

75. Isolation of *Drosophila* Genomic Clones Homologous to the Eel Sodium Channel Gene

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Introduction. Molecular basis of the membrane excitability has recently been elucidated by cloning the genes encoding the voltage sensitive ionic channels.¹⁾⁻³⁾

We report in this paper an identification of the two *Drosophila melanogaster* genes which have homologies to the eel voltage sensitive sodium channel. *Drosophila melanogaster* is an ideal organism for the study of gene regulation as well as its fine structure analysis, since any gene once cloned can be properly manipulated and delivered again into the genome by the P-element mediated gene transfer method.⁴⁾

Therefore, we now expect that the two tentative *Drosophila* ionic channel genes we cloned can be used as materials for the study of specific gene expression in excitable cells.

Materials and methods. Genomic DNA was extracted from adult flies.⁵⁾ The genomic library was made with EMBL4 as a cloning vector.⁶⁾ The 64mer probe oligonucleotide was synthesized with an automatic DNA synthesizer (ABI Model 381A), and purified with Spectra-Physics HPLC system and Nucleosil 300-7C18 reverse phase column. It was kinased with ³²P and used for the library screening.⁷⁾ Hybridization was performed at low stringency (formamide 20%, 6xSSC, at 42°C). Nucleotide sequence was determined by the chain termination method⁸⁾ using plasmids as templates (Takara 7-Deaza Sequencing Kit). To determine the sequence upstream from the 64mer hybridizing region in pNa16 (see the text of Results), a set of clones with various deletions from the 5'end of the insert were made.⁹⁾ Clones with the deletions up to near the 64mer region was used for sequencing. Other recombinant DNA experiments were carried out by the standard methods.⁷⁾ *In situ* hybridization to salivary gland chromosomes were performed according to Engels *et al.*¹⁰⁾ The deficiency stocks Df(2R)M-c^{33a} = Df(2R)60E2-3;60E11-12 and Df(2L)H68=Df(2L)36B1-2;37B1 were obtained from Umea *Drosophila* stock center and Dr. K. Mogami, respectively. These strains were crossed to the normal strain (Canton-S) and used for chromosomal preparations.

Results. To isolate *Drosophila* homologs of the eel sodium channel gene, we screened the genomic library with a synthetic 64mer oligonucleotide probe (Fig. 2). This region corresponds to the transmembrane segment IVS6 which is well conserved through the repeat I to IV within the eel gene,¹⁾ and is also conserved between the eel and rat gene.²⁾ To increase the detection sensitivity, we hybridized the probe at low stringency to the membranes on which phages had been amplified *in situ* after transfer from plates.⁷⁾ Out of 160,000 independent genomic clones, we obtained eight clones which showed positive hybridi-

zation signals. They were named as λ Na14, λ Na16, λ Na17, λ Na20, λ Na21, λ Na24, λ Na30 and λ Na31. DNA of each clone was purified and digested with EcoRI. The EcoRI fragments which hybridized with the probe were subcloned into pUC18 (Fig. 1). The subclones were named as pNa14, pNa16, pNa20, pNa21, pNa24, pNa30 and pNa31, respectively. From the patterns of the restriction enzyme digestion and from the cross-hybridization experiments of these subclones, we classified the original phage clones into four groups; I) λ Na16 and λ Na30, II) λ Na14, λ Na17 and λ Na21, III) λ Na24 and λ Na31, IV) λ Na20. The EcoRI fragments in λ Na16 and λ Na30, which had been classified into the group I, showed far more intense hybridization signals than those from other groups of clones (Fig. 1). These fragments alone hybridized also to the eel sodium channel cDNA (pSCH50)¹ at low stringency (data not shown). Therefore, we expected that these clones most likely encode a *Drosophila* sodium channel homolog.

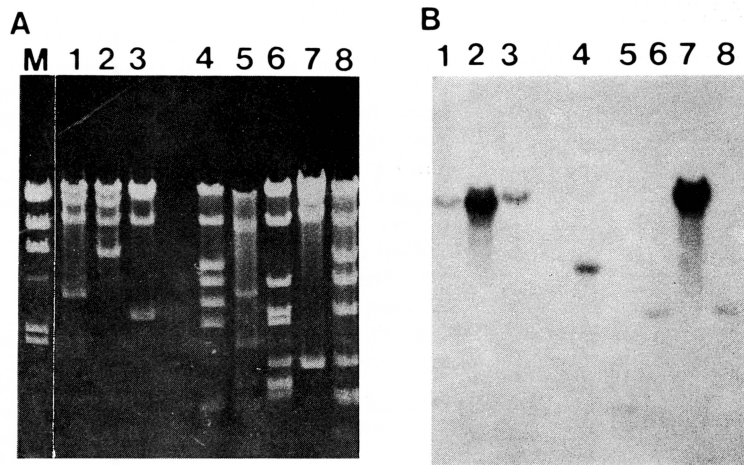


Fig. 1. DNA blot hybridization of phage clones. DNA from the phage clones (lane 1, λ Na14; lane 2, λ Na16; lane 3, λ Na17; lane 4, λ Na20; lane 5, λ Na21; lane 6, λ Na24; lane 7, λ Na30; lane 8, λ Na31) was digested with EcoRI, electrophoresed in agarose gel (A), and blotted to the filter membrane. The lane denoted as M is λ DNA digested with HindIII for molecular weight markers. It was hybridized with the ³²P-labeled 64mer and autoradiographed (B).

The EcoRI fragment subclones were digested with Sau3AI and further subcloned at random into pUC18. These clones were screened with the synthetic 64mer, and the nucleotide sequences of the positive clones were determined. The nucleotide sequences and the deduced amino acid sequences of the clones belonging to the group I and II turned out to have significant homologies to the eel IVS6 sequence.

In the group I (λ Na16 and λ Na30), the homology of the deduced amino acid sequence to that of the eel sodium channel was the most remarkable. We further determined the nucleotide sequence upstream from the 64mer hybridizing region in pNa16. The combined result is shown in Fig. 2. The amino acid sequence of this region coincides with that of the eel sodium channel at 60%. This suggests strongly that this region encodes a *Drosophila* sodium channel, and that the region corresponding to the eel VIS4 to VIS6 is not interrupted

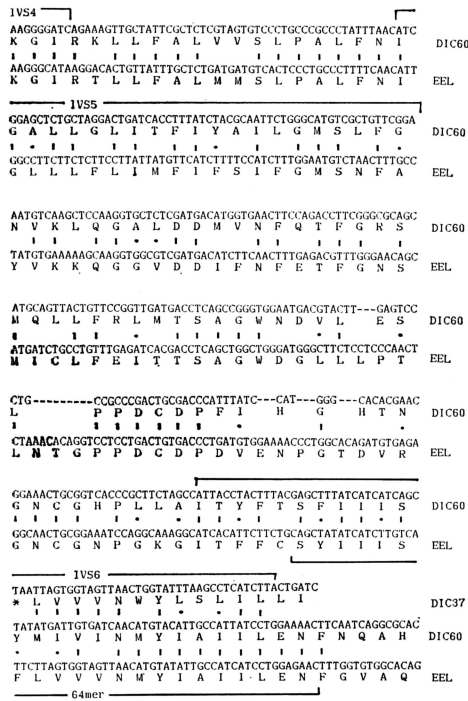


Fig. 2. Nucleotide and amino acid sequence comparison among DIC60, DIC37 and the eel sodium channel gene. The deduced amino acid sequence is shown below the corresponding nucleotide sequence for each gene. Gaps have been inserted into the sequences to achieve a maximum homology. The identical amino acids and the conservative amino acid replacements between the eel and the *Drosophila* are marked by vertical bars and dots, respectively. The transmembrane segment regions (IVS4, IVS5 and IVS6) and the probe 64-mer sequence are shown. The stop codon in DIC37 is indicated by an asterisk.

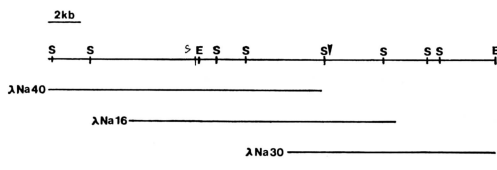


Fig. 3. The restriction map of the DIC60 gene. The arrangement of λ Na30, λ Na16 and λ Na40 is shown below the map. The direction of transcription is found to be from left to right from the sequence data. Bar=2 kb, E=EcoRI, S=SacI. The probe 64-mer hybridizes to the region indicated by an arrow head.

by introns. A new clone λ Na40 was obtained by screening the genomic library again with pNa16 and pNa30 as probes. The restriction map of the region covered by λ Na40, λ Na16 and λ Na30 is shown in Fig. 3.

In the group II (λ Na14, λ Na17 and λ Na21), a stretch of fifteen nucleotides is completely matched with a part of the 64mer probe, followed by the additional seven amino acids homologous to the eel sodium channel (Fig. 2). In this clone, however, a stop codon appears in frame just 5' to the completely matched region, but no homology is found in the adjacent upstream sequence.

We determined the chromosomal locations of the two distinct groups of clones by *in situ* hybridization to polytene chromosomes. The clones of the group I (λ Na16, λ Na30, λ Na40) are located at 60E of chromosome 2R (Fig. 4 A, B). The clones of the group II (λ Na14, λ Na17, λ Na21) are at 37B-D (Fig. 4 D, E). These results were further confirmed by using the deficiency strains (Fig. 4 C, F). No mutations assumed to have a defect in a sodium channel are located at these sites.¹¹⁾ However, the first gene may be the same with that reported by Salkoff, L. *et al.* at the Cold Spring Harbor meeting in 1986.¹²⁾ We call our two *Drosophila* ionic channel gene candidates as DIC60 and DIC37 according to their chromosomal locations.

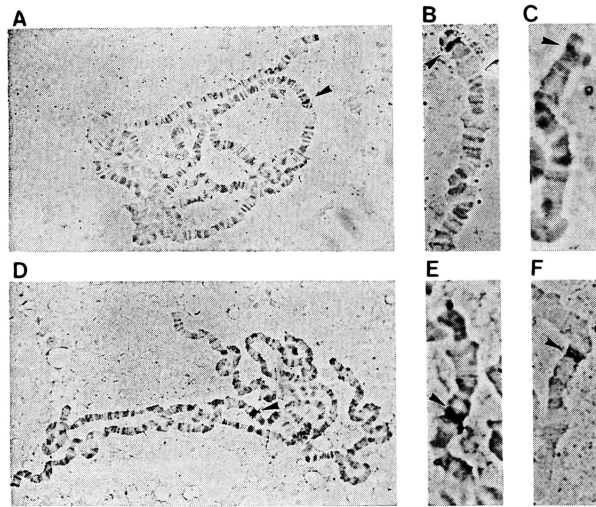


Fig. 4. *In situ* hybridization to polytene chromosomes. A and B: The pNa 16 clone was hybridized to salivary gland chromosomes of normal (Canton-S) strain (A: $\times 400$, B: $\times 1000$). C: pNa 16 was hybridized to Df(2R)60E2-3; 60E11-12 heterozygote ($\times 1000$). The tip of chromosome 2R branches off, because of the heterozygous deficiency. The signal is detected only on the longer branch (normal chromosome), but not on the shorter one (deficient chromosome). D and E: pNa 17 was hybridized to normal strain (D: $\times 400$, E: $\times 1000$). The signal is detected proximal to the 35AB puff. F: pNa 17 was hybridized to Df(2L)36B1-2,37B1 heterozygote ($\times 1000$). The signal is detected just proximal to the bending region (shown by a dot) where a pair of chromosomes are mismatched.

Discussion. We identified two *Drosophila* genes (DIC60 and DIC37) which have a homology with the eel sodium channel gene. DIC60 has a region where the amino acid sequence coincides with that of the eel sodium channel through IVS5 to IVS6 at 60%. DIC37 has a region of twelve amino acids with a high homology to the eel IVS6, but the homology is interrupted by the appearance of a stop codon in-frame just 5' to this region. Since it is unlikely that such a high homology occurs simply by chance, we expect that the homologous region in DIC37 is an exon isolated from the main part of a gene by an intron. It is not also excluded that it is a remnant of some evolutionary degraded pseudogene. We screened the oligo-dT primed cDNA library from pupae with pNa21 as a probe. Although we could get several positive clones, their inserts did not reach the 64mer hybridizing region. Therefore, we cannot yet determine whether the upstream stop codon in DIC37 is averted by splicing in the mature mRNA.

The first thing that should be elucidated with respect to the functions of our tentative ionic channel genes is to identify their ionic specificity. Recently, a candidate for a calcium channel gene was cloned.³⁾ It has a remarkably similar structure with sodium channels. Therefore, the genes we isolated cannot be considered to encode a sodium channel simply from their structural homology. One way to answer this question is to isolate the full length cDNA and to express its product in cells which lack sodium channel such as in *Xenopus* oocyte.¹³⁾ However, this system may not necessarily work for the *Drosophila* cDNA, since the eel sodium channel cDNA could not be expressed in the *Xenopus* oocyte

membrane. We may be able to overcome this difficulty in an alternative way by exploiting sophisticated technology specific to *Drosophila*. As the chromosomal locations of the genes are already known, we can study flies with a deficiency of these region. From the electrophysiological defect of such flies, we can draw inference as to the ionic specificity of our tentative channel genes. We can confirm it, if the defect is rescued by introducing the normal gene into the genome of the deficiency strain.

To examine the structure and function relationships into further details, it is necessary to accumulate information as to how the function is affected by various mutations. In the *Xenopus* oocyte system, mutations are introduced into the cDNA *in vitro*. This method can be extended in *Drosophila* to obtain transformed flies having *in vitro* mutagenized genes by using P-element mediated transformation method. We can also collect a number of mutant alleles systematically by screening the alleles which do not complement the electrophysiological defects of the deficiency strains. They may be collected either as lethal or conditional mutants. Since many temperature sensitive paralytic mutants are known in *Drosophila*, the ionic channel function may also become temperature-dependent, if a proper mutation is introduced. Such a conditional mutant is useful since the gene function can be switched on and off at cellular or even at individual levels.

Drosophila melanogaster is also the most suitable for studying the regulatory mechanism of the ionic channel gene expression. We are performing the P-element mediated transformation experiments to re-introduce the chimeric gene composed of an *E. coli* β -galactosidase and our tentative channel genes. With this system, we can visualize the tissue specific expression pattern of our genes and identify the regulatory region necessary for their proper expression.

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