An Ankyrin-related Gene (unc-44) Is Necessary for Proper Axonal Guidance in Caenorhabditis elegans

Anthony J. Otsuka,*† Rodrigo Franco,‡ Bin Yang,* Kyu-Hwan Shim,* Lan Zhao Tang,* Yu Yong Zhang,* Pratumtip Boontrakulpoontawee,* Ayyamperumal Jeyaprakash,‡ Edward Hedgecock,§ Virginia I. Wheaton,‡ and Alan Sobery*

* Department of Biological Sciences, 4120 Illinois State University, Normal, Illinois 61790-4120; ‡Department of Genetics, University of California, Berkeley, California 94720; and §Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218

Abstract. Caenorhabditis elegans unc-44 mutations result in aberrant axon guidance and fasciculation with inappropriate partners. The unc-44 gene was cloned by transposon tagging, and verified by genetic and molecular analyses of six transposon-induced alleles and their revertants. Nucleotide sequence analyses demonstrated that unc-44 encodes a series of putative ankyrin-related proteins, including AO49 ankyrin (1815 aa, 198.8 kD), AO66 ankyrin (1867 aa, 204 kD), and AOI3 ankyrin (x<4700 aa, ~<517 kD). In addition to the major set of ~6 kb alternatively spliced transcripts, minor transcripts were observed at ~3, 5, 7, and 14 kb. Evidence is provided that mutations in the ~14-kb AO13 ankyrin transcript are responsible for the neuronal defects. These molecular studies provide the first evidence that ankyrin-related molecules are required for axonal guidance.
Figure 1. (a) The proposed structures of ankyrin-related proteins. Multiple proposed forms of human erythrocyte ankyrin (Lambert et al., 1990; Lux et al., 1990), human brain ankyrin 2, a partial structure of brain ankyrin 1 (Otto et al., 1991), and partial structures of the putative nematode unc-44 ankyrin-related products are shown. Brain ankyrins 1 and 2 contain different carboxyl termini produced by alternative splicing at aa position 1444 (Otto et al., 1991). In brain ankyrin 1, alternative splicing results in insertion of 2085 aa, including 15 repeats of a 12-aa sequence, into the carboxyl-terminal domain to produce a 440-kD product (Kunimoto et al., 1991; Chan et al., 1993). For unc-44, the amino acids are numbered from the start of the partial cDNAs, while those expected in the full-length protein are noted in parentheses. In the unc-44 products, alternative splicing modifies the carboxyl terminus (at cDNA aa positions 983 and 910) in a manner similar to that found in erythrocyte ankyrin. In the linker domain, between the ankyrin repeats and the spectrin-binding domain, the DD#PAO49 product contains a 6-aa alternatively spliced microexon relative to DD#PAO66. An inversion of the nucleic acid sequence (boxed arrow) occurs in the linker domain in the DD#PAO66 cDNA clone. The ankyrin repeat domains shown as dashed lines are those expected on the basis of Northern blot and spectrin-binding-domain analysis. (b) Map of the unc-44 region. The positions and extents of the cosmid (BO350 and C44A), phage (DD#LRF1), genomic plasmid subclones (DD#PRF6, DD#PRF7, DD#PPB40, and DD#PSLR8), and cDNA clones (DD#PAO13, DD#PAO49, and DD#PAO66) are displayed. The positions of unc-44 mutations are shown above the genomic map. The ankyrin domains present on the 11-kb BamHI fragment in DD#PPB40 are shown along with the exon map (filled blocks) above the genomic map. Relevant restriction sites for BamHI (B) and SalI (S) are noted. The dashed lines extending from the cDNA clones estimate the full extent of the RNAs as determined by Northern blot analysis. The cDNA clones were obtained by screening cDNA libraries with probes corresponding to DD#PPB40 (11 kb) and DD#PSLR8 (3.7 kb). The dashed lines extending from cosmid clone C44A represent the uncertainty of nematode RNA junctions in the clone. (c) The genomic organization of the 11-kb BamHI fragment. The 11-kb region from DD#PPB40 was sequenced by the exonuclease III deletion method using the subclones DD#PRF6 and DD#PRF7. The exons are represented by boxes and the introns by a line. The ankyrin repeats divide the boxes and the strong spectrin-binding domain similarity is shown in black. The primed numbers represent breaks within the individual ankyrin repeats.
with transposons (Otsuka et al., 1987). In this paper, we report the molecular cloning and characterization of the unc-44 gene. The DNA sequence analysis, Southern and Northern blot analysis, and genetic complementation tests demonstrate that the unc-44 gene has been cloned. Analysis of six spontaneous unc-44 alleles ascertained that all were due to DNA insertions (see Fig. 1b). Reversions of these six alleles result in in-frame deletions of the transposons or secondary insertions of transposons at RNA splicing junctions.

The composite structures of the unc-44 ankyrins have been obtained from a combination of cloned genomic and cDNA sequences. These studies demonstrate that alternative splicing produces several transcripts from a single ankyrin-related gene. There is a major set of 6 kb transcripts and several minor transcripts. Paralleling the human ANK2 gene, unc-44 encodes "conventional" ankyrin isoforms (AO49 and AO66 ankyrins) with gross similarities to erythrocyte ankyrin and brain ankyrin 2, as well as a much larger form of ankyrin (AO13 ankyrin). Although AO13 ankyrin is predicted to be similar in size to vertebrate brain ankyrin 1, its carboxy-terminal domain sequence is highly acidic and distinct from that reported from brain ankyrin 1 (Chan et al., 1993).

Materials and Methods

Cloned DNAs and Nematode Strains

Nematode strains and recombinant DNA clones are listed in Table I. The insertion mutations define a single complementation group because they fail to complement in all combinations tested, i.e., the rh1042 allele failed to complement both q331 and rh1042 mutations, while the q331 allele failed to complement the rh1042 allele.

Preparation of DNA

Nematodes were cultured and DNA prepared as described previously (Brenner, 1974; Sulston and Brenner, 1974). Plasmid and phage DNAs were prepared by standard methods (Maniatis et al., 1982).

Southern Blot Hybridization

3 µg of each restriction enzyme-digested DNA were fractionated on Tris-borate 0.7% agarose gels containing ethidium bromide, and Southern blots were prepared (Maniatis et al., 1982). After prehybridizing the nitrocellulose filters (Schleicher and Schuell, Inc., Keene, NH), the hybridization was performed in 6× SSC, 0.01 M EDTA, 5× Denhardt's solution, 0.5% SDS, 100 µg of denatured herring sperm DNA/ml, and 1 µg 32P-labeled probe (10^7 cpm/µg), for 12–16 h at 68°C. The blots were washed extensively in 2× SSC and 0.5% SDS at 68°C. The TcI probe was the plasmid pCe003 (Emmons and Yesner, 1984).

Northern Blot Analysis

RNA was prepared from a mixed-stage population of N2 worms by French pressure cell disruption, lysis with a Polytron homogenizer, or grinding in liquid nitrogen in the presence of guanidinium chloride, followed by selection of ethanol precipitation (Maciuca et al., 1981). Messenger RNA was prepared from a mixed-stage population of N2 worms by French pressure cell disruption, lysis with a Polytron homogenizer, or grinding in liquid nitrogen in the presence of guanidinium chloride, followed by selection of ethanol precipitation (Maciuca et al., 1981). Poly(A)+ RNA was introduced into the EMBL3 bacteriophage lambda vector, and the resulting library was screened with a TcI probe to yield clone DDL4RF7 (Table I). The region flanking the TcI element was subcloned into a plasmid vector and used to screen a wild-type nematode genomic library in EMBL3. Six unc-44 clones were obtained (Table I) and used to identify cosmid clones (Table I and Fig. 1b).

Screening Clone Banks

Six genomic clones were obtained from a phage bank probed by standard methods (Maniatis et al., 1982). Five independent cDNA clones were obtained from ~1.4 million phage of the Barstead-Waterston bank without additional amplification (Barstead and Waterston, 1989).

DNA Sequencing and Computer Analysis

DNA was subcloned into pTZ18R, pTZ19R (Pharmacia LKB Biotechnology, Piscataway, NJ), or pBlueScript SK− (−) (Stratagene) and sequenced by the dideoxynucleotide method (Sanger et al., 1977). Appropriate subclones were obtained by using suitable restriction fragments or by the exonuclease III deletion method (Henikoff, 1984). DNA sequence was obtained from both DNA strands except in some of the introns. Sequences determined from a single strand were done at least four times. Additional analysis was done using the Pustell and MacVector DNA Sequence Analysis Programs (Eastman Kodak Co., New Haven, CT) and our own programs.

Results

Cloning the unc-44 Gene

To identify a restriction fragment length polymorphism associated with the unc-44 gene, a set of recombinants was constructed in the unc-44 region (Table I). Linkage of a TcI transposon to the unc-44 gene was demonstrated by the presence of a 12.6-kb TcI-containing EcoRI fragment in the rh1042 mutant and in recombinants retaining the unc-44 (rh1042) allele (Fig. 2, lanes 2 and 4). The wild-type N2 strain and recombinants that have lost the rh1042 mutant phenotype do not contain this fragment (Fig. 2, lanes 1, 3, and 6). Because the wild-type DNA does not contain a transposon insertion in unc-44, no unc-44-specific band appears in Fig. 2, lane 1. However, wild-type DNA did contain an 11-kb fragment when probed with an unc-44-specific probe (data not shown). In the rh1042 revertant, the characteristic 12.6-kb mutant fragment is missing and is replaced by a 14.2-kb fragment (Fig. 2, lane 3), due to the insertion of a second TcI element. Reversion of transposon-induced mutations by insertion of additional transposons has been described previously (Mount et al., 1988)

EcoRI-cleaved rh1042 DNA fragments in the 12.6-kb size range were cloned into the EMBL3 bacteriophage lambda vector, and the resulting library was screened with a TcI probe to yield clone DDL4RF7 (Table I). The region flanking the TcI element was subcloned into a plasmid vector and used to screen a wild-type nematode genomic library in EMBL3. Six unc-44 clones were obtained (Table I) and used to identify cosmid clones (Table I and Fig. 1b).
rh1042 allele is a Tcl insertion within the DD#PAO13 open reading frame. The six insertion mutations and their in-frame excision in mn259, q331, and rh1013 revertants provide proof that the unc-44 gene has been cloned. The revertants of rh1042 and st200 are secondary Tcl insertions at RNA splicing junctions and presumably restore gene activity by altering RNA splicing.

**Cloning of cDNAs**

To clone cDNAs corresponding to the regions surrounding the unc-44 DNA insertions, two genomic DNA fragments were used to probe nematode cDNA libraries: (1) the 11-kb genomic BamHI fragment flanking the Ted insertions in q331 Table 1. Nematode Strains and Cloned DNAs and rh1013 toward the 5'-end of the gene (corresponding to clone DD#PPB40 in Fig. 1 b), and (2) the 3.7-kb SalI fragment flanking the Tcl insertion in rh1042 toward the 3'-end of the gene (corresponding to DD#PSLR8 in Fig. 1 b). DNA sequencing was performed on two clones, DD#PAO49 and DD#PAO66, obtained with the 11-kb probe, and one (DD#PAO13) of the three independent clones with identical restriction patterns obtained with the 3.7-kb probe.

**The Ankyrin Repeats Are Grouped into Six Clusters**

The overall structure of the AO49 and AO66 ankyrin isoforms (Fig. 3) can be inferred from the domains present on the 11-kb BamHI genomic (DD#PPB40) and the cDNA.
The ankyrin repeat, spectrin binding, and most of the conventional regulatory domains are arranged in 12 exons on clone DD#PAO66 (Figs. 1 c and 4). The 23 ankyrin repeats are grouped into six exons containing essentially 1, 5, 8½, and 10 ankyrin repeat exons in the human ANK gene (Tse, 1990). The clustering of repeat elements and the bifurcation of individual repeats is in contrast to the large number of single nematode repeats (Fig. 5). The conservation of individual repeat sequences in organisms as divergent as humans and nematode, mouse erythrocyte, human erythrocyte, and brain ankyrins (Fig. 6) suggests stringent limits on the three-dimensional structure. The 7-aa insertion in repeat 5 of human brain ankyrin, the lengths of the corresponding nematode, mouse erythrocyte, human erythrocyte, and brain ankyrin repeats are identical. The constancy of the individual repeat lengths suggests stringent limits on the threedimensional structure. The 7-aa insertion in the human brain ankyrin repeat 5 is not present in the unc-44 genomic DNA, and therefore could not be obtained by simple alternative RNA splicing in the nematode. Repeats 2 through 6 and 8 through 12 contain the greatest number of identical residues (18 or more). There is 52% identity (396 of 755 residues) in the ankyrin repeat domains of the various ankyrins. The unc-44 ankyrin repeat domain is more closely related to brain ankyrin (13% or 96 brain-specific residues, noted by asterisks in Fig. 5) than to erythrocyte ankyrin (5% or 39 erythrocyte-specific residues, noted by equal signs in Fig. 5). A number of residues are unique to the vertebrate ankyrin (13% or 101 residues, noted by periods in Fig. 5). The unc-44 amino-terminal domain preceding the ankyrin repeats is also more similar in size and sequence to human brain ankyrin than to erythrocyte ankyrin (Fig. 5). These results suggest that the unc-44 products are more closely related to the vertebrate brain ankyrin than to erythrocyte ankyrin.

Figure 2. Southern blot analysis of the unc-44 (rh1042) allele. A Southern blot was prepared from EcoRI-digested DNA samples and probed with a TcI plasmid. The samples are as follows: (lane 1) wild-type N2; (lane 2) N3401 unc-44 (rh1042); (lane 3) N3413 unc-44 (rh1042) rev-1 revertant; (lane 4) NJ416 unc-44 (rh1042) bl-6 (sc6) recombinant; (lane 5) NJ417 unc-44 (+) bl-6 (sc6) non-Unc, Blister recombinant; and (lane 6) NJ418 unc-44 (+) (sc6-10). Note the 12.6-kb fragment in the unc-44 mutant strains (arrows in lanes 2 and 4) and absence of this band in the other strains. In the case of a revertant, the 12.6-kb band is converted to a 14.2-kb band (arrow in lane 3).

unc-44 cDNA Fragments Encode Spectrin-binding and Regulatory Domains

DNA sequence analysis of DD#PAO49 and DD#PAO66 partial cDNA clones reveals linker, spectrin-binding, and regulatory domains noted. The alternatively spliced microexon present in A049 ankyrin is underlined. At the bottom of the figure, the alternative carboxyl termini are noted. Amino acid residue positions are noted at the left margin.

Figure 3. Predicted amino acid sequences of A049 and A066 ankyrin isoforms. The predicted protein sequences of A049 and A066 ankyrins are shown with the ankyrin repeats (rl-r23), linker, spectrin-binding, and regulatory domains noted. The alternatively spliced microexon present in A049 ankyrin is underlined. At the bottom of the figure, the alternative carboxyl termini are noted. Amino acid residue positions are noted at the left margin.
Figure 4. The nucleotide sequence of the 11-kb BamHI genomic DNA fragment. The nucleotide (nt) sequence of the 11-kb BamHI genomic fragment including the ankyrin repeat, linker, spectrin binding, and a portion of the regulatory domains is shown. The ankyrin repeats are noted below the protein sequence. The point of overlap with the DDP0409 cDNA clone is noted. Amino acid residue positions are noted at the left margin. These sequence data are available from EMBL/GenBank/DDJB under accession number U21734.

it is not known whether the spectrin interaction with this region is specific (Davis and Bennett, 1990; Platt et al., 1993). The presence of the three introns in the linker domain could provide sequences for additional alternatively spliced exons.

Searches of the intron sequences for vertebrate-like sequences failed to reveal cryptic exons.

A 47% protein sequence identity (198 residues) was found between brain, erythrocyte, and nematode ankyrins in the
Figure 5. Comparison of the ankyrin repeat, linker, and spectrin-binding domains. The ankyrin repeat, linker, and spectrin-binding domains of nematode (n), mouse erythrocyte (mr) (White et al., 1992), human RBC (hr) (Lambert et al., 1990; Lux et al., 1990), and human brain (hb) (Otto et al., 1991) are compared. Identical residues are shown as inverse text. The consensus (c) sequence is shown below the various ankyrin sequences with brain-specific (*), erythrocyte-specific (=), and vertebrate-specific (.) residues noted. Ankyrin repeat numbers are listed at the left and the positions of introns in the nematode ankyrin are shown by the vertical arrows.

424-aa region of greatest spectrin-binding domain similarity (Fig. 5). The spectrin-binding domain similarity is intermediate between vertebrate brain and erythrocyte isoforms, suggesting that a single nematode gene may substitute for the multiple ankyrin genes in vertebrates. As with the ankyrin repeat domain, the spectrin-binding domain contains more brain-specific residues (12% or 50 residues) than erythrocyte-specific sequences (6% or 24 residues), and 18% (75
The AO49 and AO66 Ankyrin Carboxyl-terminal Domains

As is the case among the vertebrate conventional ankyrins (White et al., 1992), the regulatory domains of the AO49 and AO66 ankyrins are less well conserved than the ankyrin repeat and spectrin-binding domains. For this reason, the carboxyl-terminal assignments of sequence similarity are tentative. The nematode ankyrin regulatory domains are smaller than those in vertebrates. Using the regulatory binding domain boundary defined by Lux et al. (1990), the sizes of the regulatory domains are as follows: AO66, 381 aa; AO49, 324 aa; human red blood cell (RBC) 2.1 ankyrin, 497 aa; 2.2 ankyrin, 336 aa; and human brain ankyrin 2, 401 aa. Although weak, the amino acid sequence identities are greatest at the extremities of the regulatory domain (Fig. 7). Starting at aa position 1432 of full-length AO49 ankyrin, which marks the beginning of the predicted regulatory domain, a region of ~160 aa can be aligned with the vertebrate ankyrins. Beyond this point, neither AO49 nor AO66 ankyrins shows an obvious version of the band 2.1 exon that is spliced out in band 2.2 ankyrin. At position 1655, there is a short sequence in the vertebrate erythroid and nematode ankyrins that resembles a single copy of the 12-aa repeat found in brain ankyrin 1 (Otto et al., 1991). At position 1702, there is a poor similarity to a small region at the 3' end of the "2.1 exon." Downstream of this sequence at position 1755 are two copies of a sequence that shares a similarity with brain ankyrin 1. The alternative splice in AO49 ankyrin near position 1810 provides an ending that is similar to the alternative splice at the very 3' end of erythrocyte ankyrin (Lambert et al., 1990). Present at position 1815 of the AO66 ankyrin carboxyl terminus, but not in AO49 ankyrin, is a similarity to the carboxyl termini of the conventional vertebrate ankyrins.

DNA Sequence Analysis of the AO13 Ankyrin Carboxyl Terminus

The DNA sequence of DD#PAO13 reveals a potential protein fragment that is quite different from that putatively encoded by the 6-kb transcripts (Fig. 8). Northern blot analysis has shown that the DD#PAO13 cDNA clone corresponds to the 3' end of a ~14-kb RNA. The deduced protein fragment is to alternative splicing is shown below the DD#PAO66 sequence. The 3' end of DD#PAO49 follows nt 2728 of DD#PAO66. The inverted repeat sequences at the breakpoints of the inversion near the 5' end of the cDNAs (starting at nt positions 100 and 275 in DD#PAO49 and at position 74 in DD#PAO66) and a potential polyadenylation site (AAATAA) at the 3' end of DD#PAO49 are underlined. A single nt difference at position 731 results in arginine 244 (AGA) being substituted by lysine (AAA) in DD#PAO49 and is assumed to be a cloning artifact. Potential hairpin structures near the 3' ends of the cDNAs are noted by arrows. These sequence data are available from EMBL/GenBank/DDBJ under accession numbers U21731 and U21732.

Figure 6. The AO49 and AO66 cDNA and predicted protein sequences. The DD#PAO49 sequence has been broken into three parts and aligned with the DD#PAO66 sequence. The 5' end of DD#PAO49 joins the DD#PAO66 sequence at nt 97 (aa 31) just to the right of the DNA recombination site. The 18-nt insertion due
of the deduced protein fragment, the most notable feature is a repeat of the sequence S(L/V) (T/S) SL (Q/A) EFERL-EKE in the central portion (repeat A in Fig. 8). The transposon insertion in the rh1042 allele is located between these repeats (Fig. 8). A shorter set of repeats containing the sequence TDSL occurs near the carboxyl terminus (repeat B in Fig. 8).

In the 3' untranslated region of the cDNA, there are four inverted repeat sequences which may fold into hairpin structures in the mRNA. Three of the structures contain the sequence GCCCCAA in the loop of the hairpin.

**Multiple Transcripts Encode Conventional and Large Ankyrins**

Northern blot analysis revealed a major 6-kb band and several minor transcripts, including a ~14-kb transcript(s). To analyze the transcripts that arise from the 5'-half of the gene, probes from the AO49 spectrin-binding and regulatory domains were used (Fig. 9 a, lanes 1-6). When blots of wild-type poly A-selected RNA (Fig. 9 a, lane 1) or total RNA (Fig. 9 a, lane 2) were probed, a major band at 5.95 ± 0.26 kb (n=79) was observed along with several less prevalent bands, including 3.19 ± 0.13 kb (n=45), 5.07 ± 0.10 kb (n=20), 6.91 ± 0.32 kb (n=32), and 13.86 ± 1.25 kb (n=8). Of particular interest is the wild-type ~14-kb minor transcript, near the limit of detection in Fig. 9 a, lanes 1 and 2, and Fig. 9 b, lanes 1 and 2. As detailed below, the ~14-kb transcript is the only transcript affected by all the insertion mutations, and therefore alterations in this transcript are responsible for the uncoordinated phenotype.

Besides the 5.95-kb, 6.91-kb, and 13.86-kb bands that were present in wild-type RNA, additional bands differing by the 1.6-kb size of Tcl were detected at 7.64 ± 0.28 kb (n=7), 8.55 ± 0.10 kb (n=4), and 14.79 ± 0.79 kb (n=9) in rh1013 mutant RNAs (Fig. 9 a, lanes 3 and 4). It appears that the transposon is transcribed into RNA, and somatic excision of the transposon gives rise to the normal sized RNAs in the mutants (Emmons and Yesner, 1984). In the rh1042 mutant, the 5.07-, 5.95-, and 6.91-kb transcripts were unaffected by the downstream Tcl element (Fig. 9 a, lanes 5 and 6). However, as in the rh1013 mutant, the rh1042 mutant RNA revealed the ~14-kb and ~15-kb bands. The ~15-kb band accumulates in the mutants to greater levels than the ~14-kb band, making it more readily detectable.

Transcripts overlapping the 3'-end of the gene were analyzed with DD#PAO13 riboprobe (Fig. 9 a, lanes 7-10). The ~14-kb transcript was observed in wild-type RNA (Fig. 9 a, lanes 7 and 8), and both ~14- and ~15-kb bands were found in the rh1013 mutant (Fig. 9 b, lanes 9 and 10). The spanning of the entire gene by the ~14-kb RNA was demonstrated by probing blots of total RNA with the ankyrin repeat probe, stripping the blots, and reprobing the same blots with the DD#PAO13 riboprobe. Probing wild-type RNA with an ankyrin repeat probe (Fig. 9 b, lane 1) revealed a weakly hybridizing ~14-kb band at exactly the same position as that obtained by reprobing with a DD#PAO13 probe (Fig. 9 b, lane 2). Further evidence for a transcript that spans the entire gene is provided by analysis of the rh1013 and rh1042 mutant RNAs. Common bands at ~14 and ~15 kb were found in rh1013 RNA with both probes (Fig. 9 b, lanes...
Figure 8. The predicted A01 ankyrin carboxyl domain sequence. The nt and predicted aa sequences are presented as in Fig. 5. The uncertainty in the position of the TcJ element in the rh042 allele is due to the two-base-pair duplication created during TcJ insertion. The repeated motifs are noted by double underlines. Potential glycosylation sites (Glyc) and an RGD sequence (Singer et al., 1987) are noted by underlines. Potential hairpin structures are noted by arrows, and numbered, above the DNA sequences. Potential hairpin structures are as follows: N2 wild-type, lanes 1, 2, 7, and 8; unc-44 (rh013), lanes 3 and 4; and unc-44 (rh042), lanes 5 and 6. The sizes of the RNAs were determined relative to 0.24-9.49-kb RNA markers (GIBCO-BRL, Gaithersburg, MD). The sizes of the RNAs larger than 9.49 kb were determined by extrapolation from the size marker curve (Otto et al., 1991). In panel b, common bands in the ~14-16-kb region (arrow) were detected by probing blots with the ankyrin repeat probe (odd lanes), and then reprobing the stripped blot with the DDBPA049 riboprobe (even lanes). Total RNA samples are as follows: N2 wild-type, lanes 1 and 2; unc-44 (rh013), lanes 3 and 4; and unc-44 (rh042), lanes 5 and 6. In panel c, the ~14-16-kb region of the Northern blot has been expanded by running the gel for an extended period of time. A ~14-kb band is present in wild-type RNA (lane 1), while ~14- and ~15-kb bands (arrows) are present in RNAs from unc-44 (q331) (lane 2) and unc-44 (rh013) (lane 3) when probed with the ankyrin repeat probe. In a manner similar to rh013, the q331 mutation affects several of the smaller mRNAs (data not shown).

Discussion

In this paper, we report the molecular cloning of unc-44, a gene that is required for the correct targeting of axons to appropriate partners, and the identification of the putative gene
products as ankyrin-related proteins. Although the presence of ankyrin in the brain has been known for some time (Drenkhahn and Bennett, 1987), this paper provides the first evidence that anknyres are required, directly or indirectly, for axonal guidance.

The following facts demonstrate that the unc-44 gene has been definitively cloned. First, six unc-44 mutations are due to DNA insertions. Four alleles (mn259, q331, rh1013, and rh1042) are TcI insertions. The remaining two alleles (mn339 and st200) are insertions that are larger than TcI. Second, four revertants of q331, rh1013, and mn259 are in-frame excisions of TcI. Because in-frame excisions of TcI are unusual, these results demonstrate that the restoration of the reading frame is critical for the function of the unc-44 ankyrins. Third, complementation tests show that the DNA insertion mutations define a single complementation group, and that this complementation group corresponds to unc-44. Fourth, Northern blot and cDNA sequence analysis demonstrates that multiple transcripts are generated from unc-44, but that only the ~14-kb transcript(s) is affected by all the insertion alleles tested.

The isolation of several different cDNA clones demonstrated that the unc-44 gene produces several alternatively spliced transcripts, with the most abundant RNA being ~6 kb. The ~6-kb RNA is smaller than the major vertebrate erythrocyte mRNAs which range from 6.8 to 9.5 kb (Lambert et al., 1990; Lux et al., 1990). However, the unc-44 conventional ankyrins have smaller regulatory domains than the vertebrate proteins, which might account for part of the difference. The pattern of multiple mRNAs generated from unc-44 (~3, 5, 6, 7, and 14 kb) is similar to the pattern of 4, 7, 9, and 13 kb RNAs from the neuronal ANK-2 gene (Otto et al., 1991). The presence of alternatively spliced unc-44 RNAs suggests that the products of this gene may play varied roles in the organism.

From the unc-44 mutant phenotype, the complementation data, the RNA analysis, and the positions of the unc-44 mutations, we propose that the large AO13 ankyrin is required for proper axonal guidance in C. elegans. The lack of cysteines in the central region of the AO13 ankyrin fragment and the predicted highly α-helical character of the domain suggest that the carboxyl-terminal domain has an extended structure. Exact repeats of the sequence EFERLEKE may indicate a functional role for these sequences.

This is the first demonstration that ankyrin plays a functional role in neural development. A role for AO13 ankyrin in neural development is reinforced by the finding that the 440-kD ANK-2 product is present in the developing rat brain and is localized to neuronal processes (Kunimoto et al., 1991; Otto et al., 1991; Chan et al., 1993). Consequently, the predominant 6-kb unc-44 messenger RNAs and the AO49 and AO66 ankyrins may not be critical for axon guidance.

Because the nematode unc-44 ankyrin gene represents an evolutionarily primitive form, the conserved amino acid residues provide a starting point for structure-function analysis by site-directed mutagenesis and DNA transformation. The cloning and partial sequencing of the unc-44 gene provides the foundation for molecular genetic analyses which may reveal specific roles for the various unc-44 products in neurons and other cell types.

We thank the group of R. Herman for the kind gifts of the unc-44 (mn259), mn339, q331, and st200 mutants and revertants isolated in the laboratories of R. Herman, J. Kimble, J. Shaw, and R. Waterston; R. Nishiwaki and J. Miwa for the nematode genomic bank; R. Barstead, R. Waterston, I. Schauer, and W. Wood for cDNA bank; A. Coulson and J. Sulston for identifying and providing the cosmid clones; S. Emmons for the TcI clone; J. Matsuzaki and S. Weldon for discussions; H. T. Cheung for his continued encouragement; W. Li for communication of unpublished results; H. T. Cheung and H. Brockman for critically reading the manuscript; and the Caenorhabditis Genetics Center for strains.

R. Franco was a National Institutes of Health postdoctoral trainee (grant T32-HDO-7299) for a one year period. This work was funded by gifts from TONEN, Inc., Saitama, Japan, a Biomedical Research Support Grant, Illinois State University Research Grants, and grants from the American Heart Association Illinois Affiliate and the National Science Foundation to A. J. Otsuka.


Received for publication 17 November 1994 and in revised form 28 February 1995.

References


