Alteration of *Caenorhabditis elegans* gene expression by targeted transformation

**(gene targeting/homologous recombination/promoter function/transgenic/nematode)**

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**ABSTRACT** We have produced strains carrying a synthetic fusion of parts of two vitellogenin genes, vit-2 and vit-6, integrated into the *Caenorhabditis elegans* genome. In most of the 63 transformant strains, the plasmid sequences are integrated at random locations in the genome. However, in two strains the transgene integrated by homologous recombination into the endogenous *vit-2* gene. In both cases the reciprocal exchange between the chromosome and the injected circular plasmid containing a promoter deletion led to switching of the plasmid-borne promoter and the endogenous promoter, with a reduction in *vit-2* expression. Thus in nematodes, transforming DNA can integrate by homologous recombination to result in partial inactivation of the chromosomal locus. The simplicity of the event and its reasonably high frequency suggest that gene targeting by homologous recombination should be considered as a method for directed inactivation of *C. elegans* genes.

Homologous recombination between chromosomal and introduced DNA provides a powerful technique for altering the genome to study the function of genes not accessible by classical genetic analysis. This method has been used widely in budding yeast (1) and has also been reported in several other systems (2–7), including mammalian cells, where the frequency is low, but strong selective pressures can be applied. After selection, tissue culture cells containing homologously integrated constructs can be introduced into embryos, producing chimaeras carrying the modified cells, to produce strains with altered germ lines (8–11).

Germ-line transformation is an established procedure in *Caenorhabditis elegans*. Stinchcomb et al. (12) developed DNA-mediated transformation by injecting plasmid DNA into the cytoplasm of the syncytial portion of the distal gonad. The transformant progeny always carried the injected DNA in extrachromosomal high-copy-number tandem arrays. Expression from these arrays was originally reported to be incorrectly regulated (13), but correct regulation has been seen from such arrays more recently (14, 15). Fire (16) developed integrative transformation by injecting plasmids containing a suppressor tRNA gene, *sup-7*, into meiotic oocyte nuclei, rather than into the distal arm of the gonad where mitosis occurs. The presence of *sup-7* provides both a selection for transformants and a selection against large arrays since high copy number is deleterious. Integrated genes segregate in a Mendelian manner and are correctly regulated but subject to some position effects (16–19).

Our laboratory has been studying the regulation of *vit-2* expression by using transgenic nematodes. The *vit* genes of *C. elegans* form a gene family of six members, four of which are known to be functional (20; unpublished data for *vit-3*). We have used a plasmid containing a synthetic gene, *vit-2/6*, with the promoter and 5' terminus of *vit-2* and the 3' coding and untranslated regions of *vit-6* (18). The *vit-2* gene is *X* chromosome-linked while *vit-6* is autosomal (21). The plasmid thus contains homology to two chromosomes, in addition to the *sup-7* gene used for selection of transformants. In the course of our studies, we created numerous deletion and point mutations in this plasmid and selected more than 60 low-copy-number integrated transformants (19). Most of these resulted from random integration of the plasmid at nonhomologous sites. We present evidence here that in two strains homologous integration of a single copy of the plasmid occurred within *vit-2*, effecting an exchange of promoters between the endogenous and introduced genes.

**MATERIALS AND METHODS**

**Growth of *C. elegans*.** *C. elegans* strains were grown on NGM plates or synchronously in liquid S medium (22–24). For RNase protection experiments, worms were harvested just after the adult molt.

**Strain Construction and Genetic Mapping.** Transgenic strains were derived by germ-line transformation of *tra-3*(*e1107*) mutant worms. The *vit-2/6* fusion gene was cloned into a pUC-derived plasmid containing the *sup-7* gene (Fig. 1), and transformants were selected on the basis of suppression of *tra-3* (13). The pJ142 and pJ174 plasmids were as described (19). Linkage mapping of BL306 was by standard techniques (22), scoring both *sup-7* activity and expression of *vit-2/6* gene protein (p155) on SDS gels. Markers used were *lon-2*(*e678*); *dpy-5*(*e61*); *unc-4*(*e120*); *dpy-1*(*e1*); *lon-1*(*e185*); *dpy-13*(*e12*); and *lon-3*(*e2175*).

**Southern Blot Analysis.** Genomic DNA (25) was digested with restriction enzymes for 3–4 hr prior to electrophoresis on 0.9% agarose gels for 670 V-hr. DNA was blotted onto Hybond-N (Amersham) and hybridized to a PCR-amplified fragment labeled with *32*PdCTP by using a Boehringer Mannheim random-primed DNA labeling kit (19).

**RNase Protection.** Adult worm RNA was purified by disruption in 6 M guanidinium hydrochloride, ethanol precipitation, resuspension in 7 M urea, and repeated phenol/chloroform extractions (18, 26). A 354-bp *Hinc*II fragment spanning the site of the *vit-2–vit-6* fusion was subcloned into Bluescribe, downstream of the *t7* promoter to allow production of the RNA probe used for RNase protection. Hybridization was overnight at 37°C, and RNase digestion was for 1 hr at 30°C (27). Fragments were separated on 5% polyacrylamide/7 M urea gels.

**Electrophoretic Analysis of Proteins.** Worms were boiled in SDS sample buffer (28). Gel electrophoresis was by the method of Laemmli (28) with the modification described by Sharrock (29) and a further 2-fold reduction in *N*,*N*'-methylenebisacrylamide concentration to resolve ypl70a from ypl70b.

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In Situ Hybridization. Cut-worm samples were treated by a modification of Schedin et al. (30) as described in MacMorris and Blumenthal (31). An RNA probe specific for vit-2 (called VU) was prepared by T7 RNA polymerase transcription (32) using 35S-labeled UTP (>1000 Ci/mmol; 1 Ci = 37 GBq; Amersham). A 0.9-kb EcoRI fragment of vit-2 cDNA containing the unique 3' untranslated region was cloned into pTZ18U so that T7 transcription, after digestion with Sau3AI, would produce a transcript complementary to the final 71 nt of vit-2.

RESULTS

Discovery of the Homologous Integrants. Initially we determined that 247 bp of the vit-2 promoter was sufficient to produce vit-2/6 fusion gene RNA and protein at nearly the level of the endogenous vit-2 gene products when the plasmid was integrated at random locations (19, 33). We then created a deletion to position -174 that resulted in a 2-fold reduction in transgene product and a deletion to position -142 that reduced expression 5- to 10-fold (19, 33). We found, however, that one strain carrying the position -142 deletion and one carrying the position -174 deletion exhibited anomalously high expression of the transgene and concomitant reduction of vit-2 (see below). This phenotype could be produced by integration of a single copy of the plasmid into the vit-2 sequence, subjecting vit-2 to the control of the introduced promoter and the transgene to vit-2 promoter control (diagrammed in Fig. 1A). To determine whether such an event had occurred, we performed genomic Southern blot analysis and crude genetic mapping of the integrated construct.

Nature of the Homologous Integration Events. The Southern blot results indicate that the integration events are due to single crossovers between the circular plasmid and the chromosomal vit-2 gene. Kpn I and Kpn I/Xba I digests probed with the 5' end of vit-2 are diagnostic of this insertion. If a crossover occurred within the region of identity between vit-2 and the transgene, the endogenous vit-2 Kpn I fragment of 22.1 kb should be replaced by two bands of 6.5 and 23.8 kb (Fig. 1A). Furthermore, an 8.3-kb plasmid-sized band would be expected only if tandem plasmid copies were integrated. Fig. 1B shows a Southern blot of genomic DNA from the two integrant strains, BL300 (containing pJ174) and BL422 (containing pJ142), and from N2, an untransformed control. Both putative homologous integrants have the expected 6.5-kb Kpn I fragment. (The gel does not resolve the 23.8- and 22.1-kb positions.) When the Kpn I-digested DNA was further digested with Xba I, the 22.1-kb vit-2 fragment was reduced to a 9.8-kb Xba I–Kpn I fragment in N2. Xba I/Kpn I digests of BL300 and BL422 lack this 9.8-kb fragment. It is replaced by two fragments, the 6.5-kb Kpn I–Kpn I fragment and a novel 7.3-kb Xba I–Xba I fragment, as predicted in Fig. 1A. The predictions were fulfilled for both strains, indicating that homologous integration was apparently mediated by a simple recombination event between the chromosome and a single plasmid circle to generate a tandem arrangement of vit-2 and the vit-2/6 gene in each case.

Partial Genetic Mapping of the Integration Site. If the plasmids integrated into the vit-2 gene, they would be located on the X chromosome within 1–2 map units from lon-2 (34). Although vit-2 has not been genetically mapped, physical mapping results placed TCMEC2 (a Tcl element mapping close to mec-2) and vit-2 on nearby cosmid (ref. 35; A. Coulson and J. Sulston, personal communication), and mec-2 is 1 map unit from lon-2. We mapped the integrated construct in BL300. In segregants from a lon-2/sup-7 vit-2/6 heterozygote derived from BL300, one of 20 Lon hermaphrodites also carried the sup-7 vit-2/6 construct, and all 18 Lon+ individuals expressed the vit-2/6 gene product fp155. From these results and independent segregation of the transgenes with respect to several autosomal markers (dpy-1 III and unc-4 II), we conclude that the integrated construct is X chromosome-linked, ~2.5 map units from lon-2, consistent with a position near mec-2. In addition, the other strain, BL422, was used to construct double mutants with morphological markers. In those crosses the transgene segregated independently of dpy-5 I, lon-1 III, dpy-13 IV, and lon-3 V and as an X
Homologous Integration Leads to a Switch of Plasmid and Chromosomal Promoters, Resulting in a Depression of Endogenous vit Activity. A single crossover within the region of homology between the plasmid and the chromosome would lead to integration of the plasmid and switching of the endogenous and introduced promoters (Fig. 1A). Since the endogenous promoter and the truncated plasmid promoter differ in their activities (19), the homologous integrant strains should produce less vit-2 gene product and more vit-2/6 gene product compared to a strain in which the vit-2/6 plasmid had integrated randomly. We measured the protein products produced by both strains and analyzed RNA levels by RNase protection in BL300 and by in situ hybridization in BL422. Since the same RNA and protein species (synthesized from different promoters) were being compared in each case, we assumed that the stability of the products from the two promoters would not vary.

Fig. 2 shows total worm protein displayed on an SDS gel. The yolk proteins (yp170a, yp170b, yp115, and yp88) and the product of the transgene (fp155) are the predominant proteins. In lanes 1 and 4, containing protein from wild-type (N2) and the single-copy, nonhomologous-integrant strain (BL203), one of the most abundant proteins is yp170b, the vit-2 gene product. In contrast, the lanes with BL300 and BL422 exhibit abnormally low levels of yp170b compared with the other yolk proteins. Furthermore, these strains show high levels of fp155, similar to the level in BL203, a strain with an unmodified 247-bp promoter controlling the fusion gene product. This result is consistent with fp155 production by the vit-2 promoter and yp170b production by truncated less-active promoters.

RNase protection analysis was used to measure RNA levels in the pJ174-containing strain BL300. An RNA probe complementary to vit-2, vit-2/6, and vit-6 RNAs (Fig. 3A) was hybridized to total RNA to assess the relative levels of the different RNAs. The 405-bp RNA probe was made from a genomic DNA clone and spanned an intron in vit-6, giving protected fragments of 62 nt for vit-2, 116 and 190 nt for vit-2/6, and 39 and 190 nt for vit-6. The unique bands of 116 and 62 nt were analyzed to compare the levels of vit-2/6 and vit-2 mRNAs. The level of the 39-nt vit-6 gene product was not affected by the transgenic experiments and was used to roughly estimate total RNA levels for each lane. Fig. 3B shows an analysis of BL300, N2, and two strains in which plasmids were integrated at random locations. The first two lanes of Fig. 3B have roughly equivalent levels of the 39-nt vit-6 gene product. Therefore, from Fig. 3B, we can infer that chromosome-linked hemizygous marker (data not shown), also consistent with a location at vit-2.
BL300 has elevated levels of vit-2/6 RNA (116 nt) and depressed levels of vit-2 RNA (62 nt) compared to BL203 (a strain containing an unmodified 247-bp vit-2 promoter), as expected if a promoter switch had occurred in the creation of strain BL300. The third lane contains a strain with a partially debilitated promoter, randomly integrated, that demonstrates a reduced level of the 116-nt vit-2/6 fragment associated with lowered promoter function. Note that the ratio of vit-2 to vit-6 product in this lane is similar to the N2 control but very different from BL300. In the fourth lane, the wild-type control (containing less total RNA than the other lanes) displays only the fragments associated with the endogenous vit-2 and vit-6 mRNAs. Previous results suggested that the 247-bp promoter in BL203 is somewhat less active than the endogenous chromosomal vit-2 promoter and that the 174-bp promoter function is further reduced (19). The results shown in Fig. 3B are consistent with these observations since the level of the 116-nt vit-2/6 product is lower when under control of a 247-bp promoter (BL203) than when under control of the endogenous vit-2 promoter (BL300). In addition, the 62-nt vit-2 product produced by the 174-bp promoter (BL300) is less abundant than the wild-type vit-2 product in all of the other strains shown.

We previously reported in situ hybridization with a probe (called PL) complementary to the internal polylinker of the vit-2/6 RNA. In transgenic strains with vit-2/6 driven by the 247-bp promoter, the RNA was present in all intestinal cells (31). However, with promoter modifications resulting in slight reductions in expression, RNA was localized only in the middle and posterior regions of the intestine, while more severe mutations resulted in labeling only of the midintestine (31). If the proposed promoter switch had occurred in strain BL422, which has the 142-bp promoter, we would predict an uneven distribution of the vit-2 product, rather than the fusion gene product. We detected expression of the fusion gene with the PL probe and the vit-2 gene with the UV probe, specific for its 3' untranslated region. The PL probe was uniformly distributed in each of 14 BL422 worms scored (e.g., Fig. 4 A and B), whereas the UV probe showed patterns of distribution characteristic of mutated promoters (31), as predicted by the promoter-switch hypothesis. Of 78 BL422 intestinal samples tested with the UV probe, the anterior cells failed to bind probe in 57% (Fig. 4 C and D), the RNA signal was confined to the midintestinal cells in 38% (Fig. 4 E and F), and 5% lacked signal entirely. This experiment provides additional support for the conclusion that the promoters are indeed switched.

**FIG. 4. In situ hybridization to BL422 intestines.** 4',6-Diamidino-2-phenylindole-stained epifluorescence images (A, C, and E) are paired with simultaneous dark-field and epifluorescence images of hybridized RNA (B, D, and F). (B) The polylinker-specific probe (PL) that detects the vit-2/6 gene product. (D and F) The VU probe specific to the vit-2 3' untranslated region. Large arrows point to sets of four nuclei at the anterior ends of the intestines. Small arrowheads indicate remnants of the pharynx where it is still attached to the intestines. (×70.)

**DISCUSSION**

Homologous Integration in *C. elegans*. Several lines of evidence support the conclusion that, in two strains, BL300 and BL422, the injected plasmid integrated within the vit-2 gene by means of a single reciprocal crossover between a circular plasmid and the homologous region of the X chromosome. (i) Genomic Southern blot analysis demonstrated that the endogenous vit-2 bands were lost, and novel bands predicted by the hypothesis were present. (ii) In BL300, both the suppressor and the vit-2/6 transgene mapped to the appropriate location on the X chromosome. (iii) The vit-2/6 fusion gene is under control of the vit-2 promoter, while vit-2 is controlled by the mutant promoter in these strains. The protein product of vit-2 accumulated to abnormally low levels, whereas the fusion protein was made in abundance, as would be expected from the promoter-exchange hypothesis. Similarly, the RNA product of the fusion gene was expressed abundantly, as judged both by RNAse protection and in situ hybridization, whereas RNA expression from vit-2 was severely depressed. Thus, we believe these results convincingly demonstrate that these two transformant strains were formed by homologous integration of the plasmid into vit-2.

Prospects for Directed Gene Inactivation by Homologous Integration. In several organisms including *Escherichia coli*, yeast, and mouse, homologous integration has proven to be the most practical method for inactivation of cloned genes whose mutant phenotypes are otherwise unknown. In *C. elegans*, although numerous cases of transformation have been reported, until now none has been reported to occur by homologous recombination between the introduced DNA and the chromosome. It should be noted that most transformation schemes currently in use produce large extrachromosomal arrays of injected DNA that do not recombine with the chromosomes (36). The procedure we used (16) has two important differences from the most commonly used one: (i) injections were in regions of the gonad that are active in meiosis, and (ii) an amber suppressor gene, *sup-7*, was present on the injected plasmid. Because high levels of the tRNA product of *sup-7* are toxic, high-copy transfectants containing *sup-7* are selected against. Only integrated low-copy-number transfectants are viable. Previous studies of *sup-7*-containing integrated transfectants reported on a total of only about 30 strains (16–18). Thus, it is not surprising that no homologous integrants were obtained. In our study, 2 of 63 integration events, or 3%, have been shown to have occurred by homologous recombination. This represents a reasonable frequency of such spontaneous events and suggests that injection of plasmids containing mutant DNA and *sup-7* into meiotic nuclei should be considered as a method of gene inactivation in *C. elegans*. Furthermore, the frequency of homologous events might be increased by generating free ends in the plasmid DNA or by x-irradiation of injected animals, two methods that have been shown to increase recombination frequencies in other systems (37).
Comparison with Homologous Integration in Other Organisms. If our results are representative of the homologous integration frequency in *C. elegans*, then this organism can sustain an unusually high percentage of homologous events that is significantly greater than the frequency observed in mammalian cells (38, 39). The integrants selected in *C. elegans* also differ from those selected in most tissue culture analyses in that they involved recombination between a circular plasmid and a chromosome. Such exchange events might be more frequent in our analyses due to the method by which DNA was introduced. Supercoiled plasmid DNA was injected into the meiotic region of the gonad (16). Thus the DNA was present in recombinationally competent cells that were creating and resolving chiasmatas. Homologous recombination events in tissue culture cells, however, reflect the biology of mitotically dividing cells. Thomas et al. (39) recovered targeting events involving either gene conversion or double crossovers, but not single reciprocal exchanges, and hypothesized that this might reflect the predominant recombination machinery present in mammalian cells. They also never observed homologous recombination with supercoiled DNA, perhaps because the absence of free DNA ends would not be expected to signal recombination-repair functions. Interestingly, recent experiments by Mello et al. (36) indicate that coinciding large amounts of single-stranded oligonucleotide with supercoiled plasmid into the synecytial region of cytoplasm in the *C. elegans* gonad nonspecifically stimulates integration of plasmid arrays, perhaps by providing single-stranded DNA ends that stimulate recombination-repair processes. However, none of those integrants was homologous.

**Location of Homologous Events.** The *vit-2/6* plasmid has sequence identity to two loci on different chromosomes, *vit-2* on the X chromosome and *vit-6* on chromosome IV (21). In both cases, recombination occurred between the plasmid and *vit-2*, not *vit-6*. To examine the possibility that *vit-6* homologous integrants had been produced but were not recognized, since these would not result in an effective promoter switch, we examined genomic Southern blot data for the remaining 61 strains, but no evidence for *vit-6* integration was obtained (data not shown). Of course with only two events, it is possible that the fact that both homologous integration events occurred within *vit-2* was just due to chance. However, because the *vit-2/6* fusion gene shares nearly 4 kb of identity with the X chromosome, but only 1.4 kb of identity with chromosome IV, integration within *vit-2* is more probable, since target size is a critical variable in determining the frequency of homologous events (40). Additionally, recombination with the X chromosome might be regulated differently from recombination on other chromosomes (41). Thus, it is possible that homologous recombinants involving the X chromosome may be recovered more readily than those involving autosomal targets.

Finally, it is worth mentioning that the large reduction in yp170b that we observed is tolerated without any obvious reduction in embryonic viability. Perhaps yolk does not perform a vital function when the worm is growing in a nutritionally rich environment. Alternatively, the partial loss of yp170b may be compensated for by the presence of the other yolk proteins.

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