Molecular Characterization of *eag*: A Gene Affecting Potassium Channels in *Drosophila melanogaster*

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ABSTRACT

Genes encoding proteins involved in the function of the nervous system can be identified via mutations causing behavioral abnormalities. An example is *ether à go-go* (*eag*) in *Drosophila melanogaster*, which was identified originally as an *X*-linked mutation that displayed ether-induced leg-shaking behavior. Electrophysiological and genetic evidence suggests that the product of the *eag* locus is intimately involved in the normal functioning of one or more types of voltage-gated potassium channels. To initiate a molecular analysis of *eag* we first generated a collection of deletions to pinpoint its cytological location. On the basis of this location, we identified an existing inversion, $In(1)sc^{29}$, with one breakpoint at the *eag* locus and the other in the *scute* (*sc*) complex. A genomic library was prepared from $In(1)sc^{29}$ and screened with a genomic DNA fragment that spanned the *sc* breakpoint to isolate DNA from the *eag* region. Beginning from this starting point over 85 kb of DNA were isolated by chromosome walking. Three additional *eag* alleles, including two dysgenesis-induced insertion mutations and a γ-ray-induced insertional translocation, were located on the molecular map of the *eag* locus by Southern blot analysis. The molecular defects associated with these alleles encompass a total of 27 kb within the chromosome walk. A 10-kb transcript derived from this region, which is expressed most abundantly in heads, was identified on Northern blots. Two different *eag* mutations separated by over 20 kb interrupt the same transcript identifying it as the likely *eag* message. cDNAs representing a portion of this transcript have been isolated. The genomic DNA sequences from which these cDNAs are derived extend over 37.5 kb, providing a minimum estimate of the size of the *eag* transcription unit. Ultimately, sequence analysis of these cDNAs should enable us to the identify the *eag* polypeptide and to elucidate its role in membrane excitability.

A characteristic feature of neurons and muscle fibers is their electrically excitable cell membrane that enables them to transmit and process information in the form of electrical impulses or action potentials. Integral membrane proteins, called ion channels, mediate the fluxes of various ions such as sodium, potassium and calcium that underlie the generation and propagation of these electrical signals and thereby play the key roles in the function of the nervous system (HILLE 1984). Complete understanding of how the nervous system functions at the molecular level will therefore require detailed molecular characterization of the structure, function and regulation of ion channels. The genes that encode several different types of ion channels have now been cloned by a variety of different approaches (reviewed by CATTERALL 1988). One approach in Drosophila that is proving especially useful is the identification of genes that encode channels or that otherwise affect their function via mutations with behavioral defects (reviewed by GANETZKY and WU 1986). Electrophysiological characterization of mutations identified in this way has revealed that several of them cause profound alterations in the normal mechanism of neuronal signaling. Some of these loci, identified originally on the basis of their mutant phenotypes, have now been cloned and shown to represent ion channel structural genes.

For example, mutations of the *Shaker* (*Sh*) locus were discovered on the basis of their leg-shaking phenotype under ether anesthesia (KAPLAN and TROUT 1969). Electrophysiological studies showed that *Sh* mutations caused prolonged release of neurotransmitter at the larval neuromuscular junction because the nerve terminal failed to repolarize properly (JAN, JAN and DENNIS 1977; GANETZKY and WU 1982). These results implied a defect in potassium channels. Subsequent voltage-clamp experiments revealed that the function of a particular class of potassium channels, the A-type channels, was specifically disrupted by *Sh* mutations (SALKOFF and WYMAN 1981; WU and HAUGLAND 1985). Sequence analysis of the *Sh* cDNAs and expression of the *Sh* gene product in oocytes demonstrate that *Sh* is the structural locus for a family of A-type channels produced by alternative splicing (BAUMANN *et al.* 1987; KAMB, IVERSON and TANOUYE

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1987; PAPAZIAN *et al.* 1987; TEMPEL *et al.* 1987; IVERSON *et al.* 1988; KAMB, TSENG-CRANK and TANOUYE 1988; PONGS *et al.* 1988; SCHWARZ *et al.* 1988; TIMPE, JAN and JAN 1988; TIMPE *et al.* 1988). These results are especially important because prior to the cloning of *Sh* no biochemical purification or molecular analysis of potassium channels was achieved, emphasizing the significance of genetic approaches to channel structure and function. In other recent studies (LOUGHNEY, KREBER and GANETZKY 1989; RAMASWAMI and TANOUYE 1989), the *para* locus, originally identified on the basis of mutations with a temperature-sensitive paralytic phenotype correlated with a block in the propagation of action potentials (SUZUKI, GRIGLIATTI and WILLIAMSON 1971; SIDDIQI and BENZER 1976; WU and GANETZKY 1980), has been shown to represent a sodium channel structural gene. Genetic studies of *para* have led to new insights concerning the regulation of sodium channel levels in Drosophila neurons (STERN, KREBER and GANETZKY 1990).

Another gene with an important function in neuronal membrane excitability is the *ether à go-go* (*eag*) locus. Like *Sh*, the first *eag* mutation was identified on the basis of its leg-shaking phenotype under ether anesthesia (KAPLAN and TROUT 1969). Electrophysiological studies of *eag* mutations at the larval neuromuscular junction revealed striking abnormalities (GANETZKY and WU 1983, 1985). Release of neurotransmitter is enhanced in *eag* mutations, resulting in synaptic potentials (called ejps or excitatory junctional potentials) that are larger and more prolonged than normal. Moreover, in the absence of any nerve stimulation, there is a high frequency of aberrant spontaneous ejps whose occurrence is correlated with spontaneous repetitive firing of the motor axons. Double mutants between *eag* and *Sh* display a remarkable synergistic interaction. Transmitter release in double mutants persists for at least an order of magnitude longer than in either single mutant and is concurrent with a train of repetitive action potentials in the motor axon. Genetic and pharmacological experiments indicate that the basis of this synergism between *eag* and *Sh* mutations is that they separately affect two different types of potassium channels, both of which are involved in the repolarization of the nerve membrane (GANETZKY and WU 1985; WU and GANETZKY 1988). Measurements of potassium currents in larval muscles and cultured nerve cells using voltage-clamp and patch-clamp methodology has provided direct evidence for the reduction of one or more potassium currents in these cells (WU *et al.* 1983; WU and GANETZKY 1984; SUN and WU 1985; Y. ZHONG and C.-F. WU, personal communication). However, it is unclear at present whether eag^+ encodes a structural component of certain types of potassium channels or is involved at some level in regulating the expression or function of these channels.

Potassium channels play fundamental roles in establishing the characteristic electrical properties of different neurons and the regulation of these channels can have important functional consequences on the output of neuronal circuits (reviewed in LEVITAN 1988). Modulation of potassium channel activity has even been implicated in the molecular mechanisms of learning and memory (reviewed in CAREW and SAHLEY 1986). For these reasons and because existing biochemical information on potassium channels is still sparse, a more complete understanding of the *eag* locus should prove informative whatever the nature of its encoded product. In this paper we report the cytological localization of *eag*, cloning of genomic DNA containing this locus, molecular mapping of a group of *eag* mutations, identification of the probable *eag* transcript and the isolation of partial cDNAs corresponding to this transcript. These results provide the necessary foundation for elucidating the nature of the *eag* gene product and the basis of *eag* mutant phenotypes at a molecular level.

MATERIALS AND METHODS

Chromosomes: The following chromosome rearrangements were used in the cytological mapping of *eag*:

Df(1)RK2, deleted for 12D2-E1 to 13A2-5, and *Df(1)RK3*, deleted for 12E2-6 to 13A6-11, were induced in a Canton-S *X* chromosome by γ-irradiation and recovered in the screen for new *eag* alleles described below.

Df(1)RK4 and *Df(1)RK5* were both generated by recombination between overlapping inversions. *Df(1)RK4*, deleted for 12F5-6 to 13A9-B1 was recovered as a recombinant between *In(1)N282.2* = *In(1)13A9-B1;17C1-2* and *In(1)P363* = *In(1)12F5-6;17C1-2*. *Df(1)RK5*, deleted for 12E9-11 to 13A9-B1, was recovered as a recombinant between *In(1)N282.2* and *In(1)366.2* = *In(1)12E9-11;17C1-2*. The inversions *N282.2*, *P363*, and *366.2* were recovered by ENGELS and PRESTON (1984) in a screen for dysgenesis-induced chromosome rearrangements with one selected breakpoint at 17C, the location of *hdp-b* (heldup wings), and one or more unselected breakpoints elsewhere in the genome.

Df(1)KA9, deleted for 12E2-3 to 12F5-13A1 according to our cytology, which differs somewhat from the breakpoints reported by CRAYMER and ROY (1980).

Dp(1;f)LJ9 = *Dp(1;f)1B;12A6-10;13A2-5;20* was derived by HARDY *et al.* (1984) from $In(1)sc^{29}$ = *In(1)1B;13A2-5* (LINDSLEY and GRELL 1968) as an X-ray-induced deletion of most of the euchromatin leaving a centric fragment containing the tip of the *X* chromosome marked with y^+, the region around 13A, and *X* heterochromatin.

Screens for additional *eag* alleles: To generate *eag* alleles with lesions that could be detected by genomic Southern blotting experiments, new alleles were produced by γ-irradiation and hybrid dysgenesis. To generate γ-ray induced mutations, males (usually *g sd f* but in some cases Canton S) were irradiated with 4500 R from a ^{137}Cs source and crossed to *eag* females. *X* chromosomes carrying a new mutation that failed to complement *eag* were recovered. To isolate hybrid-dysgenesis induced mutations, dysgenic *g sd f* males

were generated by mating II2 males (ENGELS and PRESTON 1979) to homozygous *g sd f* (M) females. Dysgenic males were crossed to *eag* females and *X* chromosomes that failed to complement *eag* were isolated and saved in a II2 P cytotype background.

To screen for revertants of dysgenesis-induced *eag* mutations *g* eag^{hd} *sd f* (P) males were mated to $\overline{XX}$, *y w f;bw;st* (M) females to generate F_1 dysgenic *g* eag^{hd} *sd f* sons. These dysgenic F_1 males were mated to *eag* females and the offspring of this cross were screened for non-*eag* females. The paternally derived *X* chromosome from such females was isolated and saved in an $\overline{XX}$ II2 stock.

Descriptions of the marker mutations and chromosomes used in the genetic study can be found in LINDSLEY and GRELL (1968).

DNA preparations: Genomic DNA was isolated from adult flies as described by BENDER, SPIERER and HOGNESS (1983) and modified by J. HIRSCH (personal communication) to include a precipitation from 10 mM spermine-4HCl, 0.1 M NaCl before the final ethanol precipitation. A typical digest for genomic Southern blot analysis utilized 3–6 μg of DNA. DNA from plasmids and cosmids was isolated by alkaline lysis (BIRNBOIM and DOLY 1979), followed by polyethylene glycol (PEG) precipitation (according to a protocol from Promega) or $CsCl_2$ gradient centrifugation (MANIATIS, FRITSCH and SAMBROOK 1982). Bacteriophage λ were grown to confluence on a lawn of *Escherichia coli* K802, on NZYCM agarose plates. To isolate phage DNA, the phage were eluted and precipitated by PEG/NaCl precipitation (YAMAMOTO *et al.* 1970), lysed at 68° with 0.1% SDS in the presence of 5 mM EDTA, and extracted with phenol/chloroform, and DNA was precipitated with isopropanol. *Eco*RI fragments of the Drosophila DNA cloned in phage were subcloned into the plasmid vectors pSP6 or pGEM (Promega).

DNA was transferred to nitrocellulose by the method of SOUTHERN (1975) following electrophoretic separation of restriction fragments on 0.7–1% agarose gels. DNA size standards were a mixture of *Hin*dIII and *Hin*dIII/*Eco*RI cut λ DNA.

Construction of the *In(1)sc*^{29} genomic library: *Bam*HI digested *In(1)sc*^{29} DNA was size selected on 5–25% sucrose gradients (MANIATIS, FRITSCH and SAMBROOK 1982) and the fractions with DNA greater than 9 kb were ligated into the EMBL4 vector arms, prepared with *Bam*HI site-cohesive termini (FRISCHAUF *et al.* 1983). The ligation was packaged using extracts prepared by the method of SCHERER *et al.* (1981) and the library propagated in *E. coli* strain NM539.

Screening phage libraries: Phage libraries were screened by the method of BENTON and DAVIS (1977) as described in MANIATIS, FRITSCH and SAMBROOK (1982).

cDNAs were isolated from an Oregon-R head cDNA library prepared in the λZAP vector, which was generously provided to us by T. SCHWARZ, L. JAN and Y.-N. JAN. Because most cDNAs isolated from this library terminate at genomic *Eco*RI sites if they are present, methylation of internal *Eco*RI sites prior to ligation of *Eco*RI linkers was apparently incomplete during the construction of this library (T. SCHWARZ, personal communication).

Isolation and analysis of RNA: Flies were frozen in liquid nitrogen, vortexed and sieved to separate heads from bodies. Total cellular RNA from heads was isolated using acid guanidinium thiocyanate-phenol-chloroform extraction (CHOMCZYNSKI and SACCHI 1987). Polyadenylated [poly(A^+)] RNA was selected on oligo(dT)-cellulose (New England Biolabs). The RNA was separated by size on formaldehyde gels containing 0.65% agarose, and transferred to Zeta-Probe nylon membranes (Bio-Rad) by capillary action with 50 mM NaOH for 6 hr. Labeled DNA probes were prepared by extension from random hexamer primers with the Klenow fragment of DNA polymerase I (Pharmacia) using gel isolated DNA restriction fragments as templates. Prehybridization and hybridization was done as previously described (MULLINS *et al.* 1978) except 1% SDS was added to the hybridization buffer.

Screening the cosmid library: The cosmid library used in this analysis was the generous gift of J. TAMKUN. This genomic DNA for this library was isolated from an isogenic strain of Canton S constructed by J. KENNISON and inserted into the Not-Bam-Not-CoSpeR vector (J. TAMKUN, personal communication). Cosmid-containing bacteria were grown on nitrocellulose filters laid on ampicillin plates, replica-plated, lysed, denatured, and neutralized as in HANAHAN and MESELSON (1980) except that the filters were treated with 10% SDS as described in MANIATIS, FRITSCH and SAMBROOK (1982).

Filter hybridizations: Nitrocellulose filters from Southern blots and library screens were prehybridized at 68° for at least 30 min in 3 × SSC, 10 × Denhardt's solution, 20 mM phosphate buffer (pH 6.8), 0.1% Sarkosyl and 50 μg/ml denatured salmon sperm DNA. Filters were hybridized to radioactively labeled probe for at least 12 hr in the above solution, also at 68°. The filters were washed in 2 × SSC, 0.1% SDS at 68° for at least 1 hr.

Labeled DNA probes were made by nick translation (kit from BRL) or by extension from random 6-mer primers with the Klenow fragment of DNA polymerase I (Pharmacia).

***In situ* hybridizations to polytene chromosomes:** Chromosome squashes were prepared by the method of J. LIM (unpublished), described in ENGELS *et al.* (1986). Labeled DNA probes for *in situ* hybridization to polytene chromosomes were made with biotinylated dUTP by nick translation (kit from BRL) or by *in vitro* run-off transcription of linearized plasmids in the presence of [^{3}H]ATP and [^{3}H]UTP (Amersham) using T7 or SP6 polymerase (Promega). The biotinylated dUTP probes were detected after hybridization with streptavidin, biotinylated alkaline phosphatase and a nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate stain (kit from BRL). Chromosomes were then stained with acetic orcein. When using tritiated probes, signals were detected using Kodak NBT-2 photographic emulsion and chromosomes were stained using Giemsa stain (Gurr).

Electrophysiology: Intracellular recordings of ejps from larval muscles and extracellular recordings of action potentials from larval nerve bundles were performed as previously described (JAN and JAN 1976; WU *et al.* 1978)

RESULTS AND DISCUSSION

Cytological localization of *eag*: To design an appropriate strategy for cloning *eag*, it was desirable first to determine the precise cytological location of the gene. In the screen for new *eag* alleles generated by γ-irradiation (see MATERIALS AND METHODS) we identified two chromosomes, *Df(1)RK2* and *Df(1)RK3*, that failed to complement *eag* and were associated with cytologically visible deletions. From the breakpoints of these two deletions, we mapped the *eag* locus to between 12E2-6 and 13A6-11 (Figure 1). To refine this cytological mapping, we wished to obtain additional deletion endpoints that subdivided the 12E-F

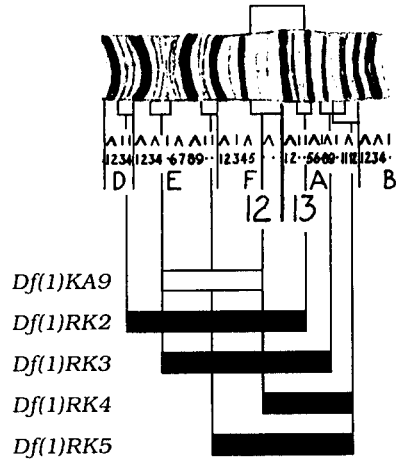

FIGURE 1.—Deletion mapping of *eag*. The drawing at the top represents a portion of the *X* chromosome, between cytological locations 12D1 and 13B9. The breakpoints of the five deletions used to determine the cytological location of *eag* are shown. Bars represent deleted material. The filled bars represent deletions that uncover *eag*, the deletion represented by the open bar does not uncover *eag*. The cytological limits of the *eag* locus defined by these deficiencies, 12F6 to 13A2-5, is marked by a bracket. Further mapping by use of a duplication (see text) places the *eag* locus precisely at 13A2-5.

region. We generated two such deletions, *Df(1)RK4* and *Df(1)RK5*, by recombination within overlapping inversions (see MATERIALS AND METHODS). Both of these deletions were found to uncover *eag*, placing it within the interval 12F6 to 13A2-5 (Figure 1). This cytological location was further confirmed by the observation that *Df(1)KA9* did not uncover *eag* (Figure 1).

A free duplication, *Dp(1;f)LJ9* (HARDY *et al.* 1984), which carries 12A6-10 to 13A2-5 from the *X* chromosome and therefore covers essentially all of the region defined by the deletion analysis as containing the *eag* locus, was tested to see if it carried eag^+. We found that *Dp(1;f)LJ9* failed to complement *eag* mutations and therefore did not carry an eag^+ allele. This observation suggested that the inversion breakpoint at 13A2-5 in $In(1)sc^{29}$ from which *Dp(1;f)LJ9* was derived (see MATERIALS AND METHODS), was just distal to the location of eag^+ or possibly within it.

$In(1)sc^{29}$ is broken at *eag*: We examined $In(1)sc^{29}$ homozygous females and hemizygous males and found that these flies displayed both the ether-induced leg-shaking behavior and the electrophysiological defect characteristic of *eag* (Figure 2) suggesting that this inversion did, in fact, disrupt eag^+. This conclusion was confirmed by complementation tests, which revealed that $In(1)sc^{29}$ failed to complement both the behavioral and electrophysiological phenotypes of *eag* mutations (not shown). The distal break of $In(1)sc^{29}$ chromosome is in the scute (*sc*) complex, which has been the subject of an extensive molecular analysis (CARRAMOLINO *et al.* 1982). On the basis of these

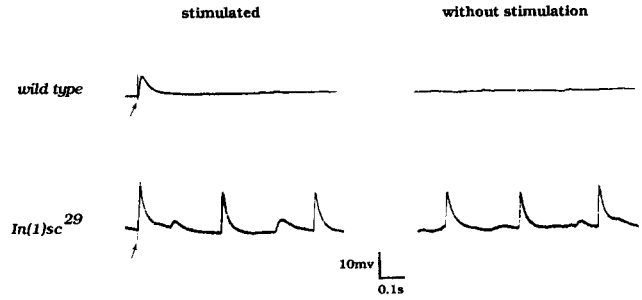

FIGURE 2.—Electrophysiological phenotype of $In(1)sc^{29}$. Oscilloscope traces of ejps recorded via a glass microelectrode inserted into a body wall muscle of $In(1)sc^{29}$ and Canton-S male larvae. The traces on the left represent responses to nerve stimulation (the arrow points to the stimulus artifact). The traces on the right represent spontaneous postsynaptic responses, *i.e.*, those occurring without any stimulus applied to the nerve. The wild-type larva shows a single small ejp in response to nerve stimulation and no ejps in the absence of stimulation. The $In(1)sc^{29}$ larva generates large multiple ejps both in response to nerve stimulation and spontaneously. This phenotype is characteristic of all *eag* mutant alleles that have been examined (see GANETZKY and WU 1983). External $[Ca^{2+}]$ concentration for these recordings was 0.2 mM.

observations we used $In(1)sc^{29}$ to initiate a chromosome walk in the *eag* region, by chromosome jumping from the *sc* region.

Use of $In(1)sc^{29}$ to isolate DNA sequences from the *eag* locus: A molecular map of the *sc* region, including the localization of the $In(1)sc^{29}$ breakpoint, has previously been published (CARRAMOLINO *et al.* 1982). The $In(1)sc^{29}$ distal breakpoint falls within genomic DNA isolated in a phage clone, λ*sc31*, which we obtained as a generous gift from J. MODOLELL. Using a subcloned *Xba*I fragment of λ*sc31* (*psc31*) as a probe in genomic Southern blotting experiments, we determined that a *Bam*HI digest of $In(1)sc^{29}$ DNA generated a *sc/eag* junction fragment of suitable size to ligate into a phage cloning vector. A library of *Bam*HI-digested $In(1)sc^{29}$ DNA was constructed in the EMBL4 vector (see MATERIALS AND METHODS). Recombinant plaques (2×10^5) were screened with a probe made from *psc31*. The distal *sc/eag* junction fragment was isolated (Figure 3) and the *eag* portion of this junction fragment was used to initiate a chromosome walk in a genomic library of wild-type DNA (MANIATIS *et al.* 1978).

Characterization of DNA in the *eag* region: Using standard chromosome walking techniques (BENDER, SPIERER and HOGNESS 1983) initially in a phage library and later in a cosmid library (see MATERIALS AND METHODS) a total of 85 kb were isolated from the *eag* region. The chromosome walk is shown in Figure 4. The walk was mapped with respect to *Eco*RI, *Bam*HI, *Bgl*II, *Hin*dIII, *Xba*I and *Xho*I restriction sites, from the distal end of the chromosome walk to coordinate +42.5. *Eco*RI alone was used to map the remainder of the walk from coordinate +42.5 to the proximal end. The map was confirmed on Southern

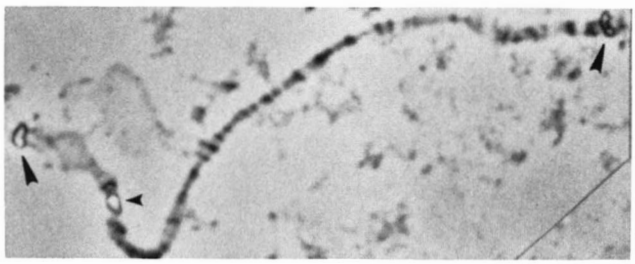

FIGURE 3.—Confirmation that the cloned $In(1)sc^{29}$ junction fragment includes sequences from both the *sc* and *eag* chromosome regions. A DNA fragment was isolated from a genomic library of $In(1)sc^{29}$ DNA on the basis of its homology to a probe from the *sc* locus. Biotinylated probe made from this cloned fragment was hybridized to wild-type polytene chromosomes and the sites of hybridization detected by an enzymatic staining reaction. Three sites of labeling can be seen. Two of the sites (large arrowheads) correspond to the breakpoints of the $In(1)sc^{29}$ chromosome at 1B and 13A. A third site of labeling (small arrowhead), at 3C1-2, represents hybridization of a probe from the *white* locus, which was included in this experiment as a positive control.

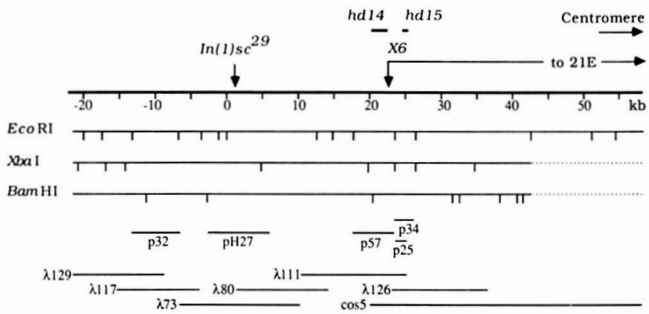

FIGURE 4.—Map of the chromosome walk in the *eag* region of the *X* chromosome. The location of sites along the genomic DNA cut by the enzymes *Eco*RI, *Bam*HI and *Xba*I are shown. All three enzymes were used to map the cloned DNA from the distal end of the walk to coordinate +42.5. *Eco*RI alone was used to map the remainder of the walk from +42.5 until the proximal end. The relative locations of the $In(1)sc^{29}$ breakpoint and three additional mutational lesions (whose molecular analysis is presented in Figure 5) are indicated at the top. The *Eco*RI site immediately to the left of the $In(1)sc^{29}$ breakpoint is defined as coordinate 0. The extents of five subclones, p32, pH27, p57, p34 and p25, are shown beneath the walk. At the bottom is shown the minimum subset of phage and cosmid clones that span the entire chromosome walk. λ126 is included because p34 was subcloned from this phage.

blots of genomic DNA using subcloned fragments of the chromosome walk as probes. The position of the walk at 12F-13A on the chromosome was confirmed by *in situ* chromosome hybridization using several portions of the chromosome walk as probes (not shown).

Localization of molecular lesions associated with mutant *eag* alleles: To delimit the *eag* locus within the cloned region, the genomic DNAs of several *eag* mutants were examined on genomic Southern blots using portions of the chromosome walk as probes. Three mutant alleles were found to be associated with detectable molecular lesions that mapped within the walk. Two of these alleles, eag^{hd14} and eag^{hd15}, were isolated in a screen for hybrid dysgenesis-induced alleles of *eag*. The third, eag^{X6}, was isolated in the screen for γ-ray induced alleles of *eag*.

Figure 5A shows that eag^{hd14} is disrupted in a fragment of the chromosome walk represented by p57 (Figures 4 and 5D). Restriction mapping (with six enzymes, data not all shown) by genomic Southern blots of the polymorphism in eag^{hd14} revealed that this allele is the result of an insertion of 3.0 kb of DNA into the 3.5-kb *Bam*HI-*Eco*RI fragment of p57. In the *Bam*HI-*Eco*RI digest of eag^{hd14} DNA, the predicted restriction fragment of about 1.2 kb, extending from the *Bam*HI site of p57 to the leftmost *Eco*RI site of the insert, does not appear on the Southern blot (Figures 4 and 5D). In contrast, in an *Xba*I digest the 1.9-kb junction fragment extending from the *Xba*I site of p57 to the *Xba*I site in the insertion hybridizes strongly to the p57 probe (data not shown). We conclude that the insertion is so close to the *Bam*HI site of p57 that the *Bam*HI-*Eco*RI junction fragment retains insufficient homology to p57 to produce a hybridization signal. The restriction map of the DNA constituting the eag^{hd14} insert (Figure 5A) is similar to the published map of the hobo transposable element (FINNEGAN and FAWCETT 1986; STRECK, MCGAFFNEY and BECKENDORF 1986), although we have not yet confirmed the identity of this insert.

Cytological examination of eag^{X6} revealed that chromosomal material between 13A1-2 and 13E4-8 was deleted from the *X* chromosome and inserted into the second chromosome at 21E. The molecular analysis of eag^{X6} is consistent with this cytological observation. In all restriction digests (examples shown in Figure 5B) one band of hybridization in the parental DNA is replaced by two new bands in DNA from the mutant. These new bands correspond to the two junction fragments, one created by the deletion of DNA from the eag^{+} locus and the other created by the insertion of this DNA into its new location. Figure 5B demonstrates that the deletion breakpoint in eag^{X6} falls within the same 3.5-kb *Bam*HI-*Eco*RI fragment of the p57 probe as the insertion in eag^{hd14}. Note that in the *Bam*HI-*Eco*RI digest (Figure 5B), the smaller of the two new bands hybridizes only faintly to the p57 probe, suggesting limited homology of this fragment to p57. We interpret this result as indicating that the eag^{X6} deletion breakpoint falls very close to the *Bam*HI site of the 3.5-kb *Bam*HI-*Eco*RI fragment of p57, and therefore close to the site of insertion in eag^{hd14}.

Figure 5C shows that eag^{hd15} generates a polymorphism in a 3.0-kb *Eco*RI genomic fragment that correponds to probe p34 (Figure 4). The insertion is at coordinate +25 (see Figures 4 and 5D). The data (not all shown) indicate that eag^{hd15} contains a 0.6-kb insertion that includes a single *Hin*dIII site. The eag^{hd15} allele reverts to wild type under dysgenic conditions at a frequency of 1.6×10^{-4} (3 revertants out of

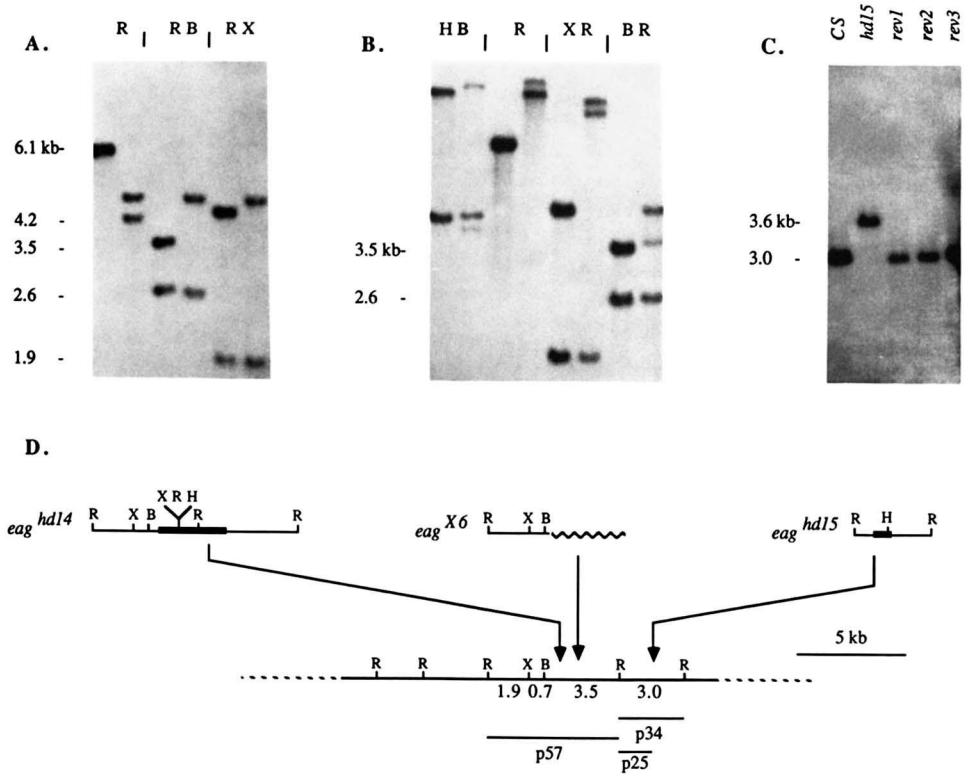

FIGURE 5.—Molecular characterization of *eag* mutant alleles. Genomic Southern blots of eag^{X6}, eag^{hd14}, eag^{hd15} and three revertants of eag^{hd15} (eag^{hd15r1}, eag^{hd15r2} and eag^{hd15r3}) are shown. In all cases, DNA was isolated from wild-type males and females, and hemizygous *eag* mutant or revertant males. Restriction sites are abbreviated as follows: *Eco*RI (R), *Bam*HI (B), *Hin*dIII (H) and *Xba*I (X). All bands present on the original autoradiograms, which would have detected fragments down to about 0.2 kb, are shown in the figure. Sizes of bands in kilobases are shown to the left in each case. (A) This genomic Southern blot demonstrates the defect in eag^{hd14}. Restriction digests are indicated at the top of the lanes. In each pair of digests, *g sd f* (the parental chromosome) DNA is on the left and eag^{hd14} DNA on the right. The blot was probed with p57 (map below). The sizes of the wild-type restriction fragments are indicated down the left side of the autoradiogram. These and other data (not shown) support the conclusion that eag^{hd14} is a 3.0-kb insertion into the 3.5-kb *Bam*HI-*Eco*RI fragment of p57, creating a new restriction pattern shown below the autoradiogram, where the heavy line represents inserted sequences. (B) eag^{X6} is an insertional translocation, that is broken in the *eag* locus and removes 13A1-2 to 13E4-8 from the *X* chromosome and inserts it into the second chromosome at 21E. This genomic Southern blot compares Canton S (wild-type) DNA to eag^{X6} DNA in four different restriction enzyme digests (enzymes named at the top of the lanes) probed with p57 (map shown below). In each pair of digests the left lane is Canton S and the right lane is eag^{X6}. In all comparisons (including others not shown here) one of the Canton S bands is replaced with two in eag^{X6} as expected from the cytological lesion. The two molecular weight sizes shown on the left of the autoradiogram correspond to the two *Bam*HI-*Eco*RI fragments in Canton S. It is clear that the same 3.5-kb *Bam*HI-*Eco*RI fragment is altered in eag^{X6} as is disrupted in eag^{hd14}. The approximate position of the eag^{X6} deletion breakpoint is indicated by the wavy line in the diagram below. (C) This genomic Southern blot compares Canton S (wild-type) to eag^{hd15} and three revertants derived from eag^{hd15}. The eag^{hd15} mutation, like eag^{hd14}, was induced in a *g sd f* background. However, Canton S and *g sd f* are identical at the level of Southern blotting in this region of the genome (not shown). The DNA was digested with *Eco*RI and hybridized to p25, a plasmid that includes the left half of the DNA in p34 (see Figures 4 and 5D). As shown, eag^{hd15} (*hd15*) contains an insert that increases the size of the genomic *Eco*RI fragment by approximately 0.6 kb relative to the Canton S (CS) DNA. In three independently isolated revertants of eag^{hd15} (*rev1*, *rev2* and *rev3*) the insert has been lost, restoring the fragment to normal length. (D) A portion of the chromosome walk is shown indicating the relative positions of the mutant lesions detected in A, B and C above. The probes used in the analysis and a coordinate bar are also shown.

18,256 chromosomes scored). In three independently derived dysgenesis-induced revertants the inserted material is absent, restoring the genomic *Eco*RI fragment to its original size (Figure 5C). The presence of a *P* element in eag^{hd15} at the cytological location of *eag* was demonstrated by *in situ* hybridization to polytene chromosomes using a *P* element probe. A *P* element at this location was not detected in similar experiments with the parental *g sd f* chromosome or with any of the three revertants of eag^{hd15}. These results provide compelling evidence that the insertion of a *P* element is the cause of the eag^{hd15} mutation.

For the three mutations eag^{hd14}, eag^{hd15} and eag^{X6}, there are no detectable lesions other than those described above from the distal end of the chromosome walk to coordinate +42.5. The lesions associated with eag^{hd14}, eag^{hd15} and eag^{X6} fall within 5 kb of each other; the midpoint of this cluster is 23.5 kb from the $In(1)sc^{29}$ breakpoint. The relative positions of these lesions are shown in Figures 4 and 5D. Southern blot analysis of four other *eag* mutations, which were newly generated by γ-irradiation, failed to reveal any detectable lesion in this region between coordinates −22 and +44.5.

Identification of the *eag* transcript: In an initial attempt to identify which portions of the *eag* region

were transcribed, genomic fragments (p32, pH27, p57 and p34) that together span the location of all the mapped *eag* mutant sites (Figure 4) were used to probe a cDNA library prepared from poly(A$^+$) RNA isolated from fly heads.

Ten cDNAs sharing regions of homology with pH27 and/or p57 and p34 were recovered. The most extensive of these cDNAs, T18, which is 2.8 kb in size, was chosen for further analysis. All ten of the cDNAs were found to be derived from the same transcript on the basis of their restriction maps and their hybridization with labeled fragments of DNA from restriction digests of T18 (not shown). To align T18 with genomic DNA this cDNA was used to probe a Southern blot containing genomic DNA from the *eag* region cut with various restriction enzymes. The results, shown diagrammatically in Figure 6, A and B, indicate that T18 contains a minimum of 7 exons derived from over 30 kb of genomic DNA. Because the genomic DNA encompassed by T18 spans the entire region defined by *In(1)sc^{29}*, *eag^{hd14}*, *eag^{hd15}* and *eag^{X6}*, it is likely that T18 represents at least a portion of the *eag* transcript.

A single additional cDNA, T13, was recovered when the cDNA library was probed with p32 (Figure 4). The 3.6-kb insert in this cDNA had a restriction map similar to that of p32 and appears to be derived from a single region of the chromosome walk (Figure 6) on the basis of Southern blot analysis (not shown). Although no overlap between T13 and T18 was found, from these data we could not rule out the possibility that these cDNAs represented different portions of a single transcript.

To test whether T18 represents at least part of the *eag* transcript and to clarify its relationship with T13, Northern blot experiments were performed. Northern blots of poly(A$^+$) RNA from whole flies, bodies and heads of wild-type flies probed with cDNA T18 revealed a major transcript approximately 10 kb in length. This transcript was not detected in RNA preparations from bodies except upon overexposure of the Northern blots (not shown). We estimate that the transcript is at least ten times more abundant in heads than bodies consistent with the expected neural localization of the *eag* transcript. Examination of poly(A$^+$) RNA isolated from the heads of *In(1)sc^{29}* and *eag^{X6}* mutants demonstrated that the 10-kb transcript was absent in both mutants (Figure 6C). Instead, a truncated 2.4-kb transcript was detected in *In(1)sc^{29}* and two transcripts of 4.5 kb and 5.5 kb were present in *eag^{X6}*. When used as a probe on Northern blots of wild-type RNA, cDNA T13 did not detect the 10-kb transcript but recognized instead two transcripts of 6 kb and 3.5 kb (not shown). Transcripts of identical size were also detected in RNA isolated from *In(1)sc^{29}* or *eag^{X6}* flies (not shown). These results indicate that T13 and T18 represent distinct transcription units.

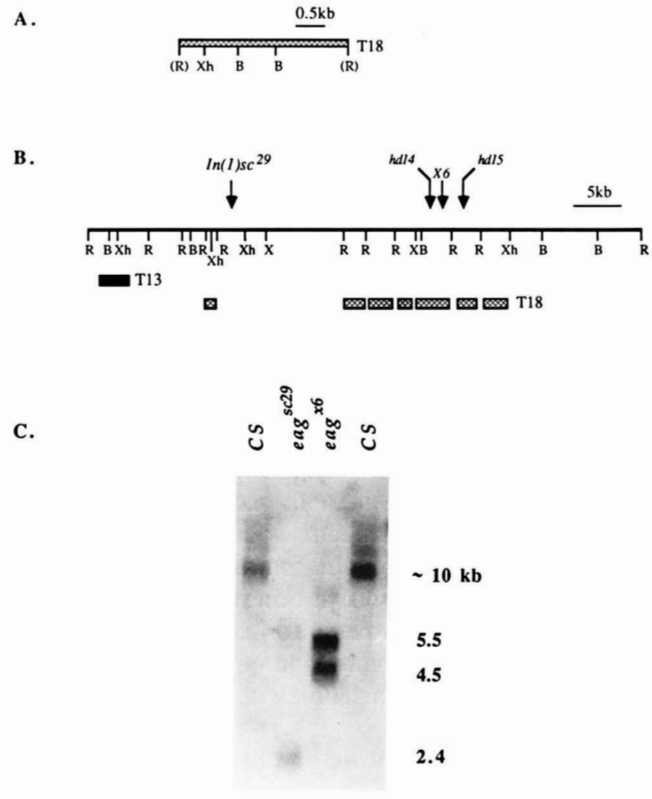

FIGURE 6.—Identification of the *eag* transcript. (A) Restriction map of cDNA T18. *Eco*RI (R), *Bam*HI (B) and *Xho*I (Xh) sites are shown. T18 did not contain any *Xba*I sites or *Hind*III sites. The bracketed *Eco*RI sites may represent either artificial cloning sites or genomic *Eco*RI sites because methylation of internal *Eco*RI sites during construction of the cDNA library was incomplete. (B) Hybridization of cDNAs to the cloned genomic DNA fragments from the chromosome walk. The chromosome walk from coordinate −13 to coordinate +42.5 is shown, with *Eco*RI (R), *Bam*HI (B), *Xba*I (X) and *Xho*I (Xh) sites diagrammed. Areas of hybridization of cDNA are denoted by the shaded boxes under the map: T18 is represented by a cross-hatched box, T13 is represented by a filled box. The presence of a box indicates only that the cDNA probe hybridizes with the corresponding restriction fragment above the box. The limits of the homology between the cDNA and each hybridizing genomic DNA fragment and the number of introns within a restriction fragment are not known. A break between boxes implies the presence of an intron in genomic DNA that is spliced out in the cDNA. Where a box spans a genomic restriction site, it indicates that the cDNA contains that restriction site (not confirmed for the *Xho*I site in T13). The sites of the molecular lesions associated with *eag* mutations are shown for reference. (C) Northern blot analysis. Approximately 10 μg of poly(A$^+$) RNA isolated from heads of Canton S (CS), *In(1)sc^{29}* (*eag^{sc29}*) and *eag^{X6}* flies was loaded onto each lane. The filter was probed with ^{32}P-labeled cDNA T18. Exposure time was 7 days.

Furthermore, we conclude that T18 represents the *eag* transcript because it spans all known *eag* mutant sites and it is the only detectable transcription unit in the region that is physically altered by *eag* mutations.

CONCLUSION

Mutational analysis of Drosophila coupled with molecular genetic techniques offers a powerful approach to identify and characterize the proteins involved in

the function of the nervous system. Recent demonstrations include analyses of the *para* and *Sh* loci, which were shown to be structural genes for sodium channels and a family of potassium channels respectively (LOUGHNEY, KREBER and GANETZKY 1989; RAMASWAMI and TANOUYE 1989; BAUMANN *et al.* 1987; KAMB, IVERSON and TANOUYE 1987; PAPAZIAN *et al.* 1987; TEMPEL *et al.* 1987; IVERSON *et al.* 1988; PONGS *et al.* 1988; TIMPE *et al.* 1988). However, at the present time only a few members of the collection of mutations known to affect electrical signaling in the nervous system of Drosophila (reviewed in GANETZKY and WU 1986) have been characterized at the molecular level. For example, despite the very striking electrophysiological defects observed at the neuromuscular junction in *eag* larvae (GANETKZY and WU 1983, 1985), the protein encoded by this gene is not yet known. Existing evidence suggests that this protein in necessary for the proper repolarization of the nerve membrane, probably acting via a potassium channel component distinct from the product of the *Sh* locus (GANETZKY and WU 1983; WU *et al.* 1983; SUN and WU 1985; WU and GANETZKY 1988). In contrast to *Sh*, for which some mutant alleles are known to eliminate the A-type potassium current completely, no *eag* allele examined, including presumptive null mutations such as $In(1)sc^{29}$ or eag^{X6}, completely eliminated any potassium current (Y. ZHONG and C.-F. WU personal communication). Therefore, eag^{+} may encode a protein that is involved in regulating or modulating the activity of one or more classes of potassium channels but not absolutely required for the function of these channels. It is known that channels can have complex subunit organizations and that post-translational modifications, such as phosphorylation, play a role in determining channel activity (reviewed in CATTERALL 1988).

In this work we have described the cloning of genomic DNA from the *eag* locus, the identification of the putative *eag* transcript, and the isolation of cDNAs derived from this transcript. Although it is clear that none of the cDNAs isolated thus far represent the entire 10-kb transcript, by isolating additional partial cDNAs it should now be possible to assemble a composite cDNA in order to determine the deduced amino acid sequence of the *eag* protein. Ultimately, the molecular characterization of the *eag* gene product will help elucidate its role in the normal mechanism of neuronal excitation.

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