm} \) mutation is on a C57BL/6 background. Rescue of the male-specific lethality shows that androgen signaling is necessary for this phenotype and explains the onset and gender-specificity of the lethal phenotype. Interestingly, the microvesicular steatosis was no longer present upon gross examination (data not shown).

The observance of androgen sensitivity of both the male-specific lethality and the microvesicular steatosis is consistent with the hypothesis that lipid accumulation in the liver results in death of the organism, although it is also possible that the coincidence of the phenotypes is not a causative relationship.

**A novel Meis1 transcript in the Tg2770 line**

Northern blot analysis was conducted to determine the expression pattern and size of transcripts generated by
the Meis1a transgene. Liver, lung and kidney RNA were isolated from FVB/NJ wild-type and FVB/NJ Tg2770 homozygous and heterozygous mice (Fig. 4). Analysis of RNA from these tissues showed the production of a novel 6.5 kb Meis1a transcript in transgene-containing mice, in addition to the 2.2 kb transcript predicted from the transgene construct and the endogenous message of 3.8 kb. The unknown Meis1a containing transcript appears to coincide with production of the 2.4 kb element in the liver and kidneys, where endogenous Meis1 expression is low. Expression of both exogenous transcripts is minimal in lung tissue, where the endogenous Meis1 locus is expressed at high levels (Fig. 4).

The unexpected 6.5 kb transcript is present in both homozygous females and heterozygous males (Fig. 4), indicating that this transcript is not responsible for male lethality or androgen sensitivity. Since Tg2770/+ mice also produce the 6.5 kb transcript, presence of this transcript is not sufficient for male lethality, whereas complete alteration of the integration locus (recessive nature of the phenotype) is necessary for eliciting the lethality. It is still possible that post-transcriptional control that varies in a gender-specific manner and is dosagesensitive is acting on the transcript. This would explain the deleterious effects in male Tg2770/Tg2770 mice but not in female mice or male Tg2770/+.

Mapping of the transgene

We were able to clone the 3’ flanking region of the transgene integration site from a subgenomic DNA library (see Materials and Methods). Sequence analysis of this region at the time (November 2001) did not reveal the chromosomal location using the public database (www.ensembl.org). However, a GA microsatellite repeat region 1 kb downstream of the transgene integration site was identified. This microsatellite marker (D1Kccl) was determined to be polymorphic between FVB/NJ Tg2770 and Mus spretus. D1Kccl was not polymorphic between FVB/NJ Tg2770 and AEJ/Gn. Analysis of a backcross panel well characterized in our laboratory between AEJ/Gn and M. spretus revealed linkage of D1Kccl with the molecular markers D1Mit65 and D1Mit4 (Fig. 5). The ratios of the number of recombinants between these markers and D1Kccl to the total number of N2 progeny scored for each locus are D1Mit65–1/88–D1Kccl–5/84–D1Mit4. The centimorgan distance ± standard error between Tg2770 and the two other loci are as follows: D1Mit65–(1.1 ± 1.1)–TG2770–(6.0 ± 2.6)–D1Mit4. Thus, the 3’ end of the transgene contains DNA from mouse chromosome 1, ~9.5 cM from the centromere.
The β-lactamase fold superfamily encompasses a diverse group of proteins, including β-lactamases, glyoxalase I and II, mRNA-processing enzymes, cyclase/dehydrodase, arylsulfatase and double-strand-break repair/V(D)J recombination protein (RS-SCID) (17–29). Comparison of LACTB2 with other proteins in the metallo-β-lactamase fold superfamily shows low overall similarity outside of the conserved domain. However, the human homologue, CGI-83 (Unigene Cluster Hs.118554), has 84% protein sequence identity and 93% sequence similarity, and correlates with mouse/human syntenic chromosome maps with a location of 8q13. This homologue exhibits broad expression based upon EST retrieval at the NCBI database. The HxHxDH motif that is invariant (17) in all active members of the metallo-β-lactamase fold superfamily is conserved in the Lactb2 gene. BLAST searches of the public protein database using protein sequence from LACTB2 outside of the metallo-β-lactamase fold provided no additional information.

Figure 5. Interspecific backcross panel results. A microsatellite marker (D1Kcc1) located 1 kb downstream from the 3′ end of the FVB/NJ Tg2770 integration site was found to be polymorphic between AEJ/Gn and M. spretus. This polymorphism was used in backcross analysis of N2 progeny from the cross (AEJ/Gn × M. spretus) F1 × AEJ/Gn. (A) Summary of the results of the interspecific backcross analysis of Tg2770. The segregating markers isolated in the analysis are listed on the left. D1Mit65 is located 8.4 cM from the centromere, while D1Mit4 is located 12.0 cM from the centromere. Closed boxes represent inheritance from AEJ/Gn, and open boxes represent inheritance from M. spretus. Numbers below the boxes represent the number of each type of chromosome inherited in the N2 generation. (B) Order of the loci examined on the region of chromosome 1 progeny from their F1 hybrid parent in the backcross analysis, with the centromere displayed at the top. To the right of each chromosome are the genetic distances in centimorgans, with the location of the loci displayed on the left.

Because transgene integrations are often associated with large-scale deletions, duplications and/or translocations (16), we needed to characterize the 5′ end of the integration site. Transgene insertion-site amplification (TISA)–PCR was used with six degenerate primers to obtain a 500 bp fragment that contained 150 bp of endogenous 5′ flanking DNA. Sequencing and BLAST searches allowed the identification of the region represented by this sequence (February 2002, www.ensembl.org). The 5′ integration site was verified by PCR amplification of homozygous Tg2770 genomic DNA using primers specific for the transgene and the 5′ flanking sequence. The integration site mapping data using TISA–PCR for the 5′ end of the transgene correlated with the backcross analysis data for the 3′ flanking region, and place the transgene insertion site on proximal chromosome 1.

Identification of the disrupted locus at the Tg2770 integration site

Sequence analysis of genomic DNA revealed that integration of the transgene caused a 21.3 kb deletion that results in the loss of exons 5–7 of a new gene that we have named metallo-β-lactamase 2 (Lactb2). 129 ESTs corresponding to Lactb2 are present in the NCBI database at the time of writing of this paper, showing that the gene is transcriptionally active. Lactb2 belongs to the metallo-β-lactamase fold superfamily spanning 34.5 kb of genomic sequence with seven exons and a 1.9 kb mature transcript (Fig. 6A). This transcript is predicted to result in a 288 aa/32.8 kDa protein.

The β-lactamase fold superfamily encompasses a diverse group of proteins, including β-lactamases, glyoxalase I and II, mRNA-processing enzymes, cyclase/dehydrodase, arylsulfatase and double-strand-break repair/V(D)J recombination protein (RS-SCID) (17–29). Comparison of LACTB2 with other proteins in the metallo-β-lactamase fold superfamily shows low overall similarity outside of the conserved domain. However, the human homologue, CGI-83 (Unigene Cluster Hs.118554), has 84% protein sequence identity and 93% sequence similarity, and correlates with mouse/human syntenic chromosome maps with a location of 8q13. This homologue exhibits broad expression based upon EST retrieval at the NCBI database. The HxHxDH motif that is invariant (17) in all active members of the metallo-β-lactamase fold superfamily is conserved in the Lactb2 gene. BLAST searches of the public protein database using protein sequence from LACTB2 outside of the metallo-β-lactamase fold provided no additional information.

Analysis of the fusion transcript

To determine the nature of the 6.5 kb transcript and what role it may play in the phenotype, we characterized the transcript at the probable fusion point between the transgene and Lactb2 using RT–PCR. Primers from the fourth exon of Lactb2 and the 5′ end of the Meis1a cDNA were used to amplify mRNA from male homozygous Tg2770 mice. The product was then sequenced, and contained Lactb2 sequence data up to the end of the fourth exon and the 5′ end of the Meis1a cDNA, including the start codon and 60 bp of 5′ flanking sequence (Fig. 6). The fact that the fusion transcript does contain Lactb2 sequence verifies that the putative gene is actually an expressed region.

Analysis of the sequence indicates that integration of the transgene caused the loss of 91 amino acids from the C-terminal end of the protein, which leaves all but the last two amino acids of the β-lactamase fold intact. These last two amino acids are conserved members of the domain, but the consequence of their deletion is unknown. The MEIS1A product is out of frame with LACTB2, resulting in the production of 86 amino acids of novel protein sequence past the fourth exon of Lactb2 before the first stop codon. Consequently, the fusion transcript produces a truncated LACTB2 protein product with 86 extra amino acids of missense protein not containing any MEIS1 domains.
Figure 6. Analysis of the integration site of the transgene. (A) Gene structure of Lactb2. The seven exons that make up the mature Lactb2 transcript are depicted in this schematic of the genomic region of Lactb2. The 21.3 kb region deleted in the Tg2770 strain is also indicated, which includes exons 5–7. (B) Protein comparison of LACTB2 with the closest members of the metallo-β-lactamase fold family. The human CGI-83 homologue is depicted, as well as the closest family members in homology in Caenorhabditis elegans, mouse, yeast, mitochondria and bacteria. The active site of the metallo-β-lactamase fold (HxHxDH) starting at amino acid 77 is conserved in LACTB2. (C) Sequence of the fusion transcript at the fusion site as determined by RT–PCR. Shown here is 415 bp of sequence flanking the fusion site of the fusion transcript between Lactb2 and Meis1a. The protein sequence resulting from translation of this mRNA is shown underneath the mRNA sequence. The shaded area represents mRNA and protein sequence derived from the Lactb2 gene. The normal start codon for the transgene is in bold, as well as the stop codon of the fusion transcript.
DISCUSSION

We have presented the results of our molecular and morphological analyses of a novel transgenic mouse line exhibiting male-specific peripubescent lethality. Necropsy of these mice indicated a liver defect. Microscopic analysis of the liver identified the presence of microvesicular steatosis in male homozygous mice and was coincident with their lethality. Using the ArTfm mutation, which renders males androgen-insensitive, we were able to rescue both the lethality and the liver defect of homozygous male Tg2770 mice. This indicated that the lethality was linked to an increase in the androgen signaling that occurs during puberty in male mice. The coincidence of the fatty liver defect with death is also consistent with microvesicular steatosis as the cause of the lethality; however, a causative role cannot be ascribed at this time.

Our molecular analysis of the transgene integration site indicates that a deletion of 21.3 kb of genomic DNA occurred (Fig. 6A), with no other major genomic rearrangements. Molecular analysis of the integration site of the transgene revealed that the deletion includes exons 5–7 of Lactb2, a new gene belonging to the metallo-β-lactamase superfamily. This family is a diverse group of proteins that are grouped together based on the presence of a protein domain that does not always have catalytic activity (17). The diverse nature of these proteins and the low similarity of LACTB2 to family members in general are very diverse, making a connection with known function is low and the roles of the family members in general are very diverse, making a connection between loss of function and phenotype difficult at this time. BLAST searches of the public database using the protein sequence outside of the conserved domain yielded no significant homologies with known proteins.

The second possibility is that the Tg2770 mutation is a gain-of-function mutation. Gain of function may be attributed to causes such as production of a truncated LACTB2 protein with a new function due to loss of a regulatory domain present in the C-terminal end or ectopic expression of Meis1a under the control of the androgen pathway due to the characteristics of the integration site. We have been able to rule out the possibility of a fusion protein containing LACTB2 domains and MEIS1A domains being the cause of the phenotype with the finding that all mRNA Meis1a sequence information is out of frame with the Lactb2 sequence (Fig. 6C). The recessive nature of the phenotype provides an additional argument against the gain-of-function hypothesis. In addition, both males and females express the 2.4 kb transgene mRNA and the fusion mRNA (Fig. 4), which indicates that mRNA expression of the gene is not sufficient for eliciting the phenotype. However, post-transcriptional regulation may be occurring that is gender-specific. Finally, despite the presence of transgene-derived Meis1a mRNA in the liver, spleen, kidney and brain, there is no additional protein produced as determined by western blot analysis (data not shown). In summary, it appears that loss of function for Lactb2 is the simplest and most probable explanation of the phenotype.

Boison et al. (33) have recently reported a mutant strain of mice exhibiting neonatal microvesicular steatosis, which is fatal. The phenotype is similar to that of Tg2770 without the male specificity and peripubescent onset. Both phenotypes have a pale discoloration of the liver, microvesicular steatosis, lethality, recessive nature, and lack of other defects of the liver associated with NAFLD. Liver failure is the result of a complete loss-of-function mutation in adenosine kinase (ADK) and, like Tg2770, exhibits no penetrance in the heterozygous mice. The author’s evidence supports a hypothesis of loss of ATP resulting in an impairment of mitochondrial lipid metabolism. Other mouse model systems that exhibit steatosis include cystathimine β-synthase (CBS)-deficient mice, which show elevated levels of plasma homocysteine (34), phosphatidylethanolamine N-methyltransferase-deficient (Pemt) mice, which have impaired lipoprotein secretion due to problems in choline biosynthesis (35–37), and adenosine deaminase (ADA)-deficient mice, which have defects in adenosine degradation (38,39). In contrast to the Tg2770 strain onset of hepatic steatosis at puberty (40 days after birth), the onset in these mutant strains varies from embryogenesis to 21 days after birth. This finding argues that the genes involved in liver function are dynamic, although it is also possible that varying stresses on the organ have an effect on onset time, as shown by Pemt mice, which only exhibit hepatic steatosis when deprived.

Evidence in support of this hypothesis is twofold. First, the recessive nature of the phenotype implies that complete loss of the integration locus is necessary for the phenotype. Second, integration of the transgene results in interruption of Lactb2 and truncation of the LACTB2 protein. The similarity of Lactb2 to members of the metallo-β-lactamase superfamily with known function is low and the roles of the family members in general are very diverse, making a connection between loss of function and phenotype difficult at this time.

First, the integration of the Tg2770 transgene may have caused a simple loss-of-function mutation in the Lactb2 gene. Our molecular analysis of the transgene integration site indicates that a deletion of 21.3 kb of genomic DNA occurred (Fig. 6A), with no other major genomic rearrangements. Molecular analysis of the integration site of the transgene revealed that the deletion includes exons 5–7 of Lactb2, a new gene belonging to the metallo-β-lactamase superfamily. This family is a diverse group of proteins that are grouped together based on the presence of a protein domain that does not always have catalytic activity (17). The diverse nature of these proteins and the low similarity of LACTB2 to family members in general are very diverse, making a connection with known function is low and the roles of the family members in general are very diverse, making a connection between loss of function and phenotype difficult at this time. BLAST searches of the public database using the protein sequence outside of the conserved domain yielded no significant homologies with known proteins.

The second possibility is that the Tg2770 mutation is a gain-of-function mutation. Gain of function may be attributed to causes such as production of a truncated LACTB2 protein with a new function due to loss of a regulatory domain present in the C-terminal end or ectopic expression of Meis1a under the control of the androgen pathway due to the characteristics of the integration site. We have been able to rule out the possibility of a fusion protein containing LACTB2 domains and MEIS1A domains being the cause of the phenotype with the finding that all mRNA Meis1a sequence information is out of frame with the Lactb2 sequence (Fig. 6C). The recessive nature of the phenotype provides an additional argument against the gain-of-function hypothesis. In addition, both males and females express the 2.4 kb transgene mRNA and the fusion mRNA (Fig. 4), which indicates that mRNA expression of the gene is not sufficient for eliciting the phenotype. However, post-transcriptional regulation may be occurring that is gender-specific. Finally, despite the presence of transgene-derived Meis1a mRNA in the liver, spleen, kidney and brain, there is no additional protein produced as determined by western blot analysis (data not shown). In summary, it appears that loss of function for Lactb2 is the simplest and most probable explanation of the phenotype.

Boison et al. (33) have recently reported a mutant strain of mice exhibiting neonatal microvesicular steatosis, which is fatal. The phenotype is similar to that of Tg2770 without the male specificity and peripubescent onset. Both phenotypes have a pale discoloration of the liver, microvesicular steatosis, lethality, recessive nature, and lack of other defects of the liver associated with NAFLD. Liver failure is the result of a complete loss-of-function mutation in adenosine kinase (ADK) and, like Tg2770, exhibits no penetrance in the heterozygous mice. The author’s evidence supports a hypothesis of loss of ATP resulting in an impairment of mitochondrial lipid metabolism. Other mouse model systems that exhibit steatosis include cystathimine β-synthase (CBS)-deficient mice, which show elevated levels of plasma homocysteine (34), phosphatidylethanolamine N-methyltransferase-deficient (Pemt) mice, which have impaired lipoprotein secretion due to problems in choline biosynthesis (35–37), and adenosine deaminase (ADA)-deficient mice, which have defects in adenosine degradation (38,39). In contrast to the Tg2770 strain onset of hepatic steatosis at puberty (40 days after birth), the onset in these mutant strains varies from embryogenesis to 21 days after birth. This finding argues that the genes involved in liver function are dynamic, although it is also possible that varying stresses on the organ have an effect on onset time, as shown by Pemt mice, which only exhibit hepatic steatosis when deprived.
of a choline-containing diet (35). The Tg2770 strain onset time
differs from any other previously described mutant strain,
indicating a new time point in liver function.

The hepatic microvesicular steatosis associated with the
lethality is classified as non-alcoholic fatty liver disease
(NAFLD). Interestingly, this disease has been found to
be more frequent in women (65–83%) (2,40–42). Other risk
factors for this disease include obesity, hyperlipidemia and
diabetes (2–8,40,43). NAFLD can result in cirrhosis of the liver
and death, but it is still unclear why some cases degenerate into
cirrhosis, while others do not. The Tg2770 model system may
prove useful in discovering factors that are related to female
susceptibility to NAFLD, since Lactb2 seems to be important
for proper homeostasis of the liver in males. By examination of
a gene such as Lactb2 that is necessary for males in
homeostasis of fatty acid levels in the liver, we may be able
to discover similar factors that are gender-specific in females
and how the gender specificity results. Additionally, further
characterization of the role of LACTB2 in lipid metabolism/
catabolism may allow for the development of treatment
strategies for NAFLD. NAFLD has the potential to become
one of the most common types of liver disease if obesity
continues to become more prevalent.

When steatosis is associated with hepatocyte ballooning and
necrosis, with or without fibrosis and Mallory hyaline,
the progression of the disease tends to be more aggressive (2–8,40,
41,43). None of these symptoms have been identified in
Tg2770 mice, but lethality still results. This observation
suggests that steatosis alone can be severely detrimental in the
mouse, which is consistent with cellular lipid accumulation
resulting in increased incidence of apoptosis (44,45). By
further examination of the role of Lactb2 in lipid level
regulation, we shall gain insight on how steatosis, the first
pathological sign of NAFLD, results and how gender can
influence lipid level regulation.

MATERIALS AND METHODS

Mice

All mice were bred at the Kimmel Cancer Center Animal
Facility (Philadelphia, PA), except for Ar^Sim mice, which were
purchased from The Jackson Laboratory (Bar Harbor, ME).
Transgenic mice were genotyped by Southern blot hybridization
using a Meis1 cDNA probe. The endogenous locus was
used as an internal control to determine transgene dosage.
More recently, we have developed a PCR assay based on the
genomic organization of the integration site to determine the
presence or absence of transgene [(cct ccc tc ct agg act g) plus
(cct gta tgg cct gaa tc) and transgene dosage [(gca gcc tat ggg
agg atg gta gag) and (atg ggg acc agg caa tca caa caa g)]. The
annealing temperature is 55°C for the former pair and 58°C for
the later. Both are performed for 35 cycles and products
visualized on 3% agarose gel.

Backcross mapping

The interspecific backcross between (AEJ/GN-a bpI(a bpII × Mus spretus)
F1 × AEJ/Gn-a bpII(a bpII has been
described previously (46). Genomic DNA extractions, restric-
tion endonuclease digestions, agarose gel electrophoresis, and
Southern blot transfers, hybridizations and washes were as
described previously (47). DNA oligonucleotides used for
detecting microsatellite markers were made using a model 3948
DNA synthesizer from Applied Biosystems (Foster City, CA).
The microsatellite marker DIKcc1 was detected by amplifying
genomic DNA from N2 animals using the primer pair [ggt ttg
gca ggc cca tgc taa g] and [ttt aag ctc cgc ccc aca gtt ac] and a
standard PCR protocol (48). Genotype data were analyzed and
correlated using Spretus Madness Part Deux software (unpub-
lished) by calculating the maximum-likelihood estimates of
linkage parameters (49).

Generation of transgenic lines

The Meis1a ORF construct was microinjected into the FVB/N
strain of mice at concentrations of 1 and 5 ng/μl. FVB/N donor
females (Taconic Farms, Germantown, NY) were superovulated
according to standard protocols (50) and mated to FVB/N
males. Plugged FVB/N females were used to isolate eggs. All
FVB/N fertilized eggs were microinjected with the Meis1a
ORF construct. Embryos were implanted after overnight
culture into pseudopregnant Swiss Webster females (Taconic
Farms). A single transgenic male founder (Tg2770) was the
progenitor of all mice analyzed for this research and was
maintained by intercrossing with FVB/J mice obtained from
The Jackson Laboratory.

RT–PCR

This was conducted using the Titan One Tube RT–PCR kit
from Roche Applied Science (Indianapolis, IN) with the
following primers at 60°C annealing temperature: [gta cag att
ctc tcc ctt c] and [cat ctt tgt gga ctc cg cat c].

Sectioning and microscopy

OsO4 staining was performed on frozen sections. H&E staining
was performed on paraffin sections by the Kimmel Cancer
Center Pathology Core Facility. Images were captured from
an ECLIPSE E600 microscope from Nikon (Melville, NY)
using a 2.3.1 camera and SPOT version 3.4.4 software from
Diagnostics Instruments, Inc. (St Sterling Heights, MI).

Northern blotting

RNA isolation, size separation and blotting were conducted as
described in (48). The membranes were prehybridized in
Church Buffer. Probes were radiolabelled using dCTP-p32x
using a Random Primed DNA Labeling Kit from Roche
Applied Science. The blot was hybridized overnight at 65°C,
and washed three times with 50 ml of 2 × SSCP with 0.1%
SDS at 65°C. Blots were developed with either X-OMAT Blue
autoradiograph film from the Eastman Kodak Company
(Rochester, NY) or Phosphor Screens from Molecular
Dynamics (Sunnyvale, CA), using a Typhoon Scanner from
Molecular Dynamics.
Subgenomic DNA library generation

We produced a subgenomic DNA library using XbaI digestion, size-fractionated with agarose gel electrophoresis. DNA fragments of 9–11 kb were extracted from agarose using a QIAquick Gel Extraction Kit from Qiagen (Valencia, CA). Standard protocols were used in all techniques (48). The Lambda FIX II/Xho I Partial Fill-In Vector kit from Stratagene (La Jolla, CA) was used in creating the DNA library.

TISA–PCR

This was used to isolate 5' flanking DNA of the Tg2770 integration site. This technique is an adapted form of VISA–PCR and was performed as described previously (51), with the following exceptions: (i) the first-round specific primer was [tgg ccg ttg ttt tac aac g]; (ii) the second-round specific primer was [gac tgg gaa aac cct ggc gtt acc]; and (iii) six different degenerate primers were employed. The six different restriction enzyme sites were chosen based upon their frequency in the mouse genome, and utilized BamHI, BglII, EcoRI, HindIII, SacI and SacII restriction sites.

Acknowledgements

We should like to acknowledge Dr Linda Siracusa for her aid in preparation and critical review of this manuscript. We should also like to acknowledge and thank Dr Hansjürg Alder for help with sequence analysis. In addition, we should like to acknowledge and thank Dr Hansjuerg Alder for preparation and critical review of this manuscript. We should also like to acknowledge and thank Dr Linda Siracusa, Judith L. Morgan, help with sequence analysis. In addition, we should like to acknowledge and thank Dr Hansjürg Alder for preparation and critical review of this manuscript.

REFERENCES


