Identification and Characterization of Lethal 6: a Chromosome 5 Embryonic Lethal Mutation in Mus musculus

Erin Stenson
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PI: Professor John Schimenti
Abstract:

Mus Musculus was randomly mutagenized using N-ethyl-N-nitrosourea (ENU). Mutations on Chromosome 5 were selected using the Rw inversion, a visibly marked, recessive, lethal inversion that covers almost fifty megabases (Mb). Multiple mutations were isolated from the subsequent three-generation mutagenesis screen, one of which was lethal 6. Using a combination of complementation tests and recombinational mapping, the lethal 6 region was narrowed down to less than 1.4 (Mb). There were thirty-eight candidate genes in this region of which 4 had already been mutated and yielded viable progeny. The coding regions of 80% of the remaining thirty-four genes have been ruled out from being lethal 6, although it is still possible that ENU affected the regulation of one of these genes. Mice homozygous for lethal 6 die around E8.0 and preliminary results from blastocyst outgrowth assays suggest that they are unable to implant.

Introduction:

The process of early mammalian development is remarkably similar between different species. Embryogenesis follows the same patterns, and the basic processes are controlled by the same conserved genes in different organisms. To discover the genes necessary for normal human development, the organism Mus musculus is a good model because approximately 93% of mouse loci are found in the same position in both mice and humans (Mouse Genome Sequencing Consortium, 2002). Mus musculus is also a good model organism because they have a short gestation period, big litter sizes, and short life cycles. Genes that are necessary for embryonic development of Mus musculus will most likely be necessary for the embryonic development of humans. The genes required for embryonic development of Mus musculus should have very similar sequences and
chromosomal locations when compared with genes necessary for the embryonic development of *Homo sapiens*.

The work of the Human Genome Project has led to the sequencing of both the mouse and human genomes. This project has also led to the identification of many expressed genes whose sequence, location, and expression patterns are known. These genes are documented in numerous databases, including UCSC Genome Browser and Ensembl.

While the locations of these genes are known, most of the genes’ functions are not known. In order to figure this out, the genome is mutated and scanned for any novel phenotypes. After narrowing down the possible region of the gene causing the novel phenotype, the gene can be matched with known expressed genes found on the above databases through sequencing. Direct experimentation is necessary to determine the gene function, and *Mus musculus* should be used as a model organism to alleviate ethical and medical concerns. This type of experimentation, called forward genetics, involves randomly mutagenizing the genome and identifying and isolating any genes that cause phenotypic abnormalities.

In this experiment, the genome was mutagenized with the chemical *N*-ethyl-*N*-nitrosourea (ENU). ENU affects the male germ line by inducing point mutations at a high frequency. Mutations within the region spanned by *Rw*, a visibly marked, lethal, recessive inversion that spans almost fifty megabases (Mb) on the 5th chromosome of *Mus musculus*, were recovered. The *Rw* inversion causes heterozygotes to be partially depigmented and homozygotes to die during embryogenesis, around age E9.5 (Bucan et al. 1995). Since the inversion suppresses recombination, *Rw* is useful as a balancer chromosome and was used in a three-generation mutagenesis screen. A balancer chromosome is used to ensure that there
is no crossing over between chromosomes, and therefore the original chromosomes (i.e. Rw, B6*) remain the same. Because the genotype of the progeny is quickly detectable due to the depigmentation caused by Rw, breeding schemes are easier and more effective than if Rw was not used.

This mutagenesis screen yielded thirty-seven mutations that caused embryonic or perinatal lethality (Wilson et al. 2005). Of these, I have been working on a mutation called \(L5\text{Jcs6}, \text{or lethal 6}\). When this gene is mutated and homozygous, it causes embryonic lethality in \(M.\mu.sculus\). \(\text{Lethal 6}\) is therefore known as an embryonic recessive lethal gene mutation. Subsequently, I explain the results of the experiment: the location and sequencing of the \(\text{lethal 6}\) gene and the time that the developing embryo dies from the effects of the mutation.

**Results:**

*Isolating the Lethal 6 mutation:*

After randomly mutagenizing the genome of chromosome 5 of a \(C57BL/6\ (B6)\) mouse with the ENU mutagen, the mutations were isolated in the following way. After the mutagenesis, the offspring were mated to a female that had the genotype \(C3H/Rw\). These genotypes refer to \(C3H\) – a strain of mice that has a slightly different genotype than \(B6\) mice do. \(Rw\) is an inversion that arose on a \(C3H\) strain. This inversion is a visibly marked recessive lethal inversion spanning almost fifty megabases (Mb), from around 26 Mb to 74 Mb. The offspring that were \(B6/Rw\) were identified by the depigmentation of their abdomens, and mated with \(Rw/Hm\). \(Hm\) (Hammertoe) is another visibly marked mutation, resulting in webbed digits. Progeny that had the \(Rw\) phenotype but not the \(Hm\) phenotype were then intercrossed (Wilson et al. 2005). The progeny of this cross could theoretically
yield three possible genotypes: \( B6/Rw, Rw/Rw, \) or \( B6/B6 \). Since \( Rw/Rw \) animals die during development, only \( B6/Rw \) animals or \( B6/B6 \) should survive if there was no mutation.

Embryonic lethal mutations were identified when this last cross yielded no animals that were \( B6/B6 \) (Figure 1). In this way, almost forty mutations were previously discovered. I have been working on one of these, L5Jcs6, or \textit{lethal 6}.

Figure 1: Three-Generation Mutagenesis Breeding Scheme: Mutated \( B6 \) mice were isolated by mating them with a combination of visibly marked mutations. Since \( Rw \) suppresses recombination, the only the mice with abdomen depigmentation could contain the \( B6^* \) chromosome.
Mapping the Lethal 6 mutation:

To narrow down the region, complementation tests were done between *lethal 6* and three deletions in the *Rw* region. These deletions, Dpp$^{6df1j}$, Qdpr$^{df3j}$, and Dpp$^{6df4j}$ span the region from 23.3 to 29.06 Mb, 40 to 51.9 Mb, and 23.3 to 27.9 Mb, respectively.

Complementation is shown when there is the presence of at least one mouse that is *B6*/Deletion. This means that for every gene that was missing from the deletion chromosome there was a normal copy of it within the *B6* chromosome. Thus, if a deletion complements with *lethal 6*, then the mutation can not be within any of the genes that were deleted. All three of these deletions were found to complement *lethal 6* (Wilson et al. 2005).

This result indicates that the *lethal 6* gene is not within any of these regions (Figure 2). These results had previously narrowed down the possible region to between 28.4 Mb and 40 Mb or between 51.9 Mb and 75 Mb.

Figure 2: Possible Gene Region based on complementation tests. Since *lethal 6* complemented with Dpp$^{6df1j}$, Qdpr$^{df3j}$, and Dpp$^{6df4j}$, the *lethal 6* mutation could not be within any of these regions, and thus the size was narrowed down to within 29 to 40 Mb or 51.9 to 75 Mb.
To further narrow down these possible gene regions, I did recombinational mapping. $B6^{*/Rw}$ mice were mated with $Cast$ mice, a different strain with a slightly different genome. The non-$Rw$ progeny ($B6^{*/Cast}$) were tail-clipped. PCR was performed several times for each DNA sample using different microsatellite markers spaced around chromosome 5. Microsatellites are highly polymorphic DNA elements. Because the mouse strains are slightly different, different alleles from either $B6$ or $Cast$ have a different number of repeats and are therefore different lengths. By PCR amplification and gel electrophoresis the different sized alleles can be separated to determine the genotype of the sample. Since $B6^{*/B6}$ causes embryonic lethality, if a marker is used and the resulting amplicon’s genotype is found to be homozygous for $B6$, then the mutation cannot be within that marker.

Because the probability of a double crossover is very small, once a $B6$ homozygote was found at a specific marker, the location was narrowed down to be between the centromere and the location of that marker. Eleven $B6$ homozygotes were found, most importantly mouse 3187 (Table 1). This mouse was found to be homozygous for $B6$ at D5Mit148, which is found at 30.4 Mb, and also homozygous at all markers through D5Mit353, at 39 Mb (Figure 3). This significantly narrowed down the possible region between 29.06 and 30.38 Mb, a region consisting of around 1.3 million base pairs (Figure 4).
Figure 3: Pictures of Mouse #3187 with markers D5Mit4 and D5Mit148. A 3% NuSieve 3:1 Gel was used. **A)** Primer D5Mit4 was used on mouse #3187 (1) and controls B6 (2) and Cast (3). A one-hundred base pair ladder is also shown (4). **B)** Primer D5Mit148 was used on mouse #3187 (5) and controls B6 (6) and Cast (7).

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Primer: D5Mit4
Expected Size of B6: 195
Expected Size of Cast: 238

Primer: D5Mit148
Expected Size of B6: 149
Expected Size of Cast: 129

Figure 4: Possible Gene Region based on Mapping Data. Mouse # 3187 was B6*/B6* for all markers between the beginning of the Qdpr<sup>df3j</sup> (40 Mb) deletion through D5Mit148 (30.4 Mb). This narrowed down the possible region to a size of less than 1.4 Mb, from 29.06 Mb to 30.38 Mb.
Table 1: Mapping Data for *lethal 6*. Mouse # 3187 was *B6*/B6* for all markers from D5Mit148, at 30.38 Mb, to D5Mit106, at 43.6 Mb. This narrowed down the possible region to a size of less than 1.4 Mb, from 29.06 Mb to 30.38 Mb.

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Finding and Analyzing Candidate Genes:

By using the UCSC and Ensembl Genome Browsers, genes that other researchers had found to be expressed and were traced back to the possible *lethal 6* gene region were found. The genomic positions of these expressed genes are known, but the phenotype remained unknown. In the possible *lethal 6* region, thirty-eight genes were found (Figure 5). Of these genes, four had yielded viable progeny when previously mutated: Emilin1, Slc30a3, Ucn, and Gckr. The remaining thirty-four genes were prioritized by their expression patterns and known phenotypes. The expression patterns were determined by researching the Unigene database and the known phenotypes from the Jackson Laboratory database. The Unigene database contains expression levels for over thirty tissues and for each developmental stage.
Genes that were ubiquitously expressed in adult tissue or in abundance in early embryonic tissue were considered to be more likely than genes that were only present in small amounts in adult tissue. This is because a gene causing embryonic lethality when mutated is expected to be an important gene.

Figure 5: Candidate Genes within the possible lethal 6 region on Chromosome 5. These genes were found using Ensembl and UCSC Genome Browsers, and are mostly genes that are known to be expressed but with unknown function.

Lethal6/Rw DNA was isolated and used to examine the candidate genes using Wave Analysis. This procedure uses temperature gradient denaturing to separate heterogeneous sequences. It enables us to quickly visualize if the gene is possibly lethal 6. If the Wave Analysis showed that there was possibly a mutation, then the gene was sequenced. Using Wave, sixteen genes were ruled out from being lethal 6: 4930471M23Rik, Cenpa, 1110039B18Rik, 9430057O19Rik, Khk, Abhd1, 0610007C21Rik, Dnajc5g, Trim54, Gtf3ct, Zfp513, Krtcap3, Zfp512, 4930548H24Rik, Mrpl33, and Rbks. Except for an exon or two, fourteen other genes were mostly ruled out: Dyps15, Mapre3, Slc5a6, Preb, Cad, Snx17,
Ppm1g, Nrbp, Ift172, Fndc4, Xab1, Supt71, Slc4a1ap, and Bre. This leaves only four genes left to analyze: 2310016E02Rik, Tcf23, Mpv17, and Eif2b4, (Figure 6).

However, it is possible that the ENU affected a non-coding region. If so, Wave Analysis would not have picked up these mutations because we only compared the coding sequences of each candidate gene. Thus, primers for real time PCR have been designed for all 34 possible genes within this region. RNA will be recovered from lethal 6/Rw mice and cDNA will be made by reverse-transcriptase PCR. Real time PCR will then be done to quantify relative gene expression as compared to the control DNA. If there is a considerable difference in the amount of lethal 6/Rw product versus control product, it is probable that ENU affected the regulatory mechanism of this gene.

Figure 6: Genes within the possible lethal 6 region and the combined Wave Analysis and Sequencing Results.
Determining the time that Lethal 6 Acts:

To determine the time that lethal 6 acts to kill the developing embryo, timed matings were done. Previous results showed that lethal 6 acts in early gestation. The first timed matings that were done were at E9.5 between B6/Rw females and B6/Cast males. This time corresponds with early to mid gestation and halfway through the normal gestation period of nineteen to twenty-one days. The pregnant female mice were sacrificed and dissected when their progeny were still in utero. At this time, normal mice should have completed turning and become C shaped. Also, early organogenesis should be underway, as evidenced by the presence of the heart and prominent pharyngeal arches.

Around thirty embryos were genotyped at this stage. Of these, all looked phenotypically normal, and when genotyped, none were B6*/B6* (Figure 7).

Furthermore, about one quarter of the decidua were empty. The cross should theoretically yield one quarter B6*/B6*, one quarter B6*/Cast, one quarter B6*/Rw, and one quarter Rw/Cast if all genotypes were equally viable. Since none of the embryos were B6*/B6*, and one quarter of the decidua were empty, the mutant lethal 6 embryos died at an earlier stage and were reabsorbed.

Next, twenty-two embryos at E8.5 were analyzed. Normal embryos at this stage are undergoing turning and are between U shaped and linear. Also, the embryos should have their first somites and pharyngeal pouches. A B6/Rw intercross was done to facilitate genotyping. All twenty-two of the collected embryos were genotypically normal (Figure 8). Also, there were seven empty decidua. This data suggests that lethal 6 acts at an earlier stage to kill the mutant embryos.
Figure 7: E9.5 Embryos. All embryos recovered at this stage looked phenotypically normal and were genotypically normal as well.

Genotypes of Embryos:

1 = B6/Cast
2 = B6/Rw
3 = Rw/Cast
4 = B6/Rw
5 = B6/Cast
6 = Rw/Cast

Blastocyst outgrowths were done to determine if lethal 6 might inhibit the earliest stages of embryo growth. Two mice were sacrificed and a total of twelve embryos were flushed out of the uteri. Of these embryos, seven were able to attach to the growth plate and
five were not. Of the seven that were able to attach and grow, two were \(Rw/Rw\) and five were \(B6^*\)/\(Rw\) (Figure 9). Based on the cross of \(B6^*/Rw\) with \(B6^*/Rw\), one quarter of the progeny should be \(B6^*/B6^*\), one quarter \(Rw/Rw\), and one half \(B6^*/Rw\) if all were equally viable. This suggests that \(B6^*/B6^*\) is unable to implant in vitro.

Figure 8: E8.5 Embryos. All embryos at this stage were both phenotypically and genotypically normal.

![Genotype: B6/Rw](image1)

![Genotype: B6/Rw](image2)

Figure 9: Blastocysts. Of the blastocysts able to implant, none were \(B6^*/B6^*\).

![Genotype: B6/Rw](image3)

![Genotype: B6/Rw](image4)
Discussion:

Forward genetics is a type of genetic screen that tries to find new genes by mutagenizing the genome and finding novel phenotypes. The mutated genes causing these new phenotypes are then isolated and cloned. In this experiment, lethal 6 was found to be an embryonic recessive lethal gene mapping within the Rw region on Chromosome 5. The Lethal 6 mutation was induced with ENU, a point mutagen affecting the male germ line. It was then isolated using a three-generation mutagenesis screen. This screen yielded no viable progeny that were B6*/B6* and thus lethal 6 was acting during embryogenesis.

After complementation tests were done to broadly reduce the possible region, PCR analysis was done. Combining the complementation tests and recombinational mapping, the possible lethal 6 region was narrowed down to a size of less than 1.4 Mb. Unfortunately, this region is very gene-rich and had thirty-eight genes within it. Four of these genes were viable when knocked-out and were thus ruled out. Another fifteen genes were ruled out during Wave analysis and sequencing. Yet another thirteen genes were almost all ruled out. Currently, six full genes need to be analyzed and it is still possible that ENU affected a coding part of these genes.

During the mapping process of the entire set of thirty-seven embryonic lethal genes that were previously isolated, another five lethal mutations (L5Jcs15, L5Jcs 16, L5Jcs 24/L5Jcs 27, L5Jcs 31, and L5Jcs 35) were found to map to the same region as lethal 6. These mutations have been tested at the same time as lethal 6, thereby increasing the number of controls and comparisons used during Wave Analysis. None of these mutations have been found either, although all have been tested with the same genes with which lethal 6 was tested. It is highly unlikely that the six lethal mutations correspond to the remaining six
candidate genes. Thus, it is probable that ENU affected a regulatory part of these genes and 
not a coding region. On the other hand, it is also possible that WAVE is not one hundred 
percent efficient and may have missed a mutation.

Therefore, the thirty-four genes that lead to unviable progeny when mutated are all 
still possibilities for being lethal 6. Real-time PCR primers have been made for all thirty-
four of the candidate genes and cDNA has been prepared as well. If ENU affected a 
regulatory part of one of these genes, then the expression profile of the affected lethal should 
be different than for the control expression data. By using RT-PCR, the amount of PCR 
product and therefore RNA present can be quantified. We can see whether a particular lethal 
has a lower amount of RNA expressed for any given gene when compared to the other lethal 
mutations and control cDNA. If so, then there is a possibility that that lethal corresponds to 
the gene being analyzed. Future work should concentrate on analyzing the expression 
profiles of these genes with all six lethal gene mutations.

Timed matings were done to determine the time that lethal 6 is required to support 
embryonic development. None of the embryos recovered at E9.5 or E8.5 were B6* 
homozygous and there were several empty decidua in each sacrificed mouse. The presence 
of empty decidua led us to believe that the mutant embryos had been resorbed at an earlier 
stage. However, since E8.5 embryos are very delicate and small, it is possible that the 
embryonic tissue was contaminated with maternal tissue. This would lead to inaccurate 
results when genotyping the embryo. More embryos at this stage of development need to be 
genotyped to verify the accuracy of the results. If more embryos are genotyped and there are 
still no B6*/B6* embryos recovered, then it is more likely that our results were accurate.
Currently, only twelve blastocysts were flushed out of the uterine horns. Of these, only seven were able to implant but none of these were $B6^*/B6^*$. It is possible that $B6^*/B6^*$ embryos are unable to implant, but more outgrowths must be performed to confirm these preliminary findings.

Overall, while a lot of progress has been made towards identifying and characterizing lethal 6, more research must be done. The remaining exons of the thirteen unfinished genes and the full six genes that have not been done still need to be analyzed via Wave to either find the lethal 6 mutation or to rule them out. Also, RT-PCR must be done for all thirty-four of these genes to determine if ENU affected a regulatory part of the genes instead of a coding part. It is likely that ENU did affect a non-coding region of one of these genes and hopefully real-time PCR will help distinguish the expression profiles. It is also possible that the lethal 6 gene is not annotated.

Also, more blastocysts must be assayed and the ones able to implant must be genotyped. In regards to determining the phenotype, more embryos must be genotyped at E8.5 and E7.5 to find out when exactly the embryo succumbs to the lethal 6 mutation. The pattern of expression must be elucidated as well. Which tissues lethal 6 affects is currently unknown.

If a greater number of blastocyst outgrowths are done and $B6^*/B6^*$ embryos fail to implant, then it is possible that the gene is required for cell growth or implantation. However, if it is found that $B6^*/B6^*$ embryos are able to implant, RNA in situ hybridization should be done using Brachyury/T as a marker. Brachyury T is a mesodermal marker, and is first expressed in the primitive streak of the embryo and later in the notochord (Herrmann, 1991). A probe will be made that hybridizes to the Brachyury T mRNA. Only cells
transcribing the Brachyury T gene will have this mRNA, and these cells will be the only ones to hybridize with the labeled probe. Therefore, the labeled probe should only be seen in cells of the primitive streak and notochord. The primitive streak begins developing around E7.0, and the late primitive streak is seen at E7.5. If lethal 6 is disrupting the mesoderm and these regions, then the B6*/B6* mutant embryos will have different spatial patterns of expression when compared to normal embryos. These patterns of expression will be helpful in determining both when and where lethal 6 acts.
Methods:

ENU Mutagenesis:
This was done previously (Wilson et al. 2005).

Complementation Tests:
This was done previously (Wilson et al. 2005).

Recombinational Mapping:

*B6*/Rw mice were mated to *Cast/Cast* mice and then the non-*Rw* progeny were intercrossed, allowing for recombination between the *B6* and *Cast* chromosomes. The tails of the F2 generation were clipped and the DNA was purified. The DNA was amplified using a number of primers spanning the *Rw* region: D5Mit348, D5Mit387, D5Mit229, D5Mit75, D5Mit353, D5Mit231, D5Mit299, D5Mit132, D5Mit82, D5Mit290, and D5Mit356. These markers are all polymorphic between *B6* and *Cast*. The PCR product was then separated on a 3% 3:1 NuSieve gel.

Wave Analysis:

DNA from all 6 lethals was purified and then amplified using the protocol Wave-HD. The PCR products were then checked on a 2% NuSieve gel run at 120 mV. The amount of product to inject into the Wave Machine was determined through comparison with the six hundred base pair band of the one hundred base pair ladder. The injection volume ranged from 5 µl to 15µl.

Sequencing:

All sequencing samples were sent to Cornell University’s Biotechnology Resource Center and analyzed using Sequencher.
**Outgrowths:**

Done according to the established protocol in *Manipulating the Mouse Embryo: A Laboratory Manual* by Nagy et al, 2003. Blastocysts were grown in 24-well plates with ES cell media.

**Timed Matings:**

*B6*/Rw mice were put into the same cage for mating. Plugs were checked for daily, and when a plug was found, this was considered day E0.5. Mice were sacrificed in the morning to get the most accurate age of embryos. Dissections were done according to the established protocol in *Manipulating the Mouse Embryo: A Laboratory Manual* by Nagy et al, 2003.
Works Cited:


Ensembl: http://www.ensembl.org/Mus_musculus/index.html

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