Structure and developmental expression of the Dα2 gene encoding a novel nicotinic acetylcholine receptor protein of *Drosophila melanogaster*

Petra Jonas, Arnd Baumann, Barbara Merz and Eckart D. Gundelfinger

ZMNH, Zentrum für Molekulare Neurobiologie, Universität Hamburg, Martinistr. 52, D-2000 Hamburg 20, FRG

(Received 20 June 1990)

Nicotinic acetylcholine receptors (nAChRs) represent a heterogeneous group of excitatory neurotransmitter receptors in the insect brain. We have characterized the *Dα2* gene of *Drosophila melanogaster*, a new member of the nAChR gene family. The protein coding region is interrupted by six introns. The positions of three of these introns are shared with all other nAChR genes. The deduced Dα2 protein shows the structural features of ligand-binding nAChR α-subunits. Cytogenetically, the *Dα2* gene maps at position 96A of the 3rd chromosome, close to the *ALS* gene which also encodes an α-like nAChR subunit. Dα2 transcripts are predominantly expressed in late embryos and in fly heads.

Nicotinic acetylcholine receptor; Nervous system; Gene structure; Development; Evolution; *Drosophila melanogaster*

1. INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are heterooligomeric membrane proteins. Their intrinsic ion channel is merely gated by the binding of acetylcholine to ligand-binding α-subunits. In vertebrates, various subtypes of nicotinic receptors are found both at the neuromuscular junction [1] and in the peripheral and central nervous system [2]. The protein subunits of nAChRs are encoded by a large family of related genes [1–12].

In insects, acetylcholine serves a vital function in the nervous system whereas synaptic transmission at nerve muscle synapses is most likely not cholinergic [13]. Evidence has accumulated for the existence of a heterogeneous population of nAChRs also in this group of organisms: two different classes of high-affinity binding sites for the snake venom component α-bungarotoxin, a potent antagonist of vertebrate muscle receptors, were detected in the *Drosophila* nervous system [14]. As in vertebrates, where the majority of neuronal AChRs is not blocked by α-bungarotoxin [2,15], additional receptors insensitive to the toxin were described [16]. Furthermore, monoclonal antibodies directed against the *Torpedo* electroplax nAChR recognize at least 4 distinct subsets of antigens in *Drosophila* neural tissue [17].

Correspondence address: E.D. Gundelfinger, ZMNH, Universitätskrankenhaus Eppendorf, Martinistr. 52, D-2000 Hamburg 20, FRG

Abbreviations: nAChR, nicotinic acetylcholine receptor; Dα2, *Drosophila* α-like subunit 2; ALS, α-like subunit; ARD, nAChR protein of *Drosophila*

Genes and cDNAs of two different nAChR subunits of the *Drosophila* nervous system have been characterized to date. The α-like subunit (ALS) shares structural features with ligand-binding subunits of vertebrate nAChRs [18] whereas the ARD protein resembles structural non-α subunits [19–21]. A more detailed study of the nicotinic cholinergic receptor system of the fly requires the identification of its further constituents. Here, we report the characterization of a gene encoding a novel α-like subunit, which was named Dα2 protein (for *Drosophila* α-like subunit 2).

2. MATERIALS AND METHODS

2.1. *Isolation and characterization of genomic clones*

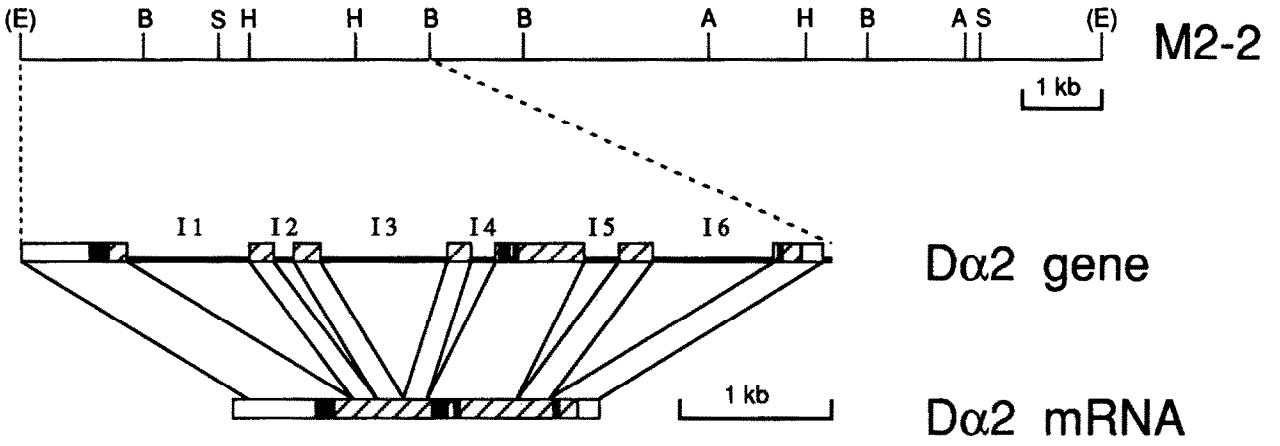
A ^{32}P-labeled fragment spanning nucleotides 1178–2244 of the ALS cDNA [18] was used to screen a *Drosophila* genomic library [22] under reduced stringency. Plaque hybridization was performed at 37°C in 42% formamide, 5×SET (1×SET: 150 mM NaCl; 1 mM EDTA; 30 mM Tris-Cl, pH 8), 5×Denhardt's solution, 0.1% SDS, 100 μg/ml denatured herring sperm DNA and 2×10^6 cpm/ml of probe. Subsequent washes were carried out in 2×SET, 0.1% SDS at 55°C. Appropriate restriction fragments of recombinant phage DNA were subcloned into pBluescript vectors (Stratagene) and sequenced using a T7 polymerase sequencing kit (Pharmacia).

2.2. *Northern analysis*

Northern blots were performed as described previously [23]. Dα2 transcripts were detected with a 45-mer synthetic oligonucleotide (5′-TCATTTGCATCTCGTCCATCAGCGCCTGTCCGAACTTG-GTCTTGC-3′) complementary to amino acid residues 357–372 of the Dα2 protein (compare Fig. 3). The oligonucleotide (20 ng) was radiolabeled to a specific activity of $\approx10^9$ cpm/μg with terminal deoxynucleotidyl transferase (Boehringer) in the presence of 80 μCi α[^{32}P]dATP (Amersham; 3000 Ci/mmol) [24]. Northern blots were hybridized for 16 h at 42°C in 20% formamide, 6×SET, 10×Denhardt's solution, 0.1% sodium pyrophosphate, 1% SDS, 100 μg/ml denatured herring sperm DNA and $\approx10^6$ cpm/ml radiolabeled probe. Final washes were in 1×SET, 0.1% SDS at 55°C.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

A



B

```
I1   GATTTG/GTAAGC.....~900bp.....AGAGCTATCCGTACATATATGCGAGTTTAATTTTTTGCAG/AATCTC
     AspLeu                                                              AsnLeu
     42

I2   CAACAA/GTAAGT.......91bp.....AAAAGCCCTGAATCAAAGCTATGTCTTCCCTTTCCTCTAG/TGCCGA
      AsnAs                                                               nAla
      94

I3   GATCAG/GTGAGT.....~810bp.....CTTTAACGGCTCCATCAACGGGGCTTTTCTCGATTCACAG/ATCGAT
     AspGln                                                              IleAsp
     154

I4   ATCCGG/GTGAGT......124bp.....TTCGTGGATTTGTAACTGCTTCAATGGCTGGACGGTTTAG/ATATCT
       ProA                                                               spIle
       208

I5   AAACAG/GTGAGT.....~180bp.....CACAAGGACACTGTGTTGACCTCTCATCTCGAACTTACAG/ATTCAG
      AsnAr                                                               gPhe
      403

I6   AATGCG/GTGAGT.....~800bp.....TAAATGTGCATAATTTACTGAAACCATTTTCCTCTTACAG/GAAGAT
     AsnAla                                                              GluAsp
     470
```

C

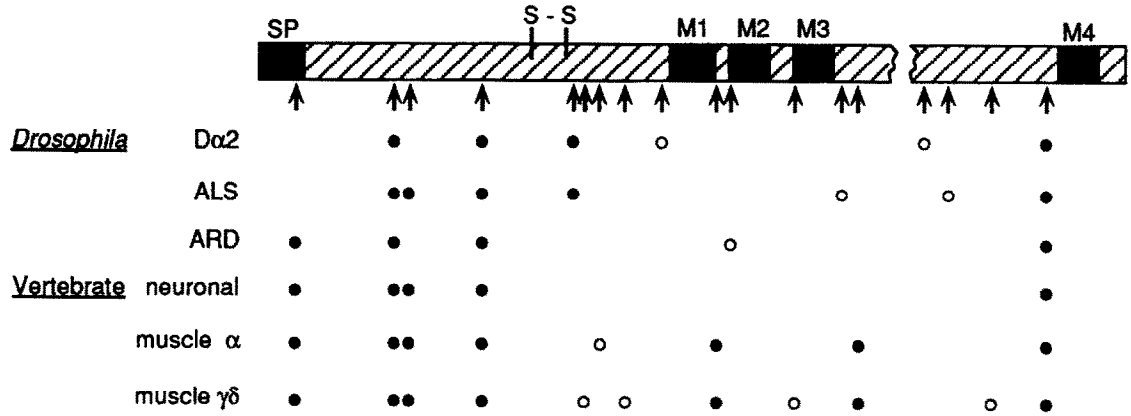


Fig. 1. Structure of the *Dα2* gene. (A) Restriction map of the genomic clone M2-2 and physical map of *Dα2* gene and mRNA. Restriction enzymes are A, *Apa*I; B, *Bam*HI; E, *Eco*RI (introduced during cloning procedure); H, *Hin*dIII; S, *Sst*I. Introns (I1–I6) are indicated as solid lines, exons are boxed (open, untranslated regions; hatched, protein coding regions; filled, hydrophobic regions, i. e. signal peptide [SP] and potential transmembrane regions [M1-M4]). (B) Exon/intron junctions. Numbers indicate the respective amino acid position within the mature Dα2 protein (compare Fig. 3). (C) Comparison of intron positions between *Dα2* and other nAChR subunit genes. Arrows indicate intron positions on a representative nAChR protein. The presence of an intron within a gene/group of genes is marked by a circle (open, intron is unique to a gene/group of genes; filled, intron is found in at least two genes/groups of genes). S-S, position of a conserved disulfide bridge [1]. Bibliography: *ALS* gene [18], *ARD* gene [20,21], vertebrate neuronal genes [6,12], vertebrate muscle genes [6,29,30,32].

2.3. *In situ hybridization to polytene chromosomes*

Preparation and in situ hybridization of salivary gland chromosomes were performed according to [25]. Digoxigenin-labeled probes were prepared using a nonradioactive DNA labeling kit (Boehringer Mannheim) and detected with peroxidase-coupled second antibodies.

3. RESULTS AND DISCUSSION

3.1. *The Dα2 gene*

Sequence homology between the various nAChR subunits is not distributed evenly along the polypeptide chain. Most notably, the amino-terminal half including the membrane spanning regions M1-M3 share ≥50% identity among neuronal receptor proteins of vertebrates and invertebrates [26]. We therefore have used a fragment of the ALS cDNA [18] spanning this conserved region to screen a *Drosophila* genomic DNA library for homologous recombinants.

The entire protein coding region of a novel nAChR gene, the *Dα2* gene, is contained within a 5.5 kb *Eco*RI/*Bam*HI fragment of the genomic clone M2-2 (Fig. 1A). Within the protein coding region, the nucleotide sequence differs in 4 positions from that of the Dα2 cDNA [27]. Only one of these microheterogeneities leads to variation of the encoded protein (Ile or Leu at position 50).

By comparison with the corresponding cDNA the exon/intron organization of the *Dα2* gene was established. The coding region is interrupted by six introns, I1 to I6, all of which are flanked by consensus splice donor and acceptor sites (Fig. 1B) [28]. The positions of introns I1, I2 and I6 are conserved in all nAChR genes analyzed so far. An intron homologous to I3 is found only in the *ALS* gene and another *Drosophila* nAChR gene currently under investigation (unpublished data). Introns I4 and I5 are unique to the *Dα2* gene (Fig. 1C).

By in situ hybridization to polytene chromosomes the *Dα2* gene was cytogenetically mapped to region 96A of the right arm of the third chromosome (Fig. 2). The *ALS* gene also maps to this chromosomal region [18] suggesting that the genes arose from gene duplication. Clustering of nAChR genes has also been described for the vertebrate muscle γ- and δ-subunit genes [29,30] and for the rodent neuronal α3-, α5- and β4-subunit genes [12].

3.2. *Structure of the Dα2 protein*

The polypeptide deduced from the nucleotide sequence of the *Dα2* gene consists of 576 amino acid residues. It shares significant sequence homology and several structural features with other nAChR subunits, i. e. two disulfide bridge forming cysteines (Cys-128, Cys-142) and 4 potential membrane spanning regions M1-M4 (Fig. 3). A comparison with homologous proteins indicates the first 41 residues as a potential signal peptide (not shown). Two consecutive cysteines (Cys-202, Cys-203) classify the Dα2 protein as a putative ligand-binding nAChR subunit [1,2]. Three potential N-glycosylation sites were found within the deduced protein sequence. One (Asn-24) is common to all neuronal nAChR subunits analyzed so far but not present in muscle receptors [1–12]. A second one (Asn-213) is located between the two consecutive cysteines and the M1 transmembrane region and a third one (Asn-529) close to the C-terminus.

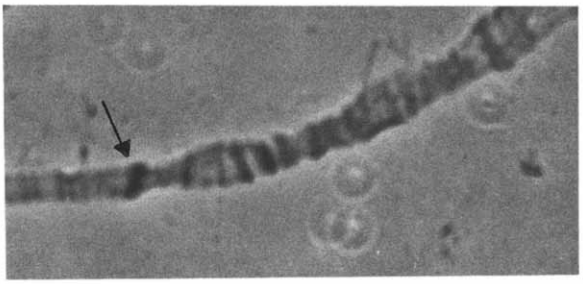


Fig. 2. Cytogenetic localization of the *Dα2* gene. The arrow indicates the site of hybridization at chromosomal region 96A.

The mature Dα2 protein shares 56% identical amino acid residues with the ALS and 40% with the ARD protein (Fig. 3). Within the highly conserved amino-terminal half comprising approximately amino acids 1–330 [26] the sequence identity with ALS and ARD protein increases to 75% and 52%, respectively. Homology values of this region between Dα2 and subunits of vertebrate neuronal nAChRs range from ≈52% (for β2-subunits [5,6]) to ≈59% (for α2-subunits [6,8]). The homology between Dα2 and muscle receptor proteins is significantly lower.

In summary, the sequence data as well as the structure of the genes (see section 3.1.) suggest that the heterogeneity of α-subunits of neuronal nAChRs has evolved independently in vertebrates and *Drosophila*.

3.3. *Developmental expression of the Dα2 gene*

The expression profile of the *Dα2* gene during different stages of *Drosophila* development was analyzed on Northern blots. To avoid cross-hybridization with other members of the nAChR gene family we employed an oligonucleotide derived from the highly variable region between membrane spanning regions M3 and M4 as a Dα2-specific probe. The mature Dα2 transcript has a size of ≈2.5 kb which comigrates with a synthetic RNA derived from the Dα2 cDNA (Fig. 4). Transcript levels were found to be highest in late embryos and in heads of newly eclosed flies, significantly lower in mid-stage embryos and pupae and lowest in second instar larvae. No Dα2 RNA was detected in 0–4-h-old embryos (Fig. 4).

The time course of the developmental expression of the *Dα2* gene shows a remarkable coincidence with that of the *ARD* gene [19,21,31]; it differs, however, significantly from the expression profile of the *ALS*

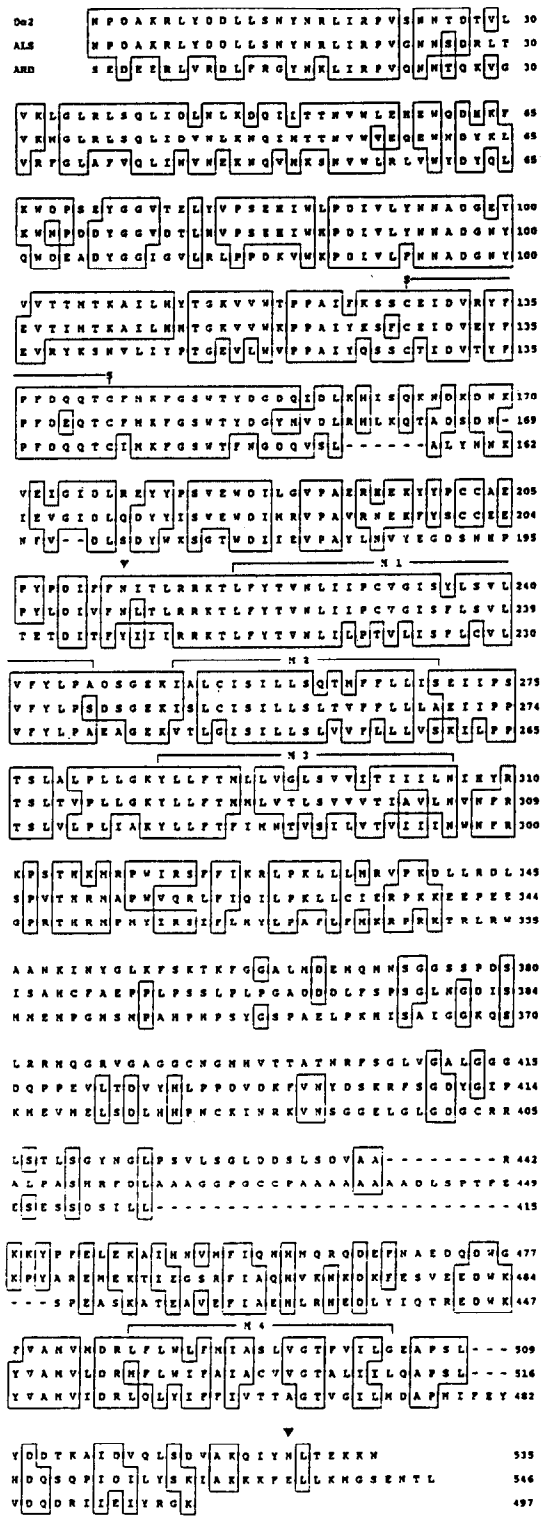


Fig. 3. Sequence alignment of the deduced mature Dα2 protein with the ALS [18] and ARD protein [19]. Identical amino acid residues are boxed. A predicted conserved disulfide bridge and 4 putative transmembrane regions (M1-M4) are indicated above the sequences. Potential *N*-glycosylation sites are marked with a black arrowhead. The nucleotide sequence complementary to the Dα2 mRNA will appear in the EMBL Nucleotide Sequence Database under the accession no. X52274.

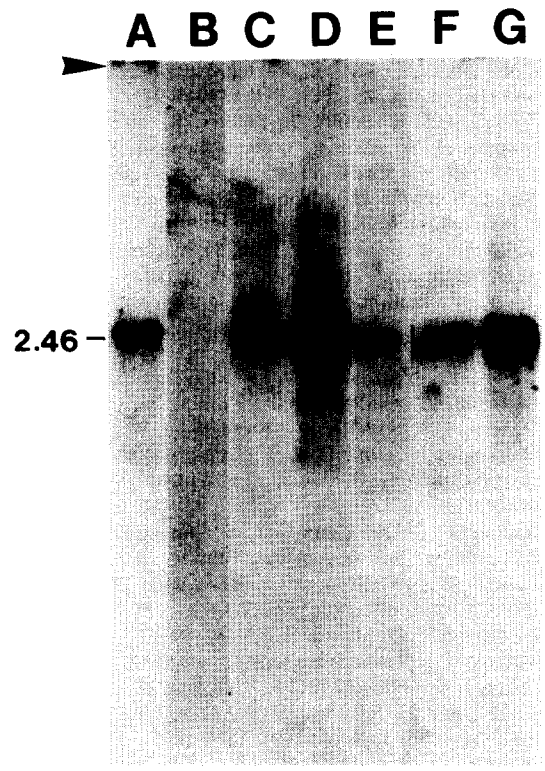


Fig. 4. Detection of Dα2 transcripts at different developmental stages by Northern analysis. A 2,461 nucleotides long cRNA (5 ng) synthesized with T3 RNA polymerase from a pBluescript vector containing the Dα2 cDNA (lane A) and poly(A)$^+$ RNA (5 μg) isolated from 0–4 h embryos (B), 2–12 h embryos (C), 10–22 h embryos (D), second instar larvae (E), 3–5-day-old pupae (F), heads of 2-day-old flies (G) were hybridized to a Dα2-specific probe. The arrowhead indicates the start site of electrophoresis.

gene which appears up-regulated in larvae [18]. In spite of their different expression profiles, immunoprecipitation experiments have shown that ALS and ARD protein are components of the same receptor complex [P. Schloß, H. Betz, C. Schröder and E.D. Gundelfinger, unpublished data]. Elucidation of the role of the Dα2 subunit - whether it is also associated with the ARD protein or a component of yet another nAChR - will require further investigation, such as comparative in situ hybridization and immunological studies.

Acknowledgements: We are grateful to B. Bossy, M. Ballivet and P. Spierer for the gift of the ALS cDNA, to Maniatis and colleagues for providing the genomic library, to N. Hess for many helpful discussions and suggestions and to C. Garner and Y. Henschel for critically reading the manuscript. This work was supported by grants of the Bundesministerium für Forschung und Technologie (BCT0395) and the Deutsche Forschungsgemeinschaft (SFB 232). It forms part of the Ph.D. thesis of P.J.

REFERENCES

[1] Hucho, F. (1986) Eur. J. Biochem. 158, 211–226.
[2] Lindstrom, J., Schoepfer, R. and Whiting, P. (1987) Mol. Neurobiol. 1, 281–337.

[3] Boulter, J., Evans, K., Goldman, D., Martin, G., Treco, D., Heinemann, S. and Patrick, J. (1986) Nature 319, 368–374.
[4] Goldman, D., Deneris, E., Luyten, W., Kochhar, A., Patrick, J. and Heinemann, S. (1987) Cell 48, 965–973.
[5] Deneris, E.S., Connolly, J., Boulter, J., Wada, E., Wada, K., Swanson, L.W., Patrick, J. and Heinemann, S. (1988) Neuron 1, 45–54.
[6] Nef, P., Oneyser, C., Alliod, C., Couturier, S. and Ballivet, M. (1988) EMBO J. 7, 595–601.
[7] Schoepfer, R., Whiting, P., Esch, F., Blacher, R., Shimasaki, S. and Lindstrom, J. (1988) Neuron 1, 241–248.
[8] Wada, K., Ballivet, M., Boulter, J., Connolly, J., Wada, E., Deneris, E.S., Swanson, L.W., Heinemann, S. and Patrick, J. (1988) Science 240, 330–334.
[9] Cauley, K., Agranoff, B.W. and Goldman, D. (1989) J. Cell Biol. 108, 637–645.
[10] Deneris, E.S., Boulter, J., Swanson, L.W., Patrick, J. and Heinemann, S. (1989) J. Biol. Chem. 264, 6268–6272.
[11] Isenberg, K.E. and Meyer, G.E. (1989) J. Neurochem. 52, 988–991.
[12] Boulter, J., O'Shea-Greenfield, A., Duvoisin, R.M., Connolly, J.G., Wada, E., Jensen, A., Gardner, P.D., Ballivet, M., Deneris, E.S., McKinnon, D., Heinemann, S. and Patrick, J. (1990) J. Biol. Chem. 265, 4472–4482.
[13] Gerschenfeld, H. M. (1973) Physiol. Rev. 53, 1–119.
[14] Schloss, P., Hermans-Borgmeyer, I., Betz, H. and Gundelfinger, E.D. (1988) EMBO J. 7, 2889–2894.
[15] Clarke, P.B.S., Schwartz, R.D., Paul, S.M., Pert, C.B. and Pert, A. (1985) J. Neurosci. 5, 1307–1315.
[16] Goodman, C.S. and Spitzer, N.C. (1979) Nature 280, 208–214.
[17] Chase, B.A., Holliday, J., Reese, J.H., Chun, L.L.Y. and Hawrot, E. (1987) Neuroscience 21, 959–976.
[18] Bossy, B., Ballivet, M. and Spierer, P. (1988) EMBO J. 7, 611–618.
[19] Hermans-Borgmeyer, I., Zopf, D., Ryseck, R.-P., Hovemann, B., Betz, H. and Gundelfinger, E.D. (1986) EMBO J. 5, 1503–1508.
[20] Sawruk, E., Hermans-Borgmeyer, I., Betz, H. and Gundelfinger, E.D. (1988) FEBS Lett. 235, 40–46.
[21] Wadsworth, S.C., Rosenthal, L.S., Kammermeyer, K.L., Potter, M.B. and Nelson, D.J. (1988) Mol. Cell. Biol. 8, 778–785.
[22] Maniatis, T., Hardison, R.C., Lacy, E., Lauer, J., O'Connell, C. and Quon, D. (1978) Cell 15, 687–701.
[23] Zopf, D., Hermans-Borgmeyer, I., Gundelfinger, E.D. and Betz, H. (1987) Genes Dev. 1, 699–708.
[24] Wisden, W., Morris, B.J., Darlison, M.G., Hunt, S.P. and Barnard, E.A. (1988) Neuron 1, 937–947.
[25] Ashburner, M. (1989) *Drosophila*: A Laboratory Manual, Cold Spring Harbor Laboratories Press, Cold Spring Harbor, New York.
[26] Gundelfinger, E.D., Hermans-Borgmeyer, I., Schloss, P., Sawruk, E., Udri, C., Vingron, M., Betz, H. and Schmitt, B. (1989) in: Molecular Biology of Neuroreceptors and Ion Channels (A. Maelicke ed.) NATO ASI Series Vol. H32, Springer, Heidelberg, pp. 69–81.
[27] Baumann, A., Jonas, P. and Gundelfinger, E.D. (1990) Nucl. Acids Res. (in press).
[28] Ohshima, Y. and Gotoh, Y. (1987) J. Mol. Biol. 195, 247–259.
[29] Nef, P., Mauron, A., Stalder, R., Alliod, C. and Ballivet, M. (1984) Proc. Natl. Acad. Sci. USA 81, 7975–7979.
[30] Shibahara, S., Kubo, T., Perski, H. J., Takahashi, H., Noda, M. and Numa, S. (1985) Eur. J. Biochem. 146, 15–22.
[31] Hermans-Borgmeyer, I., Hoffmeister, S., Sawruk, E., Betz, H., Schmitt, B. and Gundelfinger, E.D. (1989) Neuron 2, 1147–1156.
[32] Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Tanabe, T., Shimizu, S., Kikyotani, S., Kayano, T., Hirose, T., Inayama, S. and Numa, S. (1983) Nature 305, 818–823.