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Two Novel Transmembrane Protein Tyrosine Kinases Expressed during *Caenorhabditis elegans* Hypodermal Development

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We describe our characterization of *kin-15* and *kin-16*, a tandem pair of homologous *Caenorhabditis elegans* genes encoding transmembrane protein tyrosine kinases (PTKs) with an unusual structure: the predicted extracellular domain of each putative gene product is only about 50 amino acids, and there are no potential autophosphorylation sites in the C-terminal domain. Using *lacZ* fusions, we found that *kin-15* and *kin-16* both appear to be expressed during postembryonic development in the large hypodermal syncytium (hyp7) around the time that specific hypodermal cells fuse with hyp7. *kin-15* and *kin-16* were positioned on the genetic and physical maps, but extrachromosomal arrays containing wild-type *kin-15* and/or *kin-16* genes were unable to complement candidate lethal mutations. The results suggest that *kin-15* and *kin-16* may be specifically involved in cell-cell interactions regulating cell fusions that generate the hypodermis during postembryonic development.

Intercellular communication plays important roles in cell proliferation, cell migration, and cell fate choice during the development of all animals. The underlying biochemical basis of intercellular communication seems to be common to all animals, since related signal transduction proteins have been identified in organisms across the evolutionary spectrum. In *Caenorhabditis elegans*, direct and powerful methods of genetic and molecular analysis combined with the ability to examine developmental decisions with single-cell resolution enable these universal mechanisms of intercellular communication to be studied in great detail (reviewed in reference 53).

In *C. elegans*, there are two major routes to studying biological processes of interest. The primary route used thus far has been to identify genes involved in a particular process by isolating mutations that specifically disrupt the process, followed by molecular analysis to reveal possible biochemical functions. This approach has been very successful in dissecting many different processes, but it has certain limitations. One limitation is that disruption of the process under investigation must result in a distinctive and predictable phenotype. Another limitation is that a gene involved in several different processes might be difficult to identify by looking for mutations that affect only one process.

An alternative route is to clone a gene corresponding to a protein of interest, followed by genetic analysis to reveal biological functions. This approach, first used in *C. elegans* to examine the function of a myosin heavy-chain isoform (51), has become increasingly easier to apply as a physical map that is correlated with the genetic map has become available (11–13). We have begun to apply this alternative approach to the study of protein tyrosine kinases (PTKs).

The PTKs are a large family of enzymes that phosphorylate substrate proteins on tyrosine residues. All members exhibit homology in 11 conserved regions throughout a biochemically defined catalytic kinase domain of approximately 300 amino acids (19, 20). On the basis of their structures, PTKs can be divided into two subfamilies: the receptor and nonreceptor PTKs (8, 47). Receptor PTKs are transmembrane proteins containing a large (typically >200 amino acids), cysteine-rich, extracellular ligand-binding domain and a cytoplasmic catalytic domain. Most nonreceptor PTKs are cytoplasmic proteins associated with the inner plasma membrane by means of an N-terminal myristylate (10).

The multiplicity of PTKs in multicellular organisms implies that they are involved in levels of cellular coordination beyond those found in unicellular organisms (20), and indeed recent work indicates that receptor PTKs are important in the regulation of diverse developmental processes. Originally, several growth factor receptors were identified as receptor PTKs whose kinase activity was activated upon binding of a cognate peptide growth factor (reviewed in reference 56). More recently, a number of developmentally important genes, from both invertebrates and vertebrates, have been shown to encode receptor PTKs. Many of these genes, such as the *Drosophila sevenless* gene and the mouse *W/kit* locus, have been shown to regulate cell fate choice based on cellular interactions (reviewed in reference 31).

While the transmembrane structure of receptor PTKs is obviously suited for their role in signal transduction, nonreceptor PTKs also appear to be capable of participating in signal reception. In particular, the T-cell surface proteins CD4 and CD8, which are important in T-lymphocyte activation, have been shown to physically associate with Lck, a nonreceptor PTK, and under appropriate conditions to stimulate Lck kinase activity and intracellular protein tyrosine phosphorylation levels (reviewed in reference 49).

Here we present the identification and characterization of *kin-15* and *kin-16*, two *C. elegans* genes that are predicted to encode transmembrane PTKs with several unusual struc-

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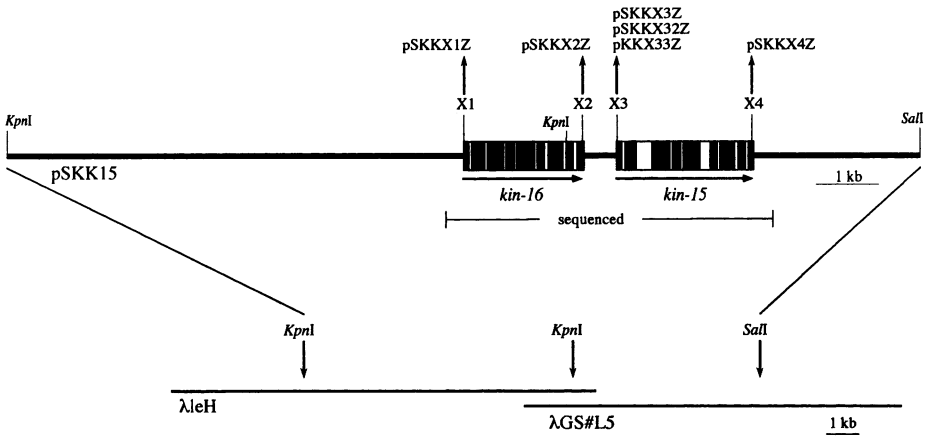


FIG. 1. Structure of the pSKK15 insert. The 16-kb *C. elegans* genomic DNA insert of pSKK15 containing the *kin-16* and *kin-15* genes (solid boxes, exons; open boxes, introns) was constructed from the genomic clones λGS#L5 and λleH (see text for details). Relevant restriction sites used in construction and the positions of *Xho*I sites (X1 to X4) introduced by in vitro mutagenesis for the insertion of *lacZ*-containing fragments (see Tables 1 and 2) and the sequenced region (Fig. 2) are also indicated.

tural features, and discuss the possible implications of these features for potential mechanisms for regulating kinase activity. We also present data indicating that these PTKs are expressed at approximately the same time that specific cells fuse with the hyp7 hypodermal syncytium during postembryonic development and discuss how this pattern of gene expression suggests possible biological functions.

MATERIALS AND METHODS

Standard recombinant DNA techniques were performed as described in reference 35 except as indicated below.

Screen for *C. elegans* PTK genes. To identify *C. elegans* PTK genes, the end-labeled oligonucleotide hybridization probe TK12 (a pool of six 17-mers; TGGATGGC[A/C/T] CCAGA[A/G]TC) was used to screen a *C. elegans* genomic library (kindly provided by Chris Link). Following plaque hybridization, high-stringency washes were performed with tetramethylammonium chloride (54). Two dozen candidate clones were initially identified. Rapid DNA sequence analysis of the genomic clones (using TK12 as a sequencing primer) or hybridization to a second oligonucleotide probe (not shown) was used to select a subset of these clones for further analysis. The detailed characterization of one clone, λGS#L5, is described in this report. The *kin-15* gene was found on this clone by direct sequencing using TK12 as a sequencing primer, and subsequent sequence analysis revealed the existence of the tandem *kin-16* gene.

TK12 was designed to target the amino acid sequence WMAPES, a motif conserved in subdomain VIII of many receptor PTKs (19, 20). Other strategies for isolating *C. elegans* PTK genes have yielded different putative PTK genes. Goddard et al. (18) and Koga and Ohshima (cited in reference 3) found PTK genes by screening *C. elegans* genomic libraries at low stringency with probes encoding the kinase domains of viral oncogenes. Kamb et al. (23) generated short *C. elegans* PTK-related sequences by using a degenerate oligonucleotide for subdomain VIII that was designed to detect amino acid sequences found primarily in cytoplasmic PTKs.

Sequence analysis of genomic and cDNA clones. For preliminary DNA sequence analysis, recombinant λEMBL4 clones were alkali denatured and annealed to end-labeled TK12 oligonucleotide. Dideoxy sequence reactions were subsequently performed, using the modified T7 DNA polymerase Sequenase (U.S. Biochemical) according to the manufacturer's instructions except that 7.5 μM dATP was substituted for [^{35}S]dATP during the extension (labeling) reaction.

Appropriate subcloned genomic restriction fragments were used to screen a λZAP *C. elegans* cDNA library (kindly provided by R. Barstead and R. Waterston [4]) by plaque hybridization. Nested sets of deletion templates were prepared by transposon-promoted deletions in *Escherichia coli* (1) or, preferably, by unidirectional digestion with exonuclease III (21). Dideoxy sequencing reactions were subsequently performed with the modified T7 DNA polymerase Sequenase (U.S. Biochemical) according to the manufacturer's instructions.

Computer-assisted DNA and protein sequence analysis was performed by using MacVector (International Biotechnologies, Inc.) and the software packages developed by the Genetics Computer Group, University of Wisconsin (14), and Staden (41). Amino acid sequence comparisons were performed at the National Center for Biotechnology Information with the nonredundant peptide sequence data base in June 1993, using the Blast network service (2). The algorithm of von Heijne (50) for the prediction of signal peptidase cleavage sites was applied by using the Macintosh program AnalyzeSignalase (27).

Northern (RNA) blot analysis. Total RNA was isolated by using general methods (35) or a method developed by Pilgrim (32). Poly(A)$^+$ RNA was purified by using commercially prepared oligo(dT)-cellulose columns [Poly(A) Quik mRNA Isolation Kit; Stratagene] or biotinylated oligo(dT) and streptavidin paramagnetic particles (PolyAttract Systems; Promega) according to the manufacturer's instructions. RNA and molecular weight markers were fractionated by formaldehyde-agarose gel electrophoresis, blot transferred to nitrocellulose filters, and hybridized to radiolabeled cDNA probes prepared by the random-priming technique (35).

Construction of *lacZ* fusion plasmids. First, pSKK15 (Fig. 1) was constructed by sequentially inserting a 6-kb *Sal*I-*Kpn*I genomic fragment (subcloned from λGS#L5) and an adjoining 10-kb *Kpn*I genomic fragment (subcloned from

TABLE 1. *lacZ* fusion gene plasmids

Plasmid[a]	*lacZ* reporter gene		
	Insertion site	Features[b]	Origin[c]
pSKKX1Z	*kin-16*, codon 10	Intron, NLS, *lacZ*, poly(A)	pBS21.28SS
pSKKX2Z	*kin-16*, codon 484	*lacZ*	pPDΔKK
pSKKX3Z	*kin-15*, codon 5	Intron, NLS, *lacZ*, poly(A)	pBS21.28SS
pSKKX32Z	*kin-15*, codon 18	Intron, NLS, *lacZ*, poly(A)	pBS21.28SS
pKKX33Z	*kin-15*, codon 25	Intron, NLS, *lacZ*, poly(A)	pBS21.28SS
pSKKX4Z	*kin-15*, codon 476	*lacZ*	pPDΔKK

[a] Each *lacZ* reporter gene is inserted into the intact 16-kb *C. elegans* genomic segment in pSKK15 (see Fig. 1) except for pSKKX32Z and pKKX33Z (see Materials and Methods).
[b] Intron, synthetic intron; NLS, simian virus 40 nuclear localization signal; *lacZ*, *E. coli trpS::lacZ* fusion gene; poly(A), polyadenylation signals from the *C. elegans unc-54* gene. See Fire et al. (17) for additional descriptions.
[c] Origin of inserted *lacZ* reporter gene fragments (see Materials and Methods).

λleH; isolated by chromosome walking) into the vector backbone of pPD16.43 (15). This 2.4-kb vector fragment was prepared by digesting pPD16.43 with *Stu*I, ligating on *Kpn*I linkers, digesting with *Kpn*I and *Sal*I, and purifying the desired fragment with Geneclean (Bio 101) following agarose gel electrophoresis.

Next, novel *Xho*I sites were created at defined sites of pSKK15 (Table 1 and Fig. 1) by oligonucleotide-directed mutagenesis (48) or polymerase chain reaction (PCR)-mediated mutagenesis (35). For the construction of all fusion plasmids except pKKX33Z, unique *Xho*I sites were created at defined sites in small cassette plasmids (<4-kb insert) containing the *kin-15* or *kin-16* gene sequence, using appropriate mutator oligonucleotides (Table 2) and the T7-GEN oligonucleotide-directed mutagenesis kit as instructed by the manufacturer (U.S. Biochemical). Using a multistep cloning procedure, mutated restriction fragments containing each novel *Xho*I site were then used to replace the corresponding wild-type fragment of pSKK15. During the construction of pSKKX32Z, a fortuitous deletion of nucleotides 3053 to 3461 occurred as a result of restriction enzyme star activity; because this region is downstream of the *lacZ* insertion site, it was not considered significant. For pKKX33Z, the novel *Xho*I site was created by PCR using the oligonucleotides MUT33 (Table 2) and L5#2 (TGATAAGCTTGACTATCTC CCG; nucleotides 730 to 751). The mutant fragment was then precisely joined to upstream sequences contained in the 10-kb *Kpn*I genomic fragment (see above). Note that pKKX33Z lacks *C. elegans* genomic sequences downstream of the novel *Xho*I site.

Finally, one of two modified *lacZ* reporter gene fragments (Table 1) was inserted in frame at each unique *Xho*I site. pBS21.28SS was produced by digesting pPD21.28 (17) with *Spe*I, filling in the ends with deoxynucleoside triphosphates and the Klenow fragment of DNA polymerase I, ligating to *Sal*I linkers, digesting with *Sal*I, and ligating the gel-purified 4.0-kb *lacZ*-containing fragment to *Sal*I-digested pBluescript KS+ (Stratagene). This fragment was released for insertion into pSKK15 by *Sal*I digestion except in the construction of pKKX33Z; in this case, for convenience, it was released using the flanking *Xho*I and *Pst*I sites of the polylinker. The latter manipulation resulted in a 6-bp insertion (TCGAGG) between the *kin-15* and *lacZ* gene sequences. pPDΔKK, a derivative of pPD16.43 lacking the 51-bp *Kpn* cassette, was created by complete digestion with *Kpn*I and recircularization (17). To purify the 3.2-kb *lacZ*-containing fragment, pPDΔKK was digested with *Stu*I, ligated to *Bam*HI linkers, and digested with *Bam*HI. For ligation, the *Bam*HI-generated ends of this insert and the *Xho*I-generated ends of the vector were partially filled in to generate complementary overhangs.

In addition to confirming gross plasmid structure by restriction enzyme analysis, critical cloning junctions were verified by sequencing single-stranded DNA templates prepared by PCR amplification and exonuclease digestion as described in reference 22.

***C. elegans* strains and culturing.** Handling and maintenance of *C. elegans* strains and the production of males by heat shock were as described in reference 43. Genotypes used for injection were wild type (strain N2 var. Bristol), *rol-6(n1270e187)* or *unc-32(e189) lin-12(n676n930)*. Strains used for complementation analysis were GS484 [*arEx2* (pRF4+pSKKX2Z)], GS485 [*rol-6(n1270e187)II*; *arEx3* (pRF4+pSKKX4Z)], GS838 [*rol-6(n1270e187)II*; *arEx20* (pRF4+pSKK15)], SP449 [*unc-4(e120) let-31(mn31)/mnC1 dpy-10(e128) unc-52(e444)II*], and SP663 [*unc-4(e120) let-240 (mn209)/mnC1 dpy-10(e128) unc-52(e444)II*].

Analysis of fusion gene expression patterns. Each *lacZ* fusion plasmid was cotransformed with a plasmid containing a selectable marker into the *C. elegans* germ line by micro-

TABLE 2. Mutator oligonucleotides

Name	Mutator oligonucleotide for:	Sequence[a]
MUT1	New *Xho*I site at nt[b] 295 (X1[c]) for pSKKX1Z construction	CATTTTCTTTGTCTTCTCGAGCTATGG
MUT2	New *Xho*I site at nt 2136 (X2[c]) for pSKKX2Z construction	AATCAAAGAAAGCTCGAGGATTGG
MUT3	New *Xho*I site at nt 2717 (X3[c]) for pSKKX3Z construction	CCTGAATGTGTTTAAACTCGAGATATGAAA
MUT32	New *Xho*I site at nt 2753 (X3[c]) for pSKKX32Z construction	CATATTACTGTTCTCGAGGATGCACCTTG
MUT33	New *Xho*I site at nt 2774 (X3[c]) for pKKX33Z construction	CGTAGAACTCGAGTAAACAAGGTGCATCAG
MUT4	New *Xho*I site at nt 4864 (X4[c]) for pSKKX4Z construction	GAGCAAATCTCGAGGATTGGATTCGG

[a] Underlined nucleotides, newly created *Xho*I site; boldface nucleotides, mutated positions.
[b] nt, nucleotide.
[c] See Fig. 1.

injection (16, 28). The selectable marker plasmid was pRF4, which contains a cloned *rol-6(su1006)* dominant allele that confers a visible Roller phenotype in a wild-type (N2) or *rol-6(n1270e187)* background (28), or p101i, which contains a cloned *lin-12*(+) gene that rescues the egg-laying defect of *lin-12(n676n930)* mutant animals (52). In general, stronger β-galactosidase expression was detected in the p101i-cotransformed strains. The selectable gene exhibited a non-Mendelian segregation pattern indicative of an extrachromosomal array. Embryos, larvae, and adults of transformed lines were stained for β-galactosidase activity at room temperature as described previously (17) except that kanamycin sulfate and 4,6-diamidino-2-phenylindole (DAPI) were generally omitted. The nuclearly localized enzyme, of pSKKX1Z for example, was more quickly detected (<1 to 12 h) than the putative membrane-bound enzyme of pSKKX2Z and pSKKX4Z (24 to >48 h). Stained animals were observed and photographed by using bright-field and Nomarski microscopy. The nuclear morphology of rapidly staining animals was well preserved and allowed the identity of individual nuclei to be assigned (see Results).

Complementation analysis using transgenic animals. The ability of a transgene to rescue the larval lethal phenotype of *let-31* or *let-240* was determined by the following genetic crosses.

(i) *kin-15*(+) rescue. GS484 Roller hermaphrodites containing the extrachromosomal array *arEx2*(pRF4 + pSKKX2Z) were mated with SP449 (*unc-4 let-31/mnC1*) or SP663 (*unc-4 let-240/mnC1*) males. F1 Roller progeny were allowed to self-fertilize individually, and the F2 progeny of *unc-4 let/++*; *arEx2* hermaphrodites were examined for the presence of viable Unc-4 Roller progeny.

(ii) *kin-16*(+) rescue. GS485 Roller hermaphrodites containing the extrachromosomal array *arEx3*(pRF4 + pSKKX4Z) were mated with SP449 or SP663 males. F1 Roller progeny were allowed to self-fertilize individually, and the F2 progeny of *unc-4 let/++*; *arEx3* hermaphrodites were examined for the presence of viable Unc-4 Roller progeny.

(iii) *kin-15*(+) and *kin-16*(+) rescue. GS838 Roller hermaphrodites containing the extrachromosomal array *arEx20* (pRF4+pSKK15) were mated with SP449 or SP663 males. F1 Roller progeny were allowed to self-fertilize individually, and the F2 progeny of *unc-4 let/++*; *arEx20* hermaphrodites were examined for the presence of viable Unc-4 Roller progeny.

Nucleotide sequence accession number. The sequences of *kin-15* and *kin-16* have been assigned GenBank accession number L03524.

RESULTS

Isolation and sequence analysis of *kin-15* and *kin-16*. To identify *C. elegans* PTK genes, a degenerate oligonucleotide hybridization probe corresponding to an amino acid motif highly conserved among receptor PTKs was used to screen a *C. elegans* genomic library (see Materials and Methods). The PTK genes found on one clone, λGS#L5, are described here.

Sequence determination of 5,217 nucleotides of the genomic clone λGS#L5 and of corresponding cDNAs identified two tandem genes, designated *kin-15* and *kin-16* (Fig. 1 and 2). The two genes are arranged head to tail and are separated by less than 400 bp. Analysis of the genomic sequence revealed that both genes contain nine *cis*-spliced exons, the last six of which have identical splice site positions (Fig. 3).

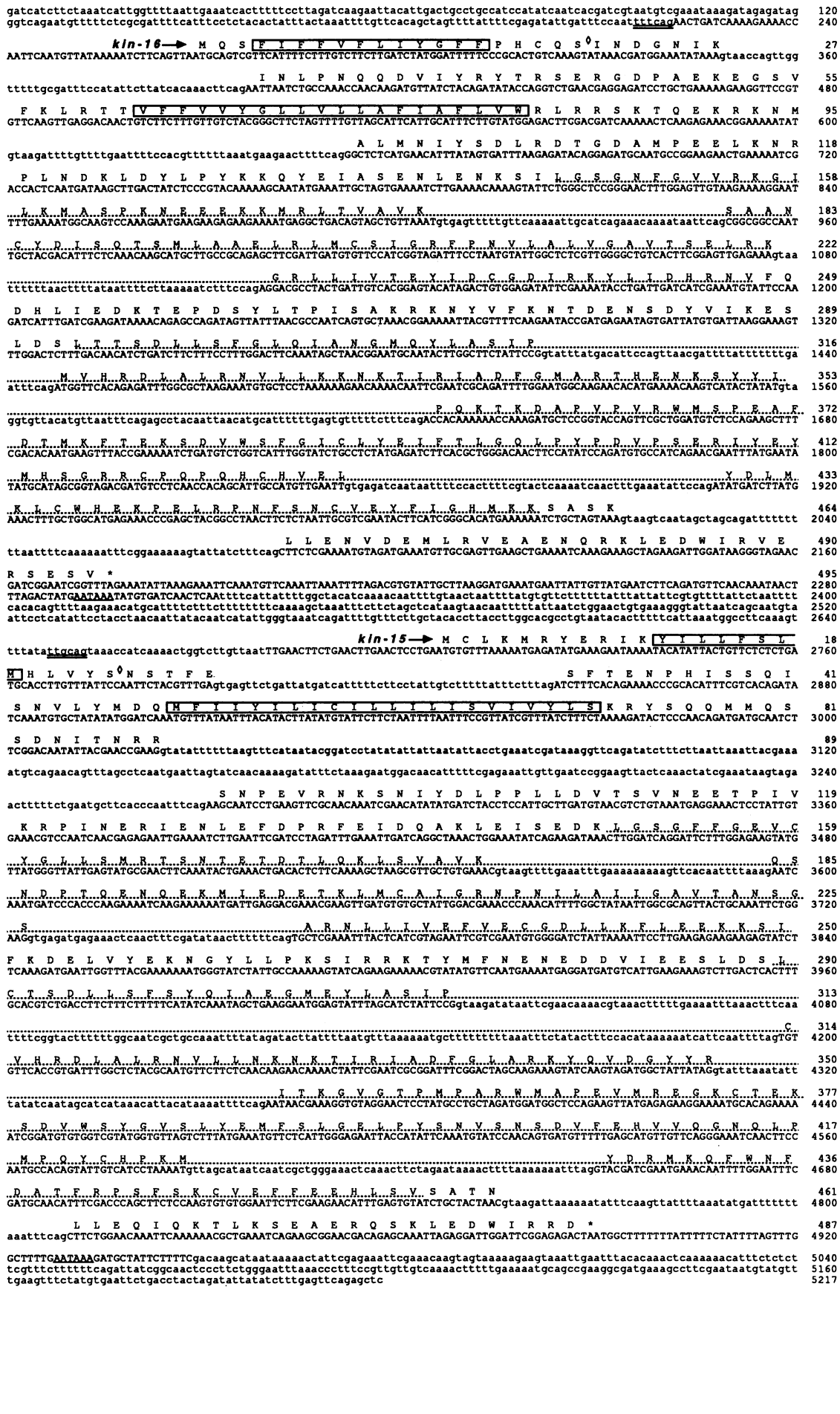
Several observations suggest that the coding sequences indicated in Fig. 2 are complete. (i) Northern blot analysis of mixed-stage *C. elegans* poly(A)$^+$ RNA using gene-specific probes identified a single mRNA for each gene of approximately 2 kb (Fig. 4 and data not shown). The longest cDNAs for each gene are therefore approximately the same size as the corresponding mRNAs detected by Northern blot analysis. (ii) The 5′ end of the longest *kin-16* cDNA matches the last 9 nucleotides of the 22-nucleotide-long transspliced SL1 leader exon found at the 5′ end of about 10% of all *C. elegans* mRNAs (reviewed in reference 7). (iii) The putative *kin-15* initiation codon is immediately preceded by a termination codon, and the approximately 350-nucleotide AT-rich *kin-16/kin-15* intergenic region does not contain an extended open reading frame. Furthermore, reverse transcriptase-PCR analysis (40) of *kin-15* mRNA indicates that the 5′ end of this transcript is transspliced to SL1 and SL2 leader exons just upstream of the putative initiation codon (57) (Fig. 2).

The *kin-15* and *kin-16* cDNAs each contain a single open reading frame which could encode a polypeptide of 487 and 495 amino acids, respectively (Fig. 2). As expected, the deduced amino acid sequence of each putative gene product possesses the hallmarks of a PTK catalytic domain (19, 20), including the nucleotide-binding consensus sequence (GXGXXG and downstream K; Fig. 3). In both proteins, the subdomain VIB motif HRDLAARN contains a previously unobserved conservative amino acid change of A→L at position 6 (Fig. 3), although substitutions have been found at this position in other PTKs (19, 20).

The predicted *kin-15* and *kin-16* gene products have receptor PTK characteristics. Hydropathy plot analysis (26) identified two hydrophobic amino acid sequences in each gene product that could function as an amino-terminal secretory signal peptide and a membrane-spanning domain (boxed in Fig. 2). In addition, the predicted Kin-15 and Kin-16 proteins share two other features unique to receptor-type PTKs: a kinase insert domain which splits the catalytic kinase domain, and the subdomain VIII motif WM(A/S)PE (Fig. 3). However, the Kin-15 and Kin-16 PTKs have predicted extracellular domains of only 25 and 41 amino acids (after removal of the presumptive signal peptide), respectively, much shorter than those of known receptor PTKs. Both extracellular domains also lack a cysteine-rich region typical of the ligand-binding domains of known receptor PTKs.

Protein data base comparisons with the Kin-15 and Kin-16 amino acid sequences revealed that the region of homology

FIG. 2. Nucleotide sequences of the *kin-15* and *kin-16* genes. Nucleotide sequences present in cDNA clones are in uppercase; intron and flanking genomic DNA sequences are in lowercase. The sequence begins at the left end of the λGS#L5 insert. The deduced amino acid sequence is indicated above the cDNA sequence. The translation start site is shown as the most upstream, in-frame ATG, although we note that in *kin-15* the second ATG is in a better context. Stretches of hydrophobic amino acids indicative of potential signal peptides and transmembrane domains are boxed; the most likely cleavage site for the removal of each signal peptide is indicated by a diamond. Canonical polyadenylation signals are underlined, the split kinase catalytic domains are dotted underlined (see also Fig. 3B), and the transsplice acceptor sites for *kin-15* and *kin-16* are double underlined.


kin-16
kin-15

(A) kinase catalytic domain:

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                          * *  * I                          II  *
Kin-15      aa# 150   LGSGFFGEVCYG.LLSMRTSNTETDTLQKLSVAVKQSNDPT
Kin-16      aa# 146   LGSGNFGVVRKG.ILKMASPKNEEEKKMRLTVAVKSAANCY
c-Kit       aa# 595   LGAGAFGKVVEATAYGL..IKSDAA....MTVAVKMLKPSA
PDGFR       aa# 606   LGSGAFGKVVEGTAYGL..SRSQPV....MKVAVKMLKPTA
FGFR        aa# 484   LGEGCFGQVVRAEAFGMDPARPDQA....STVAVKMLKDNA
c-Met       aa#1102   IGRGHFGCVYHGTLLDNDGKK........IHCAVKSLNRIT

                          III                IV                V
Kin-15      QENQEKMIEDETKLMCAIGRNPNILAIIGAVTANSGSARNLLIVEFVECG
Kin-16      DISQTSMLAAEIRLMCSIGRFPNVLALVGAVTSELRKGRLLIVTEYIDCG
c-Kit       HLTEREALMSELKVLSYLGNHMNIVNLLGACTIG...GPTLVITEYCCYG
PDGFR       RSSEKQALMSELKIMTHLGPHLNIVNLLGACTKS...GPIYIITEYCFYG
FGFR        SDKDLADLVSEMEVMKLIGRHKNIINLLGVCTQE...GPLYVIVECAAKG
c-Met       DIGEVSQFLTEGIIMKDFS.HPNVLSLLGICLRS..EGSPLVVLPYMKHG

                                      ki                VIA
Kin-15      DLLKFLEEKKSI .....(39 aa insert).... LCTSDLLSFSYQIAEGMEY
Kin-16      DIRKYLIDHRNV .....(45 aa insert).... LTTSDLLSFGLQIANGMQY
c-Kit       DLLNFLRRKRDS .....(75 aa insert).... LDLEDLLSFSYQVAKGMAF
PDGFR       DLVNYLHKNRDS .....(97 aa insert).... LTLLDLLSFTYQVARGMEF
FGFR        NLREFLRARRPP .....(15 aa insert).... LSFPVLVSCAYQVARGMQY
c-Met       DLRNFIRNETHN .....(no insert)..... PTVKDLIGFGLQVAKAMKY

                    VIB                VII
Kin-15      LASIPCVHRDLALRNVLLKNKNTIRIADFGLARKYQVDGYYCITKGVGTP
Kin-16      LASIPMVHRDLALRNVLLKNKNTIRIADFGMARTHENKSYYIPQKTKDAP
c-Kit       LASKNCIHRDLAARNILLTHGRITKICDFGLARDIKNDSNYVV..KGNAR
PDGFR       LASKNCVHRDLAARNVLLAQGKIVKICDFGLARDIMHDSNYVS..KGSTF
FGFR        LESRKCIHRDLAARNVLVTEDNVMKIADFGLARGVHHIDYYK..KTSNGR
c-Met       LASKKFVHRDLAARNCMLDEKFTVKVADFGLARDMYDKEYYSVHNKTGAK

                    VIII                IX
Kin-15      MPARWMAPEVMREGKCTEKSDVWSYGVSLYEMFSLGELPYSNVSN.SDVF
Kin-16      VPVRWMSPEAFDTMKFTEKSDVWSFGICLYEIFTLGQLPYPDVPS.ERIY
c-Kit       LPVKWMAPESIFNCVYTFESDVWSYGIFLWELFSLGSSPYPGMPVDSKFY
PDGFR       LPVKWMAPESIFDNLYTTLSDVWSYGILLWEIFSLGGTPYPGMMVDSTFY
FGFR        LPVKWMAPEALFDRVYTHQSDVWSFGILLWEIFTLGGSPYPGIPV.EELF
c-Met       LPVKWMALESLQTQKFTTKSDVWSFGVVLWELMTRGAPPYPDVNT.FDIT

                X                XI
Kin-15      EHVVQGNQLPMPQYCHPKMYDRMKQFWNFDATFRPSFSKCVEFFEEHLSV
Kin-16      EYMHSGRRCPQPQHCHVELYDLMKLCWHEKPELRPNFSNCVEYFIGHMKK
c-Kit       KMIKEGFRMLSPEHAPAEMYDIMKTCWDADPLKRPTFKQIVQLIEKQISE
PDGFR       NKIKSGYRMAKPDHATSEVYEIMVKCWNSEPEKRPSFYHLSEIVENLLPG
FGFR        SLLREGHRMDRPPHCPPELYGLMRECWHAAPSQRPTFKQLVEALDKVLLA
c-Met       VYLLQGRRLLQPEYCPDPLYEVMLKCWHPKAEMRPSFSELVSRISAIFST
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(B) kinase insert (ki) domain:

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Kin-15      aa# 252   FKDELVYEK...NGYLLPKSIRRKTYMF...NENEDDVIEESLDS
Kin-16      aa# 248   FQDHLIEDKTEPDSYLTPISAKRKNYVFKNTDENSDYVIKESLDS
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(C) C-terminus:

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Kin-15      aa# 458   SATNLLEQIQKTLKSEAERQSKLEDWIRRD*
Kin-16      aa# 461   SASKLLENVDEMLRVEAENQRKLEDWIRVERSESV*
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FIG. 3. Amino acid sequence alignments. (A) Comparison of the kinase catalytic domains of the predicted *kin-15* and *kin-16* gene products with those of four previously characterized receptor tyrosine kinases: c-Kit (55), PDGFR (9), fibroblast growth factor receptor (FGFR) (30), and c-Met (29). The 11 conserved subdomains described by Hanks et al. (19, 20) are indicated by roman numerals. The GXGXXG consensus and invariant lysine residues of the nucleotide-binding site (asterisks) and the HRDLAARN motif (overbar) indicative of PTKs are marked (see text). aa, amino acid. (B) Comparison of the kinase insert domains of the predicted *kin-15* and *kin-16* products. (C) Comparison of the C termini of the predicted *kin-15* and *kin-16* products. Residues conserved between *kin-15* and *kin-16*, and the other kinases, are shown in boldface. Gaps introduced to optimize sequence alignments are indicated by dots or numbers in parentheses. Arrowheads indicate the positions of introns conserved between *kin-15* and *kin-16*.

to protein kinases is limited to the catalytic domain and that there is no homology to other proteins. However, the Kin-15 and Kin-16 proteins do exhibit significant amino acid similarity to each other in the kinase insert (56% identity) and C-terminal (53% identity) domains, in addition to the catalytic domain (50% identity; Fig. 3 and 5). In other receptor PTKs, autophosphorylated tyrosine residues in the kinase insert and C-terminal domains have been found to be critical

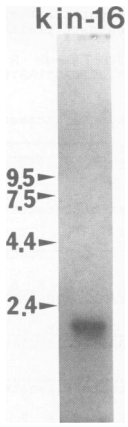

FIG. 4. Northern blot analysis of *kin-15* and *kin-16* mRNAs. Poly(A)$^+$ RNA isolated from a mixed-stage population of *C. elegans* was fractionated, transferred to a nitrocellulose filter, and hybridized with a *kin-16*-specific probe. The positions of molecular weight markers (in kilobases) are indicated.

for effector interaction and kinase regulation (8). Kin-15 and Kin-16 possess two conserved tyrosine residues in the kinase insert domain, as well as an additional nonconserved tyrosine each, but none of these are found in contexts that match known SH2-binding sites as determined by Songyang et al. (39). No tyrosine residues are present in the C-terminal domain of either gene product. The extracellular, transmembrane, and juxtamembrane domains of these two proteins display no significant amino acid homology to each other (less than 20% identity; Fig. 5).

Developmental expression patterns of *kin-15* and *kin-16*. The developmental expression patterns of the *kin-15* and *kin-16* genes were investigated by using *kin-15*::*lacZ* and

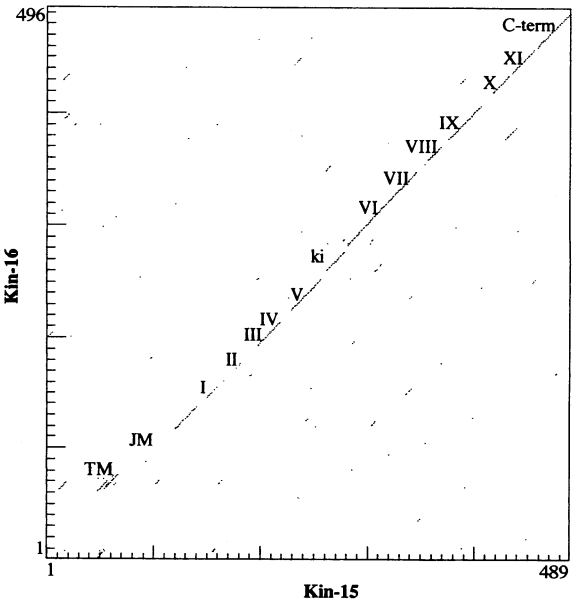

FIG. 5. Dot matrix analysis of the *kin-15* and *kin-16* proteins. Relevant protein domains are indicated. TM, transmembrane; JM, juxtamembrane; ki, kinase insert; C-term, C-terminal. Kinase subdomains are designated I to XI. The analysis was performed by using the default settings of the DIAGON program (41).

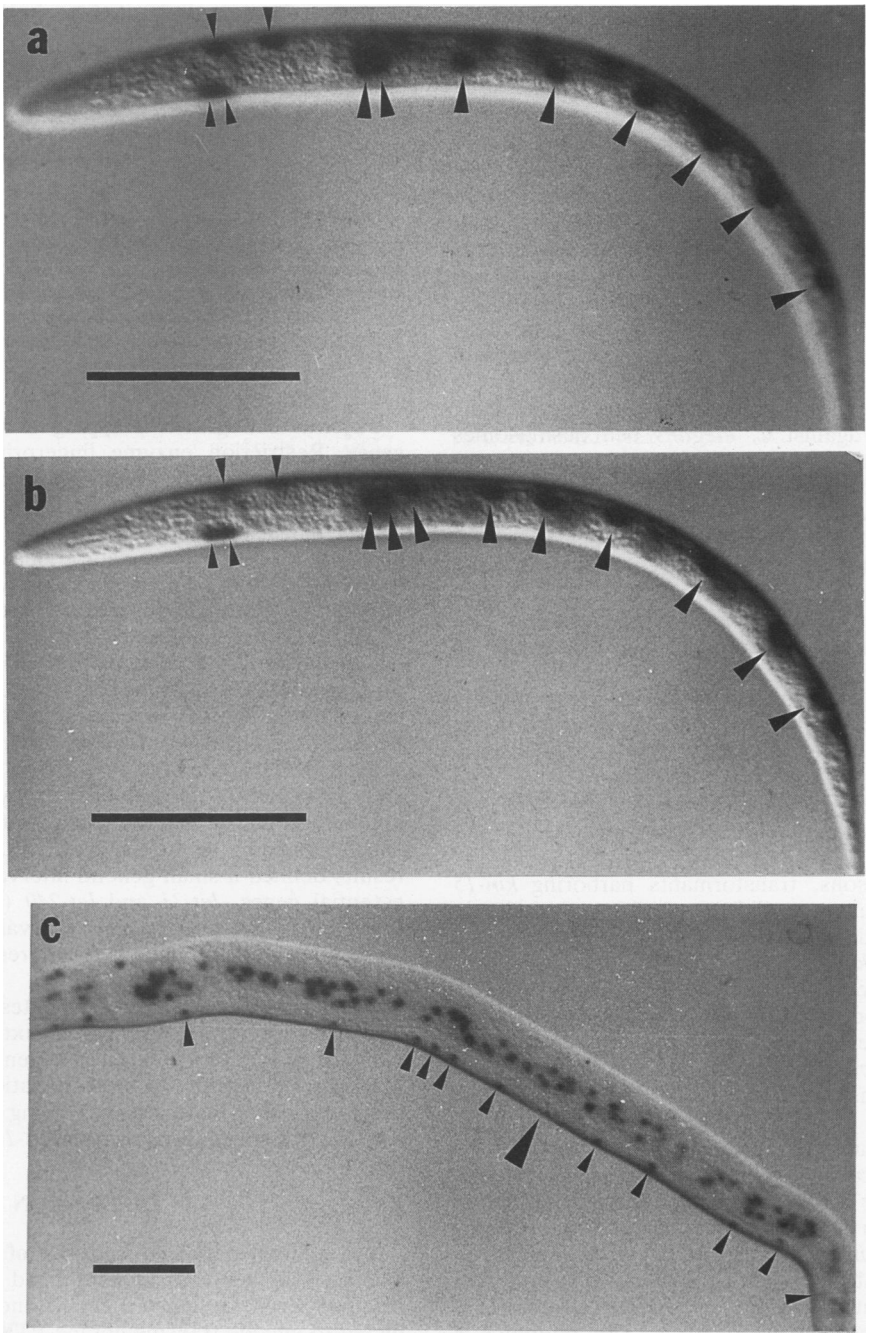

FIG. 6. β-Galactosidase staining of pSKKX1Z fusion gene transformants. (A) L1 larvae; left lateral focal plane. In addition to four anterior dorsoventral nuclei (small arrowheads), eight left lateral nuclei stain (large arrowheads). (b) Same animal as in panel a; right lateral focal plane. In addition to the same four anterior dorsoventral nuclei, nine right lateral nuclei stain. The two posterior nuclei did not stain in this animal. (c) L4 larvae; lateral view. Additional nuclear staining is observed as postembryonic cells fuse with hyp7. Most striking are the 11 Pn.p-derived nuclei (arrowheads) present along the ventral midline, six anterior (P1.p, P2.p, P3.pa, P3.pp, P4.pa, and P4.pp) and five posterior (P8.pa, P8.pp, P9.p, p10.p, and P11.p) to the vulva (large arrowhead). Anterior is left; dorsal is up. The scale bar equals 50 μm.

kin-16::*lacZ* fusion genes (17). The β-galactosidase enzyme encoded by these translational fusion genes is joined at the amino or carboxy terminus of each PTK (Table 1 and Fig. 1). The amino-terminal fusion proteins contain a nuclear localization signal which greatly assisted in the correlation of the staining pattern with the known cellular anatomy of different developmental stages. In contrast, the carboxy-terminal fusion proteins, which contain a *kin*-derived signal sequence and transmembrane domain, should be localized to the plasma membrane. Stable lines containing each gene fusion construct were established by cotransformation with a selectable marker and stained for β-galactosidase expression (see Materials and Methods).

In transgenic animals containing a plasmid with *lacZ* fused to the 5′ end of *kin-16* (pSKKX1Z), β-galactosidase staining is first detected in young L1 larvae in 23 nuclei (Fig. 6a and

b) that have the morphology typical of hypodermal cells (expanded and flat nuclei with large nucleoli [44]). This staining pattern is entirely coincident with the reported positions of the 23 nuclei which compose the hyp7 syncytium after hatching (44, 46).

The hyp7 syncytium grows during larval development by the fusion of progeny of ventral and lateral hypodermal blast cells with the existing syncytium (44). The observed pattern of *kin-16*::*lacZ* fusion gene expression from pSKKX1Z correlates completely with the dynamic pattern of cell fusions with hyp7. For example, nuclear staining of P1.p and P2.p is detected first (fusion at ~10 h posthatching), closely followed by P9.p and P10.p (fusion at ~11 h) and then P11.p (fusion at ~12 h). In L3 larvae, staining coincident with P3.pa, P3.pp, P4.pa, P4.pp, P8.pa, and P8.pp (fusion at ~30 h) is also detected (Fig. 6c). Preliminary experiments using MH27, an antibody against *C. elegans* belt desmosomes (33), were unable to determine whether fusion gene expression precedes or follows cell fusion (52). In contrast, no expression is detected in Pn.p descendants that do not fuse with hyp7: P(5-7).p descendants, which become the vulva, and P12.p descendants, which become hyp12. A total of 110 mononucleate cells are recruited during larval development, so that the adult hyp7 syncytium contains a total of 133 nuclei; a similar number of staining nuclei is observed in transgenic adults.

In addition to the hyp7 expression, the only other staining in pSKKX1Z transgenic hermaphrodites is observed in the hyp6 syncytium late in development. hyp6 is immediately anterior to hyp7 and is formed from the fusion of six cells prior to hatching. The hyp6 nuclei are consistently stained in adult hermaphrodites and occasionally as early as L3 (not shown).

For unknown reasons, transformants harboring *kin-15* N-terminal fusion constructs did not exhibit any nuclearly localized β-galactosidase activity. After *lacZ* fusion to the first possible ATG of *kin-15* (pSKKX3Z) failed, we tested the possibility that the second ATG, which is in a better context, was the initiation codon (pSKKX32Z and pKKX33Z) but still did not observe staining. However, β-galactosidase expression was detected in transformants harboring *kin-15* C-terminal fusion constructs (pSKKX2Z). As expected, the staining pattern with the presumably membrane-bound fusion proteins was generally diffuse over the surface of the animal, although localized staining was occasionally observed. Although the diffuse staining precluded the unambiguous identification of *lacZ*-expressing cells, the staining observed with the *kin-15* C-terminal fusion construct appeared similar to that of the *kin-16* C-terminal fusion construct (pSKKX4Z; data not shown), suggesting that *kin-15* is expressed in the same cells as *kin-16* is. This inference is strongly supported by the finding that *kin-15* mRNA appears to be processed from a polycistronic precursor RNA that includes *kin-16* (57).

This expression pattern is likely to reflect the expression of the endogenous gene. First, more than 7 kb of 5′ flanking sequence was present in reporter constructs, in addition to all intron sequences and extensive 3′ sequences. Much less 5′ flanking DNA has been sufficient for correct expression of many other *C. elegans* genes. Second, it is unlikely that heterologous regulatory sequences provided by cotransformation markers are responsible for the expression pattern, since the same pattern was observed with two different markers, *rol-6*, a collagen gene, and *lin-12*, a developmental control gene needed in many different cell types (see Materials and Methods).

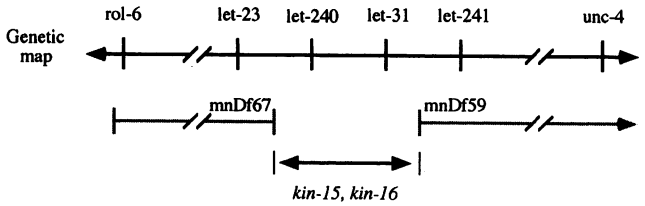

FIG. 7. Partial genetic map of *LGII*. Genes were ordered in the region by the failure of mutations to complement deficiencies (37). Horizontal lines indicate the regions deleted by *mnDf59* and *mnDf67*. The position of *kin-15* and *kin-16* in the interval defined by *mnDf59* and *mnDf67* breakpoints was inferred from correlating the genetic and physical maps as described in the text.

Physical and genetic mapping of the *kin-16* and *kin-15* genes. Restriction enzyme fingerprinting (12) of GS#L5 placed it on a contig which had been correlated with the genetic map on linkage group II between *rol-6* and *unc-4* (11). This region has been the subject of intensive genetic analysis, leading to near saturation for essential genes and the availability of many deficiencies with breakpoints in this region (37). Physical mapping of chromosomal deficiencies in this region defined a smaller genetic interval containing *kin-15* and *kin-16*. To define the right end of the interval, we probed genomic Southern blots containing DNA from deficiency heterozygotes with cosmids mapping to the right of GS#L5 and localized the left breakpoint of *mnDf59* to cosmid F35H8 (data not shown). The left end of the interval had previously been established by localizing the right breakpoint of the chromosomal deficiency *mnDf67* to a cosmid mapping to the left of GS#L5 (3). Together, these results defined a small genetic interval containing only two essential genes, *let-31* and *let-240* (Fig. 7). Mutations in *let-31* and *let-240* both result in a larval lethal phenotype, and hence they were candidates for corresponding to *kin-15* and *kin-16*.

We performed genetic crosses to test for complementation of the *let-31* or *let-240* mutation by extrachromosomal arrays carrying one or both of the *kin* genes (see Materials and Methods for details). Complementation of the lethal mutations was never observed, indicating that neither *let-31* nor *let-240* corresponds to *kin-15* or *kin-16*.

DISCUSSION

The unusual predicted structure of two *C. elegans* PTKs. We have cloned and characterized a tandem pair of *C. elegans* genes, designated *kin-15* and *kin-16*, that are predicted to encode transmembrane PTKs. Both putative gene products have all of the amino acid motifs characteristic of PTK catalytic domains, including a nucleotide-binding consensus sequence and downstream lysine. In addition, each putative PTK contains a kinase insert sequence, a characteristic of many receptor PTKs. Kin-15 and Kin-16 exhibit no homology to other PTKs outside of the catalytic domain or to other characterized proteins.

The predicted Kin-15 and Kin-16 PTKs possess several unusual features that distinguish them from most previously characterized receptor PTKs. First, the Kin-15 and Kin-16 extracellular domains are exceptionally short, less than 50 amino acids (after removal of the putative signal peptide), and are deficient in cysteine residues. Of the receptor PTKs, the Ltk protein is the most similar in this respect with an extracellular domain of approximately 100 amino acids (6),

although this transmembrane PTK appears to be regulated by a novel mechanism (see below). Second, the C-terminal tails of Kin-15 and Kin-16 are also short and lack potential autophosphorylation sites. Only members of the Trk subfamily of receptor PTKs have shorter tails, and these contain one conserved tyrosine residue, although it has not been shown to be phosphorylated (24). Finally, the Kin-15 and Kin-16 proteins possess an unusual substitution in the subdomain VI motif HRDLALRN.

Potential mechanisms for regulating Kin-15 and Kin-16 activity. Binding of a cognate ligand to the extracellular domain of a receptor PTK induces receptor dimerization and cross-phosphorylation, thereby activating the intracellular kinase domain (reviewed in references 8 and 47). Although it remains possible that Kin-15 and Kin-16 can bind extracellular ligands, the short extracellular domains prompt us to consider other possible mechanisms for regulating their kinase activities. The other known short receptor PTK, the Ltk protein, appears to be confined to endoplasmic reticulum and regulated through a redox mechanism involving the formation of disulfide-linked multimers (5). The absence of cysteine residues in the Kin-15 and Kin-16 extracellular domains precludes a similar mechanism for kinase activation.

The Kin-15 and Kin-16 proteins could function in signal reception by interacting with a cell surface protein able to bind an extracellular signal molecule. There are analogous situations that provide precedents for a PTK to function as a subunit of a receptor. For example, the Sevenless precursor protein is cleaved to produce a catalytic subunit with a short extracellular domain that associates by noncovalent interaction with a ligand-binding subunit (38). In addition, a nonreceptor PTK, the Lck protein (a Src-related PTK), interacts with cell surface proteins encoded by other genes. The Lck protein can apparently interact with T-cell surface proteins by one of two mechanisms, one possibly involving the formation of a metal ion coordination complex between pairs of essential cysteine residues on the interacting proteins (reviewed in reference 49) and another that does not (42). Again, the absence of an appropriate pair of N-terminal cysteine residues in the Kin-15 and Kin-16 proteins indicates that a metal ion coordination complex is unlikely to be the mechanism of protein-protein interaction.

Alternatively, the kinase activity of the Kin-15 and Kin-16 proteins might be regulated by an intracellular event such as phosphorylation and dephosphorylation of sites within the juxtamembrane or kinase insert domains. Both nonreceptor and receptor PTKs can be phosphorylated at cytoplasmic serine/threonine residues located in the juxtamembrane domain, and at least in the case of the epidermal growth factor receptor, such phosphorylation has been shown to inhibit kinase activity (reviewed in references 47 and 56). Phosphorylated tyrosines within kinase insert domains can be binding sites for substrates, as has been observed for the platelet-derived growth factor receptor (PDGFR) (reviewed in reference 25), and dephosphorylation of these sites may inhibit the ability of Kin-15 and Kin-16 to associate with substrate proteins.

It is also possible that Kin-15 and Kin-16 are constitutively active, since they have short extracellular domains which may be incapable of binding ligands and C-terminal regions devoid of potential autophosphorylation sites. They therefore superficially resemble the products of viral oncogenes such as v-*erbB*, v-*fms*, and v-*kit* (reviewed in reference 56). If Kin-15 and Kin-16 are constitutively active, then it is possible that the restricted hypodermal expression of *kin-15* and *kin-16* (see below) is a way to regulate kinase activity. In that case, ectopic expression of Kin-15 and Kin-16 might have deleterious consequences.

Tandem gene evolution. *kin-15* and *kin-16* seem likely to have arisen by duplication of an ancestral gene: the two genes are adjacent in the genome, and all of the splice junctions in the catalytic domain are conserved. A similar tandem gene organization has been seen for several pairs of mammalian growth factor genes, including those encoding PDGFR and colony stimulating factor 1 receptor (CSF-1R) (34).

An interesting difference is revealed from comparison of the pattern of amino acid sequence divergence between Kin-15 and Kin-16 with that of the divergence between PDGFR and CSF-1R. For PDGFR and CSF-1R, as well as other pairs of related PTKs, the juxtamembrane and kinase domains are best conserved, while the extracellular, kinase insert, and C-terminal domains are much less conserved. In contrast, for Kin-15 and Kin-16, the kinase insert and C-terminal domains, which have been implicated in substrate interaction, are best conserved. Moreover, two of the autophosphorylation sites have also been conserved in the kinase insert domain. These observations suggest that Kin-15 and Kin-16 may phosphorylate at least some common substrates, although differences between them in the potential autophosphorylation sites may indicate that the set of substrate proteins only partially overlap. The relatively greater divergence of the extracellular, transmembrane, and juxtamembrane domains between Kin-15 and Kin-16 may mean that they are regulated differently, perhaps by interaction with different proteins. On the other hand, these regions may not interact with regulatory proteins and hence are freer to diverge. The characterization of Kin-15 and Kin-16 homologs from other organisms might resolve this issue.

***kin-15* and *kin-16* gene expression patterns and biological function.** The developmental expression patterns of *kin-15* and *kin-16* were examined by using *lacZ* fusion gene constructs. Expression of both *kin-15* and *kin-16* indicates a role in the development of the hyp7 hypodermal syncytium. The nuclearly localized *kin-16*::*lacZ* fusion protein was first detected after hatching in the 23 nuclei of the L1 hyp7 syncytium. Later in larval development, the fusion protein was detected in all lateral and ventral hypodermal nuclei that fuse with hyp7, although we have not been able to determine whether the transgene is expressed prior to or after cell fusion. The pattern of expression of *kin-15* appears to be similar to that of *kin-16* (see Results).

We are intrigued by two possible roles for *kin-15* and *kin-16* in hyp7 development. One possibility is that *kin-15* and *kin-16* are involved in regulating the fusion of cells with hyp7. The complexity of the timing and spatial pattern of cell fusion with hyp7 suggests that there must be a mechanism to ensure that the correct cells fuse at the correct times. Kin-15 and Kin-16 may be involved in the reception of intercellular signals regulating fusion through interaction with a cell surface protein, or their constitutive activity might confer competence for fusion.

Alternatively, *kin-15* and *kin-16* activity may be involved in maintaining nuclei in a mitotically quiescent state once cells have fused with hyp7. There seems to be an inverse correlation between fusion and mitotic activity. For example, in wild-type hermaphrodites, the daughters of P(3,4,8).p join hyp7, while the daughters of P(5-7).p continue to divide to produce vulval cells. Interestingly, in vulvaless mutants, the daughters of P(3-8).p all join hyp7 and do not divide further, while in multivulva mutants, the daughters of P(3-

8).p do not join hyp7 and continue to divide to produce vulval cells (15, 45).

Prospects for genetic analysis. The isolation and phenotypic characterization of *kin-15* and *kin-16* mutants will be the best way to ascertain the role of these genes in hyp7 development. We mapped *kin-15* and *kin-16* to a small genetic interval and tested the genes individually and together for complementation of the zygotic lethal mutations in that interval. Although we found that the existing zygotic recessive lethal mutations in the region are not mutations in *kin-15* and/or *kin-16*, the physical and genetic localization permits the isolation of additional mutations in the region (see, for example, reference 51). We suspect that the phenotype of null mutations in *kin-15* and/or *kin-16* might be zygotic lethal or visible mutations because expression of these genes is first detected in L1 larvae. It is also possible that if the genes are functionally redundant, null mutations in each gene will be wild type, but that elimination of the activity of both genes will result in a lethal or visible phenotype. We are currently looking for zygotic lethal and visible mutations complemented by a plasmid containing *kin-15* and *kin-16*; in addition, we are using PCR to screen for transposon insertions into each gene separately, making no assumptions about the nature of the null phenotype.

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