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Genetics

Target-selected gene inactivation in *Caenorhabditis elegans* by using a frozen transposon insertion mutant bank

(reverse genetics/deletion/Tc1/sequencing/PCR)

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ABSTRACT **To understand how genotype determines the phenotype of the animal *Caenorhabditis elegans*, one ideally needs to know the complete sequence of the genome and the contribution of genes to phenotype, which requires an efficient strategy for reverse genetics. We here report that the Tc1 transposon induces frequent deletions of flanking DNA, apparently resulting from Tc1 excision followed by imprecise DNA repair. We use this to inactivate genes in two steps. (*i*) We established a frozen library of 5000 nematode lines mutagenized by Tc1 insertion, from which insertion mutants of genes of interest can be recovered. Their address within the library is determined by PCR. (*ii*) Animals are then screened, again by PCR, to detect derivatives in which Tc1 and 1000–2000 base pairs of flanking DNA are deleted, and thus a gene of interest is inactivated. We have thus far isolated Tc1 insertions in 16 different genes and obtained deletion derivatives of 6 of those.**

The DNA sequence of the genome of *Caenorhabditis elegans* is being determined (1), but at present there is no practical method to study the function of a gene by the isolation of an animal in which that gene has been inactivated or otherwise altered in a targeted fashion (2). A method has been described for the isolation of transposon insertion mutants in the fruit fly *Drosophila melanogaster* (3–5), and this method has been applied to *C. elegans* (6). It depends on a PCR, using one primer that corresponds to the ends of the Tc1 transposon (7, 8) and another that corresponds to the gene of interest; PCR products are obtained if a transposon has inserted in the area of interest. Mutant animals are recovered from complex cultures by repeated cycles of culturing of animals, DNA isolation from siblings, and analysis. The method has two problems. (*i*) The method is very laborious; it has to be repeated for every new gene. (*ii*) Most Tc1 insertions do not result in gene inactivation (6).

To solve the first problem, we took advantage of the property of *C. elegans* to survive storage in a frozen state (9), which makes it worthwhile to set up thousands of individual cultures and store each of them frozen. Forward genetic screens for Tc1-induced mutations showed for most genes frequencies of 10^{-3}–10^{-5} per generation (10, 11). We are interested only in cultures in which the mutant makes up at least 1% of the animals. This number is chosen somewhat arbitrarily to ensure that individual worms can be cultured and tested in one 96-well PCR immediately after thawing of a culture. Therefore, for a given gene (assuming an insertion frequency of 10^{-5} and a limit of 1% mutants per culture), the expected value of the number of positive cultures is $10^2 \times 10^{-5} = 10^{-3}$— i.e., 1 per 1000 cultures. This is probably an underestimate of the actual frequency, since most Tc1 insertions are in introns and do not inactivate the gene (so that the insertion frequency is higher than the mutation frequency observed in forward genetic screens). We set up 5000 cultures to ensure that even mutants of genes that are hit at low frequency are expected to be represented.

The purpose of this approach is to inactivate genes and, as mentioned above, most Tc1 insertions [those in introns but apparently also some in exons (6)] leave gene function (partially) intact. To obtain genuine knockouts, one could use the strategy that we described previously—transgene-directed double-strand break repair following Tc1 excision (12)—but this requires laborious generation of transgenic animals. Infrequent deletion derivatives have previously been detected as special cases of reversion of Tc1 insertion mutants (13–15) and of *P* element insertion mutants in flies (16, 17). We investigated whether deletions occurred at such frequencies that they can be easily recognized by PCR and isolated.

Using the two-step strategy, isolation of a Tc1 insertion allele followed by isolation of a deletion derivative, we have obtained guaranteed null alleles of several genes.

MATERIALS AND METHODS

Nematode Culture. Nematodes were cultured as described by Wood *et al.* (9). Animals were frozen for storage as follows: the strain used was MT3126 (*mut-2*), a derivative of TR679 (11), backcrossed with Bristol N2 (27). Animals were grown on 10-cm-diameter plates seeded with *Escherichia coli* OP50. Plates contained twice the concentration of peptone compared to the protocol given by Wood *et al.* (9). Since the MT3126 strain was found to be slightly thermosensitive, culturing was done at 18°C. We started cultures with nonsynchronized animals to have a continuous chance distribution for finding a mutant. Once cultures were cleared, 5 ml of M9 was added to the plates, and they were shaken overnight. This procedure allows starvation under fully aerobic conditions. The volume of the buffer is usually reduced to 2 ml by evaporation and absorption into the agar. For viable storage, 400 μl of each suspension was then transferred to identical positions of two microtiter plates (Micronics) and 400 μl was added of a solution of 30% (vol/vol) glycerol in S-basal medium. Filled trays were slowly cooled and stored at −80°C. Survival was monitored by thawing sample cultures 1 day after freezing and inspecting the progeny. The average number of survivors was 300; in the rare cases in which survival of one single culture in a series was <100, the complete series was discarded. The duplicate libraries were stored in two different freezers at −80°C. One compartment of such a freezer (60 × 40 × 20 cm) contains at present 5000 cultures and could contain 15,000.

DNA Analysis. DNA was isolated essentially as described (9). Cultures were lysed in a solution of SDS/proteinase K and 2-mercaptoethanol at 65°C. After lysates were pooled in an ordered matrix (see *Results*), DNA was purified by

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extraction with phenol and chloroform to prepare it for PCR. Once a putative address was obtained, the presence of mutant DNA in the original lysate was checked. Ten microliters of SDS/proteinase K lysate, 80 μl of ethanol, and 10 μl of H_2O were mixed at room temperature, and the mixture was spun for 20 min at room temperature at 4000 rpm in a table top centrifuge. The precipitate was taken up in 100 μl of H_2O, of which 1 μl went into a PCR. An advantage of this method is that it can be done in a microtiter plate, so that 96 samples can be prepared in parallel.

The PCR was performed with the 9600 thermocycler (Perkin–Elmer) and *Taq* polymerase (BRL). Insertions were visualized by using nested PCR. Primer concentrations were 0.2 μM for each primer; nucleotides were 0.2 mM each. The following primers were used. To detect Tc1 insertions with one orientation with respect to the gene-specific primer sequence, we used right 1 (GCTGATCGACTCGATGCCACGTCG) and right 2 (GATTTTGTGAACACTGTGGTGAAG) for the first and second PCR series. For the other orientation, left 1 (TGTTCGAAGCCAGCTACAATGGC) and left 2 (TCAAGTCAAATGGATGCTTGAG) were used. The gene-specific primers were either synthesized on a DNA synthesizer (Biosearch) or were kindly provided by collaborators, who often used them previously for determination of the genome sequence in the area. The first PCR was done in 10 μl, then 90 μl of water was added, and by touch with a yellow tip or a 96-pin "hedgehog" a very small "inoculum" was added to a second 10-μl reaction mixture. First and second PCR series were 30 cycles of 40 sec at 94°C, 40 sec at 55°C, and 2 min at 72°C. We found that by using this protocol single molecules could be amplified to plateau levels. To stay in a semiquantitative range (as in the experiment in Fig. 1*B*), we did 25 rather than 30 cycles in the second series. DNA was separated on 1% agarose gels and visualized after ethidium bromide staining. "Single worm PCR" was done as described (18).

As described in *Results*, in an alternative strategy we performed PCR on only one dimension of the orthogonal matrix (see Fig. 1*A*, rows A–H), in quadruplicate, amplifying to plateau (see Fig. 1*C*). The diluted genomic DNA that is used for this is stored in Micronics tubes in a microtiter format, so that many reactions can be initiated fast, using a multiwell pipette. If a positive partial address is obtained in quadruplicate, the second dimension (planes 1–10) is tested on DNA of lower complexity (we have purified 80 DNA samples for each set of 960 cultures: p1A–p10H). The third dimension (columns 1–12) is tested directly on the lysates of the original cultures (after a warm alcohol precipitation, as described above).

Detection and Isolation of Deletion Mutants. To detect animals with deletions, we started 100 cultures of an insertion mutant. After 2–3 generations, part of each culture was lysed as described above, and DNA was recovered from the lysate by alcohol precipitation at room temperature. This could again be done for 96 preparations simultaneously in a microtiter plate. PCR was done as described above, again in two rounds with nested primers. Primers were chosen 2–3 kbp apart in the genomic DNA sequence (i.e., without the Tc1 insertion). As a result, the insertion mutant allele is not visibly amplified, although in some cases the products of somatic excision of Tc1 (11) could be seen. Deletions that are observed usually result from loss of Tc1 and 1–2 kbp of flanking DNA. When a positive culture is encountered, one step of sib selection (6) is usually sufficient to isolate the mutant animal.

DNA Sequence Determination. DNA fragments were cut out of an agarose gel and sequenced in a cycle-sequence protocol (19). The primers used for sequencing are the same that were used for the second PCR. DNA sequences were analyzed by using the ACEDB program [J. Thierry-Mieg (Centre National de Research Scientifique, Montpellier, France) and R. Durbin (Medical Research Council, Cambridge, U.K.)].

RESULTS

Establishment of a Mutant Library. Each culture was started with 1–10 hermaphrodite animals of the MT3126 strain (in which transposon Tc1 is actively jumping). After ≈3 generations, animals were harvested and each culture was frozen in triplicate—twice for survival and once for lysis and DNA isolation. Samples were taken to check survival after freezing and thawing; the average number of surviving animals was 300. Thus, the library contains $\approx 10^6$ viable animals.

Screening of the Library. Before purification of DNA for PCR, samples from different cultures were pooled (Fig. 1*A*). To ensure homogeneity of the samples, the animals were lysed before the pooling. Lysates were pooled in a three-dimensional matrix, with each lysate contributing material to three pools (Fig. 1*A*). We did not use an approach where the presence of a Tc1 insertion is observed by a Southern blot after a single PCR (3–6), since that is laborious and does not provide the precise insertion sequence. Instead, we performed a second round of PCR with nested primers, so that amplification products could be visualized on a gel and sequenced (19). The necessary sensitivity is easily reached with this method. In a strain that contains several copies of Tc1, it will frequently occur that a new Tc1 insertion is close to another Tc1 element. We find, however, that no PCR products resulting from neighboring transposons are amplified; apparently the long (inverted) repeats at the ends rapidly fold back on each other after denaturation, and are therefore not efficiently primed. Fig. 1*B* shows a screening of 960 lysates (30 pools) for insertions in a gene of interest [the guanine nucleotide binding protein (G protein) α subunit gene *gpa-2* (20)]. In each dimension of the matrix, one insertion at the same position could be observed, and thus a unique address was obtained. Sequencing of the PCR product confirmed that indeed the Tc1 element had inserted in *gpa-2* and showed the precise insertion site. The animals from this address were thawed, and 12 individual animals were analyzed by single-worm PCR. Six of these were found to contain the mutant allele. Not each positive address was found to contain 50% mutants, but in general <100 animals had to be analyzed to obtain the mutant. The experiment in Fig. 1*B* shows that in addition to the strong signal produced by the positive address, weaker signals are also seen, presumably caused by somatic insertions (or germ-line insertions in a late stage of the culture). Therefore, the method depends on a semiquantitative PCR that visualizes the differences in intensities. An alternative approach for screening the library is shown in Fig. 1*C*. Now PCR is done in a nonquantitative manner: all samples are amplified to plateau, and the difference between weak bands and strong bands as in Fig. 1*B* is no longer observed. To distinguish germ-line from (early) somatic insertions, more dilute DNA samples are tested, each in quadruplicate. Positive samples show four identical products (e.g., band *I-G* in Fig. 1*C*). When a positive partial address is obtained, the second and third dimensions are tested. An advantage of this screening strategy is that it is not necessary to stay in the semiquantitative range for PCR (which may require different protocols for different primers), and that less DNA of the library is used for each search.

Thus far, we found 23 insertions in 16 different genes (Fig. 2). Insertions were obtained in genes encoding G-protein subunits, P-glycoproteins, homeobox proteins, kinases, etc. All of these were viable, fertile animals, with no immediately obvious phenotype. Since, as mentioned above, there can be doubt whether all interruption leads to loss of gene function, we have not proceeded to analyze these animals in much detail, but we have started using them to isolate deletion alleles.

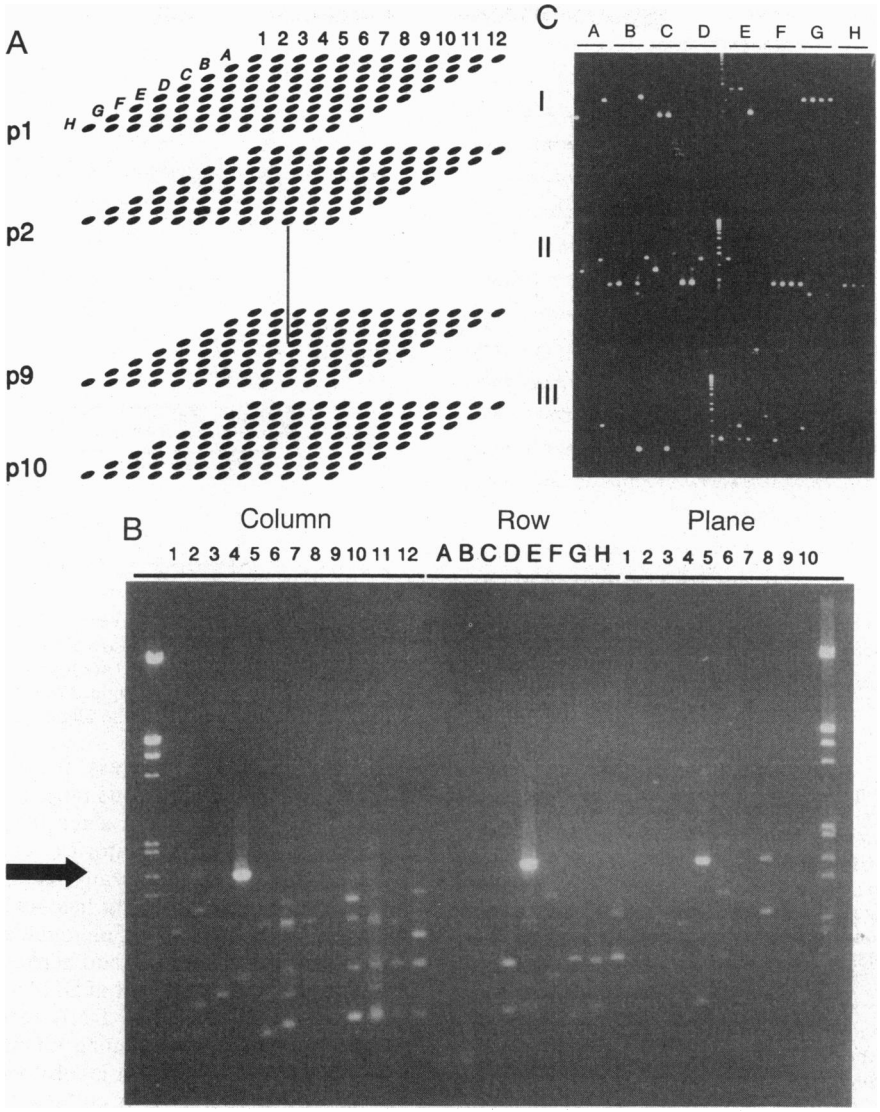

FIG. 1. (*A*) Pooling of DNA lysates before analysis. Nine hundred sixty cultures contribute to 30 DNA pools as indicated (1–12, p1–p10, and A–H). Every culture contributes DNA to 3 pools. The address of a culture containing a mutant follows from the numbers of the pools that give a PCR product. (*B*) Screening for a Tc1 insertion in the *gpa-2* gene (semiquantitative PCR). The arrow indicates a PCR product that shows up once in every dimension. The band was cut out and sequenced, and the resulting sequence confirmed that it represents a Tc1 insertion in the gene. Note that the size of the band is already an indication of the approximate position of the transposon within the gene. (*C*) An alternative screening strategy (nonquantitative PCR). The semiquantitative PCR shown in *B* is replaced by a PCR to plateau, where "real" germ-line events are distinguished from somatic events by their reproducibility. The DNA preparations for one dimension (A–H) are diluted to the point where not every sample contains one mutant molecule; if an early germ-line mutant contributes to the pool, however, then each of, for example, four samples will contain some mutant molecules, resulting in a band at four times the same position. *I, II, III* each refer to 960 cultures; lanes A–H refer to DNA pools of 120 cultures (see scheme in *A*). The quadruple in *I* (lane G) identifies a Tc1 insertion in *ceh-18*.

Detection of Deletion Derivatives. We analyzed DNA of progeny of a Tc1 insertion mutant using primers corresponding to sequences flanking the insertion at such a distance that products were only expected after loss of Tc1 plus some flanking DNA (Fig. 3). We detected a surprisingly high frequency of such deletion derivatives ($\approx 10^{-3}$). This frequency is of course determined by the choice of the primers: deletions that do not remove sufficient DNA are not amplified, nor are deletions that remove one of the primer sites. Therefore, the actual frequency of deletions will be higher than this. We isolated seven clonal lines of deletion derivatives of 6 different genes [*pgp-1, pgp-3, gpa-1, gpa-2, gpa-3* (see Fig. 3), and *ceh-13*]; deletion mutants of several other genes that were detected remain to be isolated. The sequences of the deletion derivatives in Figs. 3 and 4 show that several exons are removed, in these cases resulting in virtually guaranteed loss of function alleles. Note that in some cases the end points of the deletion coincide with a short direct repeat, as has been observed before—e.g., in *E. coli* (21)—but not in spontaneous deletions in *C. elegans* (22). Both unidirectional and bidirectional deletions were found. A unidirectional deletion with short direct repeat end points suggests the following mechanism for this class of deletion events: Tc1 excises by double-strand breaks at the transposon ends, and one of the free DNA ends scans the DNA at the other end for a short homology; when this is encountered, DNA-repair reactions ligate the two ends, thereby deleting the intervening DNA.

DISCUSSION

We describe the establishment of a mutant library and the development of a method to screen the library for insertion mutants of a given gene; we report that these insertion

gpa-2(pk19)	TTTCCGACAAATAGGACAAATAGGTATTCATTCTAAAACGAT	intr.
gpa-2(pk23)	AAAACTTCCTCGAAAGACAATATCTCCTCAAATTTTAGAAAC	intr.
gpa-2(pk24)	AGTAATTGGATTTGCCTATATACTTTTCAATTTTATCTTTAA	intr.
gpb-1(pk13)	GTGACGCTTCGGCGAAGGTATAGGATTTTTGAAAAGTTGATT	intr.
pgp-1(pk28)	GAATATAGCTATCACCGGTATATAACTTTCCAAACAAATTGC	intr.
pgp-1(pk29)	TCCACGATCTCCGACAAGAGTATTATATCCGTTTGGTAGAGT	exon
pgp-3(pk30)	CTCGGGTATCTTTCCTTCGCTAGATGTACATTTGATTTCCGG	exon
ceh-13(pk20)	AGCTTATAAGCATACCTACCTACCTGCCATAATTTTTGTTTA	intr.
ceh-13	CCACTCTCATCAATCATAGCTATGCTAGCTTATAAATTTTAA	intr.
ceh-13	TTAAAATTTATAAGCTAGCATAGCTATGATTGATGAGAGTGG	intr.
ZK637.5(pk21)	TTAAAATTACTACGGAGATATATCACCCAGACGCGAAAATTG	intr.
ZK637.5	ATTTAAAATTACTACGGAGATATATCACCCAGACGCGAAAAT	intr.
ZK637.5	GTAAATTCTAGCTTGATATATATTGAATTCAGAGATTTTAAA	intr.
prk-1(pk25)	TTTTTTGGCGTCTATATATATATATATATCTTGGTTTATTTT	intr.
prk-2(pk26)	GTGTTTTGAAACTATAAAGGTACTTAGTTGCTGAGAGATCGC	intr.
pes-9(pk22)	AATCATCATTATGCAATATGTA	?
gsa-1(pk27)	AACCCACCAATTCCTAAACATACCTGCTTATCCTTAGCCAAC	spl.
odc-1(pk32)	ACCGGTGTTTCTGCGGAACGTATCATCTATGCCAACCCATGC	exon
ceh-6(pk33)	TTTTAAATGTTCTCTACACATAACTGTAAAAAGTTAAACTTT	intr.
pgp-4(pk34)	GCCAGAAGCACCAACGGAACTATCATATCCTTCCGGTAGATT	exon
gpa-3(pk12)	CAAATCCAGAAAAGACGATTTACATGCATGAGACGTGTGGAA	exon
ceh-18(pk37)		
lin-26(pk38)		

FIG. 2. Tc1 insertion mutants recovered from the frozen library. Sequence where the transposon inserted is shown; the precise insertion site is the boldface TA sequence in the center. The sequences are aligned such that they are parallel with respect to the transposon; the insertion alleles thus all have a Tc1 integrated at the TA and are orientated such that the Tc1A gene within Tc1 runs from left to right. One insertion is outside the region for which sequence information was available, so that only the sequence at one side of Tc1 is known; two insertions have not yet been sequenced. intr., Intron area; exon, exon area; spl., splice site; ?, uncharacterized sequence. In some cases in which multiple addresses were found for insertions in the same gene, we chose one mutant to be thawed and isolated; all insertion mutants that were obtained clonally have an allele number (*pk*).

mutants can be used to isolate genuine gene knockouts. As reasoned in the Introduction, several transposon insertion alleles of the large majority of genes should be represented in the library, and this is confirmed by our results thus far. One can never be sure that all mutants are present; it is known from forward genetic studies that transposon insertion alleles of some genes have thus far not been obtained (e.g., *unc-86*, *tra-1*). We recovered mutants of all the genes that are the subject of study in our laboratory and of some genes that are being studied in other laboratories. We are nevertheless expanding the library to 10,000 cultures.

Tc1 inserts at position TA (23). This introduces a bias for intron insertions because these are known to be T+A-rich in *C. elegans*. We found indeed that the majority of Tc1 insertions are in intron sequences (see Fig. 2). Analysis of a limited number of Tc1 target site sequences has suggested a loose consensus sequence (24, 25). We address this in a detailed study of a large number of insertions (H. G. A. M. van Luenen and R.H.A.P., unpublished data). Since we found no genes without Tc1 insertions among the genes that we analyzed extensively, and since forward genetic experiments have indicated that most genes can be knocked out by Tc1 insertion, we do not expect nonrandomness of Tc1 insertion to pose general problems in this approach.

The approach described here was taken after the repeated failure of reproducible gene disruption by homologous recombination with transgenic DNA and was initially seen as a second choice. Nevertheless, it has a clear advantage over disruption with homologous targeting constructs; it is much faster and less laborious, since it requires no exchange of materials between laboratories, construction of targeting plasmids, transgenesis, or any handling of animals (until an interesting insertion mutant has been observed). It requires only the synthesis of four oligonucleotides per gene.

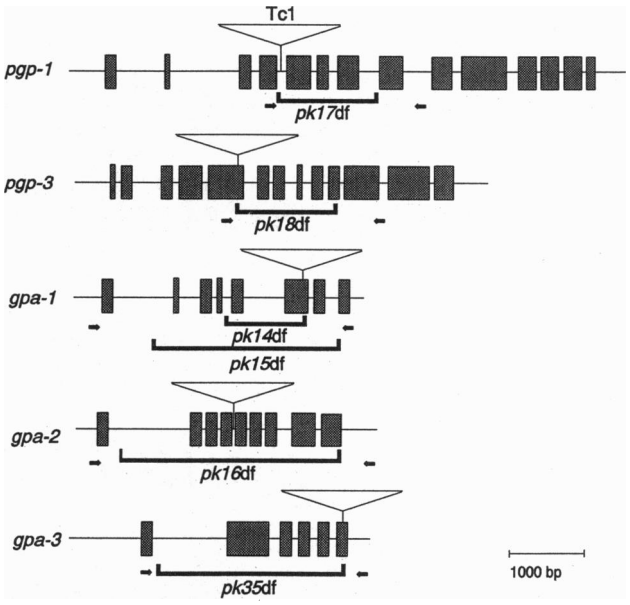

FIG. 3. Deletion derivatives of Tc1 insertion mutants of five genes. The intron/exon structure of these genes is indicated. Primers used for detection of deletions are indicated by short arrows; the Tc1 element is indicated by a triangle. For every gene, the deleted region is shown by a bracket, and the allele number is shown beneath.

The mutant bank described here is in principle for general use. However, the amount of DNA that is available does not allow free distribution of DNA to other laboratories, and therefore at present screening for insertions for other laboratories is done only in our laboratory. Amplifying the flanks of *all* Tc1 insertions in a culture would make more DNA available (and it could be distributed on a matrix filter to other laboratories for hybridization to genes of interest). The unexpected high frequency of deletion mutants in the progeny of Tc1 insertion mutants makes all insertions in or near a gene similarly useful to obtain gene knockouts, and therefore hybridization-based search strategies (that do not indicate the precise insertion site) are potentially as useful as strategies that indicate the insertion sequence. Whatever strategy is followed will be compatible with the mutant library described here.

pk14	AATCTAAACT atattcagcg---------aagctatgat ATATGTTGCT
pk15	ACTAATCAAA catcattcga---------catttctttt TGTGCATTCT
pk16	CTGAAGCTTT tctgaatttt---------tttagactat TTTGTTTTGT
pk17	CAATTTGTTT ggaaacttat---------tctcaaaaat AACTTTAAGAAACT
pk35	TTTCAAAAAA ttacttcaaa---------acgatttaca TGCATCAGAC

FIG. 4. Deletion mutants derived from Tc1 insertion alleles. Sequences around the junction sites are shown. Sequence where Tc1 is inserted prior to the deletion is boldface; for clarity, the transposon sequences were not included here. In cases of bidirectional deletions, the sites of Tc1 insertion are, of course, far removed from the end points of the deletions and are not indicated here. Sequences left after the deletion occurred are capitalized. In *pk14*, we found that only the flank of the transposon is deleted, but Tc1 itself is still present in its original site. In some cases the end points of the deletions are near short direct repeats (underlined). Allele numbers of the deletions (*pk*) correspond to those indicated in Fig. 3.

Any allele that is obtained from the "high hopper" strain should be studied with care: the strain also accumulates genetic lesions at points other than the one under study. Therefore, once an allele is obtained, it should be crossed into a "clean" genetic background, in which no further transposition takes place (e.g., the Bristol N2 strain), and the final demonstration that any observed phenotype is the result of gene inactivation is that it can be reverted by transgenesis with the cloned gene of interest.

All three *gpa* deletion mutants and the two *pgp* mutants are homozygous viable and show no obvious visible phenotype. The same is true for all the insertion mutants described in this paper. For the latter class, one can assume that in several cases the absence of a phenotype can be explained by incomplete loss of gene function; for the five deletion mutants described in Fig. 3 we have to conclude that they are dispensable for survival. A detailed study of the phenotypic consequence of inactivation of these genes remains to be done.

The approach described here, establishment of a stored mutant library, is potentially applicable to all organisms in which transposon or virus integrations can be obtained, provided that these organisms can be stored (semi) permanently. One could think of *Drosophila melanogaster*, which can be stored in a frozen state (26), and *Arabidopsis thaliana* and other plants, where seeds can be stored for many years.

The ongoing *C. elegans* genome project, at present generating megabases of sequence information per year, provides a challenge to biology—to keep up with the genome analysis by establishing genotype–phenotype relationships. Tc1-induced deletion mutants, in combination with the Tc1 mutant library, may make this possible.

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