Cytogenetic and Molecular Localization of *tipE*: A Gene Affecting Sodium Channels in *Drosophila melanogaster*

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ABSTRACT

Voltage-sensitive sodium channels play a key role in nerve cells where they are responsible for the increase in sodium permeability during the rising phase of action potentials. In *Drosophila melanogaster* a subset of temperature-sensitive paralytic mutations affect sodium channel function. One such mutation is *temperature-induced paralysis locus E* (*tipE*), which has been shown by electrophysiology and ligand binding studies to reduce sodium channel numbers. Three new γ-ray-induced *tipE* alleles associated with either visible deletions in 64AB or a translocation breakpoint within 64B2 provide landmarks for positional cloning of *tipE*. Beginning with the flanking cloned gene *Ras2*, a 140-kb walk across the translocation breakpoint was completed. Germline transformation using a 42-kb cosmid clone and successively smaller subclones localized the *tipE* gene within a 7.4-kb genomic DNA segment. Although this chromosome region is rich in transcripts, only three overlapping mRNAs (5.4, 4.4, and 1.7 kb) lie completely within the smallest rescuing construct. The small sizes of the rescuing construct and transcripts suggest that *tipE* does not encode a standard sodium channel α-subunit with four homologous repeats. Sequencing these transcripts will elucidate the role of the *tipE* gene product in sodium channel functional regulation.

BIOCHEMICAL and gene cloning studies of sodium channels (reviewed by CATTERALL 1992) from a variety of species have shown that these channels are comprised of a large α-subunit with a molecular mass of ~260 kD that forms a voltage-sensitive, ion selectivity pore in membranes. Within a species there are multiple genes encoding these α-subunits. In addition, in mammalian brain two smaller auxiliary subunits (β1 and β2) of ~36 and ~33 kD, respectively, copurify with the α-subunit. These smaller subunits play important functional roles in sodium channel expression (reviewed by ISOM *et al.* 1994). Use of a genetic approach in the study of sodium channels provides a way to analyze the interactions of these molecular components in the organism, to identify additional channel components and subtypes, and to identify molecules involved in channel regulation.

For example, analysis of behavioral mutants in Drosophila provides a method to identify genes encoding sodium channel subunits and other molecular components of membrane excitability in the nervous system (reviewed by WU and GANETZKY 1992). One specific group of mutant genes causing temperature-induced paralysis is particularly interesting because ligand-binding and electrophysiological studies have suggested that they affect sodium channels. These genes include: *para*^{ts} (*paralytic-temperature sensitive*) (SUZUKI *et al.* 1971), *nap*^{ts} (*no action potential-temperature sensitive*) (WU *et al.* 1978), *tipE* (*temperature-induced paralysis locus E*) (KULKARNI and PADHYE 1982) and *sei* (*seizure*) (JACKSON *et al.* 1984, 1985).

The *para* mutations were the first isolated with the temperature-induced paralytic phenotype (SUZUKI *et al.* 1971) and were later shown to cause a temperature-sensitive blockade of action potentials (WU and GANETZKY 1980). Subsequent gene cloning has shown that *para* encodes a sodium channel α-subunit (LOUGHNEY *et al.* 1989; RAMASWAMI and TANOUYE 1989). Ligand-binding studies of another temperature-sensitive paralytic mutation, *nap*, showed that head membranes from these flies had a decreased number of saxitoxin binding sites compared with wild type (JACKSON *et al.* 1984). Molecular cloning of *nap* revealed that it encoded a chromosome binding protein with sequence similarity to helicases. It may regulate *para* expression by binding to the *X* chromosome where *para* maps or by affecting RNA processing (KERNAN *et al.* 1991). Thus, in the *para* example the paralytic mutation defined a primary struc-

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tural component of sodium channels, while in the *nap* case it defined a protein involved in sodium channel gene regulation.

Both the *tipE* and *sei* mutations exhibit a temperature-sensitive paralysis phenotype and both mutant strains show alterations in saxitoxin-binding parameters (JACKSON *et al.* 1984, 1986). In addition, whole cell patch clamp studies on cultured embryonic neurons showed that both *tipE* and *sei* have reduced sodium currents (O'DOWD and ALDRICH 1988). Thus, cloning of these two genes is likely to identify additional molecules contributing to the structure or regulation of sodium channels. Since the molecular nature of the products of each of these genes is unknown, cloning studies must rely solely on phenotype and chromosome map position.

In this report we focus on the *tipE* mutation because of its potentially important role in sodium channel functional expression. Homozygous *tipE* flies paralyze rapidly at 38° and recover immediately when returned to 23°. Phenotypically they closely resemble the *nap* mutation (KULKARNI and PADHYE 1982; JACKSON *et al.* 1986). Double mutant studies of *tipE* with *para* or with *nap* provide additional evidence that *tipE* affects sodium channels. The combination of *tipE* with *nap* or *tipE* with various *para* alleles resulted in unconditional lethality of the double mutants at temperatures where the single mutants normally survive (GANETZKY 1986; JACKSON *et al.* 1986). Although some *para* alleles when combined with *tipE* show partial viability, other allelic combinations result in complete, unconditional lethality. Interestingly, the allele-specific lethality is not correlated with the amount of remaining *para* sodium channel activity. This suggests that the *tipE* gene product may physically interact with *para* (GANETZKY 1986; JACKSON *et al.* 1986).

The above data strongly suggest that *tipE* affects sodium channels. As a prelude to the molecular cloning of this locus, we began a cytogenetic analysis of the region on the left arm of chromosome *3* where the *tipE* locus had been mapped by recombination (KULKARNI and PADHYE 1982; JACKSON *et al.* 1986). In this paper we describe the characterization of three new chromosome aberrations isolated by their failure to complement *tipE* paralysis. We detail the use of these and other chromosome aberrations in the cytogenetic mapping of the *tipE* gene. We report a chromosome walk across the region containing *tipE* and describe the analysis of transcripts in a portion of this walk. We also report germline transformation rescue of the *tipE* paralytic phenotype with genomic DNA constructs and identify candidate *tipE* transcripts. These results provide the starting point for defining the nature of the *tipE* gene product by molecular cloning.

MATERIALS AND METHODS

Stocks and culture conditions: Drosophila cultures were grown at 21° on standard cornmeal medium (LEWIS 1960). The wild-type Canton-S strain was obtained from J. C. HALL (Brandeis University). The *tipE se* strain carries *tipE* (3–13.5) linked to *sepia* (*se*, 3–26.0), a benign eye color mutation. The *tipE se* strain was backcrossed to wild type for 10 generations to put *tipE* and *se* into a wild-type genetic background. The dominantly marked, multiply inverted third chromosomes *In (3LR) TM3, y^+ ri p^p sep Sb bx^{34e} e^s Ser* (abbreviated as *TM3*) and *In (3LR) TM6B, ss^- bx^{34e} e Tb ca* (abbreviated as *TM6B*) carry the *$tipE^+$* allele. These chromosomes were used to balance the new γ-ray-induced mutant chromosomes. The deletions *Df(3L)HR277* and *Df(3L)HR298* were provided by A. WOHLWILL (University of Illinois, Chicago) (WOHLWILL and BONNER 1991). The *$Df(3L)ems^{13}$* (GARBE *et al.* 1993) and *Df(3L)X37* stocks, isolated by M. SIMON (Stanford University), and the *Df(3L)GN19* (GARBE *et al.* 1993) and *Df(3L)GN34* isolated by R. RAWSON (University of Texas, Dallas), were obtained from J. FRISTROM (University of California, Berkeley). Descriptions of the marker mutations and chromosomes used in the genetic studies can be found in LINDSLEY and ZIMM (1992).

Mutagenesis: Wild-type males were mutagenized with 4000 rads of γ-irradiation and mated in batches of ~10 mutagenized males with ~20 *tipE se* virgin females. The F_1 progeny were screened for temperature-sensitive paralysis by placing F_1 flies (1500–2000 flies per test) onto a shelf in a preheated plexiglass box (WILLIAMSON 1971) at 38° for <8 min. Paralyzed flies were trapped on the shelf while mobile flies drowned in a mixture of vinegar and detergent at the bottom of the box. Individual paralyzed flies that recovered at 21° were crossed to *$TM3/ap^{Xa}$* flies to balance the putative mutant-bearing chromosomes against *TM3*. One new deletion-bearing chromosome *(Df(3L)TE3)* was recovered as a *T(Y;3)* translocation and was maintained over *tipE se*.

Cytological analysis and *in situ* hybridization to polytene chromosomes: Males from strains to be examined were crossed to wild-type virgin females. Salivary glands were dissected from third instar larvae in an 0.8% saline solution, rinsed by dipping in 45% acetic acid, stained for 2 min in lacto-acetic-orcein and squashed according to ENGELS *et al.* (1985). The squashes were examined using phase contrast optics and chromosome band assignments were made referring to LEFEVRE (1976). For *in situ* hybridization studies, larvae were grown at 18° and hybridizations were done as described by ENGELS *et al.* (1985) with minor modifications (MURTAUGH *et al.* 1993). DNA probes were biotinylated by nick translation using biotin-14-dATP and the BioNick Labeling System (GIBCO-BRL).

Screening libraries: Prehybridization, hybridization and washes during screening of the library on nylon membranes were done under standard high-stringency conditions (SAMBROOK *et al.* 1989). The ^{32}P-labeled DNA probes were used at a concentration of 10^6 cpm/ml. Clones with a "c" in the third position of their names (see Figure 2A) were isolated from the KT3 cosmid library (a generous gift from MAX SCOTT and JOHN LUCCHESI, Emory University) while clones with an "i" in this position were isolated from the iso-1 cosmid library (a generous gift of J. W. TAMKUN, University of California, Santa Cruz) in the Not-Bam-Not-CoSpeR vector (TAMKUN *et al.* 1992).

Genomic Southern blots: Twenty micrograms of genomic DNA [isolated by the method of JOWETT (1986)] was used for typical restriction enzyme digestions and genomic Southern blots following electrophoresis on 0.7% agarose gels. Gels were denatured, capillary-blotted onto ICN nylon membranes according to the manufacturer's protocol, and fixed by UV crosslinking using a UV Stratalinker 2400 (Stratagene). Standard high-stringency hybridization and wash conditions were used.

RNA preparation and Northern blots: Head, body and appendage (mainly legs and antennae) fractions were isolated from frozen adult flies as described previously (SCHMIDT-NIELSEN *et al.* 1977). Total RNA was prepared by the guanidinium isothiocyanate/CsCl gradient method and poly(A^+) RNA was selected by a single pass through an oligo(dT)-cellulose (type II, Collaborative Research Inc.) column (SAMBROOK *et al.* 1989). Ten micrograms poly(A^+) RNA was loaded in each gel lane. Preparation of blots and hybridization conditions were the same as previously described (ZHENG *et al.* 1995). To standardize for mRNA recovery and loading differences, blots were reprobed with a 0.6-kb rp49 cDNA fragment that encodes a widely expressed ribosomal protein (O'CONNELL and ROSBASH 1984).

Polymerase chain reaction: The 100-μl PCR reaction mixture contained 1× PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin), 0.2 mM of each of the dNTPs, 0.1 μM of each primer, 300 ng genomic DNA and 2.5 units AmpliTaq DNA polymerase (Perkin Elmer Cetus). After an initial 2 min at 94°, the following sequence was repeated 35 times: denaturation 2 min 94°, annealing 1 min 60°, extension 2 min 72°. The final extension was 10 min at 72°. Ten microliters of each PCR product was analyzed on a 1.2% agarose gel.

Germline transformation: Cosmid and plasmid DNA used for transformation were prepared by two cycles of equilibrium centrifugation in CsCl/ethidium bromide gradients (SPRADLING 1986; SAMBROOK *et al.* 1989). The cosmid clone rfi-6 (see Figures 2 and 4) in the CoSpeR transformation vector was used directly. For other transformations, genomic DNA fragments from cosmid clones were subcloned into the vector pCaSpeR2 or pCaSpeR4 (THUMMEL and PIRROTTA 1992). Both CoSpeR and pCaSpeR2/4 transformation vectors contain two *P*-element ends flanking a mini-w^+ (orange to red eye color) gene. DNA from these constructs was mixed with the helper plasmid pP[*ry*(Δ2–3)], a source of *P*-element transposase (D. READY, Purdue University) (LASKI *et al.* 1986), at a concentration of 1:0.25 μg/μl (construct:helper plasmid) and injected into *w;tipE se* homozygous embryos. Surviving G_0 adults were crossed to *w;tipE se* homozygotes and their progeny were screened for w^+ transformants. Transformants bearing a single copy of construct DNA in a homozygous *tipE* background were tested for paralysis at 38° for 2 min.

RESULTS

Isolation of new *tipE* alleles: Prior to this study the only genetic information about the *tipE* gene was recombination mapping data that placed it at 13.5 on chromosome *3* (KULKARNI and PADHYE 1982). To precisely localize the *tipE* gene cytologically in preparation for positional cloning, we screened 78,400 F_1 individuals following γ-ray mutagenesis as described in MATERIALS AND METHODS and isolated three new γ-ray-induced chromosome aberrations that gave the complementation patterns shown in Table 1. One new aberration, *Df(3L)TE1* (abbreviated as *TE1*), is a deficiency with visible breakpoints at 64A1–5 and 64B12–14. A second allele, *T(2;3)TE2* (abbreviated as *TE2*), is a reciprocal translocation between chromosomes *2* and *3* with breakpoints at 26A3 and 64B2. The third allele, *Df(3)TE3* (abbreviated as *TE3*) is a deficiency with breakpoints at 64A6 and 64B12–14. The *TE3* deletion behaves genetically as Y;3 translocation. The breakpoint on the third chromosome involved in this translocation to the *Y* has not been determined.

TABLE 1

Complementation test of new *tipE* alleles

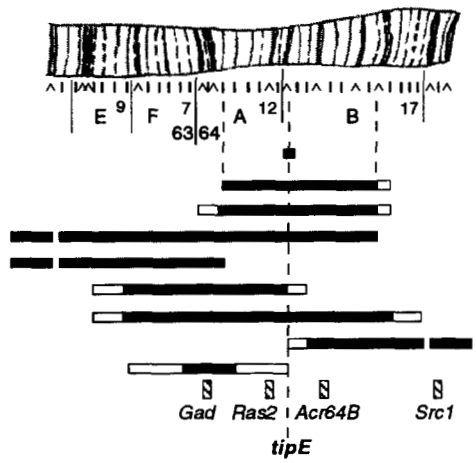
	TE1	*TE2*	*TE3*	*tipE*
TE1	Lethal			
TE2	ts	Lethal		
TE3	Lethal	ts	ND	
tipE	ts	ts	ts	ts

ts, temperature-sensitive paralysis at 38°; ND, not done; Lethal, no adults eclosed, stage of lethality not determined.

Each of these new alleles shows a temperature-induced paralytic phenotype when heterozygous with *tipE* (Table 1). This paralysis is indistinguishable from that shown by *tipE* homozygotes suggesting that the original ethylmethane sulfonate-induced *tipE* allele is a loss of function mutation. Although the deletion *TE1* and the translocation *TE2* are each lethal as homozygotes, *TE2/TE1* heterozygotes are viable and show the temperature-induced paralysis phenotype. Thus, the lethality associated with the translocation *TE2* is due to disruption of a gene other than *tipE*. Since the *TE2* lethality is not uncovered by the visible deletion *TE1* and since the *TE2* translocation breakpoint falls approximately in the middle of the *TE1* deletion, the lethality must map outside the limits of the deficiency *TE1*.

Cytological mapping of *tipE* and other genes in 64AB: The newly isolated chromosome aberrations (*TE1*, *TE2*, and *TE3*) plus six other deficiencies isolated in this region by others allowed the cytological mapping of *tipE*. The relative positions of all aberrations and their chromosome breakpoints are summarized in Figure 1. There are five deficiencies that uncover the recessive *tipE* paralytic phenotype, including *TE1*, *TE3*, *HR277*, *GN19*, and *X37*. In addition, there are three deficiencies (*HR298*, ems^{13}, and *GN34*) which fail to uncover *tipE*. Two of these deficiencies define the distal (*GN34*) and proximal (ems^{13}) limits of the gene. The most useful aberration is the *TE2* translocation with a visible breakpoint in 64B2. Since this translocation uncovers *tipE*, it provides an important chromosome landmark for positional cloning.

A number of interesting cloned genes, expressed in the nervous system, have been mapped to the 64AB region, including glutamic acid decarboxylase (Gad), two oncogene homologues (Ras2 and Src1) and a nicotinic acetylcholine receptor subunit (Acr64B) (see Figure 1 legend.) As summarized in Figure 1, we ordered these genes with respect to the aberration breakpoints and the *tipE* gene by *in situ* hybridization to salivary gland chromosomes. Gad is uncovered by *HR298* and *TE1* but lies distal to *TE3*. The Src1 gene is proximal to the *TE1*, *TE3* and *HR277* deficiencies, placing it proximal to 64B12. The Ras2 and Acr64B genes are both



	Breakpoints	Phenotype over *tipE*	Reference
T(2;3)TE2	26A3; 64B2	ts	This study
Df(3L)TE3	64A6; B12-14	ts	This study
Df(3L)TE1	64A1-5; B12-14	ts	This study
Df(3L)HR277	63B6; 64B12	ts	Wohlwill and Bonner (1991)
Df(3L)HR298	63B1; 64A6	+	Wohlwill and Bonner (1991)
Df(3L)GN19	63E6-9; 64B2-4	ts	Garbe *et al.* (1993)
Df(3L)X37	63E6-9; 64B14-17	ts	Lindsley and Zimm (1992)
Df(3L)ems13	64B2-4; 64E	+	Garbe *et al.* (1993)
Df(3L)GN34	63E9-F7; 64A7-B2	+	R. Rawson

FIGURE 1.—Cytogenetic mapping of *tipE* and other genes in the 64AB region. The black bars represent deleted regions with breakpoint uncertainties indicated by open bars. The open breaks in the black bars indicate deletions that extend beyond the limits of the chromosome diagram shown at the top. The complementation results for each chromosome aberration with *tipE* are listed as either ts (temperature sensitive paralysis) or + (wild type). The source of each aberration is listed in the Reference column. The localization of cloned genes indicated at the bottom of the figure was determined directly by *in situ* hybridization of each clone to polytene chromosomes from individuals heterozygous for each aberration. The cloned genes are glutamic acid decarboxylase (Gad) (Jackson *et al.* 1990), oncogene homologues Ras2 (Neuman-Silberberg *et al.* 1984; Mozer *et al.* 1985; Salzberg *et al.* 1993) and Src1 (Hoffman-Falk *et al.* 1983), and a nicotinic acetylcholine receptor subunit (Acr64B) (Hermans-Borgmeyer *et al.* 1986; Wadsworth *et al.* 1988).

uncovered by *TE1*, *TE3* and *HR277*. Neither Ras2 nor Acr64B is uncovered by *HR298*. The Ras2 clone hybridizes distal to the *TE2* breakpoint whereas Acr64B is proximal to *TE2* (summarized in Figure 1). Thus, Ras2 and Acr64B were identified as starting points for a chromosome walk to the *tipE* locus since they were the most closely linked, flanking clones. In addition to providing a starting point for a walk through the *tipE* locus, this work also provides useful information for screening for mutations involving the other cloned genes in the region. One example is the subsequent successful use of *TE1* and *TE3* in a differential screen for Gad mutations (Kulkarni *et al.* 1994).

Localization of *TE2* breakpoint by chromosome walking: We initiated a chromosome walk using a cosmid genomic library probed with fragments of the clones Ras2 and Acr64B that flank the *TE2* translocation breakpoint. It was soon apparent by *in situ* hybridization to salivary gland chromosomes that Ras2 was closer than Acr64B to the *TE2* breakpoint so the walk from Acr64B was discontinued. A total of 140 kb of genomic DNA was isolated (Figure 2A). At each step in the walk, fragments of the insert from each cosmid clone were used to probe genomic Southern blots made from *TE2/TE1*, *TE2/+*, and *+/+* flies looking for altered restriction fragments. Initial analysis suggested that clones rfi-6 and rfi-4 both crossed the *TE2* translocation breakpoint. This was confirmed by *in situ* hybridization to *TE2/+* polytene chromosomes that showed that clone rfi-4 hybridized across the translocation breakpoint (Figure 3). The position of the breakpoint was further localized by probing genomic Southern blots with subfragments of cosmid rfi-4. As summarized in Figure 2B, the breakpoint was localized to a 7.4-kb *Not*I/*Eco*RI fragment.

***TE2* translocation is associated with a small deletion:** Strains carrying the *TE2* translocation over the deletion *TE1* were used to simplify the restriction enzyme mapping of the translocation breakpoint since the *TE1* deletion-bearing chromosome lacks DNA in the area flanking the translocation. To precisely localize the translocation breakpoint, the 7.4-kb genomic fragment from wild-type flies in the region of the translocation breakpoint was subjected to extensive restriction enzyme mapping (Figure 2B). A series of overlapping fragments from the wild-type 7.4-kb genomic DNA fragment were used to probe genomic Southern blots from *TE2/TE1*, *TE2/+*, and *+/+* genomic DNA digested with five different restriction enzymes. Two partially overlapping probes (G7NS19 and G7PG22) detected dramatically different restriction patterns when the three genotypes were compared. As shown in Figure 2C, two restriction fragments (indicated by arrows labeled with size of the fragment) disappeared from *TE2/TE1* (lane 1) when genomic DNA was digested with either *Bgl*I, *Pst*I, *Apa*I, *Sac*I or *Ava*I, suggesting the deletion of all of these sites. In each restriction digestion shown in Figure 2C, only one new fragment appeared. These results suggested a deletion was associated with the translocation and were consistent with the model shown in Figure 2B in which the probes used (long, narrow bars in Figure 2B) did not cross the whole suspected deletion region. In this model, only the portion of each probe that lies outside the deletion hybridizes to the altered restriction fragments in *TE2*.

To further analyze this deletion and to define its

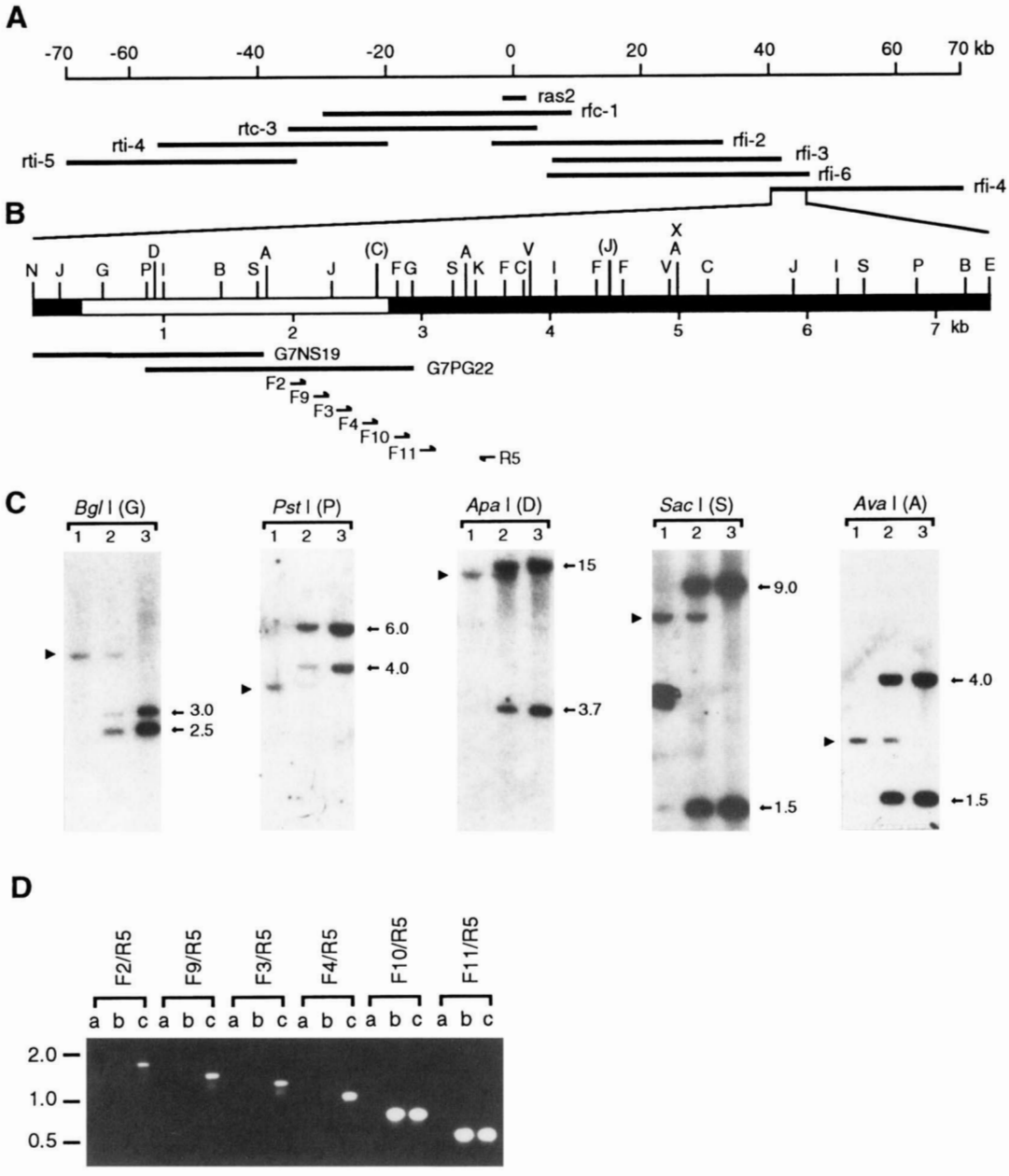


FIGURE 2.—Summary of chromosome walk and the structure of the *TE2* translocation/deletion landmark. (A) A chromosome walk was initiated using a Ras2 cDNA clone to probe cosmid libraries (TAMKUN *et al.* 1992; M. SCOTT, personal communication). *In situ* hybridization (see Figure 3) showed that clone rfi-4 crossed the *TE2* translocation breakpoint. The breakpoint was further localized to a 7.4-kb *Not*I/*Eco*RI fragment (expanded segment in part B). (B) Restriction map of the 7.4-kb genomic DNA fragment that crosses the *TE2* translocation breakpoint showing an associated deletion as a white segment within the map. Locations of two probes (G7NS19 and G7PG22) used in the genomic Southern blots (C) and primers (F2, F3, F4, F9, F10, F11, and R5) used in PCR (D) are indicated. The abbreviations used for restriction enzymes are A, *Ava*I; B, *Bst*XI; C, *Cla*I; D, *Apa*I; E, *Eco*RI; F, *Acc*I; G, *Bgl*I; I, *Bst*EII; J, *Hinc*II; K, *Kpn*I; N, *Not*I; P, *Pst*I; S, *Sac*I; V, *Eco*RV and X, *Xho*I. Restriction sites within parentheses indicate polymorphic differences. (C) Genomic DNA digested with the indicated enzyme and subjected to Southern blotting was probed with fragments from the 7.4-kb genomic segment shown in B. Blots digested with *Apa*I, *Bgl*I and *Pst*I were probed with G7NS19 while *Sac*I and *Ava*I blots were probed with G7PG22. Lane 1 contains genomic DNA from *TE2/TE1*; lane 2 from *TE2*/+; lane 3 from +/+. Arrows indicate restriction fragments disrupted by the *TE2* translocation that are reduced in *TE2*/+ and disappear in *TE2/TE1*. Triangles indicate the new bands that appear in *TE2*/+ and *TE2/TE1* due to the translocation. (D) PCR amplification using *TE2/TE1* (b lanes) and +/+ (c lanes) genomic DNA templates to verify the position of the deletion. The a lanes have no DNA template (negative control). The forward primers F2, F3, F4 and F9 are within the deleted region while primers F10, F11 and the reverse primer R5 are outside the deleted region. Molecular sizes in kilobases are indicated at arrows and lines on sides of gels.

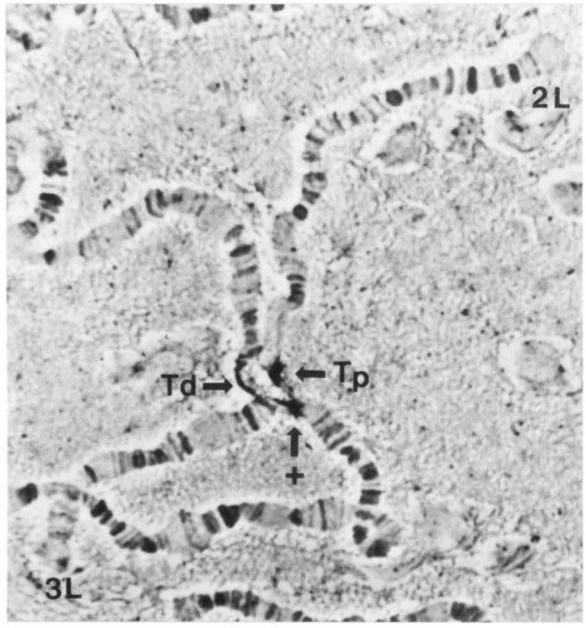


FIGURE 3.—*In situ* hybridization of cosmid clone rfi-4 to polytene chromosomes from *TE2*/+ heterozygotes. *TE2* is a reciprocal translocation between chromosome *2* and *3*. The arrows show the hybridization signal of the rfi-4 clone crossing the translocation breakpoint. The arrow with + indicates the hybridization signal to the wild-type chromosome; Tp indicates the signal from the region proximal to the breakpoint whereas Td indicates the signal from the region distal to the break. The left ends of chromosomes *2* and *3* are labeled as 2L and 3L, respectively.

proximal limit, we designed a series of primers lying within the proposed deleted region based on sequence of the 7.4-kb genomic fragment. These primers were used to amplify genomic DNA from wild type and *TE2/TE1*. As shown in Figure 2B and D, primers (F2, F9, F3, F4) that failed to amplify from *TE2/TE1* genomic DNA template (b lanes) identify the deleted region on the translocation-bearing third chromosome since the expected products were obtained using wild-type genomic DNA template (c lanes). Primers (F10, F11) that amplify with both *TE2/TE1* and wild-type templates represent regions outside of the deletion. These PCR results are consistent with the existence of the deletion deduced from restriction enzyme mapping by genomic Southern blots. Combining data from the two approaches, the size of the deletion is ~2.5 kb (open bar region in Figure 2B).

Multiple transcripts are disrupted by *TE2* translocation/deletion: Since the *TE2* translocation uncovers the recessive *tipE* phenotype, any transcripts disrupted by the *TE2* translocation/deletion are candidates for the *tipE* gene product. To identify such disrupted transcripts, Northern blots of poly(A^+) RNA from *TE2/TE1* and +/+ flies were probed with a series of three genomic DNA probes that included the *TE2* translocation/deletion and flanking regions. These and other smaller probes (data not shown) allowed us to roughly map the transcripts relative to the deletion associated with *TE2*. It should be noted that all transcripts within this region are expected to be reduced in flies bearing the *TE1* deletion, whereas observed size differences for some are likely due to disruption by the *TE2* translocation break.

As summarized in Figure 4B, the probes detected at least seven different size transcripts (7.0, 6.0, 5.4, 4.4, 3.4, 1.7, and 1.0 kb) altered by the *TE2* translocation/deletion in adults (Figure 4C). The 7.0-, 6.0- and 1.0-kb transcripts have reduced expression levels while the other four transcripts (5.4, 4.4, 3.4, and 1.7 kb) are physically disrupted by the *TE2* translocation/deletion. For these latter four transcripts, the transcripts found in the wild type (lanes 2) disappear from *TE2/TE1* flies (lanes 1) and are replaced by altered size transcripts.

In Drosophila, known sodium channel genes are confined in expression to neuronal and glial tissue (HONG and GANETZKY 1994). If *tipE* directly and specifically affects sodium channels, the *tipE* transcript should be found in body parts enriched for neuronal/glial tissue. As summarized in Table 2, the affected transcripts show a variety of expression patterns in Drosophila adult body parts. The 5.4-, 4.4-, and 1-kb mRNAs are expressed in heads, bodies and appendage fractions, although they are each in higher abundance in head and appendage fractions than in bodies. The 7.0-kb mRNA is in both heads and appendage fractions, and the 6.0- and 3.4-kb mRNA are mainly in heads. Since head and appendage fractions are enriched in neuronal tissue, these six transcripts remain possible candidates for the *tipE* gene product. In contrast, the 1.7 kb is only in bodies, making it an unlikely candidate for *tipE*. The existence of multiple transcripts in *TE2* translocation/deletion region makes it impossible to identify the *tipE* transcript simply by looking for altered transcripts.

A 7.4-kb genomic DNA rescues *tipE* paralysis: We next used transformation rescue (SPRADLING 1986) to narrow the number of *tipE* candidate transcripts. Germline transformation with the 42-kb cosmid clone rfi-6 (Figure 2A) completely rescued the temperature-induced paralysis phenotype. Various fragments from this cosmid clone and the 7.4-kb genomic fragment from cosmid clone rfi-4 (Figure 2A) were used for subsequent germline transformation. Figure 4A shows those fragments that rescue *tipE* paralysis as solid bars with an open area denoting the position of the deletion. The cross-hatched bars denote fragments that fail to rescue. Each of the rescuing fragments (42, 12, and 7.4 kb) contains the wild-type sequence in the region altered by the *TE2* translocation/deletion.

The rescue pattern shown in Figure 4A allowed us to eliminate the 7.0-, 6.0-, 3.4- and 1.0-kb transcripts as *tipE* candidates since they are each transcribed from genomic DNA outside the region of the smallest rescuing construct (7.4-kb fragment, Figure 4, A and B). The remaining 5.4-, 4.4- and 1.7-kb mRNAs are all tran-

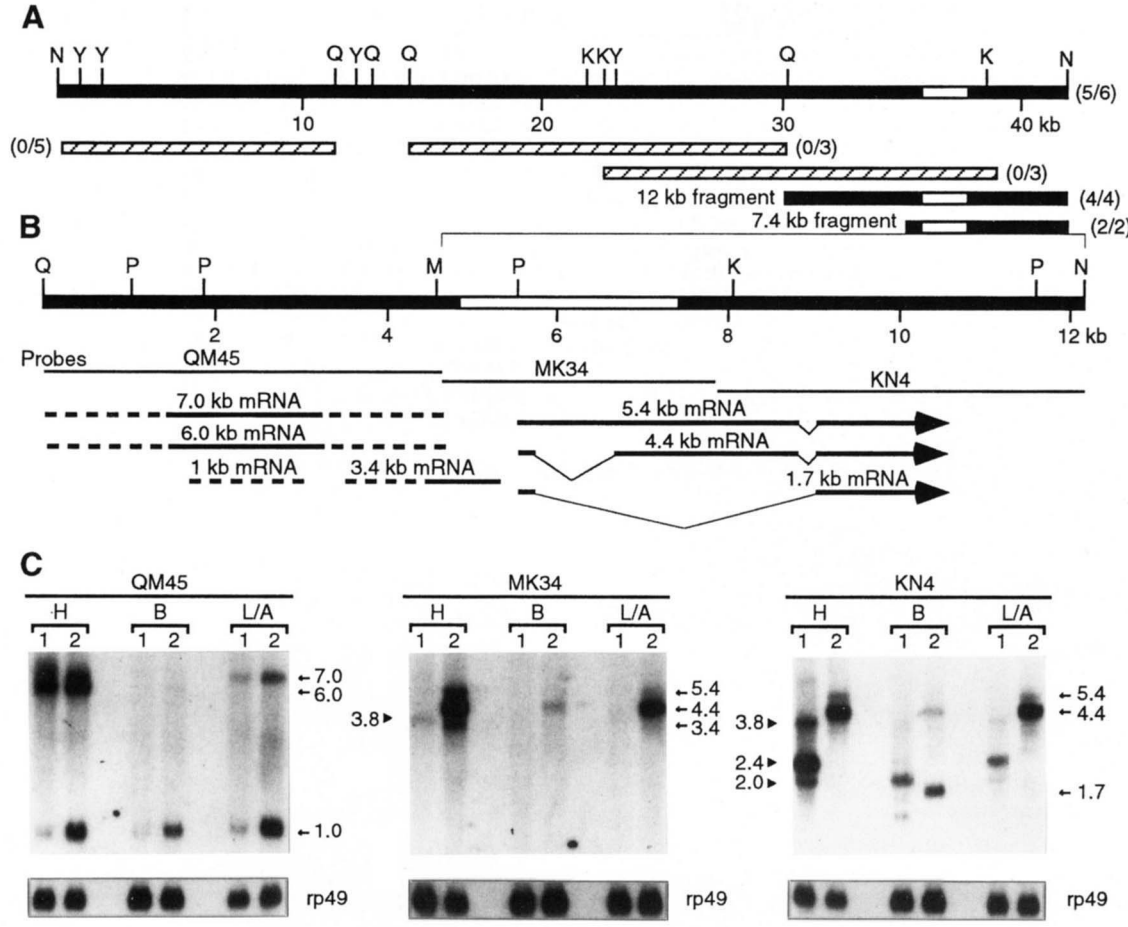


FIGURE 4.—Identification of *tipE* transcripts. (A) Transformation rescue of *tipE* paralysis. Cosmid clone rfi-6 (Figure 2A) and various fragments from this clone were used for germline transformation. Solid bars indicate DNA fragments that rescue *tipE* paralysis. The open area within the solid bar indicates the position of the *TE2* translocation/deletion. Striped bars represent DNA fragments that do not rescue *tipE* paralysis. The numbers within parentheses at the end of each bar represent (number of rescuing transformant lines/total number of transformant lines produced). The 7.4-kb fragment is from cosmid clone rfi-4 (Figure 2A). (B) Expansion of the 12-kb rescuing fragment showing positions of transcripts relative to the *TE2* translocation/deletion. The position of the 7.4-kb rescuing fragment within the 12-kb fragment is indicated by the light lines extending from the 7.4-kb bar. Dashed lines on the lower transcripts indicate uncertainty about positions of their ends. For example, the solid bar in the 3.4-kb mRNA was localized on the genomic map by a 0.9-kb subfragment of MK34. However, this transcript was not detected by any of the other probes shown in this figure, suggesting the rest of this transcript is encoded by a genomic region outside the 12-kb fragment shown. Arrow heads indicate the direction of transcripts determined by single stranded riboprobes. The 5.4-, 4.4- and 1.7-kb mRNAs are fully within the 7.4-kb genomic fragment that rescues the *tipE* paralysis. The large introns shown within the 4.4 and 1.7-kb transcripts were determined by probing Northern blots with various, small genomic DNA fragments. The small introns shown within the 5.4- and 4.4-kb transcripts were determined by a comparison of partial genomic and cDNA sequences. (C) Transcripts disrupted by the *TE2* translocation/deletion. Northern blots containing poly(A^+) RNA from heads (H), bodies (B), and legs/antennae (L/A) of *TE2/TE1* (lane 1) and wild-type (lane 2) flies were probed with QM45 (left blot), MK34 (middle blot) or KN4 (right blot). Arrows indicate the transcripts in wild-type flies (lane 2) that are affected by the *TE2* translocation/deletion. Triangles indicate new transcripts of altered size that appear in *TE2/TE1* flies (lane 1) due to the translocation/deletion. Arrows and triangles show fragment sizes in kilobases. As shown in the lower frame, Northern blots were stripped and reprobed with a cDNA encoding rp49, a widely expressed ribosomal protein (O'CONNELL and ROSBASH 1984) to determine the relative amounts of mRNA added to each lane. Abbreviations used for restriction enzymes in parts A and B are the same as in Figure 2 except that here M, *Bam*HI; Q, *Xba*I and Y, *Sac*II.

scribed from completely within the 7.4-kb genomic DNA. Based on their transcript distribution patterns, the 5.4 and 4.4 kb are the most promising *tipE* candidates since they are enriched in heads and appendages that have a high proportion of neuronal tissue. To determine the relationship among the three transcripts (5.4, 4.4, and 1.7 kb) that lie completely within the 7.4-kb rescuing fragment, Northern blots were probed with single-stranded riboprobes that showed that all three of these mRNAs are transcribed in the same direction. In addition, all three overlap extensively. Therefore, it is likely that they are alternatively spliced forms of a single transcriptional unit. Since this is the only transcriptional unit detected within the rescuing 7.4-kb genomic DNA, we conclude that these transcripts are products of the *tipE* gene.

TABLE 2

Transcripts affected by *TE2* translocation/deletion

Size (kb)	Expression pattern in wild type	Expression in *TE2/TE1*
7.0	Head, appendage	Reduced
6.0	Head	Reduced
5.4	Head, body, appendage	Disrupted
4.4	Head, body, appendage	Disrupted
3.4	Head	Disrupted
1.7	Body	Disrupted
1.0	Head, body, appendage	Reduced

DISCUSSION

Two distinct genes, DSC1 and *para*, encoding sodium channel α-subunit homologues have been identified in Drosophila (SALKOFF *et al.* 1987; LOUGHNEY *et al.* 1989; RAMASWAMI and TANOUYE 1989). In light of the fact that voltage-sensitive sodium channel α-subunits in mammals consist of a multigene family encoding at least seven structurally distinct isoforms (NODA *et al.* 1986; KAYANO *et al.* 1988; ROGART *et al.* 1989; TRIMMER *et al.* 1989; KALLEN *et al.* 1990; GAUTRON *et al.* 1992), the question remains as to whether there are additional α-subunit genes in Drosophila. Since one of the cloned Drosophila sodium channel genes was first identified on the basis of its temperature-sensitive paralytic phenotype, one way to address this question is to characterize additional mutations with similar phenotypes.

The *tipE* mutation defines one candidate for an additional sodium channel α-subunit gene since this mutation reduces the number of saxitoxin-binding sites (JACKSON *et al.* 1986) and reduces sodium currents in embryonic neurons (O'DOWD and ALDRICH 1988). However, our results suggest that *tipE* does not encode a sodium channel α-subunit. Through chromosome walking and transformation rescue experiments with genomic DNA fragments ranging in size from 42 to 7.4 kb, we identified a fragment of DNA containing the complete *tipE* gene and all of the upstream regulatory region required for its proper expression. This conclusion is based on the observation that the rescued flies all show locomotor activity indistinguishable from wild-type flies with respect to temperature sensitivity. The small size of the minimal rescuing genomic DNA construct places constraints on the maximum size of the *tipE* transcript, limiting it to <7.4 kb. Consistent with this size limitation are the data from Northern blots that showed that only three overlapping transcripts (5.4, 4.4, and 1.7 kb) were located completely within the rescuing 7.4-kb fragment. Of these three transcripts, the largest (5.4 kb) is more likely to be a splicing intermediate rather than a mature message because it shows the same general tissue distribution as the 4.4-kb mRNA but is present in much lower abundance. Furthermore, the 5.4-kb form contains an intron that is spliced out of the 4.4-kb mRNA.

Taken together, our transformation rescue and transcript analysis results rule out the possibility that *tipE* encodes a standard sodium channel α-subunit with four homologous repeats because such subunits are very large core proteins ($Mr > 180{,}000$) encoded by large transcripts ranging in size from 8 to 15 kb. No suitably sized transcripts were found to be encoded by the rescuing construct. Even if there were a very rare undetected transcript, such a large transcript alone would not fit into the rescuing genomic construct. Since many sodium channel genes have been shown to undergo extensive alternative splicing (SARAO *et al.* 1991; SCHALLER *et al.* 1992; THACKERAY and GANETZKY 1994), the actual size of a genomic fragment required for transformation rescue would be even larger due to the presence of multiple introns. Indeed, the *para* sodium channel gene in Drosophila seems to stretch over a genomic area of >70 kb based on the mapping of mutant alleles (LOUGHNEY *et al.* 1989). Consistent with our suggestion that *tipE* does not encode a sodium channel α-subunit are preliminary sequence data from cDNA clones corresponding to the candidate transcripts. The partially sequenced open reading frame does not encode a conventional sodium channel α-subunit (HALL *et al.* 1994).

Based on the effects that the *tipE* mutation has on sodium channel numbers and on sodium current levels, we suggest that the *tipE* gene product affects sodium channel functional regulation. There are several possible mechanisms by which this might occur. One would be through direct physical interaction of the *tipE* gene product with the α-subunit as would be expected for β1- or β2-type subunits (ISOM *et al.* 1994). Another would be through effects on gene expression of one or more sodium channel α-subunit genes. The latter is the proposed mechanism for *nap* effects on sodium channel numbers (KERNAN *et al.* 1991).

Although *nap* and *tipE* have very similar phenotypes with respect to the temperature-induced paralysis and recovery and effects on saxitoxin binding, there are several observations that suggest they act on different aspects of sodium channel functional expression. For example, both *tipE* and *nap* interact with *para* alleles to cause lethality even at permissive temperatures where either single mutation alone would survive. However, the pattern of specific interactions with different *para* alleles is opposite for *tipE* compared with *nap*. Thus, *para* alleles that show the strongest interaction with *tipE* show the weakest interaction with *nap* and vice versa (GANETZKY 1986). In addition, double mutants with *nap* and *tipE* interact in a synergistic fashion, with the double mutations showing more dramatic effects than either single mutation alone on temperature-sensitive paralysis, saxitoxin-binding, action potential blockade, and adult longevity (GANETZKY 1986; JACKSON *et al.* 1986). One interpretation of these results is that *tipE* affects a different step in functional expression of sodium channels than *nap*. For example, *tipE* may act at

a posttranslational step like the $\beta 2$ subunit that is required for insertion of sodium channel α-subunits into the membrane (SCHMIDT *et al.* 1985; SCHMIDT and CATTERALL 1986). Definition of the mechanism of *tipE* action will require sequencing the gene product.

When we began these studies very few genetic studies had been conducted in the region of 64AB. Recent work (WOHLWILL and BONNER 1991; GARBE *et al.* 1993; KULKARNI *et al.* 1994) has begun to develop this area. It is now estimated that there are $\geq$19 essential genes within the region of 64A3–5 to 64B12 (KULKARNI *et al.* 1994). Recently, the *pavarotti* mutation that affects the development of the peripheral nervous system has been mapped proximal to *tipE* (SALZBERG *et al.* 1994). Our chromosome walk (around 64B2) and transformation rescue constructs fall near the center of this region and our analysis has shown that the region of our walk is rich with transcripts. It is likely that the transformation strains developed in these studies will help in the identification of gene products of some additional members of this 19-member essential gene group.

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