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The *crooked neck* gene of *Drosophila* contains a motif found in a family of yeast cell cycle genes

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The *crooked neck* (*crn*) gene of *Drosophila* encodes a protein of 702 amino acids and contains 16 tandemly arranged copies of a 34-amino-acid repeat that is similar to the tetratrico peptide repeat (TPR). Multiple copies of the TPR motif have also been found in a family of yeast genes, including several members that are necessary for cell division. TPR-containing proteins encoded by the yeast genes *CDC16*, *CDC23*, and *nuc2*$^+$ are required for progression through the G_2/M transition of the cell cycle. Loss of zygotic expression of *crn* causes defects in the proliferation of brain neuroblasts and results in the absence of identified neuronal lineages in the central and peripheral nervous systems. The sequence similarity and mutant phenotypes are consistent with a cell cycle requirement for the *crn* gene product.

[*Key Words: crooked neck*; TPR motif; neurogenesis; cell cycle; *Drosophila*]

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Embryonic development in *Drosophila* begins with a series of rapid, synchronous divisions of the zygotic nuclei that occur in the absence of cell division and in the absence of detectable G_1 or G_2 phases of the cell cycle (Foe and Alberts 1983). These divisions, which consist of alternating cycles of S and M phases, are dependent on stored, maternally supplied gene products (Edgar et al. 1986). Following 13 of these divisions, the zygotic nuclei migrate to the egg cortex and become surrounded by cell membranes. Most of the embryonic cells then undergo three more asynchronous cell divisions, which occur in a precise spatiotemporal sequence and which are dependent on transcription of the zygotic genome. The majority of embryonic cells cease dividing by 5.5 hr after fertilization, although many of them become polyploid owing to continued rounds of DNA synthesis without cytokinesis. Only cells of the nervous system and germ line continue dividing during later stages of embryonic development (Hartenstein and Campos-Ortega 1985; Bodmer et al. 1989; Foe 1989).

The genetic control of the cell cycle has been analyzed extensively in yeast, but has been studied less in metazoans such as *Drosophila* in which the cell cycle can vary considerably in different cell types and during different stages of development. Screens for mutations in maternally and zygotically active genes required for progression through the cell cycle in *Drosophila* have identified a number of potentially important loci (Zalokar et al. 1975; Baker et al. 1982; Freeman et al. 1986; Gatti and Baker 1989). Sequence similarities and functional homologies between the cell cycle genes of yeast and other organisms have also been exploited to identify additional cell cycle genes in *Drosophila* (Edgar and O'Farrell 1989; Lehner and O'Farrell 1989, 1990; Whitfield et al. 1989; Jimenez et al. 1990). These examples of conservation of sequence and function suggest that the fundamental mechanisms of cell cycle regulation are universal and that other genes important for mitosis will also be conserved.

The molecular and developmental biology of *crooked neck* (*crn*) is described here. Loss of zygotic expression of *crn* causes defects late in embryogenesis and affects primarily those lineages in the nervous system that are still undergoing cell division. Molecular analysis of the *crn* gene indicates that it is related to a family of genes first identified in yeast which includes several genes required for normal progression through the cell cycle. The sequence similarity and mutant phenotypes suggest that *crn* also encodes a protein required for cell division.

Results

Cloning of the crn *gene*

The *crn* locus has been genetically mapped proximal to the maternal-effect locus *fs(1)K10* (Perrimon et al. 1984) at 2E3 on the X chromosome. Haenlin et al. (1985) cloned the genomic DNA from this region and identified a number of transcription units within it that were candidates for the *crn* gene. An X-ray-induced allele of *crn*,

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crn^{RC63}, contains a 3.6-kb insertion within a 2.3-kb transcription unit, indicating that this transcription unit corresponds to the *crn* locus (Fig. 1; Haenlin et al. 1985). P-element-mediated transformations using DNA containing the 2.3-kb transcription unit rescued *crn* mutations (Haenlin et al. 1985), further confirming this transcription unit as the *crn* locus. A 0.7-kb *Bgl*II fragment from the genomic phage 18M8 (kindly provided by E. Mohier) hybridizes to *crn* transcripts (Fig. 1) and was used as a probe to isolate *crn* cDNA clones (see Materials and methods, below).

Expression and localization of crn *transcripts during development*

Analysis of RNA isolated from different developmental stages demonstrates that a single 2.3-kb transcript from the *crn* locus is expressed throughout embryonic, larval, pupal, and adult stages at relatively constant levels (Fig. 2). The *crn* transcript is observed in RNA isolated from 0- to 1-hr embryos, prior to the initiation of zygotic transcription. This is an indication of maternal expression and is consistent with the germ-line requirement for *crn* (Perrimon et al. 1984).

Because the embryonic defects associated with *crn* mutations are tissue specific, that is, defects are confined to the nervous system and midgut (see below), the spatial distribution of *crn* transcripts during embryonic development was determined. In situ hybridization to sectioned embryos using a *crn* cDNA probe demonstrates that *crn* transcripts are uniformly distributed given the resolution of the technique, which suggests that *crn* is transcribed in all cells during embryonic development (data not shown). *crn* mRNAs are not expressed at detectably higher levels in either the nervous system or midgut.

Sequence analysis of crn

The sequence of the cDNA clone with the longest insert contains an open reading frame (ORF) of 2106 bp capable of encoding a polypeptide of 702 amino acids. The nucleotide sequence of the cDNA clone and the predicted amino acid sequence of the ORF are shown in Figure 3. The 4 nucleotides CAGC (at position 78–81; Fig. 3) immediately preceding the putative translational start site generally match the consensus sequence (C/A A A/C A/C) that precedes translation start sites of many *Drosophila* genes (Cavener 1987). Codon usage by the long ORF is in good agreement with other *Drosophila* genes (O'Connell and Rosbash 1984), suggesting that the predicted amino acid sequence is valid. Seventy-four nucleotides upstream of the cDNA cloning polylinker is the consensus polyadenylation sequence, AATAAA (Proudfoot and Brownlee 1976). A hydrophobicity plot of the predicted *crn* protein reveals no significant tracts of hydrophobic residues, indicating that it is unlikely to contain either a transmembrane domain or a signal sequence (data not shown). A potential nuclear localization signal

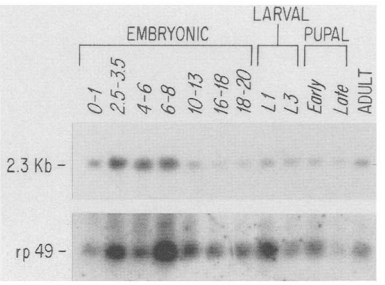

Figure 2. Developmental analysis of *crn* transcription. Ten micrograms of poly(A)$^+$ RNA, prepared from various developmental stages, was loaded in each lane, electrophoresed, transferred to nitrocellulose, and probed with a ^{32}P-labeled 0.7-kb *Bgl*II genomic fragment. Lanes are marked according to the developmental stage: Numbers during embryonic stages refer to hours of development after fertilization; larval stages L2 and L3 refer to second- and third-instar larvae, respectively; early pupae are 0–24 hr after pupation; late pupae are 96–120 hr after pupation; and adult RNA is from a mixed population of males and females. One transcript, 2.3 kb in size, is detected by the *crn* probe and is present in all stages. The blot was rehybridized with a ribosomal protein gene probe (rp49; O'Connell and Roshbash 1984) to control for RNA quantitation.

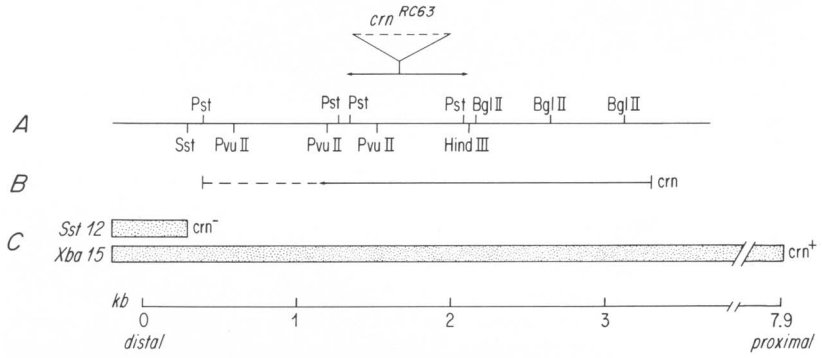

Figure 1. Organization and restriction map of the *crn* region. (*A*) Restriction map of genomic DNA encompassing the *crn* gene. The arrow above the map indicates the *Pst*I fragment, which contans a 3.6-kb insertion of unknown origin in the crn^{RC63} allele. (*B*) The line indicates the localization, as determined by Northern analysis, of the *crn* transcription unit relative to the genomic map in *A*. (*C*) Stippled boxes illustrate the genomic sequences used for P-element transformation rescue of the mutant *crn* phenotype (adapted from Haenlin et al. 1985). Xba15 transposons rescue *crn* mutants, whereas Sst12 transposons do not, indicating that the entire *crn* gene must be contained within Xba15, and at least part of the gene lies within the ~7.5 kb of DNA exclusive to Xba15.

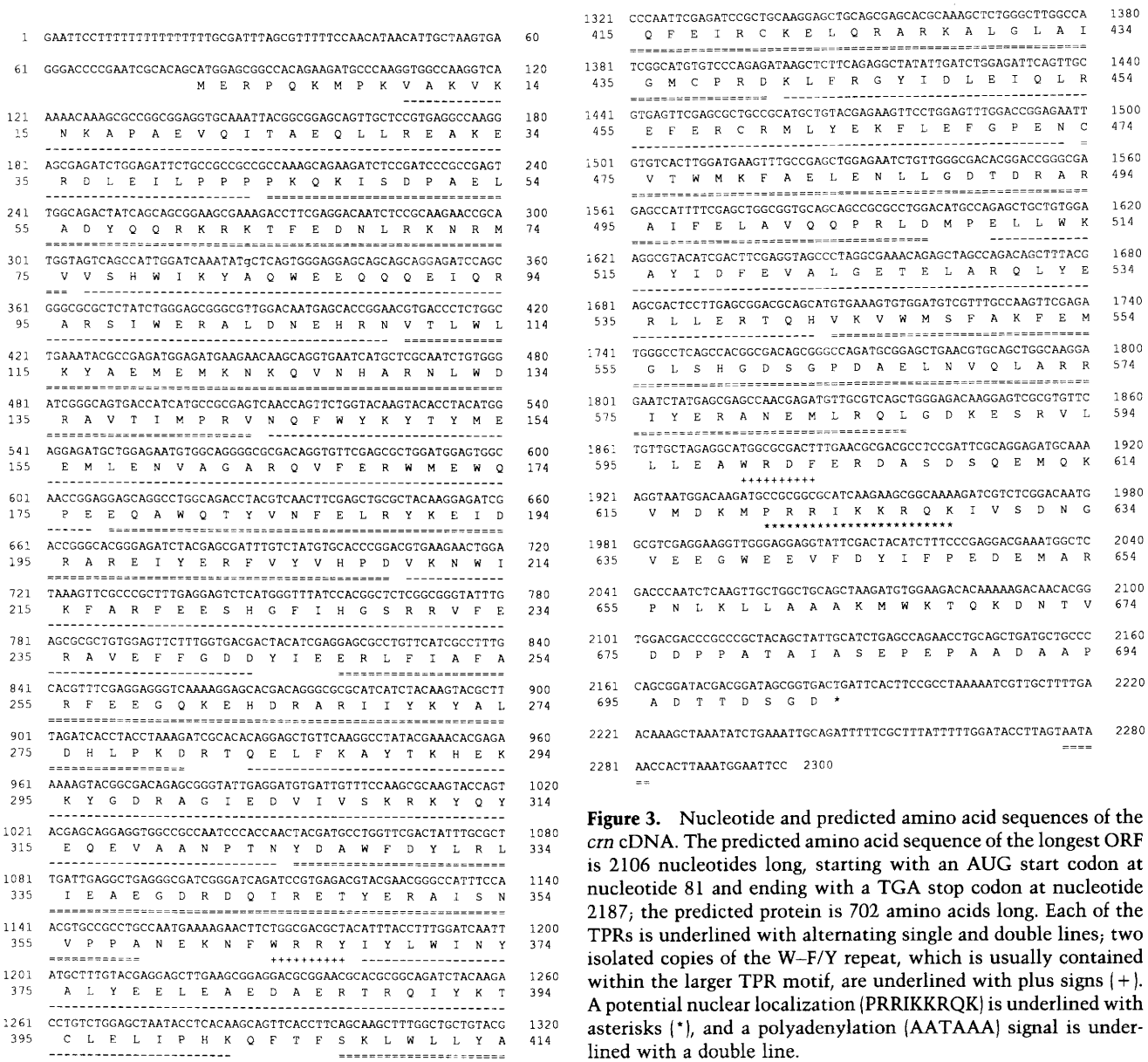

Figure 3. Nucleotide and predicted amino acid sequences of the *crn* cDNA. The predicted amino acid sequence of the longest ORF is 2106 nucleotides long, starting with an AUG start codon at nucleotide 81 and ending with a TGA stop codon at nucleotide 2187; the predicted protein is 702 amino acids long. Each of the TPRs is underlined with alternating single and double lines; two isolated copies of the W–F/Y repeat, which is usually contained within the larger TPR motif, are underlined with plus signs (+). A potential nuclear localization (PRRIKKRQK) is underlined with asterisks (*), and a polyadenylation (AATAAA) signal is underlined with a double line.

(Chelsky et al. 1989), PRRIKKRQK, is present near the carboxyl terminus of the protein (Fig. 3).

No proteins with significant sequence similarity to *crn* were found, using the FASTP program (Lipman and Pearson 1985) to search the current NBRF/PIR data base. However, a weak sequence similarity was found between *crn* and part of the homeo domain, a conserved DNA-binding motif found in a large number of developmentally important genes in *Drosophila* and other species (Scott et al. 1989). The similarity to the homeo domain is found in residues 384–443 of the *crn* protein, which are predominantly but not exclusively contained within the TPR-11 repeat (see below). Of the 61 amino acids in this region of *crn*, 27 can be found in the corresponding position of at least one of the known homeo domains. However, no one homeo domain has extensive similarity to *crn*; the homeo domain of the *fushi tarazu* (*ftz*) protein best matches *crn* and has only 14/61 identities:

crn

DAERTRQIYKTCLELIPHKQFTFSKLWLLYAQFEIRCKELQRARDALGLAIGMCPRDKLFR

DSKRTRQTYTRYQTLELEKEFHFNRYITRRRRIDIANALSLSERQIKIWFQNRRMKSKKDR

ftz

The best fit is with the 23 amino acids immediately preceding and including the first helix of the homeo domain, which includes 10 of the amino acids in common with the *ftz* sequence. Only 4 of the highly conserved amino acids of the homeo domain consensus (underlined residues above; Scott et al. 1989) are found in the appropriate positions of the *crn* sequence, and the conserved motif, KIWFQNRR, found in helix 3 of many homeo domains and important for binding site recognition, is

not found in *crn*. It seems probable that *crn* does not contain a homeo domain per se, but rather an evolutionarily convergent and functionally homologous domain that may be important for some aspect of protein function other than DNA binding, such as determination of secondary structure or interaction with other proteins.

The crn *protein contains a series of repeats similar to the yeast TPR motif*

The *crn* protein contains 16 copies of a tandemly and directly repeated motif of 34-amino-acid residues (Fig. 4A). The sequence of this motif, the presence of multiply repeated copies, and the predicted secondary structure of the motif (see below) are similar to the tetratrico peptide repeat (TPR) motif, which has been identified in a family of related fungal genes (Sikorski et al. 1990). This family includes four genes required for normal progression through the cell cycle: *CDC16* and *CDC23* of *Saccharomyces cerevisiae* (Hartwell 1976; Pringle and Hartwell 1981; Icho and Wickner 1987), *nuc2*$^{+}$ of *Schizosaccharomyces pombe* (Hirano et al. 1988, 1990), and *bimA* of *Aspergillus nidulans* (Morris 1976). Additional members of the TPR gene family that have been found in *S. cerevisiae* include a negative regulator of sucrose-inducible genes, *SSN6* (Schultz and Carlson 1987); a negative regulator of yeast killer toxin, *SKI3* (Rhee et al. 1989); the gene encoding a mitochondrial membrane protein required for protein import, *MAS70* (Hase et al. 1983; Boguski et al. 1990); a stress-inducible gene, *STI1* (Nicolet and Craig 1989; Boguski et al. 1990); and the splicing factor, *PRP6* (Legrain and Choulika 1990; M.S. Boguski, pers. comm.). The fungal genes contain between 7 and 10 copies of the TPR motif which, as in the *crn* gene, are usually tandemly arrayed. Although the overall organization of the repeats within the different proteins are similar, the *crn* protein is somewhat unusual in that it has only 9 amino acids at its amino terminus preceding the first repeat and only 126 amino acids at its carboxyl terminus following the last repeat. Between these terminal regions, the *crn* protein is composed almost entirely of repeats, with from 0 to 10 amino acids separating adjacent repeats (Fig. 3).

A consensus sequence based on finding an identical

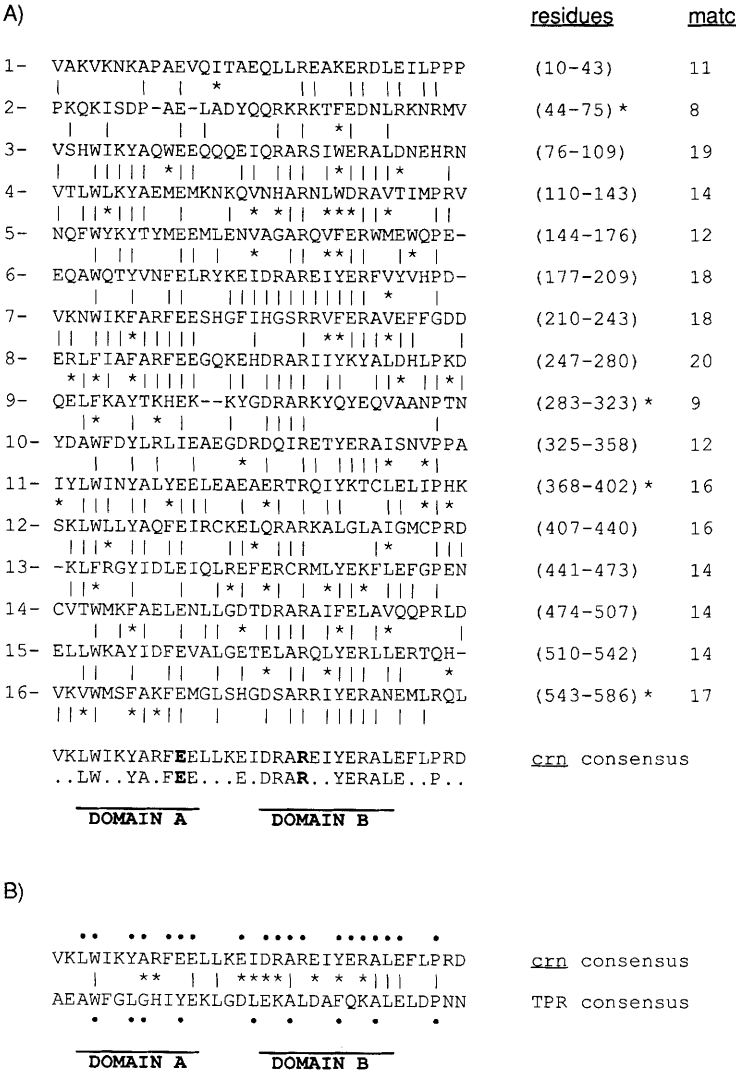

Figure 4. Alignment of the *crn* TPR units. (*A*) The 16 repeats in the predicted *crn* protein are aligned with a consensus sequence (*crn* consensus), which identifies the most frequent amino acid found at each of the 34 positions. Identities with the consensus are indicated by vertical lines below the residues, whereas conservative substitutions are marked with stars. The substitutions are based on the following groupings: (I, L, V); (K, R, H); (D, E); (W, Y, F). (Residues) The positions of the TPRs relative to the initiation methionine (residue 1); (matches) the number of identities with the consensus. The TPRs marked with stars require deletions and insertions to maintain the alignment with the consensus: two residues, indicated by dashes, have been inserted in TPR-2; a single amino acid (D at position 17, residue 384) has been deleted from TPR-11; 10 contiguous amino acids (GPDAELNVQL, residues 562–571) have been deleted from TPR-16; 2 residues have been inserted in, and 9 contiguous amino acids (GIEDVIVSK, residues 301–309) have been deleted from, TPR-9. The sequence below the consensus indicates those residues that are found in at least 40% of the repeats (M.S. Boguski, pers. comm.); these include the highlighted and nearly invariant E at position 11 and R at position 21. The highly conserved residues fall into two different structural domains, A and B. (*B*) Alignment of the *crn* consensus sequence (*crn* consensus) with a consensus sequence derived from a total of 62 TPRs found in seven fungal genes (TPR consensus; Boguski et al. 1990; Sikorski et al. 1990). The residues found most often at each position in all members of the *crn* and fungal TPRs are compared, with vertical lines indicating identities and stars designating conservative changes. The dots above the *crn* consensus and below the TPR consensus identify residues found in 40% or more of the *crn* or fungal TPRs.

amino acid at a given position in the majority of *crn* TPRs is shown in Figure 4A. None of the TPRs matches the consensus perfectly, and there is a considerable amount of variation between different TPRs, with the number of matches to the consensus varying from 8 in TPR-2 to 20 in TPR-8. Although the sequence of the TPRs may vary, the spacing of amino acids within a repeat is well conserved. Only four repeats, TPR-2, TPR-9, TPR-11, and TPR-16, require insertions or deletions to preserve the alignment. TPR-9 is a particularly divergent repeat, as it requires both the insertion of 2 residues and the deletion of 9 contiguous residues to maintain its alignment and still matches the consensus at only 9/34 positions. The two divergent repeats, TPR-2 and TPR-9, may represent functionally distinct TPRs, or may be nonfunctional copies whose similarity to the consensus is degenerating in the absence of selection.

Not all positions within the *crn* TPRs are equally well conserved. Only 19 of the possible 34 positions are so strongly conserved that an identical amino acid is found in 40% or more of the *crn* repeats (Fig. 4A; M.S. Boguski, pers. comm.). These conserved positions may be important for the structure or function of the *crn* protein. For instance, proline is frequently found near the carboxyl ends of many of the *crn* and fungal TPRs and is often found at position 32. This is particularly interesting because proline is virtually excluded from other positions within the TPRs and because proline is known to destabilize α-helices (see below; Sikorski et al. 1990). Two positions are particularly interesting in that they are nearly invariant and are unique to the *crn* TPRs: glutamic acid at position 11, which is present in 15/16 copies, and arginine at position 21, which is present in all 16 TPRs. There is no substitution for these residues by similar amino acids, suggesting that it is not simply the charged character of the amino acids that is conserved. These two highly conserved amino acids are not found in similar positions in the fungal TPRs and thus distinguish the *crn* TPRs from those found in other genes.

Comparison of many different TPRs, including those of the *crn* gene, suggest that the conserved motif can be subdivided into two more highly conserved subdomains, A and B (Fig. 4; Sikorski et al. 1990; M.S. Boguski, pers. comm.). Computer analysis by two programs (Chou and Fasman 1978; Garnier et al. 1978) of the *crn* TPR motifs predicts that each A and B domain has the potential to form α-helices (data not shown). This is consistent with a thorough computer analysis of the sequences of *CDC23*, *CDC16*, *SSN6*, and *nuc2*$^+$ (Sikorski et al. 1990), and with circular dichroism measurements of the *nuc2*$^+$ TPRs (Hirano et al. 1990), which together predict that the TPR motif forms a long α-helix punctuated by a turn at the carboxyl terminus.

crn *Mutations affect development of the nervous system and the midgut*

Embryos hemizygous for *crn* mutations die before hatching and display defects in the development of the embryonic central and peripheral nervous systems (CNS and PNS, respectively). The ventral nerve cord of a 13-hr wild-type embryo consists of a segmentally repeated pattern of neurons, whose axons are organized in a ladder-like pattern (Fig. 5A) composed of a pair of horizontal commissures in each segment and a pair of longitudinal connectives carrying axons between segments. In 13-hr *crn* mutant embryos the organization of the axon fascicles is abnormal (Fig. 5B). The commissures are present but are thinner and often fuzzy, unlike the commissures in wild-type embryos, which are precise and regular. The longitudinal connectives are reduced or absent in mutant embryos and are composed of only a few axon fascicles when present. The major nerves leading to the periphery, the segmental and intersegmental nerves, are present in mutant embryos but are also thinner and less regular than in wild-type embryos.

Mutant embryos at various stages of development were analyzed with a variety of probes to study the ontogeny of the neuronal defects (see Materials and methods). These probes demonstrate that there are no obvious defects in neurogenesis prior to germ-band retraction and axon elongation in mutant embryos. The early neuronal patterns of *even-skipped* (*eve*) and *ftz* expression appear normal (data not shown), indicating that certain identified neuronal precursors are born and divide normally. The first identified neurons to express *eve* and *ftz* (the RP2, aCC, and pCC neurons for *eve* and the MP2 and MP1 progeny for *ftz*) are born normally at 5–6 hr and, at least initially, assume their normal positions in the CNS. It is interesting that the pCC neurons are present, as these neurons normally extend axons in the longitudinal connectives; it is not yet known where the pCC axons project in mutant embryos. The ventrolateral cluster, a group of ~10 neurons per hemisegment that express *eve* at 11 hr in wild-type embryos, does not appear in mutant embryos (data not shown). Staining of mutant embryos with the SOXII monoclonal antibody (Fig. 5D) confirms the observations made with anti-horseradish peroxidase (HRP): significantly fewer axons make up the longitudinal connectives and the intersegmental and segmental nerves in embryos mutant for *crn*.

Development of the PNS is also abnormal in *crn* mutant embryos. In wild-type embryos, sensory neurons and their lineally related support cells develop in several discrete clusters in the lateral regions of each segment (Ghysen et al. 1986). Each cluster is composed of a precise number of identified neurons, which send axons ventrally to targets in the CNS via the segmental and intersegmental nerves. The sensory neurons and their support cells are missing or reduced in number in *crn* mutant embryos (Fig. 6). Typically, the earliest differentiating cluster of PNS neurons, those making up the dorsal cluster (Bodmer et al. 1989), are the least affected in mutant embryos, whereas the lateral and ventral clusters, which divide and differentiate later, are the most affected. Each of the lateral clusters in abdominal segments A1–A7 of wild-type embryos contains five characteristic stretch receptors called chordotonal organs. A wild-type chordotonal organ contains a single sensory neuron, three support cells, and a prominent scolopodial

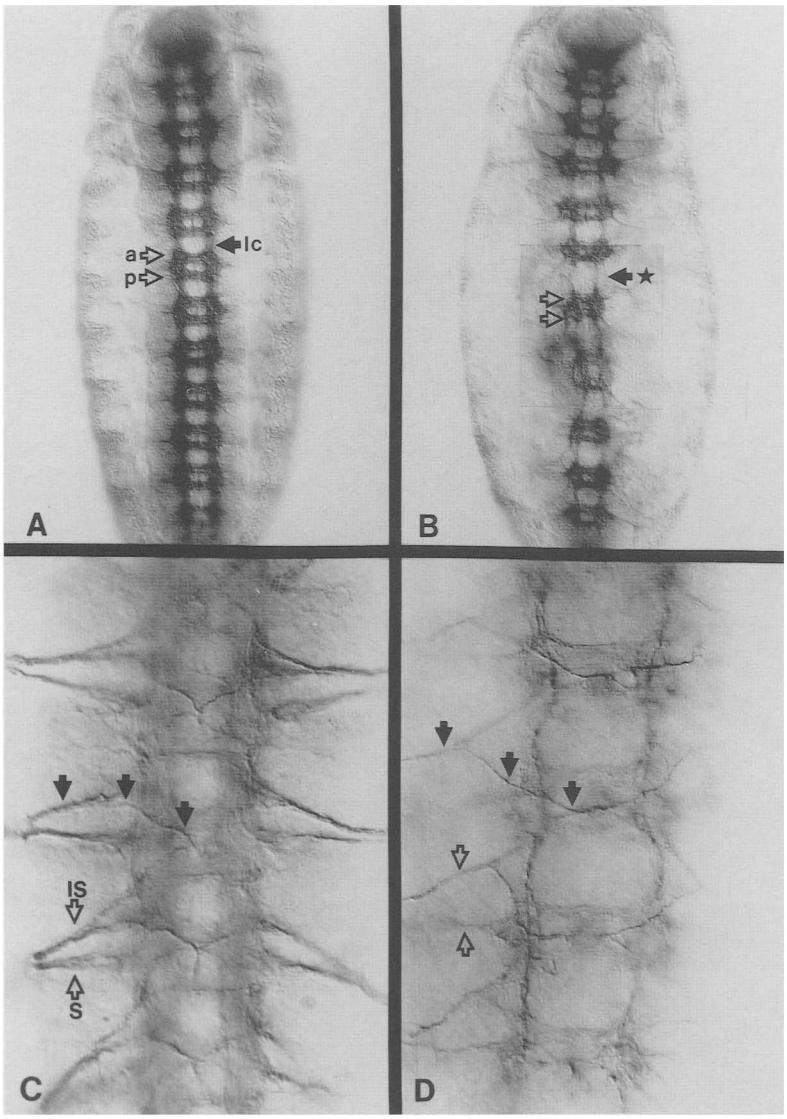

Figure 5. Development of the CNS in *crn* mutant embryos. (*A–D*) Anterior is to the *top*. (*A*) Wild-type embryo shown in ventral view and stained with a monoclonal antibody (BP102) that recognizes the membranes of CNS axons. The anterior (a) and posterior (p) commissures of a single segment are indicated by the open arrows; the longitudinal connective (lc) is indicated by the solid arrow. (*B*) A *crn* mutant embryo stained with the same monoclonal antibody. Note that a pair of commissures are visible in most segments but that they are often irregular (open arrows). The longitudinal connectives are much reduced or absent (star). (*C*) Wild-type embryo stained with the SOXII monoclonal antibody. At this stage of development (~13 hr) many neurons in the CNS stain with the antibody. These include the VUMs, a group of six identified pioneer neurons found at the ventral midline of the nerve cord in wild-type embryos. These neurons project their axons dorsally and anteriorly along the midline to a position in the anterior commissure, where the axons bifurcate and extend horizontally within a fascicle of that commissure. The axons continue to extend horizontally and bilaterally out the intersegmental nerve. The intersegmental (IS) and segmental (S) nerves from a single segment are indicated with open arrows. The axons from the VUMs are indicated by the solid arrows as they originate from the midline of the CNS and course out the CNS through the intersegmental nerve. (*D*) A *crn* mutant embryo stained with SOXII at the same stage of development. Mutant embryos have a nearly normal pattern of VUM cell bodies and axon projections. It should be noted, however, that even though the VUM axons project out the intersegmental nerve in mutant embryos, they do not always do so by normal routes; the VUM axon projections (solid arrows) are often irregular compared to wild type. Also note that there are fewer axons in the commissures and longitudinals. The segmental and intersegmental nerves are present (open arrows) but are also much reduced in size.

extension that labels strongly with anti-HRP. Mutant embryos have very few or no chordotonal organs (Fig. 6H), and cell-specific probes indicate that both the neurons and their support cells are missing (Fig. 6B and D). Staining with the SOXII antibody indicates that clusters of large cells often appear at the normal location for chordotonal neurons, but the cells within these clusters usually fail to divide and ultimately degenerate (Fig. 6F).

Mutations at the *crn* locus also cause abnormalities in the developing midgut. The wild-type midgut completely encloses the yolk at 11.5 hr of embryonic development and becomes more narrow and convoluted in later stages. By ~14 hr there are three constrictions along the length of the midgut and an additional constriction at the juncture between the foregut and midgut (Fig. 7A). It is also during this stage (11.5–14 hr) that the Malpighian tubules and the gastric caeca, respectively, posterior and anterior evaginations of the midgut, become apparent (Campos-Ortega and Hartenstein 1985). In *crn* mutant embryos the midgut develops normally until 11.5 hr. At this time, the mutant midgut encloses the yolk as in wild type and develops the anterior-most constriction where foregut and midgut meet, giving it the heart-like shape characteristic of the wild-type midgut (Fig. 7C). However, the three constrictions that normally occur along the length of the midgut do not develop in mutant embryos (Fig. 7C). Apparently as a result of this defect in midgut development, the yolk does not become uniformly dispersed and appears as a solid yolk "plug" in late embryos (Fig. 7C; Eberl and Hilliker 1988). The gastric caeca appear in mutant embryos as thickened rudiments at the anterior end of the midgut and do not evaginate as in wild type. All other internal tissues, such as the ectodermally derived foregut, hindgut, salivary

glands, and trachea and the mesodermally derived somatic and visceral musculature appear normal in *crn* mutant embryos.

crn *Mutations affect the pattern of cell proliferation*

The absence of certain neuronal lineages in embryos mutant for *crn* is consistent with a defect in postblastoderm cell division. To directly assess the effects of *crn* mutations on progression through the cell cycle, live embryos were labeled in situ with the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) at different stages of development. BrdU is incorporated into the newly synthesized DNA of cells in S phase at the time of labeling and can be detected immunologically to identify dividing cells (Bodmer et al. 1989). The pattern of BrdU incorporation is abnormal in *crn* mutant embryos. The defects are particularly obvious in older embryos, when there are rela-

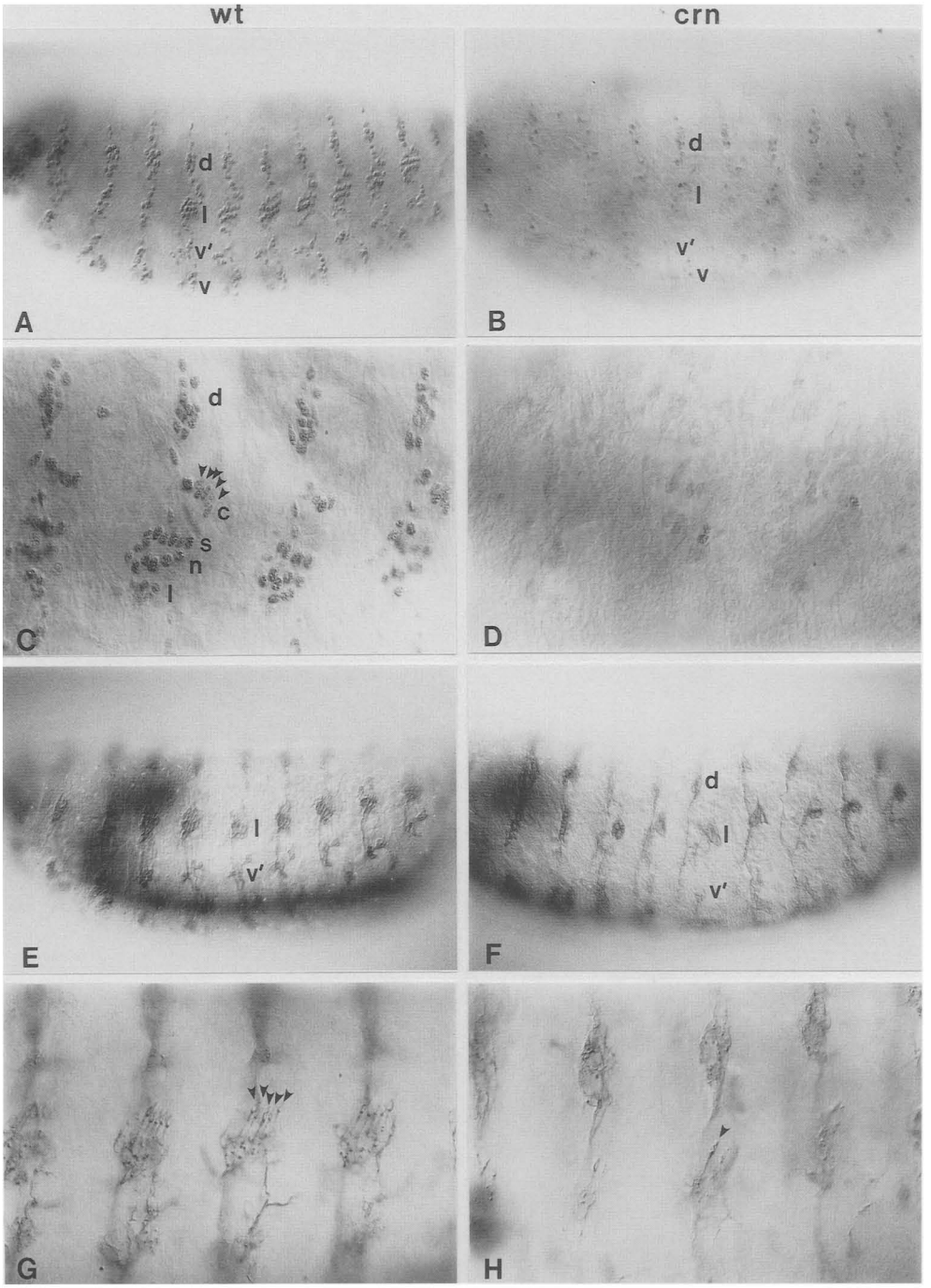

Figure 6. (*See facing page for legend.*)

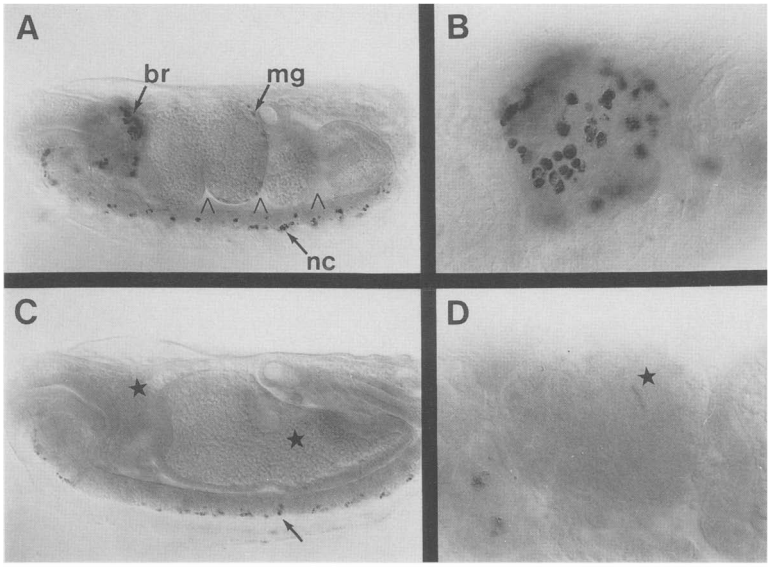

Figure 7. Cell proliferation in *crn* mutant embryos. Wild-type (*A* and *B*) and *crn* mutant (*C* and *D*) embryos incubated with BrdU at 13–14 hr of development and stained with anti-BrdU antibodies to detect proliferating cells. Both embryos are oriented with anterior to the *left*. In the wild-type embryo (*A*), labeled cells are present in the brain lobes (br), the midgut (mg), and the ventral nerve cord (nc). The arrowheads indicate the three constrictions of the midgut. In the *crn* mutant embryo (*C*), labeled cells are present only in the ventral nerve cord (arrow). There are no labeled cells in the brain or midgut (stars), and the midgut constrictions have not developed. Higher magnification demonstrates labeled neuroblasts in a wild-type brain lobe (*B*) and the absence of labeled cells in a mutant brain (*D*, star).

tively few cells still synthesizing DNA in wild-type embryos and when the genotype of mutant embryos can be unambiguously assigned on the basis of the midgut phenotype. BrdU-labeled cells in wild-type embryos at 14 hr include neuroblasts in the brain lobes, a few cells in each segment of the ventral nerve cord, and small numbers of cells in the midgut, Malpighian tubules, and gonad (Fig. 7A). Although DNA synthesis in some of these cells may reflect polyploidization rather than normal cell cycling, some cells, such as the neuroblasts of the brain (Fig. 7B), are clearly still dividing. Comparably staged embryos mutant for *crn* contain significantly fewer BrdU-labeled cells (Fig. 7C) and virtually no labeled neuroblasts in the brain (Fig. 7D), indicating that zygotic expression of *crn* is required to complete late rounds of cell division in the embryonic nervous system.

Discussion

The predicted amino acid sequence of the *crn* protein contains 16 copies of a 34-amino-acid repeat that is similar to the TPR motif found in a family of fungal genes (Boguski et al. 1990; Hirano et al. 1990; Sikorski et al. 1990). The *crn* gene represents the first member of the TPR family to be identified outside the fungi, suggesting that the motif is widespread and that additional members of the gene family will be found in other phyla. All known examples contain multiple and usually tandemly repeated copies of the TPR motif. Although the sequence of the TPR motif can vary considerably both within a gene and between genes, there is a strong conservation of amino acid type and spacing and, as a result, a highly conserved predicted secondary structure. Four of the fungal genes that contain TPR motifs have been shown genetically to be required for progression through the cell cycle. Mutations in *CDC16*, *CDC23*, *nuc2*$^+$, and *BimA* result in arrest during the G_2/M phase of the cell cycle and lead to defects in the segregation of chromosomes (Hartwell 1976; Morris 1976; Hirano et al. 1988; Palmer et al. 1990). More specifically, it has been shown that *nuc2* mutations cause arrest at the metaphase/anaphase transition (Hirano et al. 1988), suggesting that this pro-

Figure 6. Development of the PNS in *crn* mutant embryos. (*A*, *C*, *E*, and *G*) Wild-type embryos and (*B*, *D*, *F*, and *H*) *crn* mutant embryos stained with several PNS probes. (*A–H*) Dorsal is to the *top*; anterior is to the *left*. (*A* and *B*) Wild-type and *crn* mutant embryos, respectively, heterozygous for an enhancer trap/*lacZ* marker (*2A201*), which is expressed in the nuclei of neurons and support cells of the PNS; these embryos are stained with anti-β-galactosidase antibodies. In the wild-type embryo, the sensory organs comprising the two ventral clusters (v, v′), the lateral cluster (l), and the dorsal cluster (d) of abdominal segment 1 are labeled. In the mutant embryo, very few stained cells are found in the appropriate positions for the lateral and ventral clusters, whereas more cells are observed in the dorsal clusters. (*C* and *D*) Higher magnification of wild-type and mutant embryos, respectively, heterozygous for the same PNS marker. The lateral and dorsal clusters are shown in the wild-type embryo, and the nuclei of the cells that make up each of the five chordotonal organs in the lateral cluster are labeled [(c) cap cell; (s) sheath cell; (n) neuron; (l) ligament cell]. The nuclei of the five cap cells are indicated with small arrowheads. The mutant embryo shown here is an extreme example in which only a few surviving β-galactosidase-positive cells are present at the normal positions for the lateral and dorsal clusters. (*E* and *F*) Wild-type and *crn* mutant embryos, respectively, stained with SOXII monoclonal antibody. Note the small clusters of abnormally differentiated cells in the lateral clusters of the mutant embryo. (*G* and *H*) Wild-type and *crn* mutant embryos, respectively, stained with anti-HRP antisera and shown at high magnification at the level of the dorsal and lateral clusters. The five scolopidia of the chordotonal organs within a single abdominal segment are indicated by arrowheads in the wild-type embryo (*G*). In the mutant embryo (*H*), a single, well-differentiated scolopidium is observed in each of two adjacent segments (arrowhead).

tein is required for migration of chromosomes to opposite poles of the spindle. However, TPR-containing proteins are apparently involved in many diverse functions, including protein transport, RNA splicing, and the regulation of gene expression.

Although the TPR-containing proteins perform widely different roles, the phenotypes associated with *crn* mutations suggest that the *crn* protein performs a cell cycle function. Loss of maternal expression of *crn* results in the absence of the female germ line; the absence of female germ cells may be due to defects in cell proliferation. Loss of zygotic expression of *crn* leads to the absence of specific neurons in the CNS and PNS of the embryo. The most severely affected neurons are those that are born relatively late during embryonic development. Early steps in neurogenesis, such as neuroblast formation, the first divisions of neuroblasts, and the differentiation of the earliest identified neurons, appear normal in *crn* mutant embryos. Analysis of defects in the PNS indicate that both neurons and their support cells are missing in *crn* mutant embryos. The neurons and support cells that make up a sensory organ are derived from a common precursor cell, indicating that *crn* mutations cause the deletion of entire lineages of certain sense organs. The inhibition of BrdU incorporation in late-labeling brain neuroblasts of mutant embryos suggests that the absence of particular neuronal lineages in the CNS and PNS is the consequence of failed divisions of neuronal precursors. Finally, the phenotypes of embryos mutant for *crn* are similar to those of another cell cycle gene, cyclin A, particularly in the PNS where mutations in either gene result in loss of those lineages that produce the chordotonal organs (D. Smouse, unpubl.). Thus, the genetic analysis of *crn* offers strong circumstantial support for its role during the cell cycle. This, in turn, suggests that *crn* is most analogous to the *CDC16/CDC23/nuc2*$^+$ members of the TPR family.

The relatively specific effects of *crn* mutations could be due to a specific requirement for *crn* expression in just those cells and tissues affected by *crn* mutations or may more likely be due to the timing of that requirement. Maternal expression of the *crn* gene may be sufficient to allow early cell divisions to proceed normally, whereas loss of zygotic expression may affect only those divisions in the nervous system that occur after the depletion of maternally supplied *crn* gene products.

Several *Drosophila* cell cycle genes [e.g., cyclin A/*cdc13*$^+$ (Lehner and O'Farrell 1989; Whitfield et al. 1989); *string/cdc25*$^+$ (Edgar and O'Farrell 1989); *Dm cdc2/cdc2*$^+$ (Jimenez et al. 1990; Lehner and O'Farrell 1990)] have been identified as homologs of yeast cell cycle genes. The evidence presented here suggests that *crn* may also be a conserved cell cycle gene. At least three of the *Drosophila* genes are expressed maternally, yet loss of zygotic expression of any one results in embryonic lethality. This implies that the maternal endowment of these gene products is not sufficient to accomplish all embryonic cell divisions and that zygotic expression is required for completion of those mitoses. Previous screens (Baker et al. 1982; Gatti and Baker 1989) for cell cycle mutants have been based on the assumption that maternal expression of critical genes would rescue all cell divisions in the embryo and that loss of zygotic expression would result in lethality only later in development, during larval and pupal stages, when there are new rounds of division by cells of the nervous system and imaginal discs. Re-examination of mitotic defects in embryonic lethal mutations may uncover new genes required for essential cell cycle functions.

Materials and methods

Genetics of crn

The *crn* locus maps to polytene position 2E3-F1 on the X chromosome (Perrimon et al. 1984). Six alleles of *crn*, including *crn*^{RC63}, *crn*^{EA130} (Perrimon et al. 1984), *crn*^{EH160}, *crn*^{EH272}, *crn*^{EH328}, and *crn*^{EH352} (Eberl and Hilliker 1988) were examined. The four alleles isolated by Eberl and Hilliker were previously named *yolky* (Eberl and Hilliker 1988). We subsequently found *yolky* to be allelic to *crn* and have retained the original name of the locus. All of the alleles that were examined produce similar phenotypes, indicating that all are equivalent in strength. An allele that is the result of an insertion in the *crn*-coding sequence, *crn*^{RC63}, is likely to represent a null allele and was used for most of the phenotypic analysis.

Fly strains

The chromosomes carrying *crn* mutations are balanced over *FM7c*, *Binsn*, or *Binsc*; descriptions of the balancer chromosomes can be found in Lindsley and Grell (1968). The *ftz*–β-galactosidase (*ftz–lacZ*) strain from Y. Hiromi expresses β-galactosidase in embryos in a pattern similar to the pattern of expression of the native *ftz* protein (Hiromi and Gehring 1987). The strain *2A201* was generated using the "enhancer trap" technique (O'Kane and Gehring 1987) in our laboratory; it contains a P-element insert bearing the β-galactosidase gene on the third chromosome and expresses β-galactosidase in part of the midgut and in the neurons and support cells of the PNS (Perrimon et al. 1991).

Northern and Southern analyses

Total RNA was prepared from staged *Drosophila* embryo collections by the guanidinium/cesium chloride method (Maniatis et al. 1982). Poly(A)$^+$ RNAs were affinity purified on oligo(dT)–cellulose (type III, Collaborative Research). Northern blot analysis was performed by the method of Alwine et al. (1977).

For Southern analysis, DNA samples were digested with appropriate restriction endonucleases, resolved on a 0.7% agarose gel, and transferred to nitrocellulose by standard procedures (Maniatis et al. 1982).

In situ hybridization to embryos

Wild-type embryos were collected at 25°C and aged until the desired developmental stages. Fixation, O.C.T. embedding, and sectioning were performed as described by Hafen and Levine (1986). DNA probes were prepared by nick translation with ^{35}S-labeled d-CTP (NEN) to a sp. act. of $\sim 5.4 \times 10^7$ cpm/μg. Frozen sections of embryos were prepared, hybridized, and autoradiographed according to Hafen and Levine (1986). The autoradiograms were developed after 18–30 days; control hybridizations were performed with probes derived from the *ftz* gene.

Isolation of crn *cDNA clones*

cDNA clones corresponding to the *crn* locus were isolated from a λgt11 cDNA library (kindly provided by K. Zinn and C. Goodman) made from size-selected, 9- to 12-hr embryonic mRNA. A 0.7-kb *Bgl*II genomic fragment radiolabeled by random oligonucleotide priming (Feinberg and Vogelstein 1983) was used as a probe, and hybridizations were performed at 42°C in 50% formamide, 10% dextran sulfate, 1 M NaCl, 1× PE buffer [5× PE: 1% BSA, 1% polyvinyl pyrrolidone, 1% Ficoll, 250 mM Tris-HCl (pH 7.5), 0.5% sodium pyrophosphate, 5% SDS, and 5 mM EDTA], and 100 μg/ml of denatured salmon testis DNA. Filters were washed in 20× SSC, 0.1% SDS, at room temperature, and then in 0.2× SSC, 0.1% SDS, at 65°C before autoradiography. A total of 4×10^4 pfu were screened, and six positive phage clones were picked and analyzed. Two clones contain inserts of 2.3 kb and probably represent full-length cDNAs. The cDNAs were shown to correspond to transcripts from the *crn* locus by Northern and Southern analyses.

Sequencing strategy

crn cDNA clones were sequenced using the combined methods of Henikoff (1984) and Sanger et al. (1977). The insert of the *crn* cDNA was subcloned into the Bluescript(+) vector (from Stratagene), and a nested series of deletion derivatives, each diminished in size by ~200 bp, was generated using exonuclease III and SI nuclease. The ends were made blunt with the Klenow fragment of DNA polymerase I and then religated. After transformation, single-stranded DNA from each of the subclones was sequenced by the dideoxy chain-termination method. The reaction products were fractionated on 6% polyacrylamide–urea gels, fixed, dried, and exposed for autoradiography. The complete sequence was determined for both DNA strands, and ambiguous results owing to compressions were resolved using 7-deaza-dGTP in the sequencing reactions (Mizusawa et al. 1986).

Computer analysis

Translation, hydrophobicity, secondary structure prediction, and other protein structure determinations were performed with programs of the University of Wisconsin Genetics Computer Group (Devereux et al. 1984). The PIR (National Biomedical Research Foundation) protein data base was searched with the predicted ORF, using the FASTP program (Lipman and Pearson 1985).

Immunohistology

Immunohistology was performed as described in Smouse et al. (1988). Staining of *lacZ*-expressing strains was done using a mouse anti-β-galactosidase primary antibody from Promega-Biotec. Antibodies used to analyze the CNS and PNS of mutant embryos include anti-HRP (Cappel), a polyclonal antisera that labels all neuronal cell bodies and axons of the CNS and PNS (Jan and Jan 1982); SOXII, a monoclonal antibody that recognizes the cell bodies and axons of the entire PNS and a subset of CNS neurons (Goodman et al. 1984); BP102, a monoclonal antibody kindly provided by A. Bieber and C. Goodman, which labels the axons of CNS neurons; and anti-*eve*, a rabbit polyclonal antisera kindly provided by M. Frasch and M. Levine, which labels subsets of neuronal nuclei (Frasch et al. 1987; Doe et al. 1988).

BrdU labeling of embryos

Embryos were labeled with BrdU according to published procedures (Bodmer et al. 1989). Embryos were permeabilized with octane and incubated for 45 min in media containing 1 mg/ml of BrdU. Embryos were fixed immediately after labeling and processed for antibody staining as described above. The anti-BrdU antibody was a kind gift of A. Smith and T. Orr-Weaver.

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Note added in proof

Sequence data described in this paper have been submitted to the EMBL/GenBank Data Libraries.

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