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Isolation and characterization of a gene for a ryanodine receptor/calcium release channel in *Drosophila melanogaster*

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Abstract

The nucleotide sequence of a 25.7 kilobase *Drosophila melanogaster* genomic DNA segment containing a gene for a ryanodine receptor/calcium release channel homologue has been determined. Computer analysis and partial cDNA cloning revealed 26 exons comprising the protein-coding sequence in this gene. The predicted protein is homologous in amino acid sequence and shares characteristic structural features with the mammalian ryanodine receptors. In blot hybridization analysis, a ~16 kilobase RNA species was identified abundantly in a 6–12 h embryo as the transcript from this gene. In situ hybridization to polytene chromosomes indicated that this gene locates at band position 44F on the second chromosome.

Key words: Ryanodine receptor; Calcium release channel; *Drosophila*; RNA blot hybridization; Chromosomal mapping

1. Introduction

The release of Ca^{2+} from intracellular stores is an important process in many signal transduction systems. The Ca^{2+} release is mediated by at least two classes of channel proteins, the inositol 1,4,5-trisphosphate (IP_3) receptor and the ryanodine receptor, in mammalian cells [1]. The ryanodine receptor has been best characterized in skeletal muscle as the calcium-induced calcium release channel which is activated by cytoplasmic Ca^{2+}, caffeine and ryanodine [2], and as the 'foot' protein which spans the junction between the transverse tubules and the sarcoplasmic reticulum [3,4]. Recent studies have shown that three types of the ryanodine receptor, namely skeletal muscle, cardiac and brain types, exist in mammalian tissues [5–10] and the distribution of the mRNAs has also been investigated [10]. These ryanodine receptors are composed of ~5,000 amino acid residues, comprising the carboxy-terminal channel region which has four putative transmembrane segments, and the remaining portion which apparently constitutes the 'foot' structure [5,8,10]. By expression of the ryanodine receptor cDNAs, functional calcium release channels have been formed in Chinese hamster ovary cells [11,12], *Xenopus* oocytes [8] and COS cells [13].

The fruit fly *Drosophila melanogaster* is amenable to genetic analysis and provides an ideal system to examine the role of many proteins. Ryanodine, a natural plant alkaloid, was originally identified as an insecticide and then shown to have profound effects on muscle from a variety of vertebrates and invertebrates [14]. The ryanodine receptor may therefore take part in Ca^{2+} signalling of muscle and nervous systems in *Drosophila*. We present here the existence of a *D. melanogaster* counterpart of the mammalian ryanodine receptor gene.

2. Materials and methods

2.1. DNA cloning

A *D. melanogaster* genomic library [15] was screened with the 1.9-kbp *Sma*I fragment derived from clone pRR616 [5] as a probe to yield λDRRG2. The 1.9-kbp *Eco*RI fragment derived from the 5′ end of DNA insert in λDRRG2 was used as a probe for next screening to yield λDRRG27 and λDRRG33. DNA fragments generated by various restriction endonucleases from the clones were subcloned into pBluescript (Stratagene) and a total of 25,680 bp of contiguous genomic DNA from the overlapping clones was completely determined [16,17]. Nucleotide residues are numbered from the most 5′ residue in DNA insert of λDRRG27.

Protein-coding sequences were predicted by computer analysis using

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Abbreviations: IP_3, inositol 1,4,5-trisphosphate; PCR, polymerase chain reaction; ER, endoplasmic reticulum.

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the amino acid sequences of the mammalian ryanodine receptor subtypes; however, several junctions between protein-coding sequences could not be determined with the analysis. We therefore, determined them by partial cDNA cloning using polymerase chain reaction (PCR) as follows. To determine the boundary of the protein-coding sequence 9 (P9), a synthetic primer complementary to nucleotide 9,635 to 9.664 was elongated by the procedures described perviously [5] using a mixture of *D. melanogaster* poly(A)$^+$ RNA preparations from male adults, female adults and embryos. The resulting cDNAs were further amplified with AmpliTaq DNA polymerase (Perkin Elmer Cetus) using 40 pmol of 5′-synthetic primer corresponding to nucleotides 7,801 to 7,825 (sense) and 3′-primer corresponding to position 9,606 to 9,634 (antisense). Sequencing the amplified cDNA fragments showed that they divide into two different fragments in sequence and indicated that the genomic sequence corresponding to either 7,969–8,068 (P9) or 8,310–8,409 (P9′) is incorporated as an exon into the mRNA by alternative splicing. Similar procedures were repeated to determined the boundaries between P11 and P12, P12 and P13, P17 and P18, P19 and P20 and P21 and P22. In the analysis of the junction between P12 and P13, two amplified fragments that differ in length were obtained. Sequence analysis of the fragments indicated that P13 carries two splice-acceptor sites and that two forms of transcript can be generated at this region by alternative splicing (insertion/deletion of residues 12,059–12,095).

2.2. *RNA blot hybridization analysis*

Poly(A)$^+$ RNA samples, prepared by the method of Jowett [18], were electrophoresed on a formaldehyde/1% agarose gel and transfered to a nylon membrane [19]. The filters were hybridized with ^{23}P-labelled DNA insert of λDRRG2 in buffer containing 20% formamide, 5× SSC, 0.1 M sodium phosphate (pH 6.8), 0.2% bovine serum albumin, 0.2% polyvinylpyrolidone, 0.2% Ficoll (M_r 400,000), 250 μg/ml salmon DNA at 42°C. Extensive washing was done in 0.5 × SSC/0.1% SDS at 65°C.

2.3. *Chromosome in situ hybridization*

Chromosome squashes for hybridization were prepared as in [20]. The procedure for hybridization and post-hybridization was identical to the method described in [21]. The DNA insert of λDRRG2 was labelled with digoxigenin and used as a hybridization probe to polytene chromosome.

3. Results and discussion

3.1. *Gene structure from the DNA sequence and partial cDNA cloning*

Fig. 1 shows a ~30-kbp *Drosophila* genomic DNA segment from the overlapping phase clones, which was detected by its ability to cross-hybridize with the rabbit skeletal muscle ryanodine receptor cDNA. A total of 25,680 bp of the contiguous DNA segment was completely determined. Computer analysis using amino acid sequences of the mammalian ryanodine receptor subtypes and partial cDNA cloning (see section 2.1) revealed a gene with 26 exons comprising the protein-coding sequence (P1–P26). All the exon/intron boundaries assigned follow the GT/AG rule [22]. The nucleotide sequence ACGAAATGG surrounding the putative translation iniation codon (residues 344–346) agrees reasonably well with the consensus sequence for eukaryotic initiation condons CC(A/G)CCATGG [23] and the proposed *Drosophila* translation initiation sequence (C/A)AA(A/C)ATG [24]. The deduced amino acid sequence in P1, following the initiation methionine, is highly similar to amino-terminal sequences of the mammalian ryanodine receptor subtypes. Cloning and sequencing of the partial cDNA fragments indicated that multiple mRNAs can be generated by two different splicing patterns in two internal sections: (i) the mutually exclusive incorporation of P9 or P9′ into mRNA, and (ii) the choice of one out of two splice-acceptor sites in P13 (see section 2.1). The 3′-most protein-coding sequence (P26) contains the presumed stop codon (25,190–25,192) and a putative polyadenylation signal AATAAA [22] exists 260-bp further downstream. These observations suggest that the Drosophila gene can produce ~16-kb mRNAs carrying 15,378 (or 15,336 in another form) bases as the protein-coding sequence.

3.2. *Proposed structure of the gene product*

The predicted protein-coding sequence in this gene suggests a primary translation product composed of 5,216 (or 5,112) amino acid residues. Fig. 2 shows the predicted amino acid sequence of the product. Comparison of the amino acid sequence with those of the mammalian ryanodine receptor subtypes reveals 45%, 47% and 46% identities between the *Drosophila* product and the skeletal muscle type-, the cardiac type- and the brain type-ryanodine receptor, respectively. The hydropathicity profile of the product is similar to those of the mammalian ryanodine receptors. Four transmembrane seg-

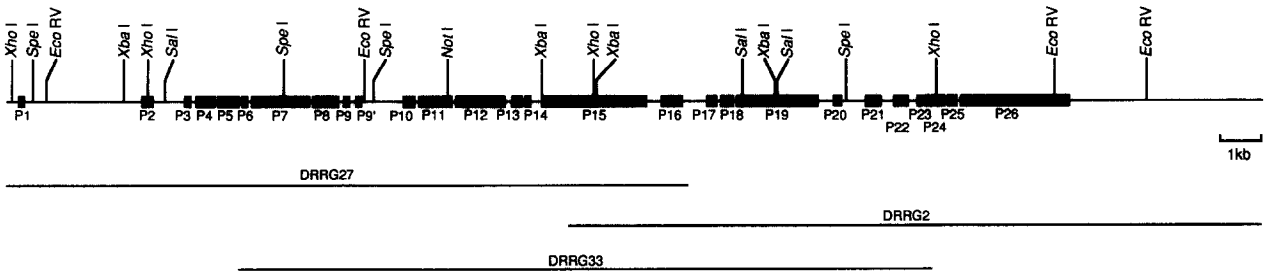

Fig. 1. Structure of the gene for the *Drosophila* ryanodine receptor homologue. The restriction map of the genomic region of the *Drosophila* ryanodine receptor homologue, the exons comprising the predicted protein-coding sequence (P1–P26) and the DNA inserts of genomic phages isolated are shown. The sequences of the exons are as follows: P1 (344–397), P2 (3,244–3,447), P3 (4,393–4,470), P4 (4,534–4,922), P5 (4,987–5,508), P6 (5,568–5,692), P7 (5,752–7,174), P8 (7,230–7,833), P9 (7,969–8,068), P9′ (8,310–8,409), P10 (9,606–9,810), P11 (9,865–10,621), P12 (10,681–11,840), P13 (12,054 or 12,096–12,248), P14 (12,321–12,436), P15 (12,743–15,015), P16 (15,524–16,003), P17 (16,604–16,741), P18 (16,895–17,181), P19 (17,243–19,341), P20 (19,690–19,802), P21 (20,439–20,784), P22 (21,007–21,307), P23 (21,592–21,702), P24 (21,760–22,179), P25 (22,248–22,449) and P26 (22,513–25,192). The sequence data for the genomic DNA segment will appear in the DDBJ/GenBank/EMBL Nucleotide Sequence Database under the accession number D17389.

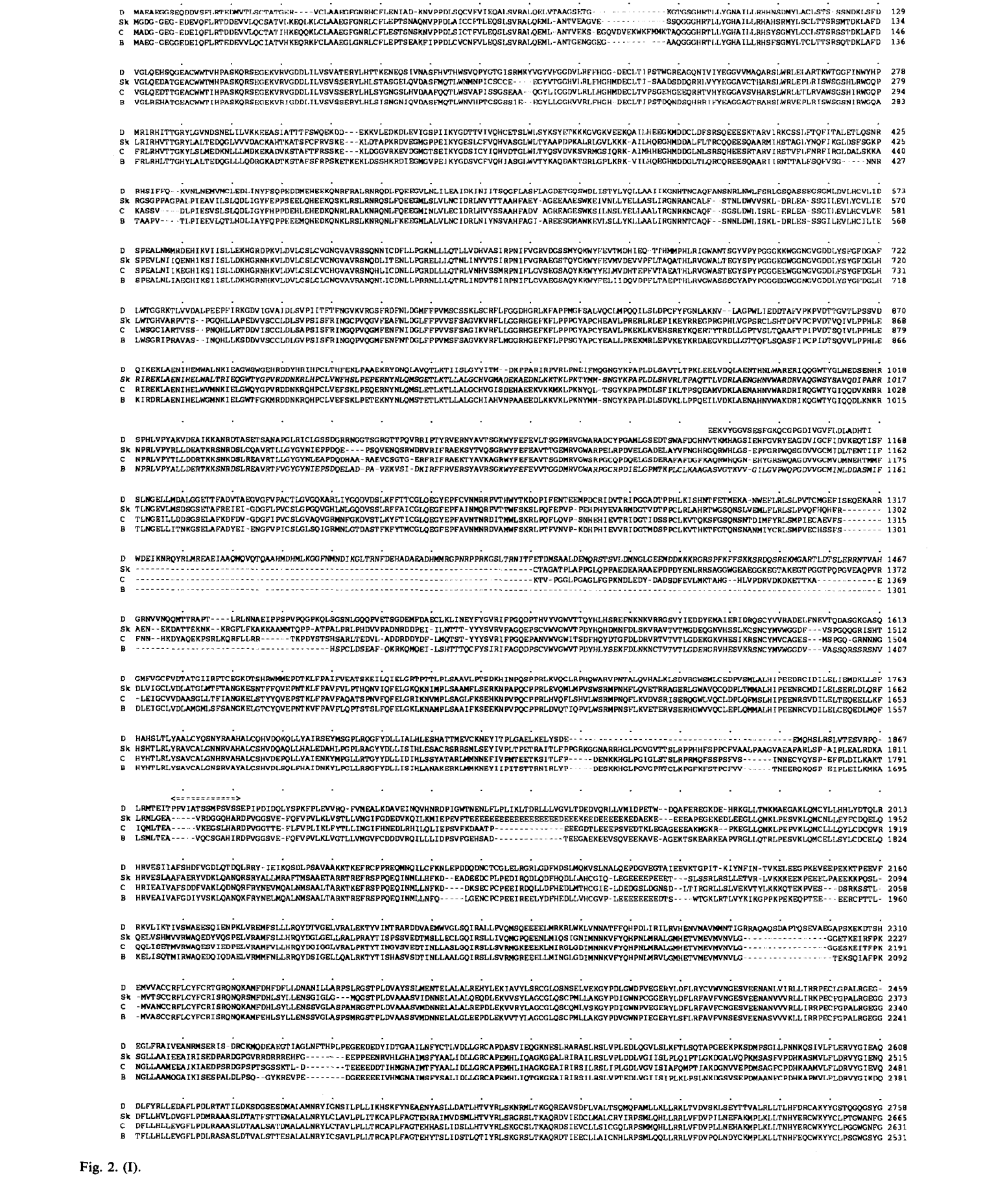

Fig. 2. (I).

Fig. 2. Predicted amino acid sequence of the *Drosophila* ryanodine receptor homologue (D) and its alignment with those of the rabbit skeletal muscle (Sk), the rabbit cardiac (C) and the rabbit brain (B) ryanodine receptor. Amino acid residues are numbered from the putative initiating methionine and the numbers at the right-hand end of the individual lines are given. Deletions are indicated by gaps (−). The putative transmembrane segments (M1–M4) are also shown; the termini of each segment are tentatively assigned. Two sets of the sequence corresponding to residues 1,134–1,166 and insertion (or deletion) of residues 1,875–1,888 were observed (see section 2.1). The sequence data for the mammalian receptor subtypes were taken from [5,8,10].

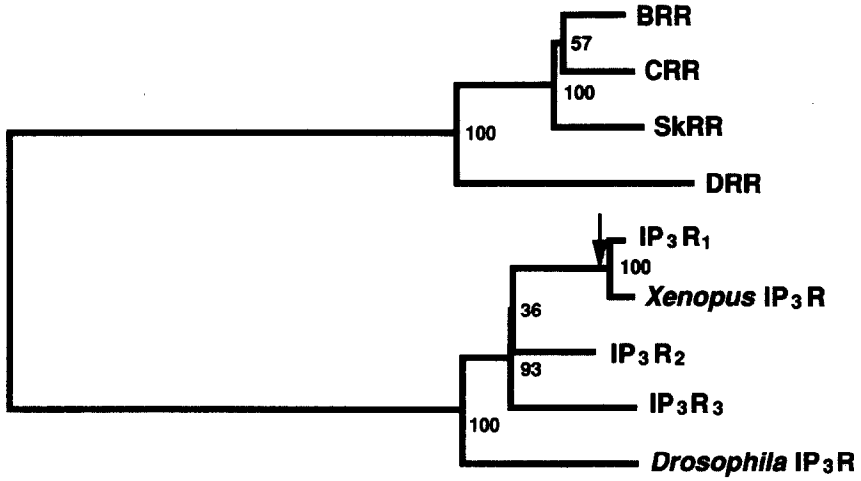

Fig. 3. Phylogenetic tree of intracellular calcium channel family. The sequences compared are as follows: BRR (rabbit brain ryanodine receptor homologue) [10], CRR (rabbit carciac ryanodine receptor) [8], SkRR (rabbit skeletal muscle ryanodine receptor) [5], DRR (*Drosophila* ryanodine receptor homologue in this study), IP_3R_1 (mouse cerebellum IP_3 receptor) [30], IP_3R_2 (rat IP_3 receptor type 2) [31], IP_3R_3 (rat IP_3 receptor type 3) [32], *Xenopus* IP_3R [33] and *Drosophila* IP_3R [28]. The arrows indicate the expected nodes of divergence between fishes and other higher vertebrates, which has been inferred by assuming 350 and 400 million years for the divergences of frog and mammals and fishes and mammals, respectively. The neighbor-joining method [34] was used for the analysis. The number at each node indicates the probability that two lineages are joined together at the node to form a single cluster, which have been estimated by a standard procedure with 1,000 times resampling [35]. Lengths of horizontal lines of the tree are proportional to estimated numbers of amino acid substitutions.

ments (M1–M4) are predicted in the carboxyl-terminal tenth of the molecule and the remaining amino-terminal portion, which shows a largely hydrophilic profile, is assigned to cytoplasmic side of the endoplasmic reticulum (ER). Thus, this product, as well as the mammalian ryanodine receptors [5,8,10], is suggested to consist of two main parts, that is, the carboxyl-terminal channel region and the large cytoplasmic region that apparently corresponds to the 'foot' structure. These observations indicate that the *Drosophila* product functions as a calcium release channel/ryanodine receptor on the ER.

In local amino acid sequence comparison between the *Drosophila* product and the mammalian ryanodine receptors, the carboxyl-terminal region including segments M3 and M4 is highly conserved whereas, the region around amino acid residue 1,400 and the region preceding segment M1 are rather divergent; these observations are consistent with previous results from the analysis of the mammalian receptor subtypes [8,10]. The *Drosophila* product also contains four repeated sequences occurring in two tandem pairs (residues 842–955, 956–1,075, 2,831–2,939 and 2,940–3,062), as shown in mammalian ryanodine receptors [6,8,10]. These region are encoded by P7–P8 and P16–P19 in the gene. However, the arrangement of these protein-coding sequences is not reminiscent of gene duplication. The gene may have been heavily diverged after the duplication.

It has been suggested that exons correlate to functional domains of the molecule for many proteins. P26 codes the carboxyl-terminal region composed of 891 amino acid residues and might represent the channel region of the product. On the other hand, more than 5 exons exist to code the ~840 carboxyl-terminal residues in the rabbit skeletal muscle ryanodine receptor gene [25]. This discrepancy suggest that the exon organization in the *Drosophila* counterpart gene is highly simplified and/or significantly different from those in the mammalian ryanodine receptor genes.

3.3. *Molecular evolution of intracellular calcium channels*

The ryanodine receptor shows amino acid sequence similarity to the IP_3 receptor and these intracellular calcium channels represent a family of structurally related gene products [1]. On the basis of a sequence alignment (data not shown), a phylogenic tree exhibiting the evolutionary relationships among known members of the intracellular calcium channel family was inferred as in Fig. 3. This tree indicates that mammalian ryanodine receptors and the *Drosophila* product arose from a common ancestor and that the three mammalian subtypes diverged at about the same time. A quite similar branching pattern was obtained in the IP_3 receptor subfamily. The tree also suggests that three subtypes of the mammalian IP_3 receptor had diverged in the early evolution of vertebrates; the time of divergence possibly antedates the divergence of fishes and other higher vertebrates. This observation is consistent with those from phylogenetic analysis in twenty different subfamilies of evolutionarily related proteins [26]. The same may hold in molecular evolution of the mammalian ryanodine receptors.

3.4. *RNA blot hybridization analysis*

Drosophila RNA preparations in various developmental stages were subjected to blot hybridization analysis

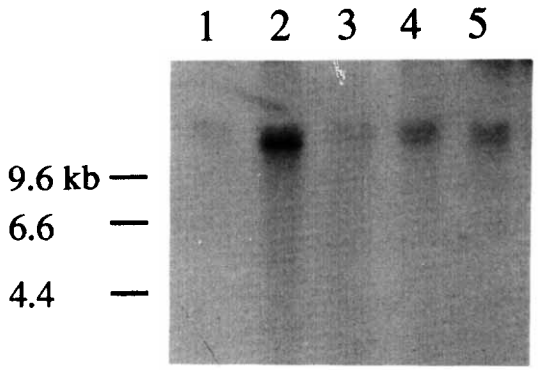

Fig. 4. Autoradiogram of blot hybridization analysis with a *Drosophila* ryanodine receptor homologue genomic DNA probe. Poly(A)$^+$ RNA preparations (20 μg) from 0–6 h embryo (lane 1), 6–12 h embryo (lane 2), 12–24 h embryo (lane 3), second instar larva (lane 4) and third instar larva (lane 5) were analysed.

with a probe specific for the ryanodine receptor counterpart (Fig. 4). A hybridizable RNA species of ~16-kb was found abundantly in a 6–12 h embryo. A low level of the transcript could be detected in second and third instar larval stages. On the other hand, no significant signal was found in pupa or in adult stages (data not shown). In a 6–12 h embryo, major morphogenic events take place; gastrulation, segmentation, germ-band elongation/contraction and primordia formation for larval organs occur successively [27]. The results obtained, together with the finding that the IP_3 receptor mRNA is highly expressed in *Drosophila* embryo [28], imply that Ca^{2+} signalling regulated by these intracellular Ca^{2+} channels may be involved deeply in morphogenic events during the embryonic development.

3.5. *In situ hybridization to polytene chromosomes*

To determine the cytogenetic locus of the gene for the *Drosophila* ryanodine receptor homologue, in situ hybridization to polytene chromosomes of larval salivary glands was performed. The probe hybridized to a single site which was identified as position 44F on chromosome 2, as shown in Fig. 5. To elucidate functional aspect of the gene, we need to isolate mutations which map to this region.

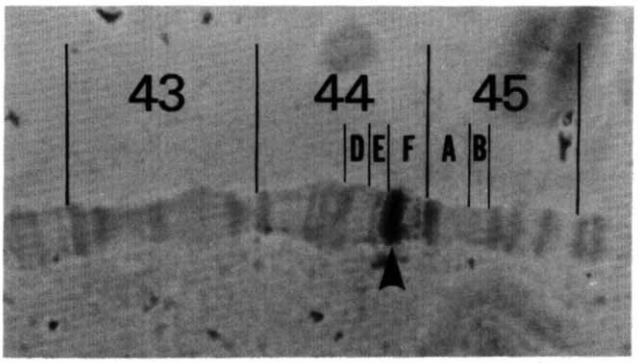

Fig. 5. Chromosomal localization of the *Drosophila* ryanodine receptor homologue gene. A hybridization signal is shown by the arrow at the 44F of the second chromosome; no other hybridizations were observed.

Recently, the isolation of a partial cDNA (~ 0.25 kbp) for a *Drosophila* ryanodine receptor homologue has been reported [29]. In the article, the gene was mapped at band position 76C-D of chromosome 3L. In spite of extensive sequence identity between their sequence and ours (85 out of 87 match in amino acids), no signal was observed at 76C-D in our analysis.

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