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Genetic and molecular analysis of chlorambucil-induced germ-line mutations in the mouse


ABSTRACT

Eighteen variants recovered from specific locus mutation rate experiments involving the mutagen chlorambucil were subjected to several genetic and molecular analyses. Most mutations were found to be homozygous lethal. Because lethality is often presumptive evidence for multilocus-deletion events, 10 mutations were analyzed by Southern blot analysis with probes at, or closely linked to, several of the specific locus test markers, namely, albino (c), brown (b), and dilute (d). All eight mutations (two c; three b; two d; and one dilute-short ear [Dfd(d se)] that arose in post-spermatogonial germ cells were deleted for DNA sequences. No evidence for deletion of two d-se region probes was obtained for the remaining two d mutations that arose in stem-cell spermatogonia. Six of the primary mutants also produced low litter sizes ("semisterility"). Karyotypic analysis has, to date, confirmed the presence of reciprocal translocations in four of the six. The high frequency of deletions and translocations among the mutations induced in post-spermatogonial stages by chlorambucil, combined with its overall high efficiency in inducing mutations in these stages, should make chlorambucil mutagenesis useful for generating experimentally valuable germ-line deletions throughout the mouse genome.

Analysis of heritable mutations at the molecular and organismal levels can provide significant insights into the structural and functional composition of the mammalian genome. Certain types of mutations, namely, chromosome deletions, translocations, and insertion-induced mutations, provide useful experimental reagents for accessing, at the molecular level, particular chromosomal segments or loci associated with specific developmental phenotypes.

In humans, chromosomal deletions have proved especially useful in the selection of DNA clones that map to genomic subregions associated, for example, with muscular dystrophy (1-3), chronic granulomatous disease (4), retinoblastoma (5, 6), and sex determination (7). In the mouse, analysis of complexes of radiation-induced deletion mutations has facilitated the development of gross functional maps of segments of the genome (8-11) and is currently providing reagents for the development of fine-structure "point" mutation maps (12) as well as for more detailed molecular maps. Consequently, the exploitation of germ-line deletion mutations has great potential for expanding our understanding of the functional and physical composition of the mouse genome. However, most of the extant heritable deletion mutations of the mouse have arisen over a period of decades in radiation-mutagenesis experiments and are limited to genomic regions including and surrounding recessive marker loci employed in the mouse visible specific locus test (13, 14) and a few dominant marker loci (15-17). Therefore, currently available deletions cover only a small percentage of the genome.

The chemotherapeutic agent chlorambucil (CHL) can induce, in certain germ-cell stages of male mice, heritable specific locus mutations at frequencies perhaps as high as $1.3 \times 10^{-7}$ mutations per locus per gamete (18). The highest frequency of mutations is transmitted by sperm derived from CHL-exposed early spermatids—i.e., in progeny sired during the third week after CHL injection. Some of the mutations recovered from these and other post-spermatogonial germ-cell stages displayed genetic attributes usually associated with chromosomal deletions and translocations (18). For example, a variant was recovered that was simultaneously mutant in d (dilute) and se (short ear), which are two closely linked [0.16 centimorgan (cM)] loci, suggesting a deletion event. Further, several lethal mutations were also identified that, on the basis of earlier findings (8, 9, 19), could be assumed to carry deletions of the marker locus along with neighboring essential loci. Semisterility (low litter size) was observed in several mutant lines, which is often indicative of the segregation of a balanced translocation (20). Furthermore, direct cytogenetic analysis also detected a three-band deletion of chromosome 4 in G-banded chromosome preparations derived from a b (brown) locus mutant as well as a translocation in a c (albino) locus mutant line (18).

If the majority of post-spermatogonially induced CHL mutations are indeed deletional in nature, this mutagenesis protocol should be a highly efficient method for producing germ-line deletion mutations in the mouse. We report here further genetic and cytogenetic analysis of the set of specific locus mutations that were recovered in the CHL-mutagenesis experiments (18), and the initial molecular characterization of 10 of them (two c, three b, four d, and one d-se). Eight of these 10 mutations were derived from CHL-exposed postgonial germ-cell stages and two were derived from exposed stem-cell spermatogonia. We demonstrate that the 8 mutations induced in postgonial stages are deleted for molecular probes at, or closely linked to, the marker loci, thereby confirming that CHL treatment of post-stem-cell stages does indeed induce deletion mutations with high efficiency. We also provide evidence that, in these same germ-cell stages, CHL induces heritable reciprocal translocations.

MATERIALS AND METHODS

Mice. All mice were bred at Oak Ridge National Laboratory. Each mutation studied was recovered in the progeny of CHL-treated (101/R1 × C3Hf/R1)F1 wild-type males that had been crossed to T-stock females of the genotype a/a; b/b; p c^h/p c^h; d se/d se; s/s (18). The mutations, along with their

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Abbreviations: CHL, chlorambucil; cM, centimorgan.

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currently known characteristics, are listed in Table 1. Fetuses produced from crosses of $c^{b}/c^{a1} \times c^{b}/c^{a2}$ heterozygotes (where $c^{a1}$ and $c^{a2}$ are any two mutations involving c) that were compound heterozygotes for albino mutations were identified at 14.5 days post coitum as those fetuses lacking eye pigment.

**Probes and Hybridization Protocols.** The MTY811 tyrosinase cDNA clone (21) was used as a probe for the c locus. Clone 23.3, a 1.4-kilobase (kb) EcoRI–HaeIII fragment derived from a region 5' to the site of integration of the Emv-23 endogenous ecotropic provirus (22), was used as an additional closely linked (0.5 cm) molecular marker for c region mutations. A 0.25-kb Pvu II fragment of the pMT4 clone (23) was used as a specific probe for the b locus (24). For the d-se region mutations, we employed clones p0.3, a 2.6-kb EcoRI–Pst I fragment derived from the region Y' to the site of integration of the d-associated provirus Emv-3 (10, 25), and p94.1, a 0.5-kb EcoRI–HindIII fragment derived from the proximal end of a 3.3-kb EcoRI deletion breakpoint fusion fragment carried in the d$^{or-22q}$ prenatal-lethal mutation (10).

High molecular weight DNA was obtained by standard methods (26, 27) from distal tail-tip biopsies from either primary mutants or their progeny. In certain instances, spleen or fetal DNAs were analyzed from progeny of genetic crosses involving specific CHL-induced mutations. Southern blot analysis of genomic DNA was performed as described (22), except that DNA was transferred to nylon in 10× SSC (instead of alkali) and was crosslinked to the membrane by exposure to UV (instead of baking). (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0.)

**RESULTS**

**Genetic and Cytogenetic Analysis of CHL-Induced Mutations.** Each specific locus mutation (m*) is recovered in heterozygosity with the corresponding recessive marker (m) contributed by the T stock. The primary mutants were crossed with appropriate stocks to confirm allelism (18), and subsequent crosses were made to generate homozygotes (m*/m*) from each mutation. In cases where m/m* is phenotypically distinguishable from m/m (e.g., c*c*e* mutants) or where the T stock supplies a linked marker (e.g., d se/d* mutants), appropriate heterozygotes to be mated for the production of homozygotes can be directly identified, and the absence of homozygotes among 30 or more of their classified offspring was taken as evidence of prenatal lethality. In other cases (s/s* or b/b* mutants), lines that could be either m/m or m*/m* were generated by outcrosses to wild type, followed by an additional outcross and backcross; when all of 12 such lines were viable, the mutation was provisionally classified as viable. [Assuming equal transmission of m* and m, the probability that all 12 lines would be m/m by chance is (0.5)$^{12}$ or $2 \times 10^{-4}$; this probability is, however, greater if transmission of m* is reduced.] Homozygous lethality/viability results are shown in Table 1, along with the fertility status of animals heterozygous for the mutation.

Since production of a reduced number of offspring can be indicative of gross chromosomal rearrangement (death of chromosomally unbalanced segregants), mutants that produced low litter size were studied cytogenetically in Giemsa-banded mitotic metaphase preparations from cultured kidney fibroblasts (28). The results of this karyotypic analysis, which

**Table 1. Heritable CHL-induced specific locus mutations used in this study**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Germ-cell stage of origin</th>
<th>Viability/lethality of homozygotes</th>
<th>Fertility of heterozygotes*</th>
<th>Karyotype</th>
<th>Deletion detectable with probe†</th>
</tr>
</thead>
<tbody>
<tr>
<td>f$^{or-7q}$</td>
<td>Spermatocytes</td>
<td>—</td>
<td>S</td>
<td>MTY811</td>
<td>23.3</td>
</tr>
<tr>
<td>b$^{or-10q}$</td>
<td>Late spermatocytes</td>
<td>Unknown</td>
<td>F</td>
<td>M4</td>
<td>0.3</td>
</tr>
<tr>
<td>b$^{or-19q}$</td>
<td>Spermatocytes</td>
<td>TNC‡</td>
<td>PF</td>
<td>Del(4C1-C3)</td>
<td>94.1</td>
</tr>
<tr>
<td>b$^{or-21q}$</td>
<td>Early spermatids</td>
<td>Lethal</td>
<td>F</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>a$^{or-8q}$</td>
<td>Late spermatocytes</td>
<td>Lethal</td>
<td>F</td>
<td>(T;?)</td>
<td>Yes</td>
</tr>
<tr>
<td>a$^{or-15q}$</td>
<td>Early spermatids</td>
<td>Lethal</td>
<td>F</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>d$^{or-18q}$</td>
<td>Spermatocytes</td>
<td>Lethal</td>
<td>SS</td>
<td>Yes</td>
<td>—</td>
</tr>
<tr>
<td>d$^{or-37q}$</td>
<td>Early spermatids</td>
<td>Lethal</td>
<td>SS</td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td>Df(d se)^s, D(34q)</td>
<td>Early spermatids</td>
<td>Lethal</td>
<td>F</td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td>s$^{or-10q}$</td>
<td>Early spermatids</td>
<td>Viable</td>
<td>F</td>
<td>T;1(17)</td>
<td>Yes</td>
</tr>
<tr>
<td>s$^{or-9q}$</td>
<td>Early spermatids</td>
<td>Viable</td>
<td>F</td>
<td>NRO</td>
<td>—</td>
</tr>
<tr>
<td>s$^{or-11q}$</td>
<td>Early spermatocytes††</td>
<td>Lethal</td>
<td>F</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>s$^{or-14q}$</td>
<td>Early spermatids</td>
<td>Lethal</td>
<td>F</td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td>d$^{or-22q}$</td>
<td>Stem-cell spermatogonia</td>
<td>Juvenile</td>
<td>F</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>p$^{or-20q}$</td>
<td>Stem-cell spermatogonia</td>
<td>Viable</td>
<td>F</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>p$^{or-19q}$</td>
<td>Stem-cell spermatogonia</td>
<td>—</td>
<td>F</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*Fertility of primary mutant and/or heterozygous descendants (unless shown otherwise): F, fertile; S, sterile; SS, semisterile (low litter size); PF, poorly fertile (infrequent matings).
†Only the c, b, and d mutations were analyzed with molecular probes. MTY811 and 23.3 are c region probes; MT4 is a b region probe; 0.3 and 94.1 are d-se region probes. —, Not done.
‡Included as a control DNA for this particular probe; see text.
¶Test not complete (TNC). Heterozygotes are ~45% weight of same-sexed littermates.
‖While the primary mutant was semisterile, semisterility subsequently segregated independently of the specific locus mutation.
‖No rearrangement was observed in karyotypes.
§Most die at 20–38 days (median, 27 days) with megacolon. Typically, pigmented areas of the fur comprise only ~10% of the surface area.
¶¶Or, differentiating spermatogonia.
††Most die at or around weaning from a type of opisthotonus resembling that seen in d$^{op}$ (8) homozygotes.
**Superscript x denotes presumed allele with expression intermediate between that of marker and wild type.
†Mutant died prior to allelism test.
are likewise summarized in Table 1, demonstrated that reciprocal translocations were also segregating within the original specific loci mutant lines. In two cases (dOR-1BQ and dOR-370), the semisterility phenotype has not yet been genetically separated from the specific locus mutation in the stock derived from the primary mutants. A T(3;6) translocation was observed in karyotypes from the dOR-1BQ line; no rearrangements could be seen in the dOR-370 line. In four cases (seOR-10Q, sOR-14AQ, cOR-8Q, and pOR-130), the semisterility phenotype segregated independently of the specific locus mutation; in three of these (seOR-10Q, sOR-14AQ, and cOR-8Q), translocations were detected that did not involve the chromosomes in which the specific locus markers reside. Although the second chromosome involved in the T(3;?) translocation in the cOR-8Q line remains to be identified, indications are that it is not chromosome 7.

Molecular Analysis of CHL-Induced e Region Mutations. Southern blot analysis of mutant DNA, utilizing probes closely linked to (or, in some cases, identical to) several of the loci employed as markers in the visible specific locus test, was used to determine whether CHL can induce molecularly detectable germ-line deletion mutations within these particular regions of the genome. Two CHL-induced c locus mutations, cOR-8Q and cOR-15Q, were tested for deletion of tyrosinase sequences (21) as well as of a closely linked (≤0.5 cM) distal DNA sequence, 23.3 (22). Since the primary mutants (cch/cOR-8Q and cch/cOR-15Q) were not available for study, we employed individuals heterozygous for the CHL-induced c mutations from the breeding stock derived from a cross of each primary mutant with a cch/cch stock (St2A).

The MTY811 tyrosinase cDNA clone recognizes five EcoRI fragments in mouse DNA (Fig. 1A). To determine whether the entire tyrosinase gene [which spans >70 kb (29)] had been deleted, carriers (cch/cOR-8Q and cch/cOR-15Q) of the two CHL-induced mutations were crossed to cch/cecch (T and St2A) spleen DNA controls, were probed with MTY811 and, as a positive control for hybridization, with the chromosome 9 clone p94.1. Fig. 1A demonstrates that both CHL-induced c mutations completely lack tyrosinase sequences but display the 2.3-kb chromosome 9 fragment. This blot was then stripped and rehybridized with the c-linked probe 23.3 (along with p94.1). Fig. 1B demonstrates that both CHL-induced mutant c chromosomes are also deleted for the 3.7-kb EcoRI fragment detected by probe 23.3.

b Region Mutations. DNAs from three primary mutants carrying CHL-induced b locus mutations were analyzed in Southern blot analysis with a 0.25-kb Pvu II subclone of pMT4, a cDNA closely associated with, and probably identical to, the b gene itself (24). A Taq I restriction fragment (5.2 kb) detected by this Pvu II fragment is present in all chromosomes carrying the standard b mutation of the mouse fancy. All other chromosomes, including C3H and 101 (both +/+/+), display two Taq I fragments of 4.0 and 1.2 kb (24). Primary mutants carrying CHL-induced b mutations carry a mutant C3H or 101 chromosome opposite a b chromosome derived from the T stock. Consequently, Taq I-digested DNAs from three primary CHL-induced b mutants, along with C3H, 101, T, and two non-b CHL-induced primary mutants (d se/Df d seOR-8Q and d se/dOR-370 +) were hybridized with the Pvu II subclone of pMT4. Fig. 2 demonstrates that all three primary CHL-induced b mutants carry only the b-associated 5.2-kb Taq 1 fragment and are deleted for the 4.0-kb/1.2-kb polymorphism carried in C3H, 101, and the two d-se-region mutants. DNAs from six homozygous b lines (b/b or b*b) set up from mutant bOR-10Q were probed with pMT4, and all were shown to be b/b (i.e., they carried only a 5.2-kb Taq 1 fragment; data not shown). If b and bOR-10Q were transmitted with equal frequency by the primary mutant, the probability that all of six lines would be b/b is only 1/64. The result is, therefore, suggestive of reduced transmission of the mutation. Since no further progeny of the primary mutant are available for test (unfortunately, bOR-10Q is now extinct), the viability/lethality of the homozygous deletion bOR-10Q/bOR-10Q will remain undetermined.

d-se Region Mutations. For this region, primary mutants carry new d, se, or Df(d se) mutations opposite a d se chromosome derived from the T stock. Therefore, new CHL-induced mutations would be recovered opposite a chromosome containing Emv-3, the provirus thought to be responsible for the original d mutation (25, 30). In wild-type DNA, clone p0.3, derived from the region of integration of Emv-3 (10, 25), recognizes a 9-kb EcoRI fragment; in d DNA, it recognizes an 18-kb fragment that represents the 9-kb wild-type fragment plus the 9-kb integrated Emv-3 provirus. Fig. 3A demonstrates that, of the five d-se region mutations that were analyzed, dOR-370 and Df(d se)OR-370 were missing the 9-kb wild-type fragment (like the radiation-induced deletion mutation Df(d se)11R45SL (10)), indicating that they are deleted for the p0.3 sequence. Three primary non-d-se region mutants (b/bOR-21Q, b/bOR-10Q, and b/bOR-10Q), used as controls, display heterozygosity for the two fragments (Fig. 3B).

Additionally, we hybridized the blot in Fig. 3A with probe p94.1 (10). In previous analyses of radiation-induced d-se
region mutations, it was noted that some prenatally lethal \( d \) mutations retain the p0.3 sequence but delete the sequence p94.1, which is derived from a chromosomal region less than 1 cM proximal to \( d \). Because there are no convenient restriction fragment length polymorphisms for the p94.1 sequence, we measured the hybridization intensity of the p94.1-associated 2.3-kb EcoRI fragment with a two-copy 3.7-kb control fragment recognized by the chromosome 7 probe 23.3. Fig. 3B presents an example of these hybridization profiles. Densitometric tracings from independent blots demonstrated that the primary mutants \( d \) se\( /dO^{OR-16Q} \) +, \( d \) se\( /dO^{OR-70Q} \) +, and \( d \) se\( /Df(d \) se\( )O^{R-34Q} \) carry only one copy of the p94.1 sequence. The primary mutants \( d \) se\( /dO^{R-30Q} \) + and \( d \) se\( /dO^{R-70Q} \) +, along with control DNAs C3H, 101, and T, carry two copies. Consequently, three \( d \)–se region mutations carry detectable deletions of DNA sequences: \( dO^{R-70Q} \) and \( Df(d \) se\( )O^{R-34Q} \) are deleted for both p0.3 and p94.1, and \( dO^{R-18Q} \) is deleted for p94.1 only.

DISCUSSION

Initial genetic and cytogenetic analyses of mutations recovered in the visible specific locus test suggested that the chemotherapeutic agent CHL could induce heritable deletions and translocations in post-stem-cell stages of spermatogenesis and that mutation yield from the most sensitive stage (early spermatids) was perhaps as high as \( 1.3 \times 10^{-7} \) mutations per locus per gamete (18). For CHL-exposed stem-cell spermatogonia, the mutation yield was not significantly greater than in controls. We have tried to generate homozygotes for 14 of the 18 mutations; 9 of 11 tested post-stem-cell mutations proved to be homozygous lethal, indicating multilocus involvement (19). The results have been further extended by our demonstration that, at the level of DNA hybridization and Southern blot analysis, all of 8 tested specific locus mutations induced in post-stem-cell stages carry deletions of specific probe sequences within the \( c \), \( b \), or \( d \)–se regions, whereas neither of the two \( d \) mutations arising in spermogonial showed detectable deletions or rearrangements with available \( d \)–se region probes.

CHL mutagenesis of post-stem cells seems, moreover, to produce germ-line deletions of substantial size. Ruppert et al. (29) have demonstrated that the coding sequences for the mouse tyrosinase gene extend over 70 kb of genomic DNA sequence. Therefore, because the two CHL-induced \( c \) locus mutations, \( cO^{R-8Q} \) and \( cO^{R-15Q} \), completely delete all sequences normally recognized by the MTSY11 cDNA clone, these deletions are at least 70 kb in size and are likely to be larger because they also delete the closely linked 23.3 sequence. Moreover, the \( Df(d \) se\( )O^{R-34Q} \) mutation is likely to be a deletion of at least 0.16 cM in genetic length (the distance between \( d \) and \( se \)) because it was originally recovered as a mutant in both the \( d \) and \( se \) loci (18) and is deleted for both the p0.3 and p94.1 sequences. Finally, perhaps most spectacular is the finding (18) that \( bO^{R-19Q} \), a mutation induced in mature spermatozoa and now known to delete sequences homologous to the putative \( b \) locus clone pMT4, is a cytologically visible deletion spanning chromosome bands 4C1–4C3, inclusive.

Exposure of post-stem cells to CHL appears to induce translocations as well as deletions. In addition to a T(X;7) found in a p-mottled variant (18), at least four other translocations were identified. All are independent of the respective specific locus mutations. That is, each of four primary specific locus mutants also carried an independent translocation [e.g., \( d \) se\( + /seO^{R-16Q} \) carried a new \( se \) mutation in chromosome 9, as well as a T(1;17) translocation]. It should
be noted that the specific locus test does not in itself score for translocations; these types of rearrangements are most often detected by the phenotype of "semisterility" (low litter size) among progeny of treated animals. For two other primary mutants that were semisterile (se/dOR-37Q+ and p/pOR-13Q), the cause of the semisterility is unknown since no cytologically detectable rearrangements were observed.

Genetic and molecular biological studies in organisms ranging from bacteriophage to humans have repeatedly demonstrated the experimental power of utilizing genomic rearrangements (and particularly deletions) for the analysis of the composition and function of whole chromosomal regions and of specific genes within particular segments of a genome. In the mouse system, the use of existing complexes of radiation-induced deletion mutations available for regions surrounding the marker loci of the visible specific locus test is facilitating the development of fine-structure physical and functional maps for regions of substantial size (8–11, 14, 19, 31). Indeed, molecular access to a specific chromosomal region and quite often to a specific gene of unknown product within a region, can be achieved by the use of deletion mutations for the mapping of DNA clones. Moreover, large deletions can be used as reagents in additional rounds of mutagenesis experiments for selecting N-ethyl-N-nitrosourea-induced presumed intragenic mutations that can aid in refining a regional functional map (12). They can also be used as reagents for simplifying the mapping of new genetic loci (especially those defined only by lethal mutations).

N-Ethyl-N-nitrosourea currently is the most effective known mutagen of mouse spermatogonial stem cells (32), where it is thought to induce primarily small intragenic changes (19, 33, 34). CHL, when applied at sensitive germ-cell stages during the post-stem-cell period, is at least as effective a mutagen as N-ethyl-N-nitrosourea (and much more potent per mole of compound) and appears to produce large lesions. The finding of eight molecularly confirmed deletions among eight specific locus mutations induced by CHL treatment of post-stem cells strongly suggests that CHL can be used to induce germ-line deletion mutations (and other chromosomal rearrangements) at a frequency that is perhaps five times what is currently achievable with classical x-ray or neutron treatments of male post-stem-cell stages or oocytes (data of W. L. Russell, cited in ref. 35). Consequently, the higher frequency of induction of germ-line deletion mutations by CHL should make possible the high-efficiency recovery of developmentally significant variants at any locus, throughout the genome, that are directly amenable to first-stage molecular access and functional analysis.

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