Induction of new mutations in a mouse *t*-haplotype using ethylnitrosourea mutagenesis

MONICA J. JUSTICE AND VERNON C. BODE

Division of Biology, Ackert Hall, Kansas State University, Manhattan, Kansas 66506 U.S.A.

(*Received 11 November 1985*)

Summary

N-ethyl-N-nitrosourea (ENU) was used to induce mutations within the t^{w5}-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^{w5}-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 lethalities from different complementation groups (t^x/t^y) (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.* 1983*b*; Fox *et al.* 1985).

A new *tf* mutation in the t^{w12}-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*-lethalities, 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, 1982*a*; Artzt, Shin & Bennett, 1982*b*; 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 $t^{w5}/+$ genotype with ethylnitrosourea to obtain t^{w5}-haplotypes with mutant alleles at one of four loci: *T*, *qk*, *tf*, or *Glo-1*. The t^{w5}-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
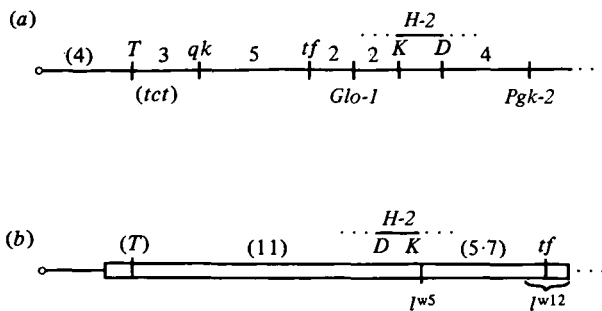
(a)

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       (4)    T  3  qk   5    tf  2    2  K   D     4
    o───────┼──┼─────┼─────┼──┼────┼──┼───┼────┼──── . . .
              (tct)                   Glo-1         Pgk-2
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$$H\text{-}2$$

(b)

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    o──┤      (T)         (11)      D   K    (5·7)    tf
       └────────────────────────┬────┬──────────┘────  . . .
                                 l^{w5}        l^{w12}
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$$H\text{-}2$$

Fig. 1. Genetic map of (a) normal and (b) t-chromatin. Distances on the two maps cannot be compared directly. See the text for an explanation of gene symbols.

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 lethalities (*l*) present in the haplotypes t^{w5} and t^{w12} 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 $T+tf/+t^{w5}+$ 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 $T+tf/+t^{ks2}tf$ strain arose as a rare recombinant in this inbred $T+tf/+t^{w5}+$ line. It retains the proximal portion of the t^{w5}-haplotype including the mutant *tct* allele present in t^{w5} but it has lost the t^{w5}-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 $T+tf/+t^{w5}+$, CBA/Ca, and C57BL/6 strains listed above.

The T^{Orl} 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 tct^k/tct^k homozygotes have normal tails, normal viability and normal fertility. Complete *t*-haplotypes, e.g. t^{w5}, contain an allele of *tct* which led to their detection.

(iii) *Ethylnitrosourea mutagenesis and mutant screening*

Males of $t^{w5}/+$ genotype were produced by mating CBA/Ca females to $T+tf/+t^{w5}+$ 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-

Table 1. *Summary of new mutations induced in the* t^{w5}-*haplotype*

Gene	Number of sperm screened[a]	Mutants found[b]
T	4869	[T^{kt1}, T^{kt2}, T^{kt3}, T^{kt*}] [T^{kt4}, T^{kt5}, T^{kt6}, T^{kt7}, T^{kt*}]
qk	2754	[qk^{kt1}] [qk^{kt*}] [qk^{kt2*}, qk^{kt3}, qk^{kt4}]
tf	2284	[tf^{kt1}] [tf^{kt3}, tf^{kt5}]
Glo-1	3174	0

[a] 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.

[b] Mutants within a given set of brackets came from the same male and presumably have identical mutant alleles.

* Died before heritability testing.

thione in 50 ml of 0·2 M phosphate buffer, pH 6·8). After 10 min, a solution of MTT tetrazolium (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), 1 mg/ml in tris-glycine buffer, was applied dropwise to the agar overlay. Protein bands with glyoxalase activity appear white in a purple background.

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^{kt} 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^{w5}-haplotype, $t^{w5}/+$ mutagenized males transmit the t^{w5} chromosome ninety per cent of the time, and the detected mutations on chromosome 17 were those induced in t^{w5}-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^{w5}-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^{w5}-haplotype and accounts for the numerous repeat mutant isolates from a given male.

(ii) *Properties of the new* T^{kt} *mutants*

It was not clear, *a priori*, what phenotype would result when a T mutation was introduced in *cis* to the resident *tct* mutation of the t^{w5}-haplotype. In normal chromatin, $T/+$ 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* $T^{kt}t^{w5}$ *with a* trans tct *allele*

Genotype of parents		Number and tail phenotype of offspring[a]			
Female	Male	Tailless	Short	Normal	Total
(1) tct^k/tct^k	× $T^{kt1}t^{w5}/++$	22	0	1	23
(2) tct^k/tct^k	× $T^{kt4}t^{w5}/++$	19	0	0	19
(3) $T+tf/+t^{k82}tf$	× $T^{kt2}t^{w5}+/+++$	13	0	0	13

[a] Due to male segregation distortion in favour of the $T^{kt}t^{w5}$ chromosome and to the lethality of T/T homozygotes, it is not unreasonable that only one normal-tailed $tct/+$ and no $+t^{k82}tf/+++$ or short-tailed $T+tf/+++$ animal was present in the limited number of offspring examined here.

Table 3. *Transmission ratio distortion in* $T^{kt}t^{w5}/++$ *males*

Genotype of Parents		Number and Tail Phenotype of Offspring			
Female	Male	Tailless	Short	Normal	Total
(1) $++/++$ × $T^{kt1}t^{w5}/++$	4	91	7	102[a]
(2) $T^{kt1}t^{w5}/++$ × $++/++$	0	27	33	60
(3) $++/++$ × $T^{kt2}t^{w5}/++$	25	62	8	95[b]
(4) $T^{kt2}t^{w5}/++$ × $++/++$	6	23	23	52
(5) $++/++$ × $T^{kt4}t^{w5}/++$	4	93	9	106[c]
(6) $T^{kt4}t^{w5}/++$ × $++/++$	0	11	11	22

[a] Data from 6 males
[b] Data from 2 males.
[c] Data from 4 males.

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^{w5}-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^{orl} 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^{kt} 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^{kt} is paired with the ENU-induced tct^k 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^{w5}-haplotype, line 3. (Note that in these '*trans*' tests the T^{kt} bearing chromosome also has the t^{w5} 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^{kt} mutants are lethal when paired with other T mutations but until we have transferred the new T^{kt} mutations into a partial *t*-haplotype that is homozygous viable, and thereby separated them from the t^{w5}-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^{kt} 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^{w5}-haplotype.

(iii) *Properties of* qk^{kt} *mutants*

Male transmission distortion and linkage to *tct* both indicate that the qk^{kt} mutations also were induced within the t^{w5}-haplotype. The new qk^{kt} 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 $qk+/qk^{kt}t^{w5}$ genotype are fertile, and homozygosity for qk^{kt} is lethal.

Because of the lethality, l^{w5}, present in the t^{w5}-haplotype, the recessive lethality of qk^{kt} mutants cannot be demonstrated in the usual manner. One way of separating the qk^{kt} lethality from the t^{w5}-lethality is to make a small region that includes qk hemizygous using the deletion present in the T^{orl} chromosome. When $T++$ $tf/+qk^{kt}t^{w5}+$ females are mated with $T^{orl}/+$ males, no $T^{orl}++/+qk^{kt}t^{w5}$ offspring are observed (e.g. 0/35 progeny with qk^{kt1}), but when $T^{orl}/+$ males are mated with control $T+tf/+t^{w5}+$ females, viable $T^{orl}+/+t^{w5}$ tailless offspring are found (9/20 progeny). This result indicates that either the qk^{kt} mutation is hemizygous lethal, or it is closely linked to a newly induced lethal. The latter is unlikely since two independent mutations, qk^{kt1} and qk^{kt4}, are hemizygous lethal. Furthermore, attempts to obtain viable quaking animals by pairing qk^{kt} alleles with a qk^k 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 qk^{kt} mutation that causes the inviability.

(iv) *The new* tf^{kt} *mutants*

The heritability and location of the tf^{kt} mutations were verified by demonstrating the transfer of the recessive tufted phenotype with transmission distortion in the male and by their linkage to the l^{w5} and *tct* markers of the t^{w5}-haplotype. When paired with a different complete *t*-haplotype, e.g. $+l^{w5}tf^{ktl}/l^{12}++$, recombination can occur between tf^{ktl} and the t^{w5}-lethality (l^{w5}) 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^{w12}-haplotype.

4. Discussion

Mutations were induced by ENU in the *T*, *qk*, and *tf* genes of the t^{w5}-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.* 1982*a*, *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^k mutations, all presumably due to single base changes, to give male fertile but quaking animals. In contrast, all three qk^k mutations, one in normal and two in *t*-chromatin, are recessive lethals. Thus, qk^k mutants complement the recessive defect in sperm production present in the *qk* mutant and it in turn complements their lethality, but these qk/qk^k 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.* 1982*b*; Condamine *et al.* 1983; Rogers & Willi-

son, 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^{w5}-haplotype was to explore this by classical genetic crosses. Crosses involving T^{kt} and qk^{kt} alleles are in progress to determine if the order of *T* and *qk* is normal in the t^{w5} 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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