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Characterization of Mutations Induced by Ethyl Methanesulfonate, UV, and Trimethylpsoralen in the Nematode *Caenorhabditis elegans*

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The genome project of the nematode *Caenorhabditis elegans* is completed. It is important and useful to disrupt nematode genes to know their function. We treated wild-type animals with potential candidates for mutagens for reverse genetics, EMS (ethyl methanesulfonate), short-wavelength UV, and long-wavelength UV in the presence of TMP (trimethylpsoralen). We estimated forward mutation rates by counting the occurrence of a marker *unc-22* mutation. We found that the forward mutation rate by TMP/UV could be comparable with EMS by improving the frequency one order higher than before. We next isolated mutants of another marker gene *ben-1* and examined the probability for the deletion mutations by PCR and sequencing. Deletion mutations were found only by TMP/UV method, which suggested TMP/UV is the choice for deletion mutagenesis among these methods. As a pilot experiment, we could isolate actual deletion mutations at a much higher frequency than previously.

***Key Words: Caenorhabditis elegans;* trimethylpsoralen; deletion mutant; reverse genetics.**

Reverse genetics is a powerful tool to understand gene function of known structure. It is especially effective for experimental systems with large amounts of genome sequence information. Now the genome-sequencing project of *Caenorhabditis elegans* is completed for the first time as a multicellular organism [1]. Moreover, full description of its morphology and cell lineage has been finished [2, 3]. Thus, isolation of stable deletion mutants of *C. elegans* is important for studying thoroughly the characteristics of each gene to get insights into the cellular processes of the products.

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Because homologous recombination is not well operated in the nematode, most of the gene disruption experiments have been done either by RNA-mediated interference [4] or deletion mutagenesis [5]. Although the RNA-mediated interference method is powerful, it is generally regarded that the effects aren't inheritable, suggesting the necessity of deletion mutants for various context of research.

Recently, the deletion mutagenesis for reverse genetics in *C. elegans* has been often done with chemical mutagens [6] instead of "transposon insertion and excision" [7]. However, efficiency of deletion mutagenesis method, even most popular TMP/UV method, has been much lower than by EMS mutagenesis [6]. To improve the forward mutation rate while not decreasing the population of deletion mutation, we examined the parameters for three methods which are potential candidates for deletion mutagenesis. To know the forward mutation rates, we scored the *unc-22* phenotype and compared the effects of mutagens and each parameter. Then, we isolated and characterized mutant alleles of a single locus *ben-1*, a β-tubulin gene of *C. elegans* by phenotypic screening and examined sizes of deletions. We propose that UV treatment in the presence of a chemical reagent TMP is the method of choice to obtain deletion mutants.

MATERIALS AND METHODS

Growth and maintenance of nematode strains. *C. elegans* strains were maintained according to Brenner (1974) [8] with a modification by Way and Chalfie [9]. We used wild-type Bristol N2 for mutagenesis as the parent strain.

Mutagenesis, scoring and isolation of mutants by phenotypes. Mutagenesis with EMS (ethyl methanesulfonate) was done as described previously [10]. Irradiation of UV at the wavelength of 254 nm was done as described previously [11]. Mutagenesis with TMP and UV at the wavelength of 365 nm was conducted as below. TMP was dissolved completely in acetone at a concentration of 0.3 mg/ml and diluted in M9 buffer just before use. Mixtures of young adults and L4 larvae were collected from the NGM agar plates and incubated for an hour at room temperature in the dark at various con-

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centration of TMP as described in the Results section. Animals were immediately transferred as 20-μl drops on the lids of Eppendorf tubes placed upside-down. The animals were irradiated with 365 nm UV with a fluorescence microscope as a UV source (Olympus BX50WI-MacroRFL, Tokyo) at various energy levels. The intensities of the UV light were calibrated with a UV luminometer and controlled by ND filters and the exposure time. Hatching rates were highly dependent on the strength of treatments. Mutagenized worms were plated on NGM agar dishes and allowed to lay eggs at 20°C. After 24 h we washed off adults and larvae to remove all the animals fertilized before mutagenesis treatment, and incubated dishes for another 24 h, while mutagenized F1 animals hatched. F1 animals were collected by centrifugation at 800 rpm for 5 min in a low speed centrifuge and re-plated on fresh NGM agar plates until they grew adults.

To score *unc-22* mutant phenotypes, we examined both F1 and F2 animals. To identify *unc-22*/+ heterozygous mutants, we soaked F1 adults in M9 buffer containing 1% nicotine and counted twitching animals as described [12]. We confirmed the *unc-22* mutation by picking up heterozygous animals and cultured until F2 animals grew, among which homozygous *unc-22* mutants segregated or reexamined the twitching phenotype of the same animals if F1 animals were sterile.

To screen for *ben-1* mutants by phenotypes, F1 adults were collected, counted and plated at 100 animals per 6 cm dish on NGM agar dishes with carbendazim at a concentration of 10 μg/ml. They were allowed to lay F2 animals. Identification of *ben-1* mutants was done as described [13].

DNA analysis. To examine possible deletion mutations, we processed DNA from animals as follows. Worms were lysed in a lysis buffer (25 mM Tris–HCl, pH 8.5, 50 mM KCl, 0.5% SDS, 1 mM EDTA and 200 μg/ml proteinase K) and incubated at 50°C overnight. We purified DNA with a DNA purification kit (MagExtractor genome kit; TOYOBO, Osaka).

We examined the sizes of PCR fragments by at least three PCR-amplification (see Fig. 2A-c, -d, and -e). The standard PCR condition was as follows. PCR buffer (10×) was 250 mM Tris–HCl (pH 8.5 at room temperature), 500 mM KCl, 10 mM 2-mercaptoethanol, 1% Tween 20. We added 1 μl of template DNA (20 ng/μl) and 20 μl of reaction mixture. The concentrations of each component in 100 μl reaction mixtures were as follows: 10× PCR buffer (10 μl), Milli-Q DW (79.5 μl), 1% gelatin (1 μl), 25 mM $MgCl_2$ (6 μl), 10 mM dNTP mixtures (1 μl), 20 μM forward and reverse primers (each 1 μl), 5 u/μl of *Taq* DNA polymerase (0.5 μl). PCR cycles were 94°C (1 min), 35 cycles of 94°C (40 sec), 60°C (30 sec) and 72°C (1 min), 72°C (7 min), and hold at 10°C. When we failed to detect amplified DNA, we further examined 12 short regions whether they were amplified or not (examples are shown in Fig. 2). Together with Southern blotting and direct sequencing by standard methods [14], deleted regions of each allele were determined.

PCR screening of deletion mutants. To screen for mutants by PCR and agarose gel electrophoresis, F1 adults of mutagenized animals were collected, singled on 8× BP NGM agar 6-cm dishes (8-fold Bacto Peptone is added) and allowed to grow for about 10 days. When animals grew nearly confluent, about half of animals were collected for DNA preparation. The others were saved for frozen stocks by the standard method [10]. After lysis of progenies of each F1 animal, we pooled the DNA solutions as matrices. In the first screening, we used 1536-genome equivalents of pooled DNA as templates. When positive signals are found, we examine 8 sets of 192-genome pools as the second screening. Then, we examine 8 sets of 24-genome pools as the third screening. We next examine 12 sets of 2-genome pools (progenies of single F1 animals) as the fourth screening.

Our PCR condition for screening was, for example, for a target size of about 3 kb was as follows. To a 100 μl-reaction mixture, we added 1 μl of template DNA (20 ng/μl) and 20 μl of reaction mixture for the first and second screenings. We added 1 μl of template DNA (diluted by 1:50 against TE) and 20 μl of reaction mixture for the third and fourth screening. First, PCR reaction mixture was 10× PCR buffer (10 μl), Milli-Q DW (79.5 μl), 1% gelatin (1 μl), 25 mM $MgCl_2$ (6 μl), 1 mM dNTP mixtures (1 μl), 20 μM forward and reverse primers (each 1 μl), 5 u/μl of *Taq* DNA polymerase (0.5 μl). First, PCR cycles were 94°C (1 min), 35 cycles of 94°C (40 sec), 60°C (30 sec) and 72°C (1 min), 72°C (7 min), and hold at 10°C. We added 1 μl of first PCR reaction products and 20 μl of second PCR reaction mixture. Second, PCR reaction mixture was 10× PCR buffer (10 μl), Milli-Q DW (79.5 μl), 1% gelatin (1 μl), 25 mM $MgCl_2$ (6 μl), 10 mM dNTP mixtures (1 μl), 20 μM forward and reverse primers (each 1 μl), 5 u/μl of *Taq* DNA polymerase (0.5 μl). Second PCR cycles were 94°C (1 min), 35 cycles of 94°C (40 sec), 60°C (30 sec) and 72°C (2 min), 72°C (7 min), and hold at 10°C. Primers used for the *ben-1* gene were C54C6#F1 (5′-ACGTGGGAATGGAACCATGT-3′), C54C6#F2 (5′-CTCCGGACATTGTAACGGAA-3′), C54C6#R1 (5′-TCTCCATTTCCTCTTCCTCC-3′) and C54C6#R2 (5′-CCCTCCATTTGAAAGAGTCC-3′). Primers used for the *mec-7* gene were ZK154#F1 (5′-GGAACCATGTTCACTGCCAA-3′), ZK154#F2 (5′-AAGGTCTCCATATGTCGGTG-3′), ZK154#R1 (5′-ACGAGGAACTAACAAGCCAC-3′) and ZK154#R2 (5′-TTATCTGGCATTCAGCTGGG-3′). Agarose gel electrophoresis was done by standard techniques [14].

RESULTS AND DISCUSSION

Forward Mutation Rates by EMS, Short-Wavelength UV, or Long-Wavelength UV/TMP

Reverse genetics of *C. elegans*, where only deletion mutations could be readily detected, has been screened by PCR reactions which detect preferentially shorter amplification products over wild-type longer fragments. It is essential to find the best way to mutagenize the nematodes which results in the efficient generation of deletion mutants. Previous studies have suggested that overall forward mutation rates multiplied by the proportion of the deletions among total mutations are the key factor for the choice of the mutagenesis protocol. Two different methods were recommended [6]: EMS which shows high forward mutation rates but low deletion rates and TMP/UV which shows low forward mutation rates but high deletion rates. We inferred that the optimization of the TMP/UV method could be obtained by increasing the forward mutation rate. In addition, we also tried another method, irradiation of short wavelength UV, which might also induce deletion mutations.

To compare the forward mutation rates, we scored the occurrence of the *unc-22* mutations by three distinct protocols above. We chose the *unc-22* gene as a marker, because it has been most frequently used for the estimation of mutagenesis in *C. elegans* [15]. We cultured F1 animals of mutagenized worms until they grew adults and scored the frequency of *unc-22* heterozygous phenotype in 1% nicotine solution, because F1 generation was simpler to estimate forward mutation rates. After EMS mutagenesis, the forward mutation rate (2.5×10^{-3}; Fig. 1) was roughly the same as that described previously ($\sim 3 \times 10^{-3}$; [16], suggesting our mutagenesis and scoring procedures reproduced the conventional method. We did not observe the sterility among the F1 *unc-22* mutant animals upon cul-

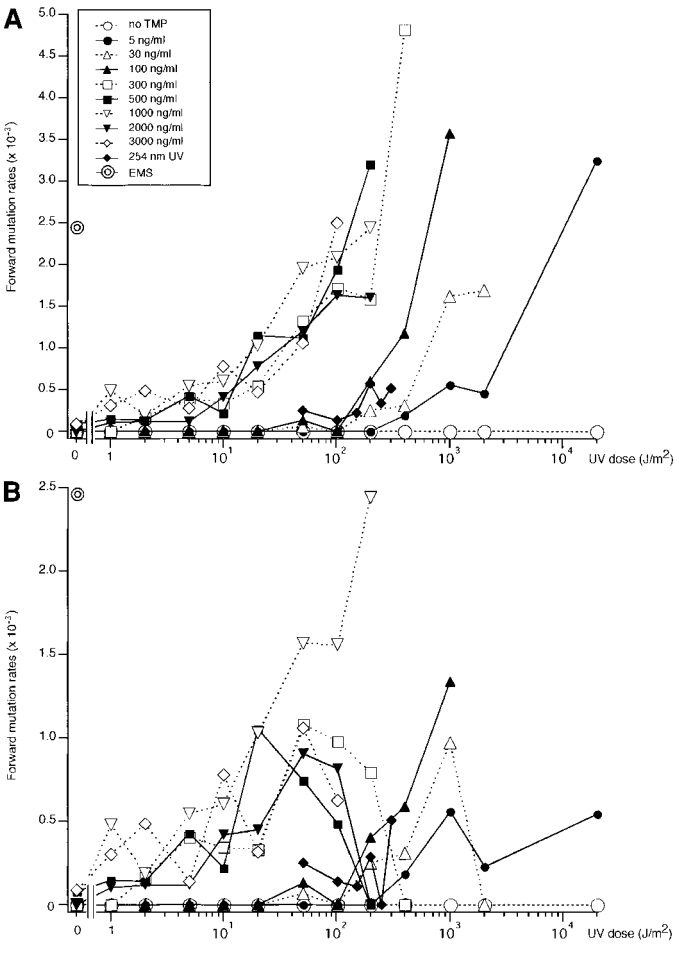

FIG. 1. Forward mutation rates by three mutagenesis methods. (A) Forward mutation rates estimated by the *unc-22* mutant phenotype. The ordinate indicates the forward mutation rate shown. The abscissa indicates the dose of UV irradiated shown by the logarithmic scale. (B) "Inheritable forward mutation rates" estimated by the *unc-22* mutant phenotype. Treatment conditions are shown by symbols: ○, 0 μg/ml TMP; ●, 5 ng/ml TMP; △, 30 ng/ml TMP; ▲, 100 ng/ml TMP; □, 300 ng/ml TMP; ■, 500 ng/ml TMP; ▽, 1 μg/ml TMP; ▼, 2 μg/ml; ◇, 3 μg/ml; ◎, EMS; ◆, short wavelength UV.

ture of EMS-mutagenized animals (compare the frequencies shown in Figs. 1A and 1B).

We next analyzed the forward mutation rates by short wavelength UV. We used the total irradiation doses between 50 and 300 J/m^2 (Fig. 1). The occurrence of *unc-22* mutants in the F1 population by short wavelength UV was less frequent than by EMS. Although the rates tended to be increased according to the UV dose, we did not examined higher doses of short wavelength UV because the hatching rates became extremely low (about 5% at 300 J/m^2). During the course of experiments, we found that a portion of heterozygous *unc-22* mutant animals were sterile. Because sterile mutants could not contribute the population to be screened by reverse genetics, we estimated the frequencies of fertile *unc-22* mutants by multiplying ratios of fertile lines and total forward mutation rates. We call these here "inheritable forward mutation rates" (Fig. 1B). Considering the sterility of the F1 *unc-22* mutants, the inheritable forward mutation rates by short wavelength UV could not be as high as other two methods.

We thoroughly examined the effects of both TMP concentration (5 ng/ml to 3 μg/ml) and doses of long wavelength UV (1–20,000 J/m^2) on the forward mutation rates estimated by the *unc-22* phenotype (Fig. 1A). When compared within the same concentration of TMP, forward mutation rates were higher at higher UV doses. Forward mutation rates appeared dependent on UV doses. Because combination of higher TMP concentrations and higher UV doses, hatching rates were too low to be analyzed and accordingly the corresponding points in Fig. 1 were missing.

The effect of the concentration of TMP was also prominent. When higher concentration of TMP was examined, the curves shifted to the left (Fig. 1A), suggesting that forward mutation rates were comparable even if lower doses of UV were irradiated. However, the effect of TMP higher than 500 ng/ml appeared saturated, and curves were overlapped with each other.

We also analyzed "inheritable forward mutation rates" of TMP/UV mutagenized animals. At higher UV doses, the F1 mutants isolated this way were sometimes sterile, making the dose–effect curve bell-shaped (Fig. 1B). With various conditions, the inheritable forward mutation rate of about 1×10^{-3} was reproducibly obtained, which was about 10-fold higher than that by the previous report (10^{-4}; ref. 5).

Phenotypic Isolation from the ben-1 Locus and Analysis of Deletion Sizes

It is necessary to make deletion mutation to be screened by PCR. After we know the inheritable forward mutation rate, we wondered how large deletions these mutagenesis conditions would give rise to. To estimate the deletion sizes by the three methods above, we chose the gene *ben-1*, a β-tubulin of *C. elegans*, because of the following three reasons [13]. First, the *ben* mutants are resistant to an anti-tubulin drug benzimidazole: mutants grow paradoxically more quickly than wild-type animals that are unhealthy, dumpy and uncoordinated in movement on selection media. Second, since all *ben* mutants have been mapped on a single locus, mutants isolated by the phenotypes should be allelic to the same gene *ben-1*. Third, the size of the coding region of the gene is about 3.3 kb (much smaller than the *unc-22* gene) and convenient for PCR analyses.

We isolated a total of 170 independent alleles from the *ben-1* locus by phenotypic screenings as previously (77 by EMS, 2 by short-wavelength UV and 91 by TMP/UV). The probability for mutant isolation by EMS

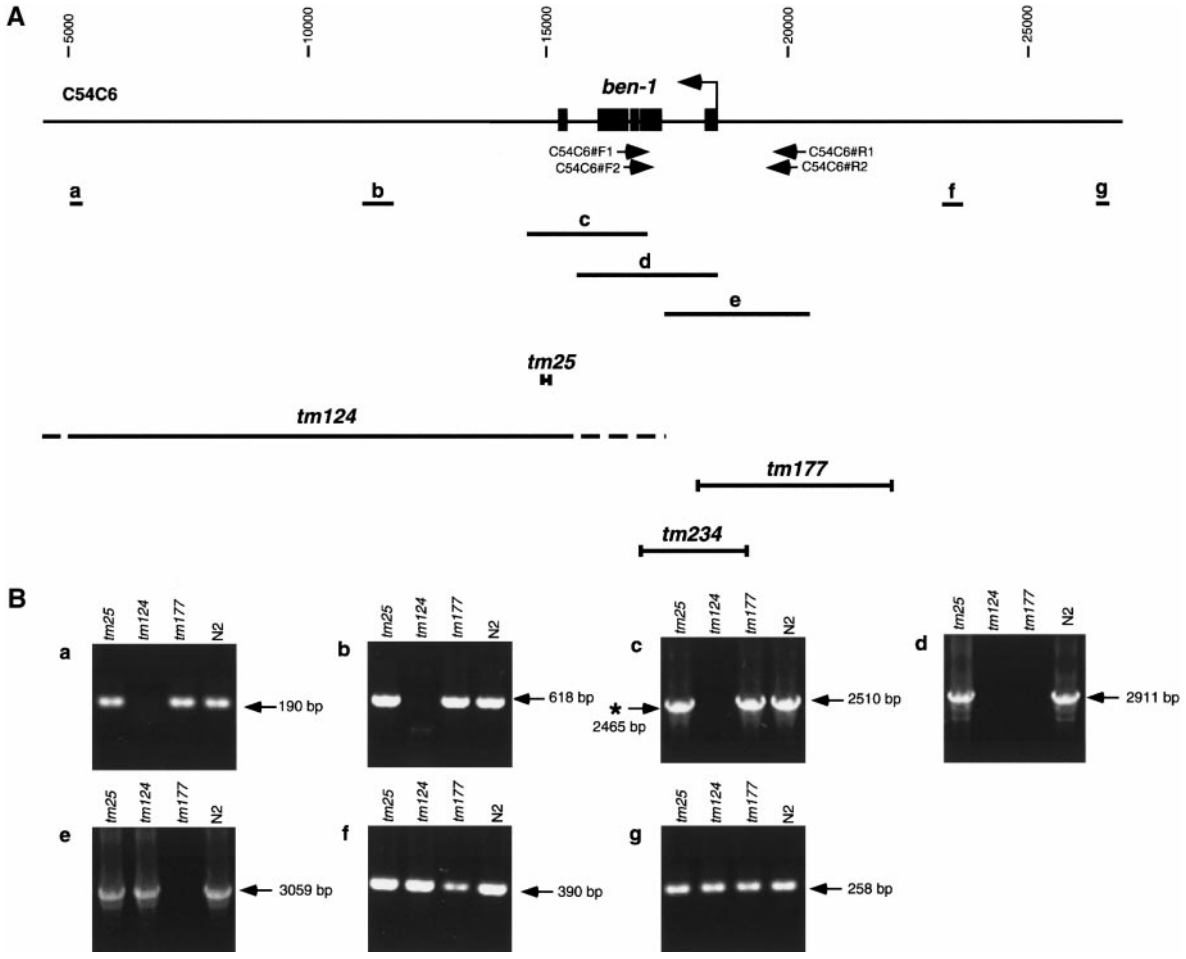

FIG. 2. Examples of PCR analysis of various alleles from the *ben-1* locus. (A) The map of the cosmid C54C6 which contains the entire *ben-1* locus. Nucleotide numbers in the cosmid are indicated at the top of the panel. The exons are depicted by solid boxes, whereas the direction of transcription is indicated by an arrow on the first exon. In this figure, 7 sets of PCR fragments are shown (bars with a–g). Deletions found by this analysis are also shown with allele names (*tm25, tm124, tm177*). Primers for PCR screening are indicated by arrows, while the deletion site found by this set of primers is indicated as a bar with the allele name (*tm234*). (B) Gel images corresponding to the DNA regions shown in (A). The lanes of the agarose gels correspond to the strain indicated in (A) and wild-type (N2). Fragment sizes are indicated beside each gel image.

(21.5×10^{-4}) was higher than those by short-wavelength UV (1.3×10^{-4}) and TMP/UV (2.8×10^{-4} on average). Because β-tubulin proteins in *C. elegans* appear very conservative, the forward mutation rate by EMS was a little higher than ordinary genes (5×10^{-4}), being compatible with the previous report (13.5×10^{-4}; Ref. 13).

We first amplified three sets of *ben-1* gene fragments (Fig. 2A, c–e) from these mutants by PCR and compared the sizes with those of wild-type fragments. When any of the fragments failed to be amplified, we examined neighboring regions (e.g., Fig. 2A, a, b, f, and g). We categorized alleles into four. First, all of the amplified fragments were indistinguishable from the corresponding wild-type fragments (data not shown). Such mutations were categorized into "transitions or very small deletions". Second, some of amplified fragments were shorter than the corresponding wild-type fragments (exemplified by Fig. 2, *tm25*). We sequenced the fragments and determined the exact sizes of deletions. Such mutations were categorized into "detectable deletions". As shown in Fig. 2, the limit for readily detectable deletions in our assay appeared nearly 50 bp in size (asterisk in Fig. 1B, c). Third, a large region (more than 10 kb) around the *ben-1* locus was resistant to the PCR amplification. For example, no amplification was observed for the left part but wild-type amplified bands are observed for the right part of the cosmid C54C6 in the allele *tm124* (Fig. 2B). Such mutations were categorized into "large-sized deletions (>10 kb)." Fourth, a number of fragments with logically unexpected sizes were amplified for some alleles (data not shown). Such mutations were categorized into "complex rearrangements."

In contrast to previous assumption [17], the probability for detectable deletions was very low among

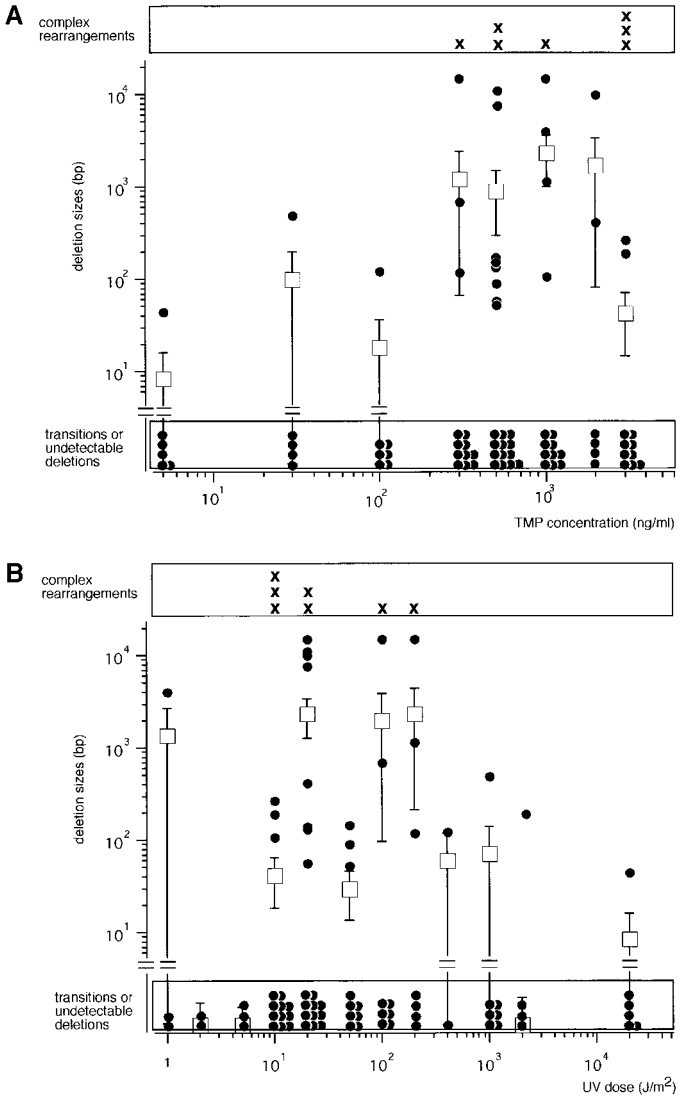

FIG. 3. Effects of TMP concentration (A) and UV dose (B) on the deletion sizes. (A) The ordinate indicates the deletion size of the *ben-1* gene shown by the logarithmic scale. The abscissa indicates concentrations of TMP shown by the logarithmic scale. Complex rearrangements are indicated on the top of the figure because we could not know the actual deletion sizes. Mean of the deletion sizes are indicated with squares and bars (SE). (B) The ordinate indicates the deletion size of the *ben-1* gene shown by the logarithmic scale. The abscissa indicates UV doses shown by the logarithmic scale. Complex rearrangements are indicated on the top of the figure. Mean of the deletion sizes are indicated with squares and bars (SE).

EMS-treated mutants (none out of 77 alleles). We also failed to find deletion mutants derived from short-wavelength UV-treatments. However, the probability of deletion mutants was high when treated with long-wavelength UV in the presence of appropriate concentrations of TMP (a total of 22 out of 91 alleles).

Estimated average deletion sizes for each TMP concentration are depicted in Fig. 3A. When animals were treated with TMP at the concentration of 100 ng/ml or lower, deletion sizes tended to be small. Average deletion sizes were around 1 kb when TMP concentrations were between 300–2000 ng/ml, when complex rearrangements were also found. UV doses did not appear to affect the deletion sizes (Fig. 3B). This was opposite to the forward mutation rates where UV doses reproducibly affected.

Pilot Screening for Deletion Mutants

To test whether the mutagenesis and screening described here indeed works, we screened for the *ben-1* mutant as a pilot experiment. When we used a target size of 3519 bp was used and screened 4608 mutagenized F1 genomes (three tubes for the first screening), we found one tube with a shorter amplified fragment (Fig. 4A, asterisk, lane 9A1-16H12). We then found one smaller pool of 192 genomes (Fig. 4B, asterisk, lane 16A1-16H12). We found the progenies of single F1 (16A11) contained the deletion mutants (data not shown). We subcultured each of such animals on separate dishes and when those animals laid eggs, we picked up adults from the dishes and asked whether they were deletion mutants or not. Among 16 animals, we found 5 animals which had deletions in this case (Fig. 4C asterisk). The phenotypes of this mutant obtained this way were indistinguishable from those of mutants isolated previously by phenotypes (data not shown). We also confirmed that this strain (tm*234*) was allelic to the *ben-1* locus by direct sequencing of the amplified fragments (2,136 bp deletion).

We also examined whether the present protocol is also applicable to other genes. We screened the same bank above for deletions of the genes mec-7, unc-86 and mec-2. Among three, we found one deletion of mec-7 which turned to be a deletion of 331 bp (only first and second screenings and single animal PCR are shown here; Figs. 4D–4F, double asterisks). Thus, our protocol appears sufficient for detection of such a small deletion. With the technology described here, we are now collecting systematically new mutants whose phenotypes are to be analyzed molecularly (Gengyo-Ando *et al.*, unpublished).

Inheritable Forward Mutation Rates by EMS, Short Wavelength UV and TMP/UV

It is important for reverse genetics that inheritable forward mutation rates are reasonably high. When we mutagenized animals by EMS, though the forward mutation rate was very high as above, no F1 *unc-22*/+ mutants were sterile (0 of 35 lines). By contrast, when we mutagenized animals by short wavelength UV at a dose of 300 J/m^2, 2 of 5 heterozygous F1 animals were sterile. In the case of TMP/UV, 1 of 12 F1 and 2 of 6 F1 animals were sterile by 0.5 μg/ml TMP + 20 J/m^2 long wavelength UV, 0.5 μg/ml TMP + 50 J/m^2 long wavelength UV, respectively. This phenomenon suggests

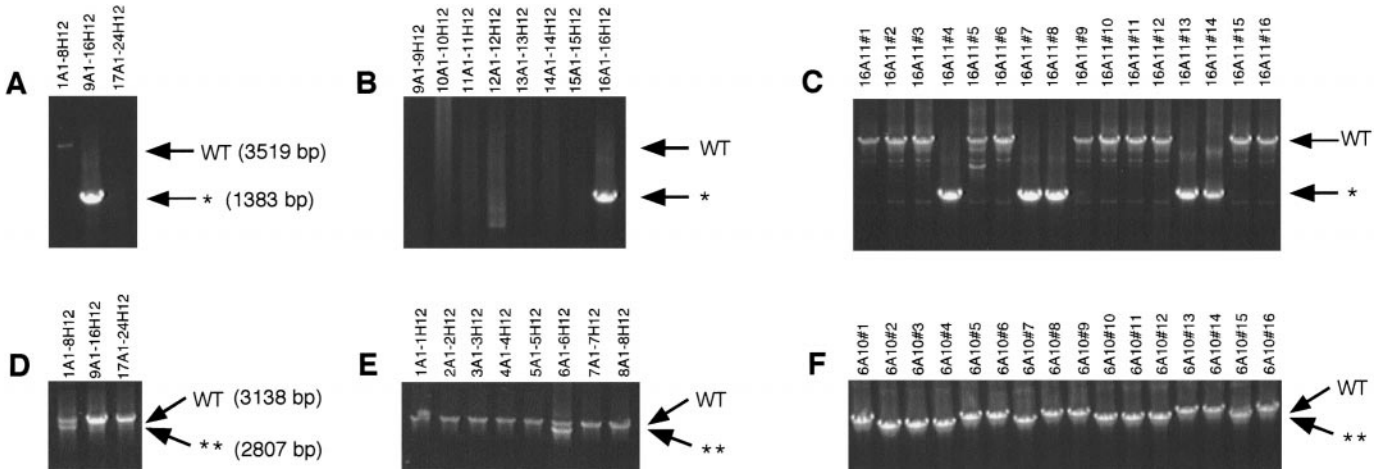

FIG. 4. Examples of screening of the deletion mutants. (A–C) First, second, and single animal screenings of the *ben-1* mutant. Agarose gel images with stock number of mutations indicated on the top of lanes are shown. Mutant bands are shorter than those of wild-type. (D–F) First, second, and single animal screenings of the *mec-7* mutant. Note that the doublet bands are seen at the earlier screenings but not at the single animal PCR in (F).

that the F1 sterility occurs at highest frequency by short wavelength UV and middle frequency by TMP/UV but rarely by EMS. The mechanisms how F1 sterility occurs are not clear at the moment. We speculate this phenomenon is related to robust chromosome rearrangement, because even very frequent point mutations by EMS rarely resulted in heterozygous sterility.

CONCLUSION

In the present study, we showed that only TMP/UV mutagenesis could efficiently yield deletion mutations. We found that the UV intensity mainly contributed to forward mutation rates, while the concentration of TMP contributed to both deletion sizes and forward mutation rates. By adjusting these parameters, one could obtain the optimal condition for deletion mutagenesis in the nematode *C. elegans* and presumably in other organisms than the nematode. Indeed, we were able to isolate mutants with a mutant bank made from clonal cultures of a rather small genome size.

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