Induction of new mutations in a mouse \( t \)-haplotype using ethynitrosourea mutagenesis

MONICA J. JUSTICE AND VERNON C. BODE
Division of Biology, Ackert Hall, Kansas State University, Manhattan, Kansas 66506 U.S.A.
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Summary
N-ethyl-N-nitrosourea (ENU) was used to induce mutations within the \( t^{neo} \)-haplotype of the mouse to make possible a further study of gene arrangement in \( t \)-mutants and to provide potential landmarks for cloning and sequence studies in the region. Two independent mutants were isolated for each of three loci in the \( t \)-region, brachyury (\( T \)), quaking (\( qk \)), and tufted (\( tf \)). The new \( T^{kt} \) alleles produce tailless mice when a \( tct \) mutation is present in \( trans \). The new \( qk^{kt} \) alleles are recessive and homozygous lethal. They are viable, male fertile, and cause seizures and quaking when paired with the \( qk \) mutation which previously defined the locus. The \( tf^{kt} \) mutations are recessive and phenotypically similar to the mutant alleles available in non-\( t \) chromosomes. The mutations were induced in the \( t^{neo} \)-haplotype at an average per locus frequency of 1 in 1500. Their isolation demonstrates the power of this technique for obtaining the specific mouse mutants that are needed to genetically dissect a complex mammalian system.

1. Introduction
Complex \( t \)-haplotypes that are isolated repeatedly from the wild exhibit a number of distinctive features. These include (1) an interaction with \( T \) to produce tailless mice, (2) an inhibition of recombination with wild-type chromatin over a region including at least \( T \) and the major histocompatibility complex (\( H-2 \)), (3) transmission distortion favouring \( t \)-bearing sperm, (4) embryonic lethality in homozygotes, and (5) sterility in males with two lethals from different complementation groups (\( t^{+} / t^{n} \)) (Bennett, 1975; Klein & Hammerberg, 1977; Sherman & Wudl, 1977; Lyon, 1981).

Genetic analysis of these intriguing mutants is difficult. Rare recombinations do occur between normal and \( t \)-chromatin but at a rate one or more orders of magnitude below normal and at sites which probably are not random. The partial \( t \)-haplotypes generated by these events do make it possible to assign determinants for the various phenotypic characteristics of \( t \)-mutants to proximal, distal, or central locations in the region (Lyon et al., 1979; Forejt, Capkona & Gregorova, 1980; Lyon, 1984). Similarly, they enable us to order or group resident genes for certain testicular cell proteins and sequences corresponding to cloned DNA restriction fragments originating from the \( t-H-2 \) region (Silver, 1982; Rogers & Willison, 1983; Shin et al., 1983; Silver et al., 1983b; Fox et al., 1983).

A new \( tf \) mutation in the \( t^{12} \)-haplotype allowed Silver & Artzt (1981) to demonstrate that two complementing \( t \)-haplotypes in \( trans \) recombine at an apparently normal frequency. Subsequent analysis of the gene order and location for various \( t \)-lethalties, the \( H-2 \) genes and the \( tf \) marker led to the realization that a gene rearrangement exists in \( t \)-haplotypes and might be the basis for the failure of \( t \)-chromatin to recombine normally with wild type (Artzt, McCormick & Bennett, 1982a; Artzt, Shin & Bennett, 1982b; Condamine, Guenet & Jacob, 1983; Rogers & Willison, 1983; Shin et al. 1983). Recently, molecular clones with DNA sequences for most of the \( t \)-region have been isolated (Rohme et al. 1984; Fox et al. 1985). These eventually should permit the analysis of this region at a molecular level. Although much has been laboriously learned, both classical genetic recombination studies and molecular sequence analyses would benefit from the availability of additional defined marker mutations in \( t \)-chromatin.

Because recombination inhibition precludes the transfer of existing mutant alleles into \( t \)-chromatin, we mutagenized male spermatogonial stem cells of \( t^{12} / + \) genotype with ethynitrosourea to obtain \( t^{neo} \)-haplotypes with mutant alleles at one of four loci: \( T \), \( qk \), \( tf \) or \( Glo-1 \). The \( t^{neo} \)-haplotype was chosen because it clearly exhibits all of the characteristic properties of \( t \)-mutants, it is frequently isolated from North American wild populations, and it is believed to be the progenitor of many other \( t \)-haplotypes. This approach was feasible only because of the efficient spermatogonial stem cell mutagenesis by ENU (Russell et al. 1979). Its success reinforces our belief that ENU-mutagenesis can be used to saturate regions of the...
mouse genome, to generate mouse models for many human diseases, and to isolate a wide spectrum of mouse mutants needed in mammalian biology.

2. Materials and Methods

(i) The t-region and markers of interest

Figure 1(a) is a genetic map of the proximal portion of mouse chromosome 17 showing only the relevant markers: T, brachyury; tct, tail interaction; qk, quaking; tf, tufted; Glo-1, glyoxalase-1; H-2, major histocompatibility complex; Pgk-2, Phosphoglycerate kinase-2. The numbers indicate map distances in structurally normal chromosomes. The gene tct is placed in parentheses because it has not been accurately mapped. Our preliminary data, obtained using an ENU-induced tct mutation (Bode, 1984), places it about 1 cM to the right of T.

Figure 1(b) is a map of the proximal portion of chromosome 17 derived from mutant t haplotypes (Artzt et al. 1982b; Artzt, 1984; Shin, Bennett & Artzt, 1984). The extent of t-chromatin is indicated by the open box. The location of the lethals (f) present in the haplotypes t<sup>nos</sup> and t<sup>nos</sup> are indicated. T is placed in parentheses because distances relative to it were determined from a T mutation in the normal chromatin of a partial t haplotype.

(ii) Mouse strains and mutants

The T<sup>+</sup>/t<sup>+</sup>+ t<sup>nos</sup>+ strain was obtained from Dr Michael Sherman, who in turn received it from Dr Dorothea Bennett. It had been inbred at Kansas State University by brother-sister mating for over twenty generations at the time of these experiments.

The T<sup>+</sup>/t<sup>+</sup>+ t<sup>nos</sup>/t<sup>nos</sup>+ strain arose as a rare recombinant in this inbred T<sup>+</sup>/t<sup>+</sup>+ t<sup>nos</sup>+ line. It retains the proximal portion of the t<sup>nos</sup>-haplotype including the mutant tct allele present in t<sup>nos</sup> but it has lost the t<sup>nos</sup>-lethality and the partial t haplotype is homozygous viable.

The CBA/Ca and C57BL/6 strains were obtained from the Jackson Laboratory, Bar Harbor, ME, but had been maintained in our colony by brother-sister mating for 6 years.

Mutant alleles of qk and tf were obtained from the Jackson Laboratory, and from Dr D. Bennett. The mutant animals used to screen for new mutations in the ENU mutagenized chromosomes had varying contributions from their original strains and from the inbred T<sup>+</sup>/t<sup>+</sup>+ t<sup>nos</sup>+ , CBA/Ca, and C57BL/6 strains listed above.

The T<sup>ori</sup> mutation was obtained from Dr Lee Silver. This mutation is reported to involve a 3 cM deletion which includes T and qk (Silver, 1981; Silver, Lukralle & Garrels, 1983a).

When present in trans with brachyury, tct mutations cause the mouse to be tailless. We recently isolated an ENU-induced tct mutation in a normal chromosome 17 (Bode, 1984). The t<sup>k</sup>/t<sup>k</sup> homozygotes have normal tails, normal viability and normal fertility. Complete t-haplotypes, e.g. t<sup>nos</sup>, contain an allele of tct which led to their detection.

(iii) Ethylnitrosourea mutagenesis and mutant screening

Males of t<sup>nos</sup>/+ genotype were produced by mating CBA/Ca females to T<sup>+</sup>/t<sup>+</sup>+ t<sup>nos</sup>+ males and selecting normal-tailed animals. At 8–10 weeks of age they were mutagenized with 250 mg ENU/kg body weight as described by Bode (1984). Beginning about 8 weeks after injection (they were sterile from week 3 to week 8–12), each mutagenized male was caged with 2–3 females carrying qk and tf mutations in various combinations, e.g. qk+/+/tf, qktf/+tf, and qktf/qk+. The males were transferred weekly to a new set of females. On the average, 200 offspring of each mutagenized male were screened. Offspring were examined for the tailless or short-tailed phenotype at birth and the quaking phenotype at weaning. They were observed during 2–3 additional weeks for the appearance of tufted and then bled to prepare red cell lysates and determine the electrophoretic mobility of their glyoxalase-1 activity.

(iv) Assay for glyoxalase-1

The electrophoretic mobility of the glyoxalase-1 enzyme was assayed using a procedure obtained from Dr Eva Eicher. Blood samples, 75 μl, were obtained from the retro-orbital sinus of 3–5 week-old mice. The sample was diluted in 1:5 ml phosphate buffered saline and the red blood cells were packed and lysed with 38 μl distilled water. The hemolysate was applied to cellulose acetate strips (Titan III, Helena Laboratories). The strips were electrophoresed in tris-glycine buffer, pH 8.5 (3·0 g Trizma® base, 14·4 g glycine per 1 distilled water) for 30 min at 200 v. (cathode to anode), and then overlayed with 5 ml of an agar solution containing 2 ml of 2% agar and 3 ml of substrate solution (0·9 ml methylglyoxal and 250 mg gluta-
New mutations induced by ENU in a t-haplotype

Table 1. Summary of new mutations induced in the t<sup>ne5</sup>-haplotype

<table>
<thead>
<tr>
<th>Gene</th>
<th>Number of sperm screened&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mutants found&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>4869</td>
<td>[T&lt;sup&gt;e5&lt;/sup&gt;1, T&lt;sup&gt;e5&lt;/sup&gt;2, T&lt;sup&gt;e5&lt;/sup&gt;3, T&lt;sup&gt;e5&lt;/sup&gt;4*]</td>
</tr>
<tr>
<td>qk</td>
<td>2754</td>
<td>[q&lt;sup&gt;kr&lt;/sup&gt;, q&lt;sup&lt;k&lt;/sup&gt;]</td>
</tr>
<tr>
<td>tf</td>
<td>2284</td>
<td>[t&lt;sup&gt;fs&lt;/sup&gt;1, t&lt;sup&gt;fs&lt;/sup&gt;2, t&lt;sup&gt;fs&lt;/sup&gt;3, t&lt;sup&gt;fs&lt;/sup&gt;4]</td>
</tr>
<tr>
<td>Glo-1</td>
<td>3174</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> The number of different mutagenized males whose offspring were tested for mutations at the specified locus is: T = 21, qk = 17, tf = 17, and Glo-1 = 18. About 200 offspring were examined from each male.

<sup>b</sup> Mutants within a given set of brackets came from the same male and presumably have identical mutant alleles.

<sup>*</sup> Died before heritability testing.

Due to male segregation distortion in favour of the t<sup>ne5</sup>-haplotype, no mutations were transmitted to the resi- dent chromosome 90% of the time, and the detected mutations on chromosome 17 were those induced in t<sup>ne5</sup>-chromatin, as was desired, rather than those induced in the normal chromatin of its wild type homologue. Previous experiments (Bode, 1984) suggested that mutagenized males produce sperm from between 50 and 150 different clones of spermatogonial stem cells. In the experiments reported here, given that a male produced sperm that was mutant at a specific locus not on chromosome 17, it was necessary to scan, on the average, 102 offspring before observing a mutant. For the mutations in the t<sup>ne5</sup>-chromatin, only 69 offspring were required, on the average, before a mutant animal was observed. This is a consequence of the transmission distortion of the t<sup>ne5</sup>-haplotype and accounts for the numerous repeat mutant isolates from a given male.

3. Results

(i) Mutations at the T, qk and tf loci were induced by ENU

Results of the mutagenesis experiment are summarized in Table 1. Nine short-tailed or tailless animals were observed, representing at least two independent occurrences of a mutation to T phenotype. Five quaking animals were observed representing at least three independent occurrences of mutation to that phenotype. Three tufted animals were observed representing at least two independent occurrences of mutation. (We isolated an additional independent tf<sup>fs</sup> allele which is not included in this data because it occurred in a group of animals that was not being routinely checked for tufted.) No Glo-1 electro- phoretic polymorphism was found among 3174 offspring of 18 different mutagenized males. We might miss such a mutant when it occurs. Many t-haplotypes have very weak Glo-1 activity (Nadeau, Phillips & Egorov, 1985) and it may be difficult to recognize an electrophoretic variant when it is heterozygous with an allele of much higher activity.

A variety of other mutants, not associated with chromosome 17, were observed during the experiment but are not listed in Table 1. These include mutations causing the following phenotypes in the heterozygotes: white-spotting, circling, curly hair, Yoda (permanent hair loss by 10 weeks and blindness at 8 months), hydrocephaly, long teeth, and two X-linked lethals.

Due to the segregation distortion associated with the t<sup>ne5</sup>-haplotype, t<sup>ne5</sup>/+ mutagenized males transmit the t<sup>ne5</sup> chromosome ninety per cent of the time, and the detected mutations on chromosome 17 were those induced in t<sup>ne5</sup>-chromatin, as was desired, rather than those induced in the normal chromatin of its wild type homologue. Previous experiments (Bode, 1984) suggested that mutagenized males produce sperm from between 50 and 150 different clones of spermatogonial stem cells. In the experiments reported here, given that a male produced sperm that was mutant at a specific locus not on chromosome 17, it was necessary to scan, on the average, 102 offspring before observing a mutant. For the mutations in the t<sup>ne5</sup>-chromatin, only 69 offspring were required, on the average, before a mutant animal was observed. This is a consequence of the transmission distortion of the t<sup>ne5</sup>-haplotype and accounts for the numerous repeat mutant isolates from a given male.

(ii) Properties of the new <i>T<sup>k1</sup></i> mutants

It was not clear, a priori, what phenotype would result when a <i>T</i> mutation was introduced in <i>cis</i> to the resident <i>ctc</i> mutation of the t<sup>ne5</sup>-haplotype. In normal chromatin, <i>T</i>+/<i>ctc</i> heterozygotes characteristically have a shortened blunt tail but this phenotype varies in different genetic backgrounds. In some strains, there are occasional normal-tailed animals, and in others a

Table 2. Interaction of <i>T<sup>k1</sup>ne5</i> with a trans tct allele

<table>
<thead>
<tr>
<th>Genotype of parents</th>
<th>Number and tail phenotype of offspring&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tailless</td>
</tr>
<tr>
<td>(1) tct&lt;sup&gt;+&lt;/sup&gt;/tct&lt;sup&gt;+&lt;/sup&gt; x T&lt;sup&gt;ne5&lt;/sup&gt;&lt;i&gt;T&lt;/i&gt;/+</td>
<td>22</td>
</tr>
<tr>
<td>(2) tct&lt;sup&gt;+&lt;/sup&gt;/tct&lt;sup&gt;+&lt;/sup&gt; x T&lt;sup&gt;ne5&lt;/sup&gt;&lt;i&gt;T&lt;/i&gt;/+</td>
<td>19</td>
</tr>
<tr>
<td>(3) &lt;i&gt;T&lt;/i&gt;+/tct&lt;sup&gt;+&lt;/sup&gt; x T&lt;sup&gt;ne5&lt;/sup&gt;&lt;i&gt;T&lt;/i&gt;/+</td>
<td>13</td>
</tr>
</tbody>
</table>

<sup>a</sup> Due to male segregation distortion in favour of the T<sup>ne5</sup> chromosome and to the lethality of <i>T</i>/<i>T</i> homozygotes, it is not unreasonable that only one normal-tailed <i>T</i>+/+ and no <i>T</i>+/tct<sup>+</sup> or short-tailed <i>T</i>+/tct<sup>+</sup> animal was present in the limited number of offspring examined here.
few per cent of the heterozygotes are completely tailless. When present in trans to a tct mutation (T+/+ + tct), T mutations cause the animal to be tailless. One might predict that tct in the t aos-haplotype would interact to give a tailless phenotype regardless of whether the T mutation was in cis or trans. However, this prediction ignores the T rol deletion, which presumably includes T and tct and results in a short blunt tailed, not a tailless, phenotype. Although a few tailless offspring do occur, the new T mutations interact with the cis tct mutation to give predominantly mice with short tails. When a tct mutation is present in trans (Table 2), all the mice are tailless. This is true both when T rol is paired with the ENU-induced tctk mutation in a normal chromosome, lines 1 and 2 of the table, and when paired with a partial t-haplotype that includes the same tct mutation that is present in cis as a part of the t aos-haplotype, line 3. (Note that in these trans tests the T rol bearing chromosome also has the t aos resident tct mutant allele in cis.)

All reported mutant alleles of T are lethal when homozygous but several of them are known to be deletions. T rol mutants are lethal when paired with other T mutations but until we have transferred the new T rol mutations into a partial t-haplotype that is homozygous viable, and thereby separated them from the t aos-lethality mutation present in the haplotype where they were induced, it is not possible to demonstrate unambiguously that they are recessive lethals or to characterize the time of their embryonic lethality.

The T rol phenotype is transmitted with distortion strongly in its favour from males but shows a normal transmission by females, Table 3. This demonstrates that the mutations were, in fact, introduced into the t aos-haplotype.

(iii) Properties of qkkt mutants

Male transmission distortion and linkage to tct both indicate that the qkkt mutations also were induced within the t aos-haplotype. The new qkkt alleles differ in at least two ways from the mutant qk allele that was available in normal chromatin before our studies, and which was used to isolate the new alleles. That mutation is recessive and gives viable, quaking animals when homozygous, but the qk/qk males are sterile (Bennett et al. 1971). In contrast, quaking males of qk+/qkkt aos genotype are fertile, and homozygosity for qkkt is lethal.

Because of the lethality, t aos, present in the t aos-haplotype, the recessive lethality of qkkt mutants cannot be demonstrated in the usual manner. One way of separating the qkkt lethality from the t aos-lethality is to make a small region that includes qk hemizygous using the deletion present in the T rol chromosome. When T + + tf/qkkt aos + females are mated with T rol/+ males, no T rol/+ +/+qkkt aos offspring are observed (e.g. 0/35 progeny with qkkt), but when T rol/+ males are mated with control T+/tf/+ t aos + females, viable T rol/+ +/+t aos tailless offspring are found (9/20 progeny). This result indicates that either the qkkt mutation is hemizygous lethal, or it is closely linked to a newly induced lethal. The latter is unlikely since two independent mutations, qkkt and qkkt, are hemizygous lethal. Furthermore, attempts to obtain viable quaking animals by pairing qkkt alleles with a qk mutation induced by ENU in normal chromatin (Bode, 1984) also failed. The results suggest that these mutations are in the same essential gene and that it is the qkkt mutation that causes the inviability.

(iv) The new tfkt mutants

The heritability and location of the tfkt mutations were verified by demonstrating the transfer of the recessive tufted phenotype with transmission distortion in the male and by their linkage to the t aos and tct markers of the t aos-haplotype. When paired with a different complete t-haplotype, e.g. + lctt tfkt / t aos, + , recombination can occur between tfkt and the t aos-lethality (t aos) at a frequency of 6% (Justice and Bode, unpublished), similar to that reported by Shin, Bennett & Artzt (1984) for the tf mutation that arose spontaneously in the t aos-haplotype.
New mutations induced by ENU in a t-haplotype

4. Discussion

Mutations were induced by ENU in the T, qk, and tf genes of the t<sub>los</sub>-haplotype at an average per locus frequency of 1:1500. This is calculated as previously described (Bode, 1984), and counts only the mutant animals that lived to maturity and with which we could demonstrate heritability. In other studies involving ENU, average mutation frequencies in the range of 1:1100–1:2700 per locus were reported for a variety of genes in normal chromatin (Hitotsumachi, Carpenter & Russell, 1983; Johnson & Lewis, 1981, Peters, 1983; Russell et al. 1979; Russell et al. 1982a, b). In our previous experiments involving the isolation of qk and tf mutations in normal chromatin, mutants were observed at a frequency of 1:1500 (Bode, 1984).

Although the number of animals screened and mutant alleles isolated in our studies are too small to warrant a detailed locus by locus consideration, there is no suggestion that any one of these loci is either particularly mutable or exceptionally refractory. Similarly, there is no indication that the mutability of the genes in t-chromatin is any different from that of some average gene or of its corresponding copy in normal chromatin.

Based on its action in Drosophila, we expect ENU to cause single base changes and not deletions or rearrangements (Vogel & Natarjan, 1979). In the two cases where it has been examined in the mouse, the reported changes are A-T and A-G (Popp et al. 1983; Peters et al. 1985). This means that the phenotype of an ENU-induced mutant should provide a relatively clean picture of the gene function in the sense that if it exhibits pleiotropic effects, these reflect the function of that gene and not the complexity of the mutational change.

The properties of the original spontaneous mutation which defined the qk locus suggest that it may be a complex mutant. It is homozygous and hemizygous viable with a recessive phenotype that includes quaking and male sterility. It interacts with the three ENU-induced qk<sup>k</sup> mutations, all presumably due to single base changes, to give male fertile but quaking animals. In contrast, all three qk<sup>k</sup> mutations, one in normal and two in t-chromatin, are recessive lethals. Thus, qk<sup>k</sup> mutants complement the recessive defect in sperm production present in the qk mutant and it in turn complements their lethality, but these qk/qk<sup>k</sup> animals still have seizures and quake. Although it appears that the original qk mutant is complex, more information is needed before the nature of this complexity or of the complex action of that gene can be specified. We routinely try to isolate at least two independent ENU-induced mutant alleles for a given locus so that allele specific components of a phenotype will be apparent.

Several laboratories have demonstrated that the positions of H-2 and tf are inverted in t-haplotypes (Artzt et al. 1982b; Condamine et al. 1983; Rogers & Willson, 1983; Shin et al. 1983), and it is important to know the arrangement of other genes in t-chromatin. One reason for isolating new mutations in the t<sub>los</sub>-haplotype was to explore this by classical genetic crosses. Crosses involving T<sup>kt</sup> and qk<sup>kt</sup> alleles are in progress to determine if the order of T and qk is normal in the t<sub>los</sub> chromosome. The properties of partial t-haplotypes indicate that these genes do lie in the proximal part of the t-region, near their normal positions, rather than distal to tufted. Nevertheless, the proximal region of t-chromatin does not recombine at a normal frequency with wild-type chromatin. If gene rearrangement is to be a general and sufficient explanation for the greatly reduced recombination between t and normal chromosomes, there must be rearrangement in this region also. The mutants described here should allow us to determine if the T-qk order is normal or inverted.

Efficient spermatogonial stem cell mutagenesis by ENU provides a new and powerful tool for mammalian biology. In the mouse, one now can employ genetic strategies which previously were only tenable in micro-organisms or Drosophila. The induction of specific mutants is a capability that will be invaluable for unraveling complex biological phenomena and dissecting mammalian developmental pathways.

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