Genetic dissection of G protein-coupled signal reduction
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Chapter 2

Transposon Tc1-derived sequence-tagged sites in *Caenorhabditis elegans* as markers for gene mapping.

Chapter 3

Transition to T-cell receptor-expressed cells in cancer progression.

Introduction to markers for gene mapping.
ABSTRACT  We present an approach to map large numbers of Tc1 transposon insertions in the genome of *Caenorhabditis elegans*. Strains have been described that contain up to 500 polymorphic Tc1 insertions. From these we have cloned and shotgun sequenced over 2000 Tc1 flanks, resulting in an estimated set of 400 or more distinct Tc1 insertion alleles. Alignment of these sequences revealed a weak Tc1 insertion site consensus sequence that was symmetric around the invariant TA target site and reads CAYATAITRG. The Tc1 flanking sequences were compared with 40 Mbp of a *C. elegans* genome sequence. We found 151 insertions within the sequenced area, a density of 1 Tc1 insertion in every 265 kb. As the rest of the *C. elegans* genome sequence is obtained, remaining Tc1 alleles will fall into place. These mapped Tc1 insertions can serve two functions: (i) insertions in or near genes can be used to isolate deletion derivatives that have that gene mutated; and (ii) they represent a dense collection of polymorphic sequence-tagged sites. We demonstrate a strategy to use these Tc1 sequence-tagged sites in fine-mapping mutations.

Within the next few years the complete genomic sequence of several organisms, including the nematode *Caenorhabditis elegans*, will be available (1-3). This will drastically alter molecular biology; all genes will be cloned and sequenced. The next challenge for biology will be to relate these genes to phenotypes and function (4). This requires efficient methods for targeted gene disruption and efficient strategies for mapping mutations of known phenotype to the sequence map. The *C. elegans* transposon Tc1 is an effective tool in both these genetic approaches: Tc1 insertions can be used to inactivate genes, and they can also serve as convenient genetic markers for mapping mutations. Tc1 is a member of the Tc1/mariner family of transposons and is present in all *C. elegans* strains analyzed (5-8). The copy number of Tc1 varies among different strains (9-11). The commonly used wild-type strain Bristol N2 has about 30 copies of Tc1; this number is stable, since germ-line transposition of Tc1 is absent in this strain (12, 13). Mutator strains show active germ-line transposition of Tc1 (12, 14-17) and are used for transposon tagging (15) and targeted gene disruption in *C. elegans* (18). Insertion of Tc1 can directly inactivate a gene. If this is not the case, the Tc1 insertion can be used to generate deletion derivatives (18). As well as using mutator strains for direct mutation, they can also be used as a source of genetic markers for mapping purposes. Mutator strains can contain large numbers of polymorphic Tc1 insertions, as high as 500 Tc1 insertions in strain Bergeroe BO (11). One approach has been to genetically identify a Tc1 insertion that maps close to a mutation of interest and to use this insertion to locate the mutation on the physical map (19). A more powerful application of polymorphic Tc1 insertions is as sequence-tagged sites (STSs) (20). Individual Tc1 insertions can be visualized by PCR using primers directed to the transposon and the flanking genomic sequence. Williams et al. (21, 22) cloned and sequenced a set of 40 strategically located Bergeroe BO Tc1 insertions. Combinations of these insertions can be detected by multiplex PCR, and linkage of a mutation to any of the Tc1 STSs can be assessed by PCR on single progeny of crosses to Bergeroe BO. Tc1 STSs have a number of advantages over conventional genetic markers (21, 22). (i) STS analysis enables genome-wide mapping of mutations. Depending on the set of PCR primers used, a mutation can be mapped to any of the six linkage groups or mapped further to a specific region. This eliminates laborious two- and three-factor crosses using conventional visible genetic markers. (ii) Multiple Tc1 STS markers can be analyzed in a single cross. Apart from allowing efficient mapping strategies, this enables mapping of multiple genes involved in complex phenotypes, such as aging (23). (iii) Tc1 STSs have no associated phenotypes, which is useful when mapping mutations with subtle phenotypes. (iv) Tc1 STS markers can be used to efficiently map lethal mutations because dead embryos and larvae can serve as substrates for PCR. (v) Tc1 STSs provide a direct link from genetic data to the physical map. A current limitation of Tc1 STSs, however, is the restricted number of Tc1 insertion site sequences that are available.

In this paper, we describe a method to shotgun sequence large numbers of polymorphic Tc1 insertions present in high Tc1 copy number strains and to map these insertions to the genomic sequence of *C. elegans*. Our approach makes optimal use of the available genome sequence; instead of using laborious genetic or physical methods, we mapped polymorphic Tc1 insertions by comparing short flanking sequences to the genomic sequence. We have obtained a Tc1 STS map with a density of about one Tc1 insertion in every 265 kb and we show an approach to using these STSs in fine-mapping mutations.

MATERIALS AND METHODS

Nematode Culture. Nematodes were cultured as described by Sulston and Hodgkin (24). High Tc1 copy number strains used in this study were RW7000, which is a derivative of Bergeroe BO (12), CB4000 [unc-1(e30) V] (J. Hodgkin, unpublished result in ref. 25), and KR1757 [unc-3(e31)] (17). Strains used in validation of the vectorette amplification approach were NL33 [pGATCCAAGGAGAGGACGCTGTCTGACGCA-3’] and NL300 [pGATCCGGAAAGAGGAACGGACCTGTGAC]. Strains used in mapping experiments were CB1489 [pGATCCGGAAAGAGGAACGGACCTGTGAC], CB164 [pGATCCGGAAAGAGGAACGGACCTGTGAC], and PB49 [pCATTAAGGAGAGGACGCTGTCTGACGA-3’]. All strains were maintained at 20°C in liquid culture on agar plates of rich nutrient media.

DNA analysis. Genomic DNA was isolated as described (18). The DNA was further purified by phenol/chloroform extraction and ethanol precipitation. Genomic DNA (100 ng) was digested with *SacI* as recommended by the supplier (New England Biolabs). After heat inactivation of *SacI* (15 min at 65°C), 15 pmol of annealed vectorette oligonucleotides (26) (top strand, pGATCCAAAGGAGGACGCTGTGACGA-3’; bottom strand, pGATCCAAAGGAGGACGCTGTGACGA-3’) were ligated with the 5’-end of the vectorette oligonucleotides and ligated into the vectorette amplification vector pQE12 V1. Products of vectorette amplification were sequenced using M13 primers and sequencing kits (Pharmacia).

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TCTCCCTTCTCGGAAACTGAACCGGTCGTCGTCGGAGGAAACGCTTGCCGTCCTTTTTCGG (as in refs. 18, 21, and 22).

RESULTS

Shotgun Sequencing of Tel Insertions. To sequence random Tel insertion sites, we generated strain- and orientation-specific libraries of Tel flanks. These libraries were constructed by cloning amplified left or right Tel flanking sequences directly into sequencing vectors. Flanking genomic sequences of Tel insertions present in the high Tel copy number strains RW7000, CB4000, and KR1787 was amplified using an anchored PCR-based method (Fig. 1A). RW7000 is a derivative of the natural isolate Bergerac BO (12) whereas CB4000 and KR1787 independently acquired mutator activity and high Tel copy numbers in a Bristol N2 background (17, 25). Genomic DNA was digested with the frequently cutting restriction enzyme Sau3A. This enzyme cut the genomic DNA in fragments ~0.2 kb in length. In addition, Sau3A cuts at known positions in Tel, leaving part of the transposon sequence attached to the flanking DNA: A double-stranded oligonucleotide containing the appropriate 5’ overhang (termed a vectorette) was ligated to the digested DNA (26). The vectorette serves as an anchor to amplify Tel flanks using one primer in the transposon terminus and one in the vectorette. The restriction sites of Sau3A in Tel are outside the terminal inverted repeats of Tel, so flanks of the right and the left side of the transposon could be amplified separately. The specificity of this amplification method was assessed by Southern analysis of the total vectorite-PCR product of a strain with a Tel insertion in the gene prk-2 and an equivalent strain without this insertion. Hybridization with a genomic pk-2 probe shows an amplified pk-2 fragment in the pk-2;+Tel strain but not in the strain without this insertion (Fig. 1B), demonstrating that this method can be used to specifically amplify the flanks of a complex mixture of Tel clones.

To clone the amplified Tel flanks, a second round of PCR with nested primers containing unique restriction sites was performed. The PCR product was cloned directly into M13-sequence vectors, and clones were sequenced using an automatic sequencer. Over 90% of these sequence tracks contained the Tel terminus and flanking genomic sequence. Sequence data were edited to remove Tel and vector sequences and obvious sequencing errors. A total of 2478 Tel flanking sequences were obtained from six different libraries: left and right flanks of strains RW7000, CB4000, and KR1787. Sequencing of random clones resulted in individual Tel flanks being represented by multiple sequence tracks. Therefore, we clustered homologous Tel flanking sequences into distinct alleles. Fig. 2 shows the distribution of the set of sequence tracks over the different Tel flanks they represent. Approximately one-half of the sequenced Tel flanks are represented by multiple sequence tracks. The other half are represented by single sequence tracks only. The distribution is clearly not random, reflecting an inherent bias of the amplification approach: some Tel flanks are amplified more efficiently than others. The distribution also shows that we have not reached saturation in sequencing all Tel flanks represented in the high Tel copy number strains RW7000, CB4000, and KR1787. Sequencing of random clones resulted in individual Tel flanks being represented by multiple sequence tracks. Therefore, we clustered homologous Tel flanking sequences into distinct alleles. A double-stranded oligonucleotide containing the appropriate 5’ overhang (termed a vectorette) was ligated to the digested DNA (26). The vectorette serves as an anchor to amplify Tel flanks using one primer in the transposon terminus and one in the vectorette. The restriction sites of Sau3A in Tel are outside the terminal inverted repeats of Tel, so flanks of the right and the left side of the transposon could be amplified separately. The specificity of this amplification method was assessed by Southern analysis of the total vectorite-PCR product of a strain with a Tel insertion in the gene prk-2 and an equivalent strain without this insertion. Hybridization with a genomic pk-2 probe shows an amplified pk-2 fragment in the pk-2;+Tel strain but not in the strain without this insertion (Fig. 1B), demonstrating that this method can be used to specifically amplify the flanks of a complex mixture of Tel clones.

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Tel Insertion Consensus Sequence. The genomic sequence surrounding the canonical TA target site of Tel was analyzed. To eliminate the noise from sequencing errors, only consensus sequences of Tel flanks with multiple sequence reads were...
aligned. We separately aligned the sequences of left and right Tcl flanks and focused on the first seven positions from the TA target site of Tcl. The base distributions of the left and right Tcl flanking sequences were not statistically different ($\chi^2 = 30.3$; site of Tel. The base distributions of the left and right Tel aligned. We separately aligned the sequences of left and right Tel. The base distributions of the left and right Tel

**Transposon-derived sequence-tagged sites**

![Graph showing the distribution of Tel insertion alleles across the genome.](image)

**FIG. 2.** Distribution of independent Tcl flanking sequence reads over different Tcl insertions. The number of reads is shown for each insertion site, with a maximum of 200 reads. The distribution is skewed towards the center, indicating a preference for symmetric insertion sites.

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To confirm that the Tcl insertions mapped in this study were present in the germ line of the strains examined and were not the result of cloning and sequencing somatic insertions, we tested 12 of the mapped Tcl insertions by PCR or Southern blot analysis. Five of these insertions were identified by both left- and right-sequenced Tcl flanks, whereas the other seven insertions were identified by one sequenced Tcl flank only. Eleven insertions were tested by PCR using a primer in Tcl and a primer in the Tel flanking sequence. Each resulted in the expected PCR fragment (Fig. 4) only in the strain in which the insertion was identified. One insertion was confirmed by Southern blot analysis (data not shown). In addition, six Bristol
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The scale is in approximate megabase pairs. Horizontal lines indicate genomic sequence used in this study is indicated by darkly shaded bars. V, and X arc represented by lightly shaded bars; the 40 Mbp of N2 (N), RW7000 (R), CB4000 (C) and KR1787 (K).

Strategy for Mapping Mutations Using Tel STSs. Tel insertions can serve as STSs; polymorphic sequences that can be visualized by PCR and can be used as genetic markers (20-22). We developed a strategy to use Tel STSs in mapping mutations. To locate a mutation to a specific region of the genome, the mutation has to be genetically linked to markers of known position. This involves scoring crossover frequencies between such markers and the mutation of interest. The closer a marker is located to the mutation, the lower the crossover frequency between the two. This poses a problem when fine mapping such markers and the mutation of interest. The closer a marker has to be genetically linked to markers of known position.

To reduce the effect of this bias, we separately amplifying Tel flanks is biased. Depending on the location of the Sau3A site with respect to the Tel insertion, Tel flanking fragments will have different sizes. A strain containing the (recessive visible) mutation should result. We describe a method to identify large numbers of transposon insertions present in high Tel copy number strains by shotgun sequencing. Flanking genomic sequence of Tel insertions was amplified using an anchored, PCR-based method, cloned in sequencing vectors, and sequenced. The total number of Tel insertions obtained depends on the efficiency with which different Tel flanks are amplified and the representation of these amplified Tel flanks in the collection of sequence tracks. The anchored PCR to amplify Tel flanks is biased. Depending on the location of the Sau3A site with respect to the Tel insertion, Tel flanking fragments will have different sizes. Consequently, large fragments will be amplified with lower efficiency, and very small fragments will be lost during cloning procedures. To reduce the effect of this bias, we separately

DISCUSSION

We describe a method to identify large numbers of transposon insertions present in high Tel copy number strains by shotgun sequencing. Flanking genomic sequence of Tel insertions was amplified using an anchored, PCR-based method, cloned in sequencing vectors, and sequenced. The total number of Tel insertions obtained depends on the efficiency with which different Tel flanks are amplified and the representation of these amplified Tel flanks in the collection of sequence tracks. The anchored PCR to amplify Tel flanks is biased. Depending on the location of the Sau3A site with respect to the Tel insertion, Tel flanking fragments will have different sizes. Consequently, large fragments will be amplified with lower efficiency, and very small fragments will be lost during cloning procedures. To reduce the effect of this bias, we separately

- Fig. 3. Distribution of sequenced Tel insertion sites mapped to the genomic sequence. The physical maps of chromosomes I, II, III, IV, V, and X are represented by lightly shaded bars; the 40 Mbp of genomic sequence used in this study is indicated by darkly shaded bars. The scale is in approximate megabase pairs. Horizontal lines indicate the location of sequenced Tel insertion sites mapped in strains Bristol N2 (N), RW7000 (R), CB4000 (C) and KR1787 (K).

- Table 1. Consensus sequence for Tel insertion

<table>
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<tr>
<th>Position</th>
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<th>G</th>
<th>C</th>
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<td>344</td>
<td>261.2</td>
<td>42.2</td>
</tr>
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</table>

*Flanking genomic sequence of left- and right-sequenced Tel flanks were aligned around the TA target site for Tel insertion. Values greater than 11.3 are significant at the 1% level; values greater than 16.3 are significant at the 0.1% level.

- PCR analysis. Linkage of a Tel STS to the mutation should result in an underrepresentation of that Tel allele in homozygous mutant animals. An example is given in Fig. 4: shown are seven RW7000 Tel insertions to position two mutations whose positions are already known, unc-36 (e251) and dpy-17 (e174), to the physical map. Marker 7 is located close to unc-36, and markers 3 and 4 are close to dpy-17. Analysis of five pools of 20 animals for each of the two genes showed crossover of all markers except 6 and 7 in the case of unc-36 and 2 and 3 for dpy-17, as was anticipated from the location of these mutations on the physical map. As expected, when a Tel marker close to one of the mutations did crossover onto the mutant chromosome, so do more distal markers. Using this mapping strategy, a mutation can be mapped to the resolution of the Tel STS map using only a single cross and analyzing only a limited number of pools for informative crossovers.

- Fig. 4. PCR amplification of polymorphic Tel insertions mapped in high copy number strains. Tel flanking fragments were amplified using a primer in Tel and a primer in the flanking genomic sequence. In each case, the first lane shows the PCR product using template DNA of the high copy number strain in which the insertion was identified, and the second lane shows the PCR product using Bristol N2 DNA. Markers pIP17 to pIP40 are in RW7000; markers pIP65 and pIP83 are in CB4000, and marker pIP41 is in KR1787. A 1-kb DNA ladder (GIBCO/BRL) was used as a DNA fragment size marker.
amplified the left and right flanks of Tc1 insertions. In addition, some of the flanking sequences of strain RW7000 were derived from genomic DNA digested with *MslIII* instead of *Sau3A*. Clustering of identical sequence tracks resulted in 378 left and 340 right Tc1 flanks. Approximately one-half of these were sequenced more than once. The other half was defined by single sequence tracks only, indicating that we probably did not sequence all Tc1 flanks represented in the different libraries.

Computer searches against 40 Mbp of genome sequence resulted in matches for 176 of the flanking sequences, defining 151 Tc1 insertions (in 25 cases, both left and right flanks were sequenced). Extrapolation from these numbers suggests that approximately 616 (718 x 151/176) different insertions are represented in this study. Assuming a genome size of 100 Mbp (1), this would predict an average density of one insertion every 160 kb whereas the observed frequency in the 40 Mbp compared directly was about one every 265 kb. A factor that may have contributed to this difference was the stringency used in examining the alignments between Tc1 flanking sequences and the genome sequence. Alignments that contained multiple mismatches were discarded. Therefore, Tc1 insertions may have been missed. To minimize the risk of mislocating STSs, we also excluded matches to repetitive sequences. This will result in an underestimation of both the total number of Tc1 insertions and the duplication between the sets of sequenced left and right Tc1 flanks. It is also possible that the density of Tc1 insertions is lower in the part of the genome sequenced so far, which concentrates on the central parts of the autosomes and on the X chromosome. However, the distribution of identified sites within the sequenced regions appeared to be uniform (Fig. 3).

The relatively low frequency of matching left and right flanks of any particular insertion site (25/181) again confirms that we did not identify all insertion sites in the strains studied. There appears to be a discrepancy between the degree of coverage estimated from this approach and that obtained by comparing the estimated number of sites sequenced (616) with the number of sites estimated experimentally: 700 total, made from ~500 for RW7000 (11), ~150 for CB4000 (J. Hodgkin, unpublished result in ref. 25) (data not shown), and ~60 for KR1787 (17). This may reflect either an underestimate in the previous experimental results or incompleteness in finding all matching sequences as described above.

Six of the eight Bristol N2 insertions present in the 40 Mbp of genome sequence were identified as well. Screening of the sets of Tc1 flanks against the genomic sequence of *C. elegans* resulted in only 27 insertions that mapped to multiple regions within the genome. This is a reflection of the relatively low abundance of repeated sequences within the *C. elegans* genome (1).

Apart from germ-line transposition, Tc1 is also active in somatic tissues (33, 34). This results in a background noise of somatic Tc1 insertions. The PCR approach used to amplify Tc1 flanks is biased toward germ-line insertions; in the mixture of digested genomic DNA, germ-line Tc1 flanks are present in a much higher template concentration than somatic insertions. Therefore, we did not expect to clone and sequence somatic insertions. Indeed, all 12 Tc1 alleles tested proved to be germ-line insertions. Nevertheless, before a Tc1 insertion mapped in this study is used for further experiments, it is advisable to check first that it is indeed an insertion that is present in the germ line of the strain in which it was identified.

We analyzed the genomic sequence surrounding the canonical TA target site of Tc1 insertion. Previous studies based on small numbers of insertion sites suggested a variety of related consensus sequences that were approximately palindromic (25, 35). Alignment of the 344 consensus flanks confirmed by
multiple reads revealed no statistically significant difference between the left and right flanks. When all flanking sequences were combined, a significant bias was seen in the four bases directly flanking the TA target site (Table 1). The resulting consensus sequence is consistent with previously reported results but is now based on the largest set of random germ-line insertions analyzed so far. The symmetry of the Tel insertion consensus sequence is a reflection of the orientation independence of Tel insertion (26). This is not surprising because Tel ends have perfect inverted repeats that are sufficient for insertion when transposase is provided in trans (37).

As a result of the high gene density of the C. elegans genome (1), most of the sequenced Tel insertions will be located in or close to genes. These Tel insertions can be used to obtain mutations in these genes (18). Deletions of flanking genomic sequence occur as a side product of Tel transposition; excision of Tel results in a double strand break in the chromosome, and repair of this break can result in loss of flanking genomic sequence. Consequently, the ability to induce deletions depends on an intact Tel element combined with a genetic background that allows germ-line Tel transposition. Tel elements are structurally invariant (9), so most Tel elements should be competent for excision. Also, the strains used in this study show germ-line Tel transposition (12, 17, 25). Therefore, it is, in principle, possible to use the set of mapped Tel insertions for deletion mutagenesis. Scaling up the sequencing of polymorphic Tel insertions could provide Tel insertion alleles of all genes in the C. elegans genome. Such Tel alleles could be used directly to delete any gene of interest. The current limitation lies in the isolation of more strains with a high Tel copy number.

The other application of polymorphic Tel insertions is gene mapping. The set of Tel insertions forms a dense collection of polymorphic sequence tagged sites. Each Tel insertion can be visualized by PCR using a primer in Tel and a unique primer in the flanking genomic sequence. Extending the work of Williams et al. (21, 22), we have demonstrated an efficient method to use Tel STSs in fine mapping mutations. The mutation in a Bristol N2 background is crossed with one of the high Tel copy number strains and mutant F2 progeny are analyzed for linkage to any of the Tel STSs. To fine-map mutations, we pooled independently segregated homozygous mutant progeny instead of analyzing single animals. Pooling of single animals allows rare, informative crossovers of Tel STSs to be readily detectable. Instead of analyzing many single animals, using PCR on a limited number of pools is sufficient. Depending on the mapping resolution required, the complexity of these pools can be varied. To fine map mutations to the resolution of the Tel STS map, in principle, pools of as many as 100 or more animals can be analyzed. Given the density of the Tel STS distribution over the genome, a mutation can now be located with a resolution of ~265 kb to the physical map, a region corresponding to about 10 cosmids, which is small enough to directly attempt to identify the cosmid containing the mutant gene by transgenesis experiments.

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