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Site-Selected Insertion of the Transposon Tc1 into a *Caenorhabditis elegans* Myosin Light Chain Gene

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We used the polymerase chain reaction to detect insertions of the transposon Tc1 into *mlc-2*, one of two *Caenorhabditis elegans* regulatory myosin light chain genes. Our goals were to develop a general method to identify mutations in any sequenced gene and to establish the phenotype of *mlc-2* loss-of-function mutants. The sensitivity of the polymerase chain reaction allowed us to identify nematode populations containing rare Tc1 insertions into *mlc-2*. *mlc-2::Tc1* mutants were subsequently isolated from these populations by a sib selection procedure. We isolated three mutants with Tc1 insertions within the *mlc-2* third exon and a fourth strain with Tc1 inserted in nearby noncoding DNA. To demonstrate the generality of our procedure, we isolated two additional mutants with Tc1 insertions within *hlh-1*, the *C. elegans* MyoD homolog. All of these mutants are essentially wild type when homozygous. Despite the fact that certain of these mutants have Tc1 inserted within exons of the target gene, these mutations may not be true null alleles. All three of the *mlc-2* mutants contain *mlc-2* mRNA in which all or part of Tc1 is spliced from the pre-mRNA, leaving small in-frame insertions or deletions in the mature message. There is a remarkable plasticity in the sites used to splice Tc1 from these *mlc-2* pre-mRNAs; certain splice sites used in the mutants are very different from typical eukaryotic splice sites.

Genetic analysis is important for determining the in vivo functions of proteins. The phenotype of an organism in which a particular protein is missing or altered can indicate the normal functions of that protein. In a superior experimental organism, genes that are defined by mutations can easily be cloned, and those that are cloned can easily be altered in vivo. Together, these two methods allow an investigator to identify the components of a process and to systematically alter those components in vivo.

Methods for cloning *Caenorhabditis elegans* genes previously defined by mutations are well established. Clones of interest are routinely isolated either by transposon tagging (34, 52) or by positional cloning, using an integrated genetic and physical map of *C. elegans* (11, 17, 18, 69). In contrast, in vivo disruption of molecularly cloned *C. elegans* genes remains difficult. Methods for disrupting genes via homologous recombination, such as those for yeast and mammalian cells (for examples, see references 10, 37, 39, 71, 74, 77, 78, and 79), or for the targeted disruption of genes by transposons (4, 36) are only now becoming available for *C. elegans* (40a, 60). As the number of cloned genes lacking genetic characterization increases, the need for such methods becomes more pressing.

As in many organisms, spontaneous mutations in *C. elegans* are often caused by the insertion of transposable genetic elements (for reviews, see references 8, 42, and 72). The *C. elegans* genome contains at least five different families of transposable elements (28, 54). Most transposon-induced alleles that have been characterized to date are caused by the Tc1 family of elements. Tc1 elements are present in the genomes of all known *C. elegans* strains, but Tc1 copy number and frequencies of transposition vary widely in different isolates (22, 27, 46, 53). Mutator genes that are present naturally in certain wild-type strains (3, 53, 56) or that are mutagen induced (15) can elevate the frequency of Tc1 transposition. The mutator mutant *mut-2(r459)* activates Tc1 transposition and excision to particularly high levels (15). Most spontaneous mutations isolated in the presence of *mut-2* are caused by insertion of Tc1 (15), although this may not be true for all genes (84).

Myosin is a hexameric protein composed of two myosin heavy chains and four myosin light chains. Heavy chains have two structural and functional domains: a globular head portion and a rod-like portion. One regulatory and one alkali (also called essential) light chain are noncovalently bound to each globular head of myosin. In muscle cells, myosin-containing thick filaments and actin-containing thin filaments interact to generate force (35). The myosin light chains regulate interactions between actin and myosin in response to changes in intracellular calcium. Specifically, the regulatory myosin light chain proteins are believed to regulate the actin-activated ATPase of myosin (for reviews, see references 1 and 38).

We have developed a procedure using mutator strains to isolate Tc1 insertions within *mlc-2*, one of two *C. elegans* regulatory myosin light chain genes. In theory, this method could be used to identify transposon insertions within any target gene whose sequence is known. We selected *mlc-2* as a target for several reasons. First, while genetic analysis of *C. elegans* myosin heavy chains is well developed (for reviews, see references 2 and 81), little is known about nematode myosin light chains and their roles in muscle function. Specifically, no mutants of *mlc-2* were known to exist. Second, the *C. elegans* regulatory myosin light chain genes, *mlc-1* and *mlc-2*, have been cloned and sequenced (20), so the reagents for this work were available. Finally, *mlc-1* and *mlc-2* encode nearly identical proteins; it is quite possible that they perform redundant functions in vivo. Therefore, mutations in either gene might not be identified by conventional genetic screens. Indeed, animals homozygous for a deletion of *mlc-1* are essentially wild type (20a).

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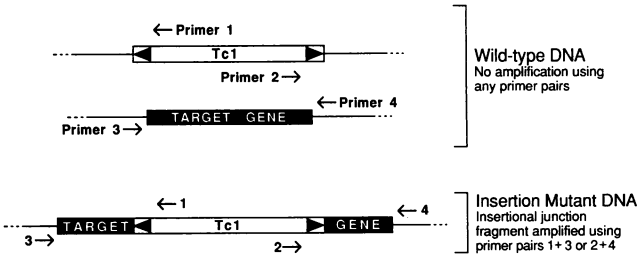

FIG. 1. General approach for detecting transposon insertions.

We describe here the isolation, molecular characterization, and phenotypes of *mlc-2::Tc1* mutants.

MATERIALS AND METHODS

Strains. Conditions for growth and maintenance of *C. elegans* are described by Brenner (12). *C. elegans* MT3126, genotype *mut-2(r459);dpy-19(n1347)*, was constructed by and obtained from M. Finney (32). MT3126 contains the mutator activity of strain TR674, genotype *mut-2(r459) unc-54(r323::*Tc1) (15), crossed 13 times into a Bristol genetic background. The mutator activity of MT3126 maps to the middle of LGI, tightly linked to *unc-13* (32).

Amplification reactions. Polymerase chain reaction (PCR) (70) amplification mixtures (25 μl) contained 0.125 U of *Taq* polymerase (Perkin-Elmer/Cetus), 10 to 100 ng of genomic DNA (extraction procedure is described below), 200 μmol of each deoxynucleoside triphosphate (dNTP), and 40 to 100 pmol of each oligonucleotide primer in a standard PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl [pH 8.3], 0.1% gelatin). Reaction mixtures were overlaid with mineral oil (Sigma) and processed in a Coy Tempcycler. Samples were heated to 93°C for 2 min and processed through 30 cycles of 93°C for 0.5 min, 55°C for 1 min, and 72°C for 3 min, completed by a 10-min incubation at 72°C.

Oligonucleotide primers (20 to 40 nucleotides [nt] long) were synthesized by the University of Wisconsin Biotechnology Center. To screen for insertions in *mlc-2*, we selected primers that anneal outside of the *mlc-2* protein-coding sequences. Tc1 primers were selected from a region of the element internal to the 54-bp terminal inverted repeats (Fig. 1). We used internal primers to avoid amplification of the extrachromosomal closed circular copies of Tc1 (63, 68). Our primer nomenclature is gene name (mlc2 or Tc1), followed by the coordinate of the first 5′ nucleotide (from the published *mlc-2* and Tc1 sequences [20, 64]), followed by primer length in parentheses. The 5′-to-3′ orientation of each primer is shown in Fig. 2A. In Fig. 2A, Tc1 nucleotide coordinate 1 is on the right, and nucleotide 1610 is on the left. Primers used in this study are as follows: A, Tc1-1275 (24 nt); B, Tc1-517 (20 nt); C, Tc1-395 (40 nt); D, mlc2-1360 (21 nt); E, mlc2-1590 (40 nt); F, mlc2-2334 (20 nt); and G, mlc2-2634 (19 nt).

DNA extraction and Southern hybridizations. Procedures for nematode DNA extraction have been previously described (23, 76). DNA samples were electrophoresed through 0.8 to 1.0% agarose gels and alkaline blotted to Nitroplus 2000 (Micron Separation Inc.) as previously described by Southern (75), with only minor modifications. Filters were hybridized with radiolabeled *mlc-2* plasmid TR#126. TR#126 contains a 1.0-kb genomic *Bam*HI-*Eco*RV fragment including most of *mlc-2* (Fig. 2A). Plasmid DNA was radiolabeled by primer extension of random hexanucleotides (30), as suggested by the manufacturer (Pharmacia).

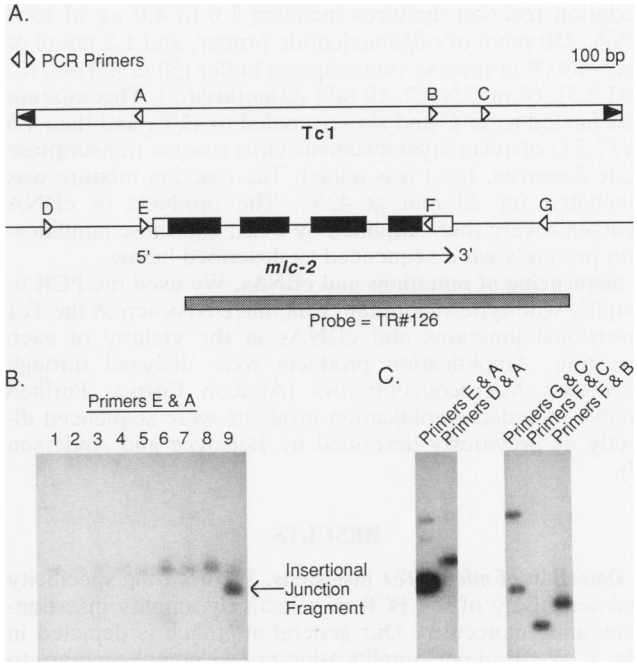

FIG. 2. Detection of transposon insertions. (A) Positions of the PCR amplification primers (A through G) within Tc1 and *mlc-2* (see Materials and Methods for primer nomenclature). Open arrowheads indicate 5′-to-3′ primer orientation; closed arrowheads represent the Tc1 inverted repeat sequences. Shaded boxes of *mlc-2* correspond to protein-coding regions; unshaded boxes correspond to 5′ and 3′ untranslated regions. The arrow below the *mlc-2* genomic region indicates the direction of *mlc-2* transcription. (B and C) Autoradiograms of Southern blots. Amplification products were separated on a 1% agarose gel, transferred to a membrane, and hybridized with radiolabeled *mlc-2* plasmid TR#126. (B) Amplification products from the first cycle of screening for *mlc-2* mutants. DNA from nine independent populations was tested by using primers E and A. Population 9 was found to contain Tc1-induced mutants; a 900-bp product is amplified from *mlc-2::Tc1* molecules (arrowed). (C) Amplification products from population 9, obtained by using a variety of primer pairs. Products of the predicted sizes were amplified with each primer pair. A 1.1-kb fragment was amplified by using nested primers D and A. Opposite-strand primers amplified a 850-bp fragment (primers G and C), a 550-bp fragment (primers F and C), and a 700-bp product (primers F and B).

RNA extractions and Northern (RNA) analysis. We prepared RNA from nematodes by using the procedure described by Ross (66) and modified by B. Saari (cited in reference 20). For Northern transfers, 16 μg of total RNA was denatured with glyoxal and dimethyl sulfoxide and electrophoresed through 1.2% agarose gels in 0.01 M NaH_2PO_4 (pH 7.0) (49). Samples were then transferred to nitrocellulose and hybridized with radiolabeled plasmid TR#115 as described above. TR#115 contains a 5.8-kb genomic *Eco*RV-*Bgl*II fragment including all of *mlc-1* and *mlc-2* (see Fig. 5A). We used a Betagen beta emissions detector (Betascope blot analyzer; Betagen Corp.) to quantify mRNA levels. Average mRNA levels were calculated for each mutant on the basis of four independent measurements of a single RNA preparation.

Reverse transcriptions. First-strand cDNA synthesis was primed with mlc2-2334 (primer F; Fig. 2A). Reverse tran-

scription reaction mixtures included 1.0 to 4.0 μg of total RNA, 250 pmol of oligonucleotide primer, and 1.2 mmol of each dNTP in reverse transcriptase buffer (50 mM Tris-HCl [pH 8.3], 60 mM NaCl, 10 mM dithiothreitol). This mixture was heated to 95°C and slowly cooled to 42°C, and then 4.5 to 17.5 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc.) was added. The reaction mixture was incubated for 30 min at 42°C. The products of cDNA synthesis were then amplified by PCR, and these amplification products were sequenced as described below.

Sequencing of mutations and cDNAs. We used the PCR to amplify wild-type and mutant genomic DNAs across the Tc1 insertional junctions and cDNAs in the vicinity of each insertion. Amplification products were dialyzed through Centricon Microconcentrators (Amicon Corp.). Purified double-stranded amplification products were sequenced directly as previously described by Bejsovec and Anderson (7).

RESULTS

Detection of *mlc-2::Tc1* insertions. We used the specificity and sensitivity of the PCR to selectively amplify insertion-containing molecules. Our general approach is depicted in Fig. 1. We used one amplification primer complementary to a site flanking the target gene (for example, primer 3) in combination with a primer complementary to a site in the transposon (for example, primer 1). Because wild-type DNA molecules contain only one primer-annealing site within the target gene (primer 3), they will not be amplified. If, however, Tc1 inserts into (or near) the target gene, then two primer-annealing sites (primers 1 and 3) are brought close together. Such DNA molecules are substrates for amplification. A detectable amplification product from any DNA sample indicates that insertion molecules are present and therefore that the population of animals from which the DNA was prepared contains insertion mutants.

We tested the specificity and sensitivity of this process in reconstruction experiments using a Tc1-induced allele of *unc-54*, a *C. elegans* myosin heavy chain gene. We mixed small amounts of *unc-54(r323::Tc1)* genomic DNA (22, 24) with an excess of wild-type genomic DNA and tested these mixtures in amplification reactions. Amplification products were electrophoresed, transferred to a membrane, and hybridized with an *unc-54* radiolabeled probe. We detected the expected insertional junction product when *unc-54 (r323::Tc1)* genomic DNA was present at a 10^{-5} or greater fraction of total DNA (data not shown). The sensitivity of detecting *unc-54::Tc1* molecules is probably typical for insertion alleles of most genes. Since we could readily screen 10 to 100 independent populations, we should be able to identify insertions affecting any target gene provided they occur at frequencies in excess of 10^{-6} to 10^{-7}. Although the frequency of Tc1 insertion into different genes varies widely, it is likely that insertions into most genes occur at frequencies greater than 10^{-7} in mutator strains (15).

We used the mutator strain MT3126 (32), genotype *mut-2(r459);dpy-19(n1347)*, to isolate *mlc-2::Tc1* mutants. For our purposes, MT3126 offers two advantages compared with mutator strains that have higher frequencies of transposition, such as strain TR679 (15). First, the Tc1 copy number in MT3126 is substantially lower than in TR679. We estimate that MT3126 contains about 60 to 80 copies of Tc1 (75a). As a result, there are fewer competing Tc1 priming sites, and the background of non-*mlc-2* amplified products is reduced. Second, the frequency of Tc1 transposition is considerably lower in MT3126 than it is in TR679 (13a). Because of the very high frequency of transposition and excision in TR679, large numbers of recessive lethal, semilethal, and sterile mutations accumulate in populations of TR679. Many subpopulations or single animals isolated from TR679 reproduce poorly or not at all. We were repeatedly unable to recover *mlc-2::Tc1* mutants from TR679 populations, although we could detect their presence in large populations. We believe that the high lethality and sterility of TR679 made it difficult to enrich for *mlc-2* mutants by a sib selection procedure. In contrast, MT3126 accumulates many fewer background mutations, and the frequency of Tc1 transposition was adequate for our purposes. We estimate that the frequency of *unc-22* mutations in MT3126 is approximately 2×10^{-4} (13a), which is about 2- to 4-fold higher than in *C. elegans* variety Bergerac but about 10-fold lower than in TR679 (15, 53).

Sib selection of *mlc-2::Tc1* mutants. We established multiple independent populations of MT3126 and screened DNA from those populations for *mlc-2::Tc1* insertional junction molecules by using amplification primers E and A (Fig. 2A). The amplification products were electrophoresed, transferred to a membrane, and hybridized with an *mlc-2* radiolabeled probe. Figure 2B shows a typical result from a screen of nine independent populations of about 8,000 to 10,000 animals each. Population 9 yielded a 900-bp amplification product that hybridized strongly with the *mlc-2* probe (Fig. 2B, lane 9). The remaining populations did not yield strongly hybridizing products, although various weakly hybridizing products were evident. Only when our initial population yielded a strongly hybridizing product could we successfully isolate *mlc-2::Tc1* mutants by using the sib selection protocol (see below). It is possible that the weakly hybridizing products are amplified from somatic, rather than germ line, insertions. The frequency of Tc1 somatic excision is much higher than the frequency of Tc1 germ line excision (22, 25, 26, 52). If this were also true for somatic insertion, then most populations would be expected to contain a heterogenous collection of insertion molecules that cannot be enriched.

To confirm the structure of this putative *mlc-2::Tc1* insert, we tested DNA from population 9 by using a variety of amplification primers. Amplification with nested primers, those designed to amplify the same insertional junction but from different priming sites, yielded fragments of the expected sizes. For example, primers D and A yielded a 1.1-kb fragment (Fig. 2C). Amplification with opposite-strand primers, those designed to amplify the other insertional junction, also yielded fragments of the predicted sizes. For example, primers F and C yielded a 550-bp fragment (Fig. 2C). Although a substantial number of amplification products can hybridize to the *mlc-2* probe, we verified true *mlc-2::Tc1* amplification products by testing DNA samples with several opposite-strand and nested primer pairs before we initiated the sib selection protocol.

A specific example of the sib selection protocol used to isolate *mlc-2(r926::Tc1)* is charted in Fig. 3A. Nine independent populations of MT3126 were established and grown to approximately 8,000 to 10,000 animals. We screened DNA prepared from half of each population and maintained the remainder as living animals. We identified population 9 as containing *mlc-2::Tc1* mutants (Fig. 2B, lane 9) and used the following sib selection protocol to progressively increase the frequency of the *mlc-2(r926::Tc1)* allele in subdivided populations. Population 9 was subdivided into 39 subpopulations, each established with 10 to 100 animals. These subpopulations were grown for one to two generations, yielding about 3,000 animals per population. We prepared DNA from half

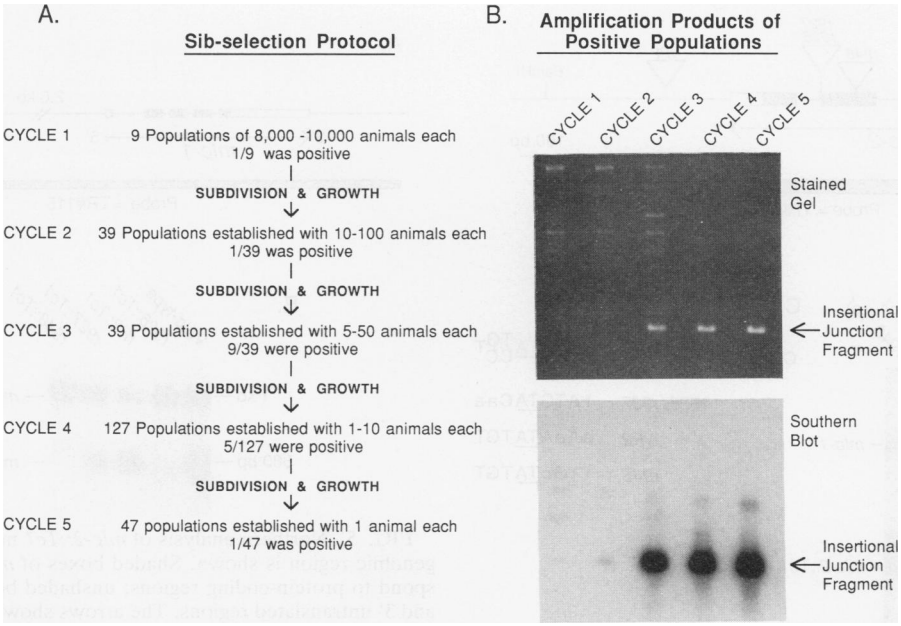

FIG. 3. Isolation of *mlc-2(r926::Tc1)*. (A) Five sequential cycles of subdivision and screening. In the first cycle of screening, one of nine populations was found to contain *mlc-2::Tc1* mutants. In the fifth and final cycle of screening, one population, started with a single animal, contained the same *mlc-2::Tc1* mutants. (B) Autoradiogram of a Southern blot showing the amplification products from positive *mlc-2::Tc1*-containing populations. DNA from positive populations from each cycle of subdivision was amplified by using primers D and A. The products of amplification were separated on a 1% agarose gel and stained with ethidium bromide (upper panel), and filters were hybridized with radiolabeled *mlc-2* plasmid TR#126 (lower panel).

of each population and rescreened these populations for the presence of *r926::Tc1*. One of the 39 subpopulations contained the same mutants. We further subdivided this positive population and repeated the screening process as described in Fig. 3A. At each cycle, we established subpopulations with fewer animals. Therefore, with each cycle of subdivision, the mutants comprised a progressively higher fraction of the total animals. After five cycles of subdivision and rescreening, we identified a positive population that had been established with a single *r926::Tc1* animal. The products of amplification from a positive population at each cycle of sib selection are shown in Fig. 3B. With each cycle of sib selection, the amount of product amplified from *r926::Tc1* molecules increased and the amount of background products decreased. After the second round of screening, Southern blot hybridizations were no longer required to detect positive populations; amplification products were easily detected on ethidium bromide-stained agarose gels.

We screened a total of 102 independent populations of MT3126, each containing 3,000 to 10,000 animals. From these populations, we isolated four homozygous mutants with Tc1 insertions within or near *mlc-2*. Three of the mutants, *mlc-2(r926::Tc1)*, *mlc-2(r927::Tc1)*, and *mlc-2 (r948::Tc1)*, have Tc1 insertions within the *mlc-2* third exon. In the fourth mutant, *rP2::Tc1*, Tc1 is inserted adjacent to *mlc-2*. Further analysis of these *mlc-2* mutants is described below. In addition to the three *mlc-2::Tc1* mutants that we isolated, we detected a number of putative *mlc-2::Tc1* mutants within the populations that we screened. Some of these putative mutants were detected in a first cycle of screening, but they could not be detected in subpopulations. Because other putative mutants had Tc1 insertions at positions identical (or very close) to those that we had previously isolated, they were not pursued. Since we did not isolate the animals corresponding to these putative mutants, we cannot confirm that the products we detected were amplified from genuine *mlc-2::Tc1* insertions. Therefore, a conservative estimate of the frequency of the *mlc-2::Tc1* mutations (based only on the three *mlc-2* mutants we did isolate) is about 9×10^{-6}.

Molecular analysis of insertion alleles. We used Southern blot analysis to confirm the structures of the Tc1 insertions (Fig. 4). Genomic DNA cut with *Bam*HI, which does not cut within Tc1, was electrophoresed, transferred to a membrane, and hybridized with an *mlc-2* radiolabeled probe. We detected a novel *Bam*HI restriction pattern in each mutant; the 1.2-kb wild-type restriction fragment was altered to a 2.8-kb fragment (Fig. 4B). This 1.6-kb increase is consistent with an insertion of the transposable element Tc1.

To determine the precise site of Tc1 insertion in each mutant, we sequenced the DNA across both the 5′ and the 3′ insertional junctions. We amplified genomic DNA across each insertional junction and sequenced the amplification products. At least 30 nt of the termini of Tc1 were sequenced for each insertional junction. The regions of Tc1 that we sequenced are identical to the canonical Tc1 element, pCe (Be)T1 (64). Figure 4A shows the positions of the insertions relative to *mlc-2*. In both *r926* and *r927*, Tc1 is inserted at nt 2157 within the *mlc-2* third exon, but the insertions are in opposite orientations. In *r948*, Tc1 is inserted at nt 2051, also within the *mlc-2* third exon. In *rP2*, Tc1 is inserted at nt 2579, approximately 180 bp 3′ of the *mlc-2* predicted cleavage and polyadenylation signal. As shown in Fig. 4C, sequences surrounding each *mlc-2::Tc1* insertion are similar to the Tc1 insertion site consensus sequence (24, 55).

To establish that the *mlc-2::Tc1* mutants are loss-of-function alleles, we compared *mlc-2* mRNA levels in wild-type and mutant strains by Northern blot analysis. Total RNA was electrophoresed, transferred to a membrane, and

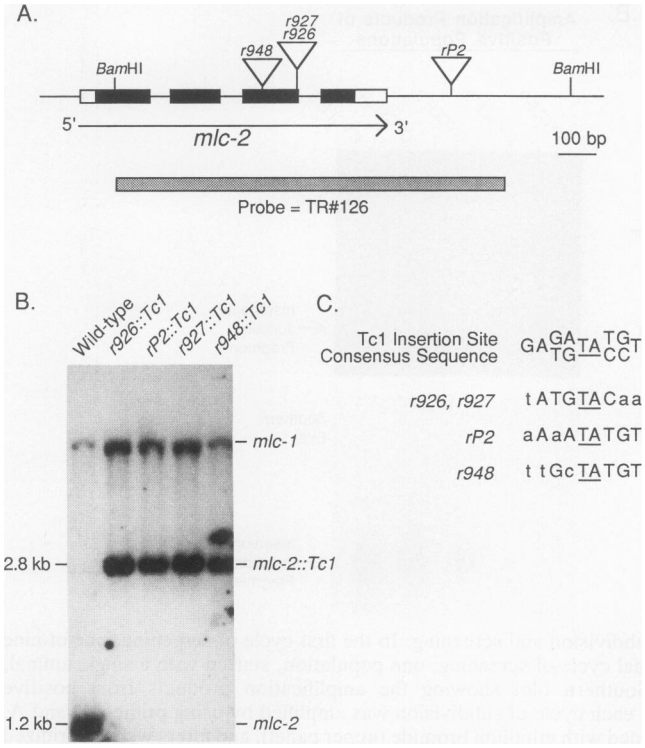

FIG. 4. (A) Tc1 insertion sites. Shaded boxes of *mlc-2* correspond to protein-coding regions; unshaded boxes correspond to 5′ and 3′ untranslated regions. The arrow indicates the direction of *mlc-2* transcription. For each mutant, the site of Tc1 insertion is indicated. (B) Autoradiogram of a Southern blot showing Tc1-induced alterations in *mlc-2::Tc1* mutants. Genomic DNA was cut with *Bam*HI, separated on a 0.7% agarose gel, transferred to a membrane, and hybridized with radiolabeled plasmid TR#126. Because of the homology between *mlc-1* and *mlc-2*, plasmid TR#126 detects both *mlc-1* and *mlc-2* genomic sequences. (C) Sequences of Tc1 insertion sites within *mlc-2*. The TA insertion dinucleotide is underlined. Uppercase letters correspond to matches to the Tc1 insertion site consensus sequence (24, 55).

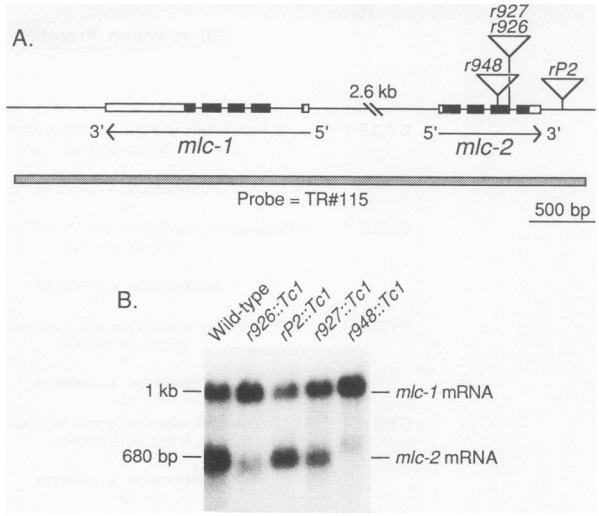

FIG. 5. Northern analysis of *mlc-2::Tc1* mutants. (A) The *mlc-2* genomic region is shown. Shaded boxes of *mlc-1* and *mlc-2* correspond to protein-coding regions; unshaded boxes correspond to 5′ and 3′ untranslated regions. The arrows show the direction of *mlc-1* and *mlc-2* transcription. Tc1 insertion sites are indicated. (B) Autoradiogram of a Northern blot. Total RNA was separated on a 1.2% agarose gel, transferred to a membrane, and hybridized with radiolabeled plasmid TR#115.

hybridized with a radiolabeled probe that detects both *mlc-1* and *mlc-2* transcripts. We measured *mlc-2* message levels relative to control *mlc-1* mRNA levels. Surprisingly, all three *mlc-2::Tc1* mutants accumulate *mlc-2* mRNA of approximately normal size, although the abundance of these mRNAs is reduced relative to the wild-type level (Fig. 5B). We estimate that *r926::Tc1* has 17 ± 7%, *r927::Tc1* has 43 ± 9%, *r948::Tc1* has 10 ± 11%, and *rP2::Tc1* has 91 ± 24% of the wild-type amount of *mlc-2* mRNA (n = 4 for all strains). We did not detect the presence of mRNAs containing the entirety of Tc1 inserted within *mlc-2*, as has been observed for a number of Tc1-induced alleles of the *C. elegans unc-22* gene (51).

To determine how *mlc-2::Tc1* mutants could produce mRNAs of approximately normal size, we sequenced mutant and wild-type cDNAs in the region of the insertions. cDNAs were reverse transcribed from total RNA and amplified by PCR, and the amplification products were sequenced directly (without cloning). *r926::Tc1* and *r927::Tc1* have identical cDNA sequences. In these mutants, Tc1 is inserted 6 nt upstream of the *mlc-2* third intron. In both mutants, the normal 5′ splice site of the *mlc-2* third intron is not used. Instead, a cryptic 5′ splice donor (nt 2145) 18 nt upstream of the wild-type 5′ splice donor is spliced to the normal 3′ splice acceptor of the third intron (Fig. 6). Utilization of these splice sites removes all of Tc1 plus 18 nt of *mlc-2* from the mature mRNA. The resulting mRNA is translatable in frame to yield a *mlc-2* protein deleted for six amino acids. The cryptic 5′ splice donor utilized in *r926* and *r927* is not used (at detectable levels) in wild-type animals. We sequenced the wild-type cDNA in this region and confirmed the previously predicted *mlc-2* splice sites (20). In *r948::Tc1*, Tc1 is inserted in the middle of the *mlc-2* third exon. In this mutant, a 5′ splice donor within the left inverted repeat of Tc1 (nt 1573 of the canonical Tc1 sequence) is spliced to a 3′ acceptor within the right inverted repeat of Tc1 (nt 10) (Fig. 6). Utilization of these splice sites removes most of Tc1 but leaves 46 nt of the transposon plus the TA target site duplication (total of 48 nt) inserted in the mature *mlc-2* mRNA. This 48-nt insertion contains no stop codons. The resulting mRNA, therefore, is

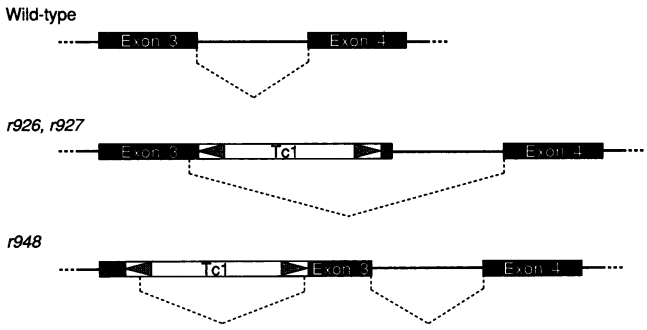

FIG. 6. Utilization of cryptic splice sites in *mlc-2::Tc1* mutants. *mlc-2* and Tc1 are not drawn to scale. Exons 3 and 4 of the *mlc-2* genomic region are shown. Shaded boxes correspond to exons; thin lines correspond to introns. Dashed lines connect the major 5′ splice donor and 3′ splice acceptor site used in wild-type and mutant mRNAs.

translatable in frame to yield an *mlc-2* protein with an insertion of 16 amino acids. The 3′ acceptor site used within the right inverted repeat of Tc1 in *r948::Tc1* is unusual. The terminal dinucleotide of the Tc1 intron is GG rather than the canonical AG of *C. elegans* (9, 31) or other eukaryotic introns (57, 58). Because Tc1 transposons are polymorphic, we confirmed that the Tc1 element of *r948::Tc1* contains GG at this position by sequencing *r948::Tc1* genomic DNA in this region.

Phenotypes of mutants. To eliminate unlinked mutations, we backcrossed *r926*, *r927*, *r948*, and *rP2* four times with a wild-type Bristol strain and resegregated the homozygotes. The phenotypes of all three *mlc-2::Tc1* mutants are very nearly wild type and indistinguishable from each other. Behavioral phenotypes requiring muscle function (motility, pharyngeal pumping, male mating, and hermaphrodite egg laying) are normal. The ultrastructures of the body wall, pharyngeal, and sex muscles (both male and hermaphrodite) are normal, as judged by polarized light microscopy. The mutants are not temperature sensitive or cold sensitive for growth. The brood sizes of the *mlc-2::Tc1* mutants are slightly smaller than for the wild-type strain N2, but *rP2* broods are similarly reduced. Therefore, this difference probably does not result from the mutations within *mlc-2*. We have detected only one difference between *mlc-2::Tc1* mutants and the wild-type strain N2. The development of *mlc-2::Tc1* mutants is delayed. We measured the length of postembryonic development (defined as the time from hatching until the onset of egg laying) in wild-type and mutant animals. The lengths of postembryonic development, at 20 to 22°C, are 73 ± 1.0 h for the wild-type strain N2, 73 ± 0.3 h for *rP2*, 85 ± 6.7 h for *r926*, 89 ± 1.4 h for *r927*, and 85 ± 5.6 h for *r948* (n = 4 for all strains). Thus, the *mlc-2::Tc1* mutants *r926*, *r927*, and *r948* require about 12 to 16 h more than wild-type or *rP2* animals require to develop from newly hatched larvae to egg-laying adults.

Detection of *hlh-1::Tc1* insertions. To demonstrate the generality of our procedure, we isolated Tc1-induced mutations of *hlh-1*, the *C. elegans* homolog of the vertebrate MyoD gene family (CeMyoD) (43). We successfully isolated two *hlh-1::Tc1* mutants. One insertion, *r955::Tc1*, is within the *hlh-1* first intron; the other insertion, *r1010::Tc1*, is within the *hlh-1* second exon. The motility of both *r955* and *r1010* homozygotes is essentially wild type, indicating that these mutants have little, if any, body wall muscle abnormalities. Although we have not determined whether *r955* and *r1010* contain *hlh-1* mRNA, muscle nuclei of both mutants immunostain with the anti-CeMyoD polyclonal antibody HM-1 (13, 43; data not shown). Thus, each of these mutants expresses detectable CeMyoD protein. We suspect that, as with the *mlc-2::Tc1* pre-mRNAs, all or part of Tc1 is spliced from some fraction of the *hlh-1::Tc1* pre-mRNAs yielding translatable mRNA.

DISCUSSION

We describe a general method for isolating Tc1-induced mutants of sequenced *C. elegans* genes. We used this procedure to isolate three Tc1-induced mutants of the regulatory myosin light chain gene *mlc-2*. One of our goals was to establish the *mlc-2* loss-of-function phenotype. We wanted to know whether the in vivo functions of *mlc-2* are redundant, as they appear to be for *mlc-1*. Animals homozygous for a deletion of *mlc-1* are essentially wild type (20a). *mlc-1* and *mlc-2* are the only muscle regulatory light chain genes that are detectable in the *C. elegans* genome (20). Although their predicted protein products are essentially identical, *mlc-1* and *mlc-2* have some striking dissimilarities outside of their protein-coding regions. For example, *mlc-2* is *trans* spliced, whereas *mlc-1* is not (20). The definitive test of redundancy is to eliminate *mlc-1* and *mlc-2* individually and in combination. Unfortunately, *mlc-1* and *mlc-2* are very close together (separated by only 2.6 kb); the recombination events that might generate an *mlc-1 mlc-2* double mutant would be extremely rare. Therefore, we cannot easily construct the double mutant by a traditional crossover.

All three of our *mlc-2::Tc1* mutants are essentially wild type, suggesting that *mlc-2* functions, like those of *mlc-1*, are redundant. Our conclusions, however, must remain somewhat tentative. Although three of these mutants have Tc1 insertions within *mlc-2* exons, these may not be null alleles. For all three mutants, a fraction of the *mlc-2::Tc1* pre-mRNA is spliced to remove all or most of Tc1 from the mature message. In all cases, the spliced products maintain the *mlc-2* translational reading frame. We assume that these mRNAs are translated, yielding myosin light chain proteins that have either a 6-amino-acid deletion or a 16-amino-acid insertion. We do not know a priori whether such mutant light chain proteins are functional in vivo. The mutant light chains may be nonfunctional or unstable in vivo, in which case these mutations are, in fact, loss-of-function alleles. Alternatively, the mutant light chain proteins may be partially or completely functional. Thus, we cannot unambiguously interpret the wild-type phenotype of these mutants. We must derive unequivocal null mutations to be certain of the *mlc-2* loss-of-function phenotype.

It is unlikely that the mutant *mlc-2* mRNAs that we characterized are transcribed from Tc1 somatic excision DNA or that they are PCR-generated artifacts. The *mlc-2::Tc1* mutants were backcrossed four times to a Bristol strain prior to analysis; Tc1 somatic excision is substantially lower in Bristol strains than in Bergerac mutator strains. By Southern blot analysis, we did not detect somatic excision DNA in any of the mutant strains (Fig. 4). By Northern blot analysis, however, *mlc-2* mRNA is present in all of the mutants (Fig. 5). Therefore, it is unlikely that the *mlc-2* mRNAs that we detect are transcribed from somatic excision DNA. In addition, these mutant *mlc-2* mRNAs do not appear to be in vitro artifacts generated by PCR. The relative size differences of the mutant mRNAs, as predicted by PCR analysis, are also evident by Northern blot analysis (Fig. 5). *r926* and *r927* mRNAs are slightly smaller than wild-type *mlc-2*, while *r948* mRNA is slightly larger than wild-type *mlc-2*.

Splicing to remove transposons from pre-mRNA has been reported for a number of transposon-induced alleles in maize and *Drosophila melanogaster* (for examples, see references 21, 33, 40, 61, 62, 73, and 82). In these organisms, as in *C. elegans*, a variety of cryptic splice sites can be used within either the target gene or the transposon. Multiple splice sites are often activated, yielding several different mRNA products from a single transposon-containing pre-mRNA (33, 62, 82). In our analysis, we sequenced the PCR amplification products directly (without cloning). Therefore, we characterized only the most abundant spliced product of each pre-mRNA. We would not have detected other, low-abundance mRNAs spliced from alternative sites within *mlc-2::Tc1* pre-mRNAs. The 3′ acceptor splice site used within Tc1 in *mlc-2(r948::Tc1)* is quite unusual. The terminal dinucleotide of this novel intron is GG rather than the canonical AG typical of most eukaryotic splice acceptors (9, 31, 57, 58).

Why is the abundance of *mlc-2::Tc1* mRNAs reduced? In

many organisms, mRNAs that contain nonsense mutations and are not translated throughout their entire length are unstable (for examples, see references 6, 41, 47, 48, 50, and 80). In *C. elegans*, mRNAs containing premature termination codons are also unstable (61a). If a variety of different splice sites are used to remove Tc1 from these mutant *mlc-2::Tc1* pre-mRNAs, then mRNAs spliced in a manner that leaves nonsense or frameshift mutations within the message should be unstable. The small amounts of in-frame *mlc-2* mRNAs that are present in *mlc-2::Tc1* mutants are presumably stable because they are translated throughout their entire length. Alternatively, it is possible that Tc1 affects *mlc-2* transcription in these mutants. Transposons in a number of organisms are known to affect the transcription of target or adjacent genes (for examples, see references 16, 19, 44, 59, 67, and 83).

It is possible that many Tc1 insertions within genes do not cause functional defects. Most previously analyzed Tc1-induced mutations were identified because they do cause functional defects. The procedure that we developed identifies Tc1-induced mutations on the basis of their structural properties, regardless of mutant phenotype. As seen for *mlc-2*, splicing of Tc1-containing transcripts may often yield partially or fully functional protein products. If this is true, it may be difficult to isolate loss-of-function mutations by using this procedure alone. However, a two-step site-directed mutagenesis procedure could be used to isolate such mutants. First, a Tc1-induced mutant could be isolated by using the method that we describe here. Then, an altered transgene could be reintroduced at the site of Tc1 insertion via DNA gap repair following Tc1 excision (5, 29, 60).

In theory, this sib selection method can be used to isolate mutations in almost any cloned *C. elegans* gene. In addition to the *mlc-2* and *hlh-1* mutants that we isolated, Tc1-induced mutations affecting *gpa-1* and *gpa-2* (85) and *pgp-3* (12a) have been isolated by using this method. Although Tc1-induced mutations of a number of genes have been isolated, mutations of certain genes may be difficult to obtain. Tc1 insertion is site specific (24, 55, 65), and some genes may lack the required Tc1 insertion sites. However, *C. elegans* has at least four additional families of transposable elements that actively transpose in the germ line (Tc2, Tc3, Tc4, and Tc5; 13a, 14, 45, 84). This procedure could detect mutations caused by the insertion of any of these elements. Populations could be screened with a variety of transposon-specific primers to increase the chances of isolating a mutant.

Clearly, this technique is not limited to *C. elegans*. In *D. melanogaster*, similar procedures have been used to isolate P-element insertions near a gene expressed in the eye (4) and within the singed (*sn*) locus (36). Our results indicate that the technique is not limited to *D. melanogaster* but is also applicable to *C. elegans* and, presumably, to many other organisms. Mutations in cloned genes could be isolated in any organism in which known transposable elements are active and in which large numbers of independent populations of animals or cells can be easily manipulated.

The *mlc-2* and *hlh-1* mutations that we isolated are homozygous viable and fertile, but our method should be effective for isolating mutations in essential genes as well. Tc1 insertions that are lethal when homozygous can be enriched for as heterozygotes during the sib selection protocol. Although each generation of self-fertilization results in a 2-fold reduction in the allele frequency of a recessive lethal mutation, each cycle of sib selection results a 10- to 100-fold enrichment of the mutant allele. Sib selection should more than compensate for homozygous lethality. For mutations that result in a dominant or semidominant decrease in fecundity, enrichment of the mutant allele through sib selection will be modest but still possible. This method should be especially useful for isolating mutations in genes whose functions are redundant or for which the loss-of-function phenotype is wild type. Indeed, *mlc-2* may be such a gene.

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