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 \(^{32}\)P and used for the library screening.\(^7\) Hybridization was performed at low stringency (formamide 20\%, 6×SSC, at 42°C). Nucleotide sequence was determined by the chain termination method\(^8\) 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\(^{32a}\) =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-
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They were named as λNa14, λNa16, λNa17, λNa20, λNa21, λNa24, λNa30 and λNa31. 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) λNa16 and λNa30, II) λNa14, λNa17 and λNa21, III) λNa24 and λNa31, IV) λNa20. The EcoRI fragments in λNa16 and λNa30, 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) 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, λNa14; lane 2, λNa16; lane 3, λNa17; lane 4, λNa20; lane 5, λNa21; lane 6, λNa24; lane 7, λNa30; lane 8, λNa31) was digested with EcoRI, electrophoresed in agarose gel (A), and blotted to the filter membrane. The lane denoted as M is λDNA digested with HindIII for molecular weight markers. It was hybridized with the 32P-labeled 64mer and autoradiographed (B).](image)

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 (λNa16 and λNa30), 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.
by introns. A new clone λNa40 was obtained by screening the genomic library again with pNa16 and pNa30 as probes. The restriction map of the region covered by λNa40, λNa16 and λNa30 is shown in Fig. 3.

In the group II (λNa14, λNa17 and λNa21), 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 colon 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 (λNa16, λNa30, λNa40) are located at 60E of chromosome 2R (Fig. 4 A, B). The clones of the group II (λNa14, λNa17, λNa21) 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. However, the first gene may be the same with that reported by Salkoff, L. et al. at the Cold Spring Harbor meeting in 1986.

We call our two *Drosophila* ionic channel gene candidates as DIC60 and DIC37 according to their chromosomal locations.
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. 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. 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.

Fig. 4. *In situ* hybridization to polytene chromosomes. A and B: The pNa16 clone was hybridized to salivary gland chromosomes of normal (Canton-S) strain (A: ×400, B: ×1000). C: pNa16 was hybridized to Df(2R)60E2-3; 60E11-12 heterozygote (×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: pNa17 was hybridized to normal strain (D: ×400, E: ×1000). The signal is detected proximal to the 35AB puff. F: pNa17 was hybridized to Df(2L)36B1-2,37B1 heterozygote (×1000). The signal is detected just proximal to the bending region (shown by a dot) where a pair of chromosomes are mismatched.
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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