Cloning within the unc-43 to unc-31 interval (linkage group IV) of the Caenorhabditis elegans genome using Tc1 linkage selection

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The region around the twitcher gene, unc-22, flanked by unc-43 on the left and by unc-31 on the right, has been intensively studied in our laboratory over the period of the last 8 years. In this paper we describe the identification and isolation of probes specific for several restriction fragment length differences (RFLDs) which lie within this region. Many RFLDs in Caenorhabditis elegans are caused by the insertion of a transposable element, Tc1. The method we used involved the isolation of Tc1-containing genomic fragments. These were recovered from a λgt10 library of DNA from a specially constructed genetic strain containing the unc-43 to unc-31 interval from the BO strain and the rest of the genome from N2. Because the BO strain is rich in Tc1 insertion sites and the N2 strain has few, the majority of Tc1-bearing genomic fragments in the constructed strain were derived from the unc-22 region. Of nine such Tc1-bearing genomic fragments isolated, six were found which mapped within the region of interest. The 350 kilobases of genomic sequences isolated as a result of these studies are being used to study the molecular organization of this region. The method described here for Tc1 linkage selection is one that is rapid, general, and may be targeted to any genetically characterized region of the C. elegans genome.

Key words: Tc1, unc-22, restriction fragment length differences, Caenorhabditis elegans, linkage selection.


La région autour du gène de contraction (saccades), unc-22, flanquée sur la gauche par le unc-43 et sur la droite par le unc-31, a été étudiée de façon intensive au cours des huit dernières années. Dans ce document, les auteurs décrivent la méthode d’identification et d’isolation de sondes spécifiques pour plusieurs fragments de restriction de différentes longueurs (RFLDs) qui sont situés dans cette région. Plusieurs RFLDs chez C. elegans sont causés par l’insertion d’un élément transposable, le Tc1. La méthode utilisée implique l’isolation de fragments génomiques contenant du Tc1. Ceux-ci furent recueillis d’un feuillet λgt10 d’ADN chez une souche génétique spécifiquement développée qui contient l’intervalle unc-43 à unc-31 de la souche BO et le reste du génome de la souche N2. Puisque la souche BO est riche en sites d’insertion de Tc1 et que la souche N2 n’en a que peu, majorité des fragments génomiques porteurs de Tc1 chez la souche développée fut dérivée de la région unc-22. Sur neuf tels fragments génomiques isolés porteurs de Tc1, six se sont avérés appartenir à la région d’intérêt. Les 350 kilobases de séquences génomiques isolées comme résultat de ces recherches sont utilisées aux fins d’une étude sur l’organisation moléculaire de cette région. La méthode décrite ici, soit celle de la sélection de linkages de Tc1, en est une rapide, générale qui peut être appliquée à toute région génétiquement caractérisée du génome de C. elegans.

Mots clés: Tc1, unc-22, fragments de restriction de différentes longueurs, Caenorhabditis elegans, sélection de linkage.

[Traduit par le journal]

Introduction

In this paper we describe the targeted isolation and partial characterization of cloned probes defining restriction fragment length differences (RFLDs) around the region flanking the unc-22 gene in Caenorhabditis elegans. Previously we described a method for obtaining cloned molecular probes for genetically defined genes whose products had not been biochemically identified (Rose et al. 1982). That method involved the two closely related C. elegans strains, var. Bristol (N2) and var. Bergerac (BO) (Emmons et al. 1979) and cloned probes for two regions of the genome were identified: pCe s18 which identifies a strain polymorphic site (sp1) in the unc-15 region of linkage group (LG) I (Rose and Bailie 1980; Rose et al. 1982; Harris and Rose 1984) and pCe s102 (sp2) which maps into the highly polymorphic region surrounding the heat shock genes on LG IV (Smfrac and Bailie 1983, 1984;
targeted cloning of genes. Our method makes use of a special class of RFLD caused by the insertion of the transposable element, Tcl (Emmons et al. 1983). Unlike our previous approach of mapping randomly obtained RFLDs, here we describe a method for the directed cloning of a specific region of the genome.

The region we selected is the interval between unc-43 and unc-31 on LG IV. This region contains the twitcher gene unc-22. The organization of this gene (Moerman and Baillie 1979; Moerman 1980) and the region around the gene (Rogalski et al. 1982; Rogalski 1983; Rogalski and Baillie 1985; L. Donati and D. L. Baillie, unpublished results) has been the object of intense genetic analysis. Mutational analysis of this region has revealed a cluster of 16 essential genes to the left of unc-22, whereas in the same interval to the right of unc-22 only 4 essential genes were identified (Rogalski and Baillie 1985). The isolation of cloned probes for the unc-22 region of LG IV makes possible studies of the molecular organization of this region.

Materials and methods

Caenorhabditis elegans strains and genetic crosses

Wild-type and mutant strains were maintained and mated on nematode growth medium (NGM) streaked with Escherichia coli OP50 (Brenner 1974). Crosses were carried out on 10 x 35 mm culture plates. All experiments were performed at 20°C. The wild-type strain N2 and some mutant strains of C. elegans var. Bristol were originally obtained from the MRC stock collection in Cambridge, England. Others were received from the Caenorhabditis Genetics Center at the University of Missouri, Columbia. Caenorhabditis elegans var. Bergerac (BO) was obtained from D. Hirsh in Boulder, Colorado. The following mutant genes and alleles were used: unc-22 (h3), unc-22 (s7), unc-31 (e109), and unc-43 (e266).

Ethyl methanesulfonate mutagenesis

Individuals from the Bergerac strain, BO, were treated with 0.01 M ethyl methanesulfonate in M-9 buffer for 4 h. The F2 descendents were screened for mutant phenotypes and a twitcher recovered. This allele (h3) failed to complement a known unc-22 allele, s7.

Preparation of C. elegans DNA

DNA was isolated from the strains of C. elegans as previously described (Rose et al. 1982).

Construction of λgt10 library

A λgt10 vector, a gift from T. Huyhn, was used to construct a library of unc-22(h3); N2/BO DNA in the following way. unc-22 DNA (0.1 μg) was digested to completion with EcoRI and ligated in the presence of 1 μg of EcoRI-digested λgt10 vector. The ligation mix was packaged according to the method described in Davis et al. (1980) using packaging extracts prepared by T. Snutch. Approximately 10⁶ phages/μg of DNA were recovered. Restriction digests were done under conditions recommended by the supplier (Bethesda Research Labs). T4 ligase was prepared from an E. coli lysogen.

unc-22 BO
unc-22 BO
≈300 Tc1s

30 Tc1s

unc-22 BO + +

unc-22

N2 female

Wildtype: twitcher

3 : 1

pick single twitcher × N2♂

repeated backcrosses and segregation

unc-22

BO Tc1s captured in unc-22 region of an N2 genome

Fig. 1. The genetic protocol followed to generate the unc-22 hybrid strain. BO DNA is shown by wavy lines and N2 DNA by straight lines.

Phage purification

The probes used to isolate Tcl-containing phages from the λgt10 library were Tcl (compliments of S. Emmons) and Tcl (Hin), a variant which differs by the presence of a HindIII site (Rose et al. 1985). A pUC13 vector (Viera and Messing 1982) containing Tcl(Hin) was nick translated (Davis et al. 1980) to a specific activity of 10⁶ - 10⁷ cpm/μg. Phages (10⁶) were screened for homology to the probe by the method of Benton and Davis (1977). Plaque-purified phages were amplified and 10⁷ phages were plated on media prepared in 25 × 35 cm lasagna trays. After 8 h of growth at 37°C the tray was flooded with 70 mL of λ diluant (10 mM Tris pH 7.5; 10 mM MgSO₄) at 4°C. The phages in λ diluant were left overnight at 4°C, collected, and purified by centrifugation in a CsCl gradient (Davis et al. 1980). Phage DNA was extracted by the formamide method described in Davis et al. (1980).

Subcloning

Subcloning was carried out as described by Snutch (1984). One microgram of λgt10 carrying the fragment to be cloned and 0.1 μg of plasmid vector pUC13 were mixed in 100 μL of EcoRI restriction buffer and restricted with 2 units of EcoRI for 2 h. The reaction was stopped by heat inactivation at 70°C for 5 min. After cooling to room temperature, 0.1 μg of DNA ligase was added. Ligation was performed at 16°C for 2 h followed by a sixfold dilution and stored at 4°C overnight. Fifty microlitres of ligation mix were used to transform competent E. coli strain JM83 by the method of Morrison (1979). Transformed cells were plated on XGal, IPTG, and ampicillin-containing L-broth plates, and white colonies were se-
backcross BO mutant to N2 to capture BO TcIs
↓
prepare DNA
↓
genomic blot to identify captured TcIs
↓
gt10 library of DNA
↓
screen for TcI - hybridizing clones
↓
purify on CsCl
↓
subclone into pUC13
↓
EcoRV cut and religate
to obtain unique flanking sequence
↓
position on genetic map: use as probes for cloning genes

Fig. 2. Flow diagram of the cloning procedure.

From 10 mL overnight cultures by a method described in Davis et al. (1980).

Flanking sequences of the TcI s were isolated by digesting the subcloned fragment with EcoRV, which cuts in the terminal repeats of TcI. The ends were then religated and transformed into JM83. Single colonies were isolated and screened for the absence of TcI. These were then grown to maturity, and the DNA that was isolated was used to map the RFLPs.

Blot hybridization
Genomic DNA was digested with restriction endonucleases and electrophoresed in submerged horizontal agarose gels. DNA fragments were transferred to nitrocellulose filters (Schleicher and Schuell) by the method of Smith and Summers (1980). Prehybridizations were carried out in 5 × SSPE (SSPE = 0.18 M NaCl, 1 mM Na₂EDTA, 10 mM phosphate buffer, pH 7.0) and 0.3% SDS at either 62 or 68°C for at least 1 h. Overnight hybridizations were done under the same conditions after adding 10⁶ cpm of [³²P]-labelled probe DNA per millilitre of hybridization solution. Filters were washed in three changes of 2 × SSPE at 62°C and air dried prior to autoradiography. Hybridizations to phage or plasmid DNA were done for 1–4 h.

Construction of three-factor mapping strain DNA
RFLPs were initially mapped to LG IV. The LG IV mapping DNA was N2 for LG IV in a mixed N2/BO genome To position the TcI-induced RFLPs with the 2 map unit interval defined by unc-43 and unc-31, we constructed N2–BO recombinant chromosomes in either the unc-43 to unc-22 or the unc-22 to unc-31 region. These recombinants were used to prepare DNA. This was done as follows. The cis-heterozygote, unc-22 unc-31 (N2)/+ + (BO), was constructed and six Unc-31 crossover individuals were isolated from the self-cross progeny by T. Rogalski. From each of the six Unc-31 individuals, + (BO) unc-31 (N2)/unc-22 unc-31 (N2), a single individual homozygous for the crossover chromosome was identified. Homozygous recombinant individuals were grown separately for DNA. In a similar manner, nine Unc-43 recombinants were isolated by L. Turner and grown. DNA from each recombinant was studied separately, or in some cases, equal amounts of DNA from individual isolates of one recombinant class were pooled for genomic blot hybridization experiments.

Results
Genetic strains that contained BO DNA in the unc-22 region of linkage group IV and N2 DNA for the rest of the genome were constructed using a Bergerac mutant allele, h3. Individuals homozygous for unc-22 (h3) were outcrossed to N2 males, and heterozygous hermaphrodite progeny resulting from this mating were
was isolated as the progenitor of the first backcross strain. This procedure was repeated six times, such that approximately 98% of the h3 genome was replaced with N2 DNA except for the region immediately adjacent to the unc-22 gene (Fig. 1). Because each backcross diluted the remaining BO portion of the genome by 50%, then after six backcrosses the BO portion is \((1/2)^6\), that

on 100-mm culture plates and the DNA was prepared. This DNA was referred to as “unc-22” DNA.

The flanking DNA from sites containing Tc1 insertions in the BO strain provided probes for RFLDs which were mapped within the unc-22 region. A flow diagram of each of the steps to obtain the RFLD probes is shown in Fig. 2. A number of BO Tc1s were present in the “unc-22” DNA as can be seen in Fig. 3. In N2, 30 or more bands can be identified, whereas in the unc-22 strain considerably more (greater than 60) are present. As described in the Materials and methods, a λgt10 library was prepared from the “unc-22” DNA. Using Tc1(Hin) as the probe, the library was screened for Tc1-hybridizing clones. After plaque purification of the identified phage, phage bands were collected from CsCl gradients. Thirteen phages were isolated in this way. Each of the phages were digested with EcoRI to identify the insert sizes. Nine different inserts were observed (four fragments were independently isolated twice) and are shown in Fig. 4. In this sample of Tc1-containing phages the fragment sizes ranged from 1900 to 4300 base pairs (bp). EcoRI-digested phage inserts were subcloned into the plasmid vector pUC13. DNA from pUC13 subclones was prepared using 10 mL overnight cultures, and digested with EcoRV which cuts in the Tc1 terminal repeats (Rosenzweig et al. 1983) producing a 1600-bp fragment (Fig. 5). All the subclones but one, lane 4, gave a 1600-bp band as expected. Further analysis of this subclone is not described in this paper. The two largest inserts each had an additional EcoRV site in the flanking sequence. (The additional EcoRV fragments did not hybridize to Tc1.) One (lane 8) gave a 1600-bp doublet and another (lane 9) also gave a 1600-bp doublet plus an 1100 bp fragment. (In addition both has pieces attached to the pUC13 vector.)

In order to clone flanking sequence after Tc1 elimination, a portion of the EcoRV digestion mix for each of eight subclones was diluted 10-fold to favor a ring-closure reaction and religated. Following transformation into E. coli JM83, individual white colonies were isolated. DNA was prepared from 10-mL overnight cultures and digested with EcoRI. If the Tc1 sequence had been removed, each subclone should now contain and EcoRI fragment of the original size minus 1600 bp. This was observed for six of the cloned Tc1s. In the case of the two largest, which had additional EcoRV sites in the flanking sequence, one of them (pCE s237) has 700 bp of pUC13-attached flanking sequence. The other, pCE s238, has 100 bp of pUC13-attached flanking sequence plus the 1100-bp EcoRV fragment. EcoRI digests of these probes demonstrated that the expected fragment sizes had been cloned. In this manner, we obtained flanking sequence probes for RFLDs between the N2 and BO strains.
mic blots were prepared using N2, BO, the "unc-22," and LG IV mapping DNA. The LG IV mapping DNA was N2 for LG IV in a mixed N2/BO genome and was prepared as described previously (Rose et al. 1982). These blots were probed with the plasmids containing flanking sequence DNA. Two of the probes were not linked to unc-22. One, pCe s230, was an insertion into the N2 strain. Another, pCe s238, a BO insertion, gave a complex banding pattern and did not map to LG IV. Further analysis of these two clones is not described in this paper. Five of the plasmids which were BO insertions were unique sequences, whereas one (pCe s235) was a low repetitive sequence. All of the plasmids generated RFLDs and were linked to unc-22. Tight linkage to unc-22 is indicated by the presence of only an N2 DNA. No indication of a BO band was observed for experiments done with pCe s229 or pCe s233. Weak bands could be seen with the others.

In order to more accurately position these sites with regard to unc-22, blot hybridizations were done using pooled EcoRI-digested DNA from the recombinants of the three-factor crosses described in the Materials and methods. Homozygous recombinants from the 1 map unit interval either to the right or the left of unc-22 were grown individually for DNA and equal amounts of each were pooled for genomic blot hybridization experiments. The pooled DNA was used for the initial positioning; then DNA that was prepared from individual recombinant events was tested. For example, the polymorphism sp6 was mapped by using the flanking se-
EcoRI-digested genomic DNA from nine Unc-43 recombinants was probed. These Unc-43 recombinants were derived from an unc-43 unc-22 (N2) chromosome over a + + (BO) chromosome and the homozygous recombinant used to prepare DNA. The resulting autoradiograph is shown in Fig. 6. As can be seen, eight recombinants showed the N2 (1700 bp) and one showed the BO (3300 bp) fragment size. Thus, the sp6 site lies close to unc-43, one-ninth of the way across the interval between unc-43 and unc-22. In agreement with this position, the Unc-31 recombinants clearly showed the sp6 site to be to the left of unc-22. These results are summarized in Table 1. In a similar manner, Unc-31 and Unc-43 data placed the sites which were identified by pCe s234 (sp5), pCe s231 (sp7), and pCe s237 (sp8) clearly to the left of unc-22. Furthermore, sp7 and sp8 were very close to or to the left of unc-43. pCe s229 was used to position the sp3 site. Data based on the Unc-43 recombinants placed the RFLD site at or to this site was half-way between unc-22 and unc-31. The Unc-31 mapping data places the site identified by pCe s233 (sp4) to the left of unc-22, whereas the Unc-43 data places it to the right. Therefore, sp4 must be very tight to unc-22. A summary of the results of the mapping data is given in Table 1 and illustrated in Fig. 7 which gives a detailed map of the unc-22 region (Rogalski and Baillie 1985).

In addition to the above results, we hybridized the LG IV probes to a second Bergerac strain, FR. We had shown previously that FR had the same fragment size as N2 for the sp4 site identified by pCe s18 on LG I (Rose et al. 1982). The results of genomic blot hybridizations using EcoRI-digested DNA from the FR strain are shown in Table 2. All of the RFLDs to the left of unc-22 showed the BO pattern, whereas all those to the right including the 70 kd heat shock region RFLDs showed an N2 pattern. Thus in the FR strain, one portion of LG IV was N2-like and another BO-like.
Fig. 6. Autoradiograph of a genomic blot hybridization probed with the flanking sequences from pCe s235. Each of the lanes contained DNA from one of nine Unc-43 recombinants described in the Materials and methods. The upper arrow indicates the position of the 3300-bp BO fragment size. The lower arrow indicates the position of the 1700-bp N2 fragment size. Lane 1 is underloaded relative to lanes 2–9.

<table>
<thead>
<tr>
<th>Name of plasmid</th>
<th>Original fragment size</th>
<th>Both RV sites</th>
<th>Insert size (bp)</th>
<th>Inserted strain</th>
<th>Unc-31*</th>
<th>Unc-43*</th>
<th>Name of site</th>
</tr>
</thead>
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<tr>
<td>pCe s229</td>
<td>1900</td>
<td>+</td>
<td>300</td>
<td>BO</td>
<td>3/6</td>
<td>9/9</td>
<td>sP3</td>
</tr>
<tr>
<td>pCe s230</td>
<td>1950</td>
<td>+</td>
<td>350</td>
<td>N2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCe s231</td>
<td>2300</td>
<td>+</td>
<td>700</td>
<td>BO</td>
<td>6/6</td>
<td>0/9</td>
<td>sP7</td>
</tr>
<tr>
<td>pCe s232</td>
<td>2350</td>
<td>1 only</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>pCe s233</td>
<td>2600</td>
<td>+</td>
<td>1000</td>
<td>BO</td>
<td>6/6</td>
<td>9/9</td>
<td>sP4</td>
</tr>
<tr>
<td>pCe s234</td>
<td>2800</td>
<td>+</td>
<td>1200</td>
<td>BO</td>
<td>6/6</td>
<td>2/9</td>
<td>sP5</td>
</tr>
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<td>pCe s235</td>
<td>3300</td>
<td>+</td>
<td>1700</td>
<td>BO</td>
<td>6/6</td>
<td>1/9</td>
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<td>700</td>
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<td>6/6</td>
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<td>1100</td>
<td>BO</td>
<td>6/6</td>
<td></td>
<td>Not on LG IV</td>
</tr>
</tbody>
</table>

*Number of crossovers with the BO band size from either Unc-31 or Unc-43 crossover products derived, respectively, from either unc-22 unc-31 (N2)/+ × (BO) or unc-43 unc-22 (N2)/+ × (BO) heterozygotes.

Discussion

We describe here the targeted isolation of cloned DNA from the region flanking unc-22. We have used a rapid and general method that can be used for any genetically characterized region of the C. elegans genome. An outline of the method is shown in Fig. 2. Certain aspects deserve comment. First, the generation

to screen N2 genomic libraries and a set of overlapping Charon 4 phages have been obtained using these probes. Cosmids that overlapped these phage have been obtained compliments of J. Sulston, MRC Hills Road, Cambridge. At present, approximately 350 kb of DNA sequence covering the interval between unc-43 and unc-31 have been obtained. Extensive characterization of
repeated backcrosses to N2 in order to construct the appropriate genetic strain. Second, the use of the inser-

fractions that are smaller than 7000 bp and indeed, the largest fragment we have identified in this library was 6000 bp. Further, the Tc1 hybridization pattern was examined on a genomic blot of unc-22 DNA. As can be seen in Fig. 3, many additional Tc1-hybridizing bands were present as would be expected. Many, but not all, of the unc-22 Tc1s cloned by us were readily distinguishable on this autoradiograph. Apparently there were cryptic Tc1 insertion fragments which were masked by the N2 bands. Fortunately of the nine Tc1 insertions isolated, seven were BO insertions and of these, six mapped to the unc-22 region. The fact that all cloned Tc1 insertion sites examined were found to be occupied in one or the other of the parental lines indicated that none of these Tc1 insertions resulted from mobilization of the element during the construction of the strain. Although there are several EcoRV sites in λgt10, it is possible by visual examination of agarose gels to determine whether or not a 1600-bp band is present in the purified λgt10 phage. EcoRV sites exist
band (more precisely 1572) by EcoRV digestion is a simple way of confirming the presence of an intact Tc1 in the cloned phage. In this study, all the insertions into the BO strain that we mapped had intact EcoRV sites. We suspect that the one lacking an EcoRV site is a N2 insertion.

Lastly, and more importantly for the purpose of this study, the presence of the EcoRV sites suggested a rapid method for obtaining flanking sequence probes from the cloned Tc1-containing plasmids. The original cloned EcoRI fragments (containing Tc1) can’t be used for genetic mapping because of the many Tc1 inserts in BO which would result in a smear on an autoradiograph. Although the unique flanking sequence fragment could have been purified from phage preparations, subcloning into a plasmid was done in order to facilitate subsequent manipulations. In our approach the EcoRI band was easily subcloned into a plasmid vector and immediately grown using overnight plasmid cultures. EcoRI and EcoRV restriction digests provided confirmation that the desired fragment had been subcloned. The EcoRV digested DNA was immediately used to prepare flanking sequence probes. By digesting with EcoRV, diluting the digestion mix and re-ligating, we were successful in obtaining flanking sequence probes from the plasmid subclones which could be used as [32P]-labelled probes for genomic blots. (Although these probes contained 38 bps of Tc1 terminal repeat sequence this did not cause a problem under the hybridization conditions described.) It is unlikely that we have identified all the unc-22 Tc1-insertion sites in BO. In a sample of 13 isolated Tc1s, four were represented twice and five were represented only once. It is likely that a number of yet-unidentified Tc1-containing BO sites are present in this region.

All the RFLDs which map to the left of unc-22 showed a BO pattern in the FR strain, whereas all those to the right showed an N2 pattern. These results are consistent with the interpretation that FR was derived from a genetic hybrid of the N2 and BO strains of C. elegans. The phenotype of FR is also consistent with this suggestion. FR hermaphrodites are more coordinated and FR males more fertile than BO (A. M. Rose, unpublished results). We propose that the Bergerac FR strain is a mixture of N2 and BO and that on LG IV an exchange event occurred between these two genomes near the unc-22 locus between sp5 and sp4. The recombinant chromosome produced is composed of BO DNA to the left and N2 to the right of unc-22.

The focus of our analysis has been the unc-22 region. This region has been the object of an extensive and on-going genetic analysis (Moerman and Baillie 1979; Moerman 1980; Rogalski et al. 1982; Rogalski 1983; Rogalski and Baillie 1985; L. Donati and D. L. Baillie, flanking unc-22 has been accomplished (Rogalski and Baillie 1985). The isolation of 350 kb of cloned and mapped DNA from this interval has initiated an intense investigation into the molecular organization of this region. The sp3 site is located to the right of unc-22 in the midst of a mutationally quiescent region. Analysis of cloned DNA around sp3 shows, however, that this region is functionally active. The sp4 site has provided a series of cosmids which have been used to identify the breakpoints of deletions flanking the let-56 gene, immediately to the left of unc-22. Further to the left of unc-22, sp5 and sp6 are situated in the essential gene cluster described by Rogalski and Baillie (1985). Attempts are currently underway to identify coding regions and make assignments to the identified genes in this interval.

The method described in this paper, that of Tc1 linkage selection, is rapid, general, and may be targeted to any genetically characterized region of the C. elegans genome.

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