The *Drosophila* Couch potato protein is expressed in nuclei of peripheral neuronal precursors and shows homology to RNA-binding proteins

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Through enhancer detection screens we have isolated and cloned an essential gene that is expressed in the neuronal precursors and their daughter cells in the *Drosophila* embryonic peripheral nervous system (PNS). The gene is named *couch potato* (*cpo*), because several partial loss-of-function alleles cause hypoactive behavior in adults. Here, we present evidence that the structure of the *cpo* locus is unusually complex: It spans >100 kb, encodes three different messages, is differentially spliced, lacks an AUG initiation codon, and may encode three different proteins. Two putative Cpo proteins contain similar but nonidentical RNA-binding domains that are most homologous to the RNA-binding domains of the *Drosophila* embryonic lethal *abnormal vision* (*elav*) gene and a human brain protein that has been implicated in a paraneoplastic sensory neuropathy. Polyclonal antibodies raised against a fusion protein localize Cpo to the nucleus. Immunocytochemical studies demonstrate that the *achaete-scute* and *daughterless* genes are required for proper expression of *cpo* in the PNS but not in other cells that express *cpo*. On the basis of our observations, we present a model in which *cpo* is controlled by genes that determine cells to become PNS cells. Cpo, in turn, may control the processing of RNA molecules required for the proper functioning of the PNS.

[Key Words: *couch potato*; enhancer detection; peripheral nervous system; neural precursors; RNA-binding protein]

Received June 24, 1992; revised version accepted August 12, 1992.

To unravel the genetic and molecular pathway by which neurons differentiate, we have chosen to study the developing peripheral nervous system (PNS) of *Drosophila*. The cells of the embryonic PNS differentiate from neural precursor cells named sensory mother cells (SMCs). The SMCs are selected from small groups of lateral neuroectodermal cells, the proneural clusters. The SMCs delaminate from the proneural clusters into a subectodermal position and divide in a characteristic pattern to form sensory organs (Bodmer et al. 1989). The genes encoded by the Achaete-Scute Complex (AS-C) (Ghysen and Dambly-Chaudiere 1989a) and the *daughterless* (*da*) locus (Caudy et al. 1988a,b) are known to play key roles in determining which cells in the proneural clusters become competent to form SMCs. Embryos that lack AS-C or *da* genes lack many or all SMCs and PNS organs, and these genes have therefore been named proneural genes (Ghysen and Dambly-Chaudiere 1989a,b; Romani et al. 1989). The products encoded by the AS-C genes and the *da* gene are helix-loop-helix (HLH) proteins, which act as heterodimeric transcription factors (Murre et al.

1989). It seems likely that these HLH proteins initiate the expression of a cascade of differentiation genes to form the sensory organs. Preliminary evidence indicates that similar mechanisms may also operate in the development of vertebrate PNS (Johnson et al. 1990; Lo et al. 1991; Johnson et al. 1992).

It is possible that the identity of a specific sensory organ is controlled by genes that act on subsets of SMCs. However, some genes may be required for all SMCs, controlling aspects of differentiation specific to the PNS. In an attempt to identify such PNS genes we have screened for genes that are expressed in most or all SMCs and their daughter cells using enhancer detector screens. This has permitted us to isolate an essential gene, *couch potato* (*cpo*), that is expressed in all SMCs (Bellen et al. 1992). Behavioral and genetic studies have indicated that partial loss-of-function *cpo* alleles cause aberrant adult behavior because mutant *cpo* flies exhibit abnormal geotaxis, phototaxis, and flight behavior (Bellen et al. 1992). In this report we describe the structure of the *cpo* gene, the sequence of multiple cDNAs, the characteristics of the Cpo protein, and the distribution of the protein in different mutant backgrounds. Our observations suggest that *cpo* may play a role as a PNS differentiation gene by

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regulating the processing of nervous system-specific transcripts.

Results

Structure of *cpo*

The *cpo* gene was identified through P-element-mediated enhancer detection. P-element insertion strains were selected that express β -galactosidase in the SMCs of the embryonic PNS and more differentiated cells of the PNS. Of 22 strains that express β -galactosidase in all SMCs (of 4500 lines screened), 15 were shown to contain an enhancer detector P element at cytological subdivision 90D (Bellen et al. 1989, 1992; Bier et al. 1989; Wilson et al. 1989). Using plasmid rescue we isolated unique genomic fragments flanking the enhancer detectors and initiated the bidirectional walk shown in Figure 1. Approximately 110 kb of genomic DNA from cytological subdivisions 90D and 90E have been cloned.

We isolated 62 cDNAs by screening $\sim 10^6$ plaques (Zinn et al. 1988; Bellen et al. 1992). The three longest cDNAs, 61.1 (3.4 kb), 17 (3.0 kb), and 61.2 (2.5 kb), were selected for further study. The cDNAs were hybridized to the genomic phages to construct a low resolution map. However, because the cDNAs as well as phages of the walk contain OPA repeats (Wharton et al. 1985), the high resolution map shown in Figure 1 was established by sequencing the three cDNAs (see Fig. 2) and the corresponding genomic fragments to which the cDNAs hybridized.

Sequence analysis of these cDNAs shows that they differ at both their 5' and 3' ends. The structure of the

three cDNAs is shown in Figure 1 and described in the legend. The sequence of the three cDNAs is shown in Figure 2. We have also sequenced the exon-intron boundaries for the 61.1 cDNA. Most splice donor sequences are consistent with consensus sequences flanking *Drosophila* introns (Mount et al. 1992), with the possible exception of the splice donor at the boundary of exon 3-intron 3 (see 3A and 3B at bottom of Fig. 2A sequence).

As shown in Figure 3, Northern analysis of 3- to 24-hr-old embryos with unique probes derived from the 3' end of 61.1 shows that the message from which the 61.1 cDNA is derived is ~ 7.8 kb long. A probe derived from the 61.2 leader recognizes two messages of ~ 4.5 and 5.0 kb, suggesting that another alternatively spliced product exists that does not correspond to an isolated cDNA. The Northern blot data show that the 61.1 and 61.2 cDNAs are not full length. No typical polyadenylation signal is found upstream of the poly(A) tail, suggesting that they are the result of internal oligo(dT) priming. As shown below, preliminary evidence suggests that the sequences missing from 61.1 are confined to the 3' end.

Three different transcripts encode three different putative proteins

Sequence analyses of three *cpo* cDNAs show that they contain three distinct, but overlapping, long open reading frames (ORFs). The 5' ends of these ORFs are shared by the three cDNAs, but the ORFs differ at their 3' end owing to alternative splicing. *Drosophila* codon usage is respected throughout most of the common ORFs, but it

Figure 1. Structural map of the *cpo* locus. Map of genomic DNA and *cpo* transcription units. Genomic phage inserts spanning the region are shown above an *Eco*RI and *Xba*I restriction map. (As there are many *Eco*RI and *Hind*III sites in phages 1:1 and 1:3, not all sites have been mapped). The boxes below the map show the approximate size and location of the *cpo* exons of three different cDNAs (61.1, 61.2, and 17). Solid boxes represent leader and trailer sequences; open boxes represent ORFs. The leader of 61.2 (A) consists of three exons, A0, A1, and A2; and the leader of 61.1 (B) contains two exons, B1 and B2. The A2 and B2 exons splice to a common third exon containing most of the ORF. The ORF initiates at the 5' end of exon 3 and extends into exons 4, 5, and A6 for 61.2 cDNA, and exons 4, 5, B6, B7, and B8 for 61.1 cDNA. Exon B6 is included in exon A6, but exon B6 splices to B7 in the middle of exon A6. The third exon is large for an internal exon (1.5 kb) and is followed by a 42-kb intron. All of the remaining exons and many of the introns are small, except the last exons. The 17 cDNA contains only a single exon, C3, which is almost identical to the genomic DNA sequence. This cDNA is 15 nucleotides longer than A3 at the 5' end and extends ~ 1.7 kb, 3' of exon 3. The phage to which exon A0 maps has not been linked to the walk yet. All the enhancer detectors map at or close to position 0. The sizes of introns and exons are shown in Fig. 2.

[illegible]

Int.ron#:	Exon-Int.ron	Int.ron-Exon
1	TATTT-GTAAATCAAAAC	TGATCTCTGGGAC-AAG
2	AAAG-GTAAATATTTC	TGCTCTTTGGGAC-GTG
3A	GAGC-CTAGATGACAGCTT	GTCAATCAITGGAC-TTC
3B	AGAG-GTGAGTCAAGCATT	TGTCATCAATGAC-GTT
4	TATC-GTGAGTTTTTCAT	TACACACACACAG-AGC
5	ATCC-GTGAGTAAAGGTT	AAATATTTTACAG-CGC
6	GGAG-GTAAATATGACGA	TCAITTTACAGCAG-GST
7	GGAC-GTAGTGAAGAAAC	TCTGGACTCGGAG-ATG
8	TCAA-GTAGTGGAAAGC	CCCCACTTTGAC-ATG

[illegible]

C

0001 GCTCTTTTGGCAGTTCACACTGA...start of ORF same as 61.1 until base 2394...
1369 GTG AGT CAC AGC TTC CAT TCC GGC ATC CCA AAA GAT AAA CGA AAT AAA AAG CTG
450 val ser his ser phe his ser ala ile pro lys asp arg asn lys lys leu
1423 CAG ATA AAG ATA GCG TTG GCG GGG CAA AAA CGA AAA GCT CGC CCC AGC AGC
468 gln ile lys ile ala val gln gly gln lys arg lys gly ser gly pro ser ser
1477 GAA ATG CGA CAA AAG TGA CAGCTTGGGGATACAGAGTCTGCTGACTGAATATTTGCATAAAAT
486 glu met arg gln lys ***
1541 CGGTGGAAAGTCCCGCAAACTCTCTCGCGACGTCTTAAGTGAGTACTAGAAATTTGGCGTAGG
1611 ATTTCATTTTGATGATACAAAGCAAGGTCCTCAATGAAATGTGTTCTGGGATATATCTTTGGGGGGGGGG
1681 TCGTTGGATTTTCAGAAATTTGCTAGTACTAAATTTGGCAGCTACCATCTTAAATTTAAAGCTTTTGTGA
1751 CAGTTTTCATCTTTGAAATTTTCGCGTAGCAACAAATATTAATTTATTCATTTCTTTCATAGGTTATCCCT
1821 TCTAAAAAAATACCCCTTTTCTCTCCCTTCACAGCTTGATTTTATGAGTCTCAAAATTTAAAGGTTCAATA
1891 AATTCAATCCAGATGTGTGAAAGATCTCTTTCAATCAATTCGCTTTCGACTGAATCAATTTAAAGACTTTTC
1961 GCAAGATTTTCGCTGCATATGAAGATATGATCGATATGTCAGTGTGTTCTGGCCCAATACAGCAACCAAC
2031 CACTTGGAAATATCCCTGAAATATGTGTGCGCTACACACAGCTGGCAGCTCGGGCGTAGAATTTTGTGG
2101 GCGCCCTTTGTGTTTGGCCAAATAAAGGGGGGGGCTGCTGTTTCTTAAAGCGGAAAGCTGGG
2171 GAGGCGAGGGAAGTTGTGGGCGATGCTTGCAGCTTGSTGTAGTGGGATGTGCACCATTTGTTGTGTCTAC
2241 TCGTGTTCGAATCGGCTGTGTCTGCTTACATTTTGATTTTATGAGTGGCTGTGTTCTATGTCACAGATATTTAC
2311 TTTCCTTCGCGACAGCGTGCCACGCGATATACCGCGGCGAGTGGCGCGAGCCACACCCATCAAGCAGCA
2381 GCTGCTTCCCTTTTCATACAGCTGTACCCACGCTACACCGACCAACACCGACCCACCATGCTTTGTCGTGCT
2451 GCATCGTGTTCATGTTCACGTTGGTGAAGTGGCTTACACCGAAGCAAGCGGCGTGTGTTGAGAGGGGGGT
2521 TGGTTTAAAAAAAGGGGGGGGGGCTTACCACCTACTACCGAGCGATGGTTTAAATTTGATTTTATCCCTT
2591 GATTAATAATAGTCTCTCTATTTTGTGTTTACTTACACCTTCCACATTCGTTATGATTTTATGATTTTATTCGCG
2661 GTAGATTTTGGGAATTTTATTTGAAGTCTTACTCCGACAGGTTTTCCTGAATAGTTGCGAGAGCTTATTTGGCA
2731 TGGGAAAGTTTGACATGAAGATTTATGTTTAAAGCGGGCGAAGATTCGACAGCATATATTTAAGATATTT
2801 TAATATCTGATATTCACCTACGCGATGCTGATTTTTCGACAGACAGATGACAGAGAAACCTTTTCCGCCCTT
2871 GCTGCTGATTTTAGTCCACGGAAATTCGATTTAAAAATGCATCAATCACTTGGTTCGCCCTTGCCTCACT
2941 TCACCTTCCCGACAGCTCTGCAGTTTCTCGGCCAAATTAAGAGCGAAGGAAATTCAGTTCGGTGGAGAT
3011 AAGATTGCACATGAAAAAAGGAAAT

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is respected poorly at the 5' and 3' ends of the ORFs. A number of much shorter ORFs can be identified in the three cDNAs, but these have generally poor *Drosophila* codon usage. Although the first methionine codon within the ORFs occurs at codon 423, several lines of evidence suggest that translation is initiated upstream of this site in embryos. First, the context of codon 423 does not conform well to the *Drosophila* consensus for translation initiation (Cavener 1987). Second, *Drosophila* codon usage is maintained upstream of codon 423 and drops abruptly between codons 30 and 40 of the ORFs, suggesting that most of the ORF is translated. Third, the products of translation initiating at codon 423 have predicted molecular masses of 22 kD or less, far smaller than the 50-kD products detected on immunoblots. Fourth, in vitro translation shows that the three cDNAs can be translated, albeit at low efficiency, into several proteins, including a 50-kD protein (data not shown). Fifth, extensive sequencing of genomic fragments encoding the sequences for the cDNA did not reveal sequencing mistakes. These observations suggest that translation is initiated upstream of codon 423, possibly between codons 30 and 40.

The putative proteins encoded by the three ORFs can be subdivided into four domains on the basis of amino acid composition (Fig. 2). These domains are a polyalanine-rich stretch, followed by a proline- and histidine-rich domain containing a putative bipartite nuclear translocation signal (Robbins et al. 1991) and seven P-V/I/A-S-V/A/H-P repeats of unknown significance (1–193); an OPA repeat (194–296) (Wharton et al. 1985); a serine-rich region with two homopolymeric stretches and serine-alanine repeats (297–426); and a domain that is different in each clone. This domain contains an 80-

amino-acid core sequence in 61.1 and 61.2 cDNAs: the ribonucleoprotein consensus-type domain (RNP-CS-type) found in many RNA-binding proteins. The Cpo RNP-CS-type domains are most homologous to a human brain protein (HuD) (Szabo et al. 1991) and its *Drosophila* homolog *embryonic lethal abnormal vision* (*elav*) (Robinow and White 1988). Both ELAV and HuD are expressed in the nervous system and contain three RNP-CS-type domains, whereas *cpo* cDNAs only contain one RNP-CS-type domain. The two Cpo RNP-CS-type domains differ in 15 amino acids at their carboxyl terminus owing to differential splicing (italicized in Fig. 4B).

P-element enhancer detectors are inserted in key regulatory sequences

Genetic evidence indicated that key regulatory sequences of *cpo* are present in the vicinity of the enhancer detectors. Southern blot analysis showed that all of the P elements are clustered in a 200-bp genomic fragment just upstream of the 5' end of the 61.1 cDNA. To show that the sequences surrounding the enhancer detectors contain *cpo* regulatory sequences, a 10-kb *Xba*I genomic fragment (derived from wild-type DNA present in phage 12:4; see Fig. 1) carrying these putative regulatory sequences, as well as the B1 and B2 exons and the first intron, was fused to a heterologous promoter driving expression of the *lacZ* gene (Hiromi and Gehring 1987). Embryos of two independently transformed strains were stained with an anti- β -galactosidase antibody. As shown in Figure 5, the expression pattern of *lacZ* essentially corresponds to the expression pattern observed in the enhancer detector strains. However, staining in many

Figure 2. (A) Nucleotide sequence of the *cpo* 61.1 cDNA, its 5'-flanking genomic DNA, the insertion sites of the enhancer detectors, and the predicted amino acid sequence of the *cpo* ORFs. Putative binding sites for transcription factors in genomic flanking sequences are shadowed or shown in small, bold uppercase letters. They correspond to three GAGA protein-binding sites, a C/EBP-binding site, and three Zeste-binding sites. The single bold underlined uppercase letter corresponds to the first nucleotide of the longest of three independently isolated cDNAs of the 61.1 type. The bold A in TTCAGTTT corresponds to the putative transcription start site. The insertional location of 10 enhancer detectors is shown. Enhancer detectors that are in the same orientation (5' → 3') as *cpo* transcription are shown between angle brackets (<>), those inserted in the opposite orientation (3' → 5') are shown between inverted angle brackets (><). (l) Lethal; (cp) Couch potato phenotype; (v) viable [see Bellen et al. (1992)]. The insertional consensus for the 8 nucleotides next to the 10 P elements shown here is GXTCAGGC. O'Hare and Rubin (1983) found GGCCAGAC. Exon-intron and intron-exon boundaries are hyphenated and followed or preceded by 13 genomic nucleotides. The sizes of the introns are indicated. The longest ORF is translated. In the absence of an AUG initiation codon, the first codon was numbered 1. The splice donor and splice acceptor sites are tabulated below the sequence. The boundaries of the third intron cannot be determined and the two alternative possibilities are shown as 3A and 3B (Fig. 2). In either case, the splice donor or the splice acceptor site does not conform to the consensus. Two and possibly three splice acceptor sites do not conform to the consensus (Mount et al. 1992). Those of introns 4 and 5 contain few pyrimidines when compared with most splice acceptor sites. The estimated molecular mass of the protein if the entire ORF is translated is 64 kD. Differences were observed between the cDNA sequence and the genomic cDNA: at 1115, the genomic sequence is C; at 1398, the genomic sequence is T; at 1578–1582, the genomic sequence is TGAC; at 1671, the genomic sequence is A; at 1767, the genomic sequence is G. Between 1926 and 1927, the 61.2 cDNA has an additional CAG repeat, which is not present in the genomic DNA and the 61.1 cDNA. At 2208, the genomic sequence is T. (B) Nucleotide sequence of the 61.2 cDNA. Only the sequences that are not shared between the 61.2 and 61.1 cDNAs are shown. The leader consists of at least three exons (A0, A1, and A2), but the boundary between exon A0 and intron 0 has not been determined precisely. This cDNA shares exons 3, 4, and 5 with 61.1. Exon A6 is only partly shared by the two cDNAs. A6 remains contiguous with the genomic DNA, whereas the 61.1 cDNA splices to exon B7 at 2588. The estimated molecular mass of the protein if the entire ORF is translated is 56 kD. (C) Nucleotide sequence of the 17 cDNA. The 17 cDNA consists of a single exon, C3. It contains 15 bp upstream of exon 3 of the 61.1 cDNA and 1.7 kb downstream of the site where 61.1 and 61.2 are spliced off. It shares only exon 3 with 61.1 and 61.2. The estimated molecular mass of the protein if the entire ORF is translated is 51 kD.

Figure 3. *cpo* Northern analysis and Cpo immunoblot. (A) Northern blots. Ten micrograms of poly(A)⁺ RNA isolated from 3- to 24-hr-old embryos was run on an 0.8% agarose gel, transferred to a membrane, and hybridized with different probes. Northern analyses with a probe derived from 61.2, which contains sequences shared by the three cDNAs (A0, A1, A2, and 500 nucleotides of A3), detects three transcripts of 4.5, 5.0, and 7.8 kb in 6- to 22-hr-old embryos (data not shown). This indicates that *cpo* encodes at least three embryonic transcripts. We detect faint or no hybridization signals with this probe at later developmental stages, except in pupae. The Northern blots shown here were hybridized with a 400-bp *Bgl*I fragment of 61.1 and with a 150-bp *Cl*aI fragment for 61.2. Both probes are unique. No signal has been observed on the same Northern blots with probes derived from the 3' trailer of the 17 cDNA. However, this 17 cDNA fragment identifies a transcript in situ that is expressed faintly in many PNS cells early in development. At later stages of development, this transcript is expressed only in the neurons of the chordotonal neurons (see Fig. 7B). 18w is a *Drosophila* gene that encodes a 5.8-kb transcript (B. Eldon, pers. comm.). It is shown as a molecular mass marker. (B) Immunoblots of bacteria that express the Cpo fusion protein and 3- to 24-hr-old Canton-S embryos. The anti-Cpo antibody recognizes a single band in embryonic extracts that corresponds to a protein of ~50 kD.

cells of the external sensory organs is much fainter than in other cells that express the β -galactosidase. Hence, <10% of *cpo* contains most regulatory sequences used in embryogenesis.

To further characterize putative regulatory sequences the genomic region immediately upstream of the 5' end of the 61.1 cDNA was sequenced. As shown in Figure 2, this sequence contains many putative binding sites for known *Drosophila* transcription factors: three GAGAGA-binding sites (Biggin and Tjian 1988); three partially overlapping CGAGC/TG sites or Zeste-binding sites (Benson and Pirrotta 1988; Biggin et al. 1988); and a CCAAT box or C/EBP-binding site (Johnson et al. 1987; Friedman et al. 1989). There is also a *Drosophila* consensus sequence for a transcription initiation site (TCAGTT) 22 nucleotides upstream of the first nucleotide of the longest cDNA (Hultmark et al. 1986; Pirrotta et al. 1987; Burtis et al. 1990). The positions of 10

different enhancer detectors were determined by sequencing the P-element boundaries. Insertions that cause homozygous lethality are indicated with *l*, those that cause a *cpo* phenotype and fail to complement *l* insertions are abbreviated *cp*, whereas others are abbreviated *v* (Fig. 2; Bellen et al. 1992). As shown in Figure 2A, eight enhancer detector insertions (*cp1*, *cp2*, *l2*, *l3*, *l6*, *v2*, *v1*, *v3*) are located between the putative zeste-binding sites and the putative transcription start site. Six P elements (*cp1*, *cp2*, *l2*, *l3*, *l6*, *v2*) have inserted at exactly the same position (0 in walk shown in Fig. 1) but cause very different defects: The *cp1* and *cp2* insertions are 17-kb P[*lArB*] insertions (Wilson et al. 1989) and cause behavioral defects in adults; *l2*, *l3*, and *l6* insertions are 10-kb P[*lacZ*, *w*⁺] insertions, which cause homozygous lethality; *v2* is a P[*lacZ*, *w*⁺] insertions, inserted in the opposite orientation relative to the other five, and no obvious phenotype has been associated with this insertion. Previous studies have shown that the *cp1* and *l2* P elements can be excised leading to a concomitant reversion of the phenotypes (Bellen et al. 1992). Hence, the phenotype associated with enhancer detectors that are inserted at exactly the same position is dependent on size, type, or orientation of the P element.

Cpo is a nuclear protein

To characterize the Cpo protein, an antibody was generated against a TrpE-Cpo fusion protein. As shown in Figure 6, this antibody recognizes an antigen that is most abundant in the neurons and support cells of the PNS, less abundant in a number of glial cells of the PNS and central nervous system (CNS), and barely detectable in cells of the gut of some embryos. Some faint and diffuse staining is also observed in the CNS.

The β -galactosidase expression pattern in the enhancer detector strains and the anti-Cpo staining pattern in double-labeled embryos are very similar. In addition, some *cpo* mutant embryos lack the protein recognized by the anti-Cpo serum in most cells in which it is normally found. Hence, the histochemically detected protein most likely corresponds to the Cpo protein. As shown in Figure 3B, the anti-Cpo serum detects a single band of ~50 kD on an immunoblot of embryonic extracts.

Double-labeling experiments with the anti-Cpo antibody and the anti- β -galactosidase antibodies (Fig. 5B; data not shown) show that Cpo is localized to the nucleus. Little or no label is observed in the cytoplasm. This indicates that the protein is actively transported into the nucleus and/or that its presumed affinity for pre-mRNA sequences leads to nuclear retention (Swanson et al. 1987). Nuclear localization is observed in the cells of the PNS, some glial cells of the CNS, and the cells of the gut (see Fig. 6C). We have been unable to exclude the possibility that *cpo* may be present at low levels in the cytoplasm and nuclei of CNS neurons.

Cpo expression in wild-type and mutant embryos

The developmental expression pattern of *cpo* mRNA has

Figure 4. (A) A novel type of repeat sequence in Cpo: PVSVP. A consensus repeat sequence, PVSVP, can be derived by comparing the amino acid sequence in the carboxyl terminus portion of domain 1. (B) Comparison of the putative RNA-binding domains of the Cpo protein with other known RNA or single-stranded DNA-binding proteins. Identical and conserved amino acids are boxed. The italicized stretch of carboxy-terminal amino acids are different between 61.1 and 61.2. The two most conserved domains correspond to the ribonucleoprotein consensus 1, or RNP1 (LFVSGLP), and RNP2 (PVGFXF) found in many RNA-binding proteins. The RNP-CS-type domains of Cpo is more homologous to two proteins that contain three RNP-CS-type domains and are expressed in the nervous system: ELAV and HuD (Monte Carlo score, Cpo 61.1-ELAV = 6.87; Cpo 61.1-HuD = 12.26; Cpo 61.2-HuD = 13.92; Cpo 61.2-ELAV = 7.42; a score >6 means probable homology). Other RNA-binding proteins (with GenBank accession number) shown are Potato spliceosomal U2B protein (M72892), *Drosophila* D25 small nuclear ribonucleoprotein (M89775), human U1 small nuclear RNA-binding protein (X06347), and *Xenopus* nucleolin RNA-binding protein (X63091).

been described previously (Bellen et al. 1992). The expression pattern of the Cpo protein in wild-type embryos is shown in Figure 6. Briefly, Cpo expression is initiated in the SMCs at maximal germ-band extension after the first signs of segmentation appear (Fig. 6A). During germ-band retraction, the pattern becomes complex: Many precursor cells as well as more differentiated cells of the PNS express Cpo. Later, during embryogenesis, most cells of the PNS express Cpo (Fig. 6B,E). Some support cells of the extra sensory organs express Cpo very weakly or not at all. Other cells that express Cpo are located on the dorsal and ventral sides of the CNS (Fig. 6D). The positions and shapes of these cells suggests that they are glial cells. The expression pattern in the nervous system corresponds to the expression of *cpo* messages (Bellen et al. 1992).

Cpo protein expression is also seen in the developing gut. Staining in these cells is faint and easily overlooked. As shown in Figure 6C, most of the expression in late embryos is confined to the more posterior parts of the midgut. Contrary to the expression in the PNS and the CNS, *cpo* expression in the gut was not observed in whole-mount in situ hybridizations (Bellen et al. 1992).

The Cpo protein distribution was analyzed in several mutant strains that carry different *cpo* alleles. As shown

in Figure 7A, embryos homozygous for the most severe *cpo* alleles [the 11 enhancer detector insertion or a small deficiency that removes some of the regulatory sequences (Bellen et al. 1992)] lack the protein in all cells that normally express Cpo except the chordotonal neurons. These neurons only express Cpo late during embryonic development, whereas wild-type embryos express Cpo earlier in the SMCs and their progeny. The protein expression pattern in these two mutants corresponds to the RNA in situ expression pattern seen in these mutants (Bellen et al. 1992). It also corresponds to the pattern observed when a probe specific for the 17 cDNA is used (Fig. 7B). The Cpo protein was also immunocytochemically localized in many other *cpo* mutants described previously (Bellen et al. 1992). Embryos homozygous for all other lethal alleles tested, with the exception of 19, show reduced levels of Cpo expression, consistent with the decreased mRNA levels observed in these mutants (data not shown). However, no visible changes in levels or pattern of expression were observed in homozygous viable *cpo* mutants or homozygous 19 embryos. The 19 insertion is ~120 nucleotides upstream of the transcription start site, and on the basis of genetic criteria this allele was classified as a weak hypomorphic homozygous lethal allele (Bellen et al. 1992).

Figure 5. Expression of β -galactosidase in embryos carrying a *cpo/enhancer/lacZ* fusion construct. [A] Whole-mount stage-16 embryos (for staging, see Campos-Ortega and Hartenstein 1985) carrying a 10-kb *Xba*I fragment (derived from phage 12.4 shown in Fig. 1) upstream of the *lacZ* gene in pHZ50 (Hiromi and Gehring 1987). β -galactosidase is expressed in all cells of the chordotonal organs, the cells of the CNS, and cells in the midgut (not shown). Other cells than chordotonal cells in the PNS only label faintly or not at all. [B] Fragment of the lateral wall of an embryo stained with an anti- β -galactosidase antibody (brown and cytoplasmic) and the anti-Cpo antibody (gray and nuclear). Note that the chordotonal neurons and their support cells stain the darkest and are brown (arrow 1). Nuclei of external sensory neurons are gray (arrow 2). Some external sensory neurons (gray) are surrounded by a brown haze.

Figure 6. Expression of the Cpo protein in developing embryos. Whole-mount embryos (A–D) are shown anterior to the *left*, dorsal *up*. Embryos were immunocytochemically stained with a polyclonal anti-Cpo antibody. (A) An early stage-11 embryo. Note the expression of Cpo in a pair of cells or a single cell in the posterior section of each segment. In some segments a faint labeling cell or pair of cells appears in the anterior portion. These cells correspond to neural precursors of the PNS. The Cpo protein is not observed before segmentation, whereas the *cpo* message is. (B) A stage-16 embryo. The focal plane is just below the cuticle. Note the regular expression pattern in each segment and the punctate nuclear localization. Almost all PNS cells express Cpo. (C) A stage-16 or -17 embryo. The focal plane is on the gut. Note the nuclear localization in the cells of the midgut. (D) Detail of the anterior section of the ventral nerve cord of an embryo. Note the nuclear staining in a small subset of cells of the CNS. (E) Detail of the Cpo distribution in two abdominal segments and the corresponding diagram of the PNS (redrawn from Bodmer and Jan 1987). (es) External sensory organ; (ch) chordotonal organ; (md) multiple dendrite neuron; (n) neuron; (th) thecogen cell; (tr) trichogen cell; (to) tormogen cell; (li) ligament cell; (s) scolopale cell; (c) cap cell; (da) dendritic arbor neuron; (bd) bipolar dendrite neuron; (td) trachea innervating cell. From dorsal to ventral: The dorsal cluster, the lateral cluster, and the most dorsal of the two ventral clusters are shown in a single focal plane.

To establish whether *cpo* expression depends on the expression of the *da* and AS-C genes, we localized Cpo in mutant embryos that lack almost all genes of the AS-C or the *da* gene. Homozygous *da/da* embryos do not express Cpo in the PNS at any stage of development. However, expression of Cpo in the cells of the CNS and a small cluster of cells around the posterior spiracle is not affected (Fig. 7C). In addition, some cells of the gut also express Cpo in *da/da* embryos, although this pattern is different from the wild-type pattern. From these observations we conclude that *da* is required for proper expression of *cpo* in the PNS. In AS-C mutant embryos most of the cells of the PNS except the chordotonal neurons and their support cells, as well as few other neurons, fail to express Cpo (Fig. 7D). The remainder of the *cpo* expression pattern, glial cells of the CNS and gut, is not obviously affected. These observations show that genes of the AS-C are required for proper expression of *cpo* in many cells of the PNS but not in non-PNS cells. Because *cpo* is not expressed in many SMCs of AS-C mutant embryos (data not shown), and because genes of the AS-C are expressed in SMCs (Cabrera et al. 1987; Brand and Campos-Ortega 1988), the genes of the AS-C also control expression of *cpo* during early neurogenesis (see Discussion). However, after stage 13, expression of T3, T4, T5, and T8 transcripts of the AS-C cannot be detected in the cells of the PNS (Cabrera et al. 1987; Brand and Campos-Ortega 1988; data not shown). It is therefore possible

that once *cpo* expression is initiated, the Cpo protein initiates a number of events leading to transcriptional autoregulation. Two lines of evidence argue against this hypothesis. First, no difference was observed in the β -galactosidase expression pattern among *l1/l1* or *l1/+* embryos from the *l1* enhancer detector strain at all stages tested, indicating that Cpo is not required for *cpo* transcription. Second, β -galactosidase staining of homozygous amorphic mutant embryos carrying a P element that contains most of the regulatory sequences of the *cpo* gene upstream of the *lacZ* gene (see Fig. 5) expresses the marker gene in the same cells as nonmutant embryos at all developmental stages.

Discussion

To further dissect the molecular aspects of nervous system differentiation we have screened for genes that are expressed in most or all neural precursors and the more differentiated cells of the PNS during embryogenesis. The goal of these studies is to identify genes that are controlled by the proneural genes and to unravel the molecular cascade of events that lead to a fully developed and functional PNS. Using a reverse genetic approach based on enhancer detection (for review, see Bellen et al. 1990), we have identified several genes that are expressed in all precursors of the PNS. A genetic analysis of one of these genes, *cpo*, indicates that the gene is essential for

Figure 7. Expression of Cpo in mutant embryos. Whole-mount embryos (A–C) are shown anterior to the left, dorsal up. Embryos were immunocytochemically stained with a polyclonal anti-Cpo antibody (A,C,D). (B) A wild-type whole-mount embryo from an in situ hybridization with a probe specific for the 17 cDNA trailer. (A) A homozygous *l1/l1 cpo* mutant embryo at stage 15. Note the lack of staining in all cells of the PNS except the chordotonal neurons. (B) Expression pattern of the transcript that corresponds to the 17 cDNA in a stage-16 embryos. (C) A homozygous *da²/da²* embryo. These embryos show many morphological abnormalities [Caudy et al. 1988a], but Cpo expression can be detected in the nuclei of specific cells of the CNS, as well as in some midgut cells and small cluster of cells at the posterior end of the embryo. (D) Detail of the posterior and ventral portion of an *sc^{B57}/sc^{B57}* embryo stained with the Cpo antibody. Shown are the lateral and ventral clusters. Only one cell labels in the dorsal cluster of these embryos. (Left) A schematic drawing of the PNS. The most ventral band in the photograph is the ventral nerve cord. The cells that are most closely associated with the ventral

nerve cord correspond to the nuclei of the peripheral glia. The nuclei that label in the ventral and lateral cluster belong primarily to cells of the chordotonal organs. With few exceptions, other neurons or support cells cannot be identified.

embryonic development and required for normal adult behavior. However, no obvious morphological defects have been observed in the PNS. Here, we present molecular data that suggest a possible role for *cpo* in PNS differentiation.

Structural analysis of *cpo* shows a complex organization. The most conspicuous features are the presence of a very large intron with nontypical splice acceptor or donor sites [Mount et al. 1992], extensive alternative splicing, and a large exon flanked by small exons. Our analysis also indicates that *cpo* has two promoters upstream of exon A0 and B1. The large 42-kb exon is alternatively spliced, and an unusual splice donor or splice acceptor at the boundary of exon 3–intron 3 or intron 3–exon 4 may be responsible for the splicing pattern. All enhancer detector insertions map to a 200-bp fragment, and six enhancer detectors have inserted at exactly the same position. These six P-element insertions cause very different phenotypes depending on the type or size of the P elements or their orientation. Sequence data indicate that eight insertions are located between enhancer-like sequences and the promoter of one of the transcripts, suggesting that they may interfere with the transcriptional machinery. This interference may rely on complex spatial and structural requirements for the interaction between the upstream regulatory sequences of *cpo* and its transcription initiation site.

Sequence analysis reveals that none of the *cpo* cDNAs

nor the corresponding genomic DNA sequences contain a typical AUG initiation codon in the ORFs. Our observations suggest that a non-AUG initiation codon located at the beginning of the ORF is used for translational initiation. It should be noted that in vitro studies have shown that an AUG codon is not an absolute requirement [Peabody 1987] and that at least one other nervous system-specific *Drosophila* gene [Shugihara et al. 1990] and a mammalian gene [Hann et al. 1988] do not contain an AUG initiation codon.

We have arbitrarily subdivided the Cpo proteins in four domains on the basis of the primary amino acid sequence, the distribution of particular residues, and homopolymeric stretches. The significance of these homopolymeric runs in many proteins is unknown, although they are often found in neuronal transcription factors [for discussion, see Vaessin et al. 1991]. The similarity between the auxiliary domains of proteins that contain RNP-CS-type domains and the auxiliary domains of transcription factors has been pointed out previously [for review, see Bandzulis et al. 1989].

Homology of the carboxy-terminal domains of some putative Cpo proteins to proteins containing RNA-binding domains like the HuD protein is interesting as HuD has been implicated in a sensory neuropathy in some cancer patients [Szabo et al. 1991]. A mammalian homolog of *cpo* has recently been cloned and the Cpo RNP-CS-type of mouse and *Drosophila* are very similar [T.

Saito and D. Anderson, pers. comm.). The high degree of amino acid sequence conservation of these domains between such evolutionarily divergent phyla as arthropods and chordates suggests a functional role for these domains beyond mere RNA binding.

The Cpo protein is localized to the nucleus in all PNS cells. Nuclear localization is in agreement with the presence of a proposed nuclear localization sequence (NLS) in the protein (90–114 amino acids). This NLS also contains the SPKR sequence. This sequence is similar to the consensus sequence that is phosphorylated by the *cdc2* kinase, and phosphorylation of such sites may increase the rate of nuclear accumulation of the protein (for discussion, see Robbins et al. 1991). However, a NLS may not be required because the pre-mRNA may act as nuclear retention sequences. Whichever mechanism is responsible for the nuclear localization, the presence of the protein in the nucleus agrees with the proposed function.

Analysis of Cpo protein expression in embryos that lack the proneural AS-C or *da* genes shows that both genes are required for proper expression in the SMCs of the PNS at germ-band extension. We have not demonstrated direct transcriptional regulation by the proneural genes. These genes are expressed in the SMCs at the same time that *cpo* expression is initiated (stages 9 and 10). Moreover, contrary to the CNS in which cell death is visible after stage 11, no cell death has been reported in the PNS of proneural mutants (see Brand and Campos-Ortega 1988). Thus, the proneural genes may directly control expression of *cpo*, either by forming a transcriptional block that is derepressed when their concentration decreases or by acting as positive transcriptional regulators when their expression becomes concentrated in the SMCs (Cubas et al. 1991; Skeath and Carroll 1991). It is unlikely that this type of regulation is taking place at later developmental stages because the proneural genes are not expressed in the PNS at these stages (Cabrera et al. 1987; Brand and Campos-Ortega 1988). Our data suggest that Cpo does not initiate an autoregulatory cascade that could maintain Cpo expression.

There are some striking similarities between *cpo* and the *Sex-lethal* (*Sxl*) gene. Both encode proteins with RNP-CS-type domains, and both produce through alternative splicing messages that do not contain this domain and are likely to be nonfunctional (Bell et al. 1991). Genetic evidence also indicates that both are controlled by proneural genes of the AS-C (Torres and Sanchez 1989) and the *da* gene (Cline 1983). Finally, both genes seem to have two (or more) promoters (Keyes et al. 1992). *Sxl* regulates its own splicing as well as that of the *transformer* gene, thereby initiating a cascade of events in sex determination and dosage compensation (for review, see Baker 1989). Because mutations in *cpo* do not cause obvious morphological defects in the PNS or the CNS and because *cpo* is required for viability, we propose that *cpo* regulates RNA processing of one or more genes that are required in the PNS and/or other cells where it is expressed. The majority of the genes that are expressed in the *Drosophila* nervous system are alternatively spliced, and even single-base editing of RNA has been shown to

have dramatic effects on the properties of some channels (Sommer et al. 1991). Hence, alternative processing of RNAs may have an important role in nervous system function, and proteins like HuD (Szabo et al. 1991), ELAV (Robinow and White 1988), and Cpo may fulfill these roles.

Materials and methods

Molecular techniques

Plasmid rescue from fly DNA was performed as described by Wilson et al. (1989). Other cloning techniques, Northern and Southern analysis, and chromosomal walking were performed by standard procedures as described in Sambrook et al. (1989). For sequencing, fragments were subcloned in Bluescript KS and SK. Single- or double-stranded DNA was sequenced by the dideoxy chain-termination method (Sanger et al. 1977), with the automated fluorescence procedure (Applied Biosystems) as described by Smith et al. (1986). DNA sequence analysis was carried out by the Nucleic Acids Core in the Institute for Molecular Genetics at Baylor College of Medicine.

Fly strains and culture

All *cpo* fly strains used in this study are described in Bellen et al. (1992). The AS-C deficiency strain, *Df(1)sc^{B57} w sn³ /FM6, y^{3ld} dm B*, was obtained from E. Grell and Y.-N. Jan (University of California at San Francisco). The *y; d da² /CyO* and *Df(2L)27 /SM1* strains were obtained from A. Ghysen (University of Brussels, Belgium). Flies were cultured on standard fly food supplemented with dry yeast.

Rabbit polyclonal anti-Cpo antibody

A 949-bp *Xho*II fragment from the 61.2 cDNA, which corresponds to amino acids 164–479 shown in Figure 2A, was cloned into the *Bam*HI site of the polylinker of the pATH 1 vector (Dieckman and Tzagoloff 1985). The construct was transformed in RR1 *Escherichia coli*, and the fusion protein was induced as described. The fusion protein was isolated by preparative PAGE, electroeluted, dialyzed, dried, and resuspended in Freund's adjuvant. Purified protein (100–200 µg) was injected twice into two New Zealand white rabbits. The preimmune and immune sera were tested on embryos and Western blots.

In situ hybridization to embryos

Digoxigenin-labeled probes were prepared as described [Boehringer Mannheim (DNA labeling and detection kit: nonradioactive)]. In situ hybridizations to whole-mount embryos were performed essentially as described previously (Tautz and Pfeifle 1989).

X-gal staining and HRP/alkaline-phosphatase immunocytochemistry

X-gal staining, and anti-β-galactosidase staining with an affinity-purified polyclonal antibody (Cappel) were performed as described by Bier et al. (1989). Immunocytochemistry with other antibodies was performed in a similar manner. The secondary antibodies used in this study are an anti-mouse or an anti-rabbit antibody conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP). Most antibodies were first preabsorbed to 0- to 3-hr-old embryos at a concentration 10× higher than the final concentration for 2–3 hr at room temperature before use. The

following rabbit polyclonal antibodies (RPAb) were used: RPA anti-Cpo at 1 : 7,000–10,000; RPA anti-sense or anti-T8 at 1 : 5000 (Vaessin et al. 1991; M. Brand, pers. comm.). Double-labeling experiments were performed by first completing the entire procedure with a primary and the anti-HRP secondary antibody, followed by signal detection with HCl-diaminobenzidine in 0.12 M Tris (pH 7.6), which leads to formation of a brown precipitate. The entire procedure was subsequently repeated with another primary antibody, again followed with an anti-HRP secondary antibody. The signal was detected with HCl-diaminobenzidine in Tris (pH 7.6) 0.12 M containing 4% of 8 M lithium chloride, leading to the formation of a black precipitate.

Immunoblotting

Immunoblotting was essentially performed as described by Towbin et al. (1979). Embryonic extracts were separated by 10% PAGE and transferred onto nitrocellulose. Primary antibody was used at a final concentration of 1 : 5000. Signal detection was performed with goat anti-rabbit conjugated to AP at 1 : 1000.

Acknowledgments

We thank Dan Strumpf and Linus Tsai for helping us out in the last phases of this project. We thank Harald Vaessin, Ethan Bier, Susan Sheperd, Ed Grell, and Yuh Nung Jan for help, suggestions, antibodies, and fly strains. We thank Alain Ghysen for fly strains; Ron Davis and Kai Zinn for genomic and cDNA libraries; Ron Richman for suggestions and advice; and Beth Eldon, Sue Berget, Jim Lupski, David Anderson, Juan Botas, Arthur Kania, Troy Littleton, and Karen Schulze for comments on the manuscript. This research was supported by a grant from the Muscular Dystrophy Association to H.J.B. H.J.B. is an assistant investigator of the Howard Hughes Medical Institute.

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Note added in proof

Sequence data in this paper have been submitted to the EMBL/GenBank data libraries under accession numbers Z14311 for *cpo* 17, Z14312 for *cpo* 61.2, and Z14974 for *cpo* 61.1.

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The *Drosophila* couch potato protein is expressed in nuclei of peripheral neuronal precursors and shows homology to RNA-binding proteins.

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Genes Dev. 1992, **6**:

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