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Genetics

Trimethylpsoralen induces small deletion mutations in *Caenorhabditis elegans*

(mutagenesis/restriction fragment polymorphisms)

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ABSTRACT **To examine the mutagenic spectrum of 4,5′,8-trimethylpsoralen (TMP) in *Caenorhabditis elegans*, we isolated mutations in the *unc-22* and *pal-1* genes following TMP mutagenesis and analyzed them for restriction fragment length polymorphisms by Southern blot. Eleven of 21 *unc-22* mutations exhibited restriction fragment length polymorphisms, 8 of which were deletions of between 0.10 and 15 kb in length. Both of two *pal-1* mutations were also small deletions within this size range. Comparison of our results with previous studies on mutagenesis by γ-rays and x-rays suggests that the mutagenic spectrum of TMP may be similar. TMP should be useful in generating mutations that cause complete loss of function of single genes and that are likely to result in allele-specific DNA polymorphisms.**

The DNA cross-linking agent 4,5′,8-trimethylpsoralen (TMP) is activated by near ultraviolet light (1). Interstrand DNA cross-links are believed to be repaired by sequential nucleotide excision and recombinational repair (2, 3), which in *Escherichia coli* is an error-prone process that often results in deletion mutations (4). Earlier studies of the mutagenic properties of psoralen compounds in *E. coli* reported that they produce primarily transitions and single base-pair deletions (5), but more recent work showed that TMP also produces larger deletions (4).

To examine the usefulness of this mutagen in the nematode *Caenorhabditis elegans*, we have analyzed mutations at the *unc-22* and *pal-1* loci that were isolated after TMP mutagenesis. The *unc-22* gene encodes a 753-kDa protein required for normal muscle function (6, 7). It was chosen for this study for several reasons. The *unc-22* mutant phenotype is easily recognizable, and because the gene is >50 kb in length, *unc-22* mutations are frequent. Homozygous mutant animals have abnormal muscle cells and twitch continuously. Heterozygotes also exhibit the twitching phenotype when placed in 1% nicotine (8). Therefore, both homozygotes and heterozygotes can be easily identified and recovered after mutagenesis. The locus has been cloned and sequenced in its entirety (6), which greatly facilitates the mapping of restriction fragment length polymorphisms (RFLPs). Structural features of the *unc-22* gene are shown in Fig. 1.

A possible disadvantage to *unc-22* is the presence in its DNA sequence of repeated motifs (6), which might cause a bias toward deletion formation during cross-link repair. Therefore, we also analyzed TMP-induced mutations in another gene, *pal-1* (9), which does not appear to contain internal repeats (L.G.E., S. Carr, and W.B.W., unpublished data). Our analyses show that many of the mutations in the *unc-22* and *pal-1* genes following TMP mutagenesis are small deletions of <15 kb in length.

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MATERIALS AND METHODS

Strains of *C. elegans*. All strains were from our collection or were obtained from the *Caenorhabditis* Genetics Center, and all were derived from the wild-type Bristol strain N2 (10). The mutant alleles and chromosomal rearrangements used are listed below. The *mnC1* chromosome serves as a balancer for the left approximately two-thirds of linkage group (LG) II (11). The *qC1* chromosome carries a rearrangement with a breakpoint in the *glp-1* gene and serves as a balancer for LG III (12); *sDf22* is a large deletion on LG IV (13).

LG II: *mnC1*[*dpy-10*(*e128*)*unc-52*(*e444*)]
LG III: *pal-1*(*e2091, ct224, ct281*), *qC1*[*dpy-19*(*e1259*)*glp-1*(*q339*)]
LG IV: *dpy-20*(*e1282*), *unc-22*(*ct38, ct156*, and consecutively numbered alleles *ct163–ct182*), *sDf22*.

Genetic and Biochemical Methods. Standard techniques were used for growth and breeding of *C. elegans* (10, 14), isolation of *C. elegans* genomic DNA (14), growth and preparation of phage and plasmids (15), gel electrophoresis and Southern blotting (16), and the polymerase chain reaction (PCR) (17). Probes were prepared with random hexamer priming and were used under appropriate hybridization conditions (15, 16). Plasmids containing probe DNAs for the *unc-22* analysis were kindly provided by D. G. Moerman (DM17, DM18, DM20, and DM22) and G. Benian (pSL13 and pSL14). Probes used for the *pal-1* analysis were derived from the plasmid pWK14, which was kindly provided by C. Kenyon.

Method of Mutagenesis. One 15-cm plate of slightly starved N2 worms was cut into quarters, and each quarter was placed on a fresh plate. The four plates were then placed in an incubator at 20°C for 24 hr. Worms were harvested by washing the plates with sterile M9 medium (14) and then collected by low-speed centrifugation in conical tubes. After washing twice with M9 medium, as much liquid as possible was removed and the worms were resuspended in 10 times their volume of a solution prepared by diluting a 3 mg/ml stock solution of TMP (Sigma) in dimethyl sulfoxide with M9 medium to a concentration of 30 μg/ml. The tube was wrapped in foil to screen out light and placed on a rocker for 15 min. The worms were then poured onto a large uncovered Petri dish and irradiated with a UV lamp (Blak-Ray lamp, model UVL-21) at an intensity of 340 $\mu W/cm^2$ for 60 sec. After irradiation they were placed on an agar plate spread with *E. coli* and incubated at 20°C in the dark. After 5 hr, 400 L4 hermaphrodites were transferred onto 100 plates (4 per plate) at 20°C for production of F_1 progeny.

The irradiation dose used was calibrated in separate experiments by measuring F_1 embryonic lethality (scored as unhatched embryos) and production by the surviving F_1s of

Abbreviations: TMP, 4,5′,8-trimethylpsoralen; RFLP, restriction fragment length polymorphism; LG, linkage group; EMS, ethyl methanesulfonate.

F_2 embryonic lethal mutations on LG II balanced by *mnC1*. Percentages of F_1 lethality and F_2 LG II lethals were proportional over the range of 10–90% F_1 lethality. Some variability in dose–response was found with different stock solutions of TMP. Under the conditions described above, F_1 embryonic lethality was 10%, and the frequency of F_2 embryonic lethals was 4.1% for LG II, giving an estimate of about 30% for the genome as a whole.

Screening for Mutants. F_1 progeny were washed off of the plates with M9 medium and resuspended in a 1% solution of nicotine in M9 medium, in order to identify animals heterozygous (or homozygous) for *unc-22* mutations by their twitching phenotype (8). Potential *unc-22* mutations were checked for ability to complement a known allele, *unc-22(ct38)*; all failed to complement.

The two TMP-induced *pal-1* alleles were isolated in separate screens for nonmaternal-effect embryonic lethals (L.G.E., S. Carr, and W.B.W., unpublished data); both failed to complement the previously described allele *pal-1(e2091)* (9).

RESULTS

Isolation and Analysis of *unc-22* Mutants. Twenty-three independent *unc-22* mutant strains were isolated after TMP mutagenesis in a screen of 1×10^5 F_1 animals (2×10^5 genomes). This mutation frequency of about 10^{-4} is about 100-fold higher than the previously observed rate of $\leq 10^{-6}$ for spontaneous mutations at the *unc-22* locus (18). Each mutant strain was tested for noncomplementation with the nearby allele *dpy-20(e1282)*, which maps 0.2 centimorgan to the left of *unc-22*. None failed to complement, indicating that none of the recovered TMP-induced mutations are large deletions extending through the *dpy-20* gene. Seventeen mutations were homozygous viable, and six were originally homozygous lethal. Two of these (*ct180* and *ct181*) gave homozygous viable *unc-22* mutant offspring after backcrossing, and a third (*ct182*) was balanced over a chromosome carrying the deficiency *sDf22* (13). The three remaining homozygous lethal strains were lost when an incubator malfunctioned before further analysis could be performed.

The 20 remaining mutations and an additional *unc-22* mutation (*ct156*) isolated in a previous TMP mutagenesis were examined by Southern blot for RFLPs, using the probes diagrammed in Fig. 1*D*. Eleven of the strains exhibited visible polymorphisms (Table 1), approximately located as shown in Fig. 1*A*. Data for three representative polymorphisms are shown in Fig. 2.

Eight of the 11 polymorphisms could be clearly identified as deletions, ranging in size from 100 bp to about 15 kb, and all were homozygous-viable. Of the remaining three, one was a 3.5-kb insertion, and two, including the one homozygous-inviable *unc-22* mutation, appeared to be more complex rearrangements. For the remaining 10 alleles, no polymorphisms were detected by Southern blot analysis. These strains must carry either rearrangements too small to be detected, point mutations, or mutations outside the region examined.

Isolation and Analysis of *pal-1* Mutants. Two alleles of the *pal-1* gene (*ct224* and *ct281*) were isolated after TMP mutagenesis in a screen for nonmaternal-effect embryonic lethal mutants (L.G.E., S. Carr, and W.B.W., unpublished data). Each of these alleles was balanced over the *qC1* chromosome (12), and genomic DNA was prepared from heterozygous animals. When analyzed by Southern blot as above using a *pal-1* genomic probe, DNA from both alleles exhibited novel bands (data not shown). RFLP analysis indicated that both *ct224* and *ct281* are deletions of 4.2 and 4.7 kb, respectively.

DISCUSSION

Of the 21 analyzed *unc-22* mutations isolated after TMP mutagenesis, 8 are deletions ranging in size from 0.10 to 15 kb, with an average deletion size of 2.8 kb. These deletions appear to be clustered in the 3′ end of the gene. Although this clustering might indicate mutational hot spots, it is more

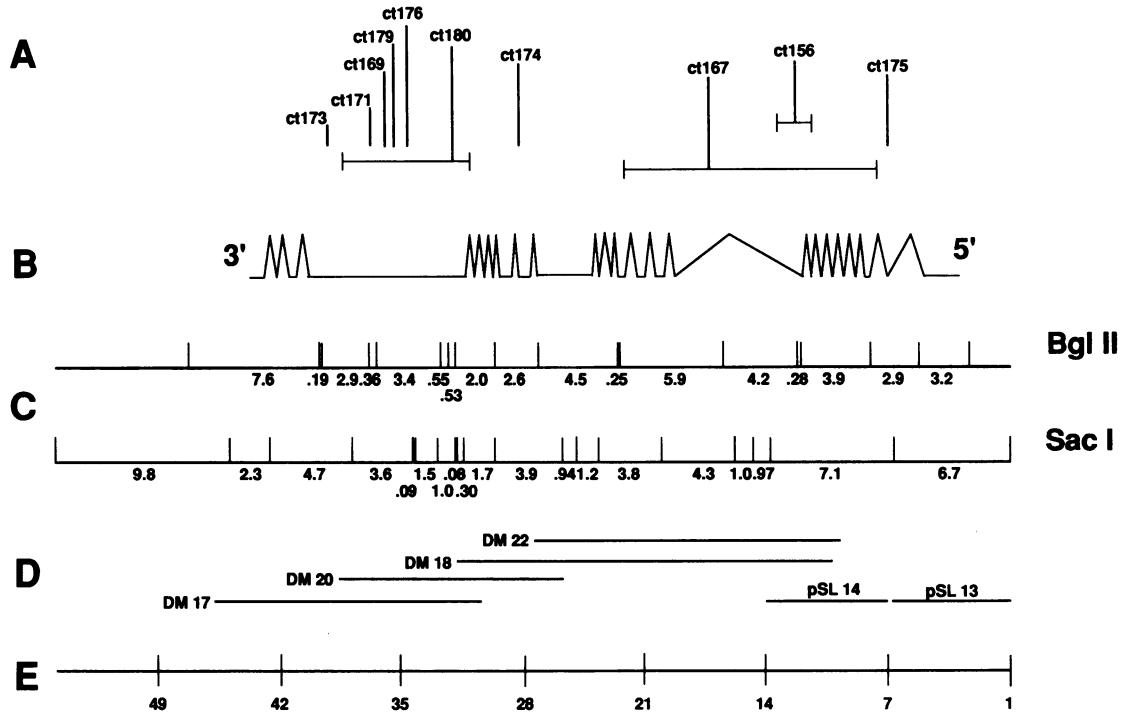

FIG. 1. Structure of the *unc-22* gene (adapted from ref. 19), showing approximate positions of RFLPs and probes used in the analysis. (*A*) Approximate positions of *unc-22* polymorphisms; uncertainties are indicated by horizontal bars. (*B*) Intron/exon boundaries of the gene. (*C*) *Sac* I and *Bgl* II restriction maps of the *unc-22* gene (6). (*D*) Regions covered by probes used in the analysis. (*E*) Size of genomic region in kb.

Table 1. Mutations in the *unc-22* and *pal-1* genes isolated following TMP mutagenesis

Allele	Type of polymorphism	Approximate size of polymorphism, kb	Viability of homozygote
unc-22(ct156)	Deletion	2.0	+
unc-22(ct163)	ND	—	+
unc-22(ct164)	ND	—	+
unc-22(ct165)	ND	—	+
unc-22(ct166)	ND	—	+
unc-22(ct167)	Deletion	15	+
unc-22(ct168)	ND	—	+
unc-22(ct169)	Deletion	3.5	+
unc-22(ct170)	ND	—	+
unc-22(ct171)	Deletion	0.1	+
unc-22(ct172)	ND	—	+
unc-22(ct173)	Deletion	0.4	+
unc-22(ct174)	Deletion	0.8	+
unc-22(ct175)	Deletion	0.2	+
unc-22(ct176)	Deletion	0.6	+
unc-22(ct177)	ND	—	+
unc-22(ct178)	ND	—	+
unc-22(ct179)	Insertion	3.5	+
unc-22(ct180)	CR	≤10	+
unc-22(ct181)	ND	—	+
unc-22(ct182)	CR	Unclear	−
pal-1(ct224)	Deletion	4.2	−
pal-1(ct281)	Deletion	4.7	−

ND, none detected; CR, complex rearrangement.

simply explained by the intron/exon distribution within the *unc-22* locus. As can be seen in Fig. 1, the 5′ region of the gene consists almost entirely of intron sequences. The 3′ end of the gene, however, is comprised predominantly of exon sequences (19). Most mutations within introns would not be expected to generate an *unc-22* phenotype and be recovered in our screen.

The partially repetitive nature of the *unc-22* DNA sequence suggests the possibility that mutation at this locus could be inherently biased toward production of deletions, which might be expected to result from homologous recombination between direct repeats during TMP cross-link repair. However, a previous study of mutations in a highly repetitive *C. elegans* gene found no evidence for such a bias. Dibb *et al.* (20) sequenced an x-ray-induced, a formaldehyde-induced, and ethyl methanesulfonate (EMS)-induced deletions in the *unc-54* myosin heavy chain gene of *C. elegans*. The x-ray-induced mutation (*e903*) was found to be a deletion of two consecutive adenines, a finding consistent with excision of a thymidine dimer. The two EMS-induced alleles (*e675, e190*) and the formaldehyde-induced allele (*s291*) were found to contain deletions of 270, 401, and 1275 bp, respectively. The *unc-54* gene sequence contains 187 direct repeats and 106 inverted repeats longer than 11 bp (20). The deletion junctions of *e190* and *s291* do not correspond to any of the direct repeats within the *unc-54* sequence, leading the authors to conclude that these deletions are not the product of homologous recombination between repeated sequences within the gene. Thus, a *C. elegans* gene with repetitive DNA sequences does not seem to be especially predisposed to produce deletions by homologous recombination between internal DNA repeats.

Both of the two mutant *pal-1* alleles isolated after TMP mutagenesis also contain small deletions that fall within the size range of deletions at the *unc-22* locus. In addition, other investigators using our protocol have succeeded in isolating small deletion mutations. Shivakumar and Kenyon (S. Shivakumar and C. Kenyon, personal communication) found two small deletions of 1.5 and 1.7 kb following TMP mutagenesis in a *C. elegans* homologue of the *wnt-1* gene. Thus small deletions have now been isolated using this procedure in at least two other genes beside *unc-22*. These findings support the view that the high frequency of small TMP-induced deletions recovered at the *unc-22* locus does not result from some unusual property of this gene.

However, TMP also appears to induce large deficiencies. Three mutations isolated at the *lag-2(V)* locus following TMP mutagenesis were all large deficiencies of >100 kb (E. Lambie, D. Gao, and J. Kimble, personal communication), as was a single mutation isolated at the *unc-101(I)* locus (21).

The spectrum of mutations produced by TMP appears very different from that produced by the commonly used chemical mutagen EMS, which is known to induce primarily GC → AT transition mutations (22). Sequencing of 23 EMS-induced mutations in the *unc-54* gene of *C. elegans* revealed 19 point mutations and only 4 deletions, all ≤2 kb (20, 23). A Southern blot analysis of 23 EMS-induced mutations in the *HPRT* gene of the Chinese hamster cell line v79-4 (24) showed that none resulted in RFLPs, suggesting that most of the mutations were either point mutations or very small deletions.

Whereas EMS appears to produce predominantly point mutations in eukaryotic organisms, other chemical mutagens such as diepoxyoctane and formaldehyde produce a high percentage of large deficiencies. A genetic analysis showed that mutations isolated following diepoxyoctane mutagenesis in *C. elegans* were predominantly multilocus deficiencies (25). Analysis of mutations in the *C. elegans unc-22* locus recovered after formaldehyde mutagenesis showed 33 to be multilocus deficiencies and the remainder to be putative point mutations (26), and analysis of 26 formaldehyde-induced mutations on LG V of *C. elegans* showed that 15 were either large deficiencies or more complex rearrangements (27). Likewise, an analysis in *Drosophila melanogaster* showed that 12 of 18 formaldehyde-induced mutations at the *Adh* locus were multilocus deficiencies (28). In contrast, the mutagen diepoxybutane appears to produce smaller deletions at about the same frequency and in the same proportion as EMS, but with a lower proportion of deletions than TMP; RFLP analysis of 47 mutant alleles recovered at the *C. elegans unc-54* and *unc-22* loci following diepoxybutane treatment showed that 7 were deletions ranging in size from 0.5 to 3.5 kb (29).

Somewhat different results have been obtained in studies of mutations induced by ionizing radiation, such as γ-rays, α-particles, and x-rays. An extensive study of γ-ray- and α-particle-induced mutations at the hamster *HPRT* locus determined that 30/43 of the γ-ray-induced mutations and 11/15 of the α-particle-induced mutations were either deletions or rearrangements (30, 31). Analysis by Southern blot showed that over half the deletions and rearrangements were small intragenic deletions. A comprehensive analysis of 28 x-ray-induced mutations in the *Adh* locus of *D. melanogaster* showed that they consisted almost exclusively of deletions, with a continuous size distribution from 2 bp to large deficiencies encompassing more than one locus (32–34).

Unfortunately, a similarly unbiased study of mutations induced by ionizing radiation at a specific *C. elegans* locus has not been reported. Most of the mutations recovered after γ- and x-irradiation have been large deficiencies and other gross rearrangements (e.g., refs. 35–37). However, in each of these studies the genetic screens used were biased in favor of recovering such mutations. In contrast, among 12 mutations isolated at the *unc-93* locus after γ-irradiation, 6 exhibited polymorphisms, of which one was a 0.3-kb deletion and the rest were apparently local complex rearrangements. However, the screen employed would have recovered only homozygous viable mutations and thus would have been unlikely to yield large deficiencies (38).

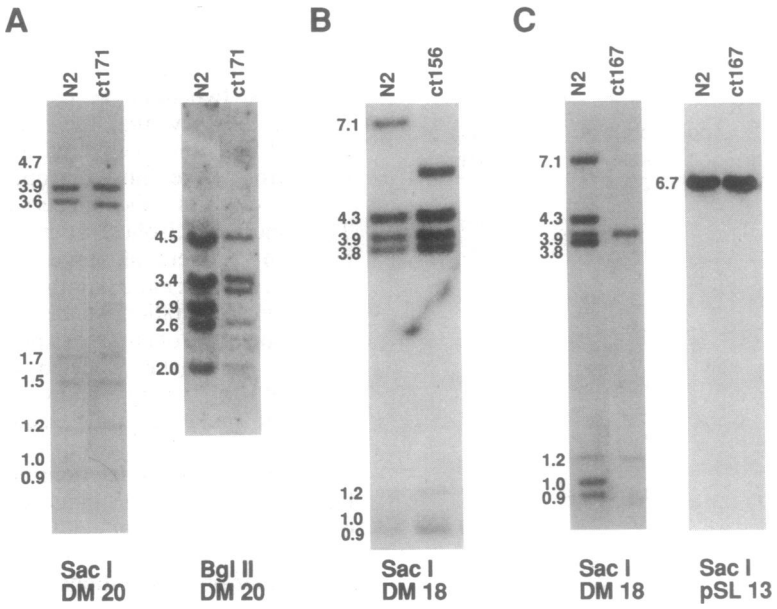

FIG. 2. RFLP mapping data for three TMP-induced alleles of *unc-22*. Genomic DNA was made from each mutant, digested with the restriction enzymes *Sac* I and *Bgl* II, and probed with a series of genomic probes (Fig. 1) covering ≈50 kb of sequence. (*A*) Evidence that the strain *ct171* contains a deletion of ≈100 bp. A *Sac* I digest of *ct171* genomic DNA probed with DM20 shows a barely detectable shift in the 3.6-kb band relative to a digest of N2 DNA. A *Bgl* II digest of *ct171* genomic DNA probed with DM20 shows that the wild-type 2.9-kb band is missing and a new 3.2-kb band has appeared. This shift is best explained as the result of a small deletion of <100 bp from the neighboring 0.36-kb fragment (see Fig. 1), which removes the intervening *Bgl* II site, thereby fusing these two fragments to generate the observed 3.2-kb band. (*B*) Evidence that *ct156* is a deletion of ≈2 kb. A *Sac* I digest of DNA from strain *ct156* probed with DM18 shows an ≈2-kb decrease in size of the 7.1-kb band seen in N2. The size of this deletion was confirmed by PCR using several primers specific to this region of the *unc-22* gene (data not shown). (*C*) Evidence that *ct167* is a deletion of ≈15 kb. DNA from the *ct167* strain digested with *Sac* I and probed with DM18 is missing the 7.1-, 4.3-, 3.8-, and 1.0-kb bands seen in N2. Additionally, the unresolved doublet that runs at 0.94 and 0.97 kb in N2 is present at half the intensity, consistent with absence of the 0.97-kb component of the doublet. The 3.9-, 1.2-, and 0.94-kb bands, all three of which lie 3′ to the missing bands (see Fig. 1), are present and of the expected size. Thus, the 3′ deletion breakpoint must lie somewhere near the 3′ end of the 3.8-kb *Sac* I fragment. The pattern of *ct167* digested with *Sac* I and probed with pSL13 (Fig. 1) is identical to that of N2. Thus, the 5′ deletion breakpoint lies 3′ to the region covered by pSL13, indicating that *ct167* is a deletion of ≈15 kb.

Therefore, on the basis of the limited available evidence, the spectra of mutations produced in *C. elegans* by ionizing radiation and TMP may be similar to each other and to the spectrum produced by ionizing radiation in *Drosophila*. These mutagens are all believed to produce single- and double-strand breaks in DNA. Repair of such lesions is postulated to proceed by recombination, either according to the Meselson–Radding model (39) or via double-strand break repair (40), with deletions probably resulting from incomplete repair. TMP cross-links, at least in *E. coli*, are thought to be repaired by means of sequential nucleotide excision and recombinational repair (3, 4). TMP cross-links were found to produce a high frequency of deletions in plasmids carrying the *E. coli gpt* gene when maintained in a host carrying a chromosomal deletion of this gene, but an almost 100-fold lower frequency when maintained in a wild-type host, indicating that TMP-induced deletions arise much more frequently when homologous recombination is suppressed (3). Evidence from studies in *Saccharomyces cerevisiae* (41) suggests that a similar mechanism may exist in eukaryotes for repair of TMP cross-links. Thus, similarities between the mutagenic spectra of γ-rays, α-particles, x-rays, and TMP may be a result of the fact that all four mutagens give rise to deletions by means of failed double-strand break repair.

In *C. elegans*, where mutations isolated after treatment with ionizing radiation have been primarily large deficiencies or complex rearrangements, TMP mutagenesis may provide a better method than γ-ray or x-ray mutagenesis for induction of small deletions. Of the 23 TMP-induced alleles we have analyzed at the *unc-22* and *pal-1* loci, 10 are deletions of <15 kb. Thus, TMP can be useful for the generation of mutations likely to cause complete loss of single gene functions and to result in allele-specific DNA polymorphisms helpful in gene cloning.

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