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Expression of a *Drosophila melanogaster* Acetylcholine Receptor-Related Gene in the Central Nervous System

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We isolated *Drosophila melanogaster* genomic sequences with nucleotide and amino acid sequence homology to subunits of vertebrate acetylcholine receptor by hybridization with a *Torpedo* acetylcholine receptor subunit cDNA probe. Five introns are present in the portion of the *Drosophila* gene encoding the unprocessed protein and are positionally conserved relative to the human acetylcholine receptor alpha-subunit gene. The *Drosophila* genomic clone hybridized to salivary gland polytene chromosome 3L within region 64B and was termed AChR64B. A 3-kilobase poly(A)-containing transcript complementary to the AChR64B clone was readily detectable by RNA blot hybridizations during midembryogenesis, during metamorphosis, and in newly enclosed adults. AChR64B transcripts were localized to the cellular regions of the central nervous system during embryonic, larval, pupal, and adult stages of development. During metamorphosis, a temporal relationship between the morphogenesis of the optic lobe and expression of AChR64B transcripts was observed.

Cholinergic neurotransmitter systems are important components of excitatory neurotransmission. The peripheral nicotinic acetylcholine receptor (AChR) of vertebrates represents a particularly well characterized element of such systems because of its relative abundance in the electric organ of *Torpedo* and *Electrophorus* species (5, 30). The primary sequences of the AChR subunit proteins and the pentameric organization of the subunits in the functional receptor are highly conserved among vertebrate species (5, 30). An experimentally useful property of the peripheral AChR is that the alpha subunit binds the acetylcholine antagonist, alpha-bungarotoxin, essentially irreversibly. This property of peripheral AChR has been exploited to define its distribution in various tissues and to probe its localization within tissues. From such studies and parallel pharmacological studies, it has been established that the peripheral AChR represents the alpha-bungarotoxin-binding component localized to the postsynaptic region of electromotor synapses and vertebrate neuromuscular junctions (5, 7, 30). In neuronal tissue, the precise correspondence of alpha-bungarotoxin-binding activity and AChR is presently unclear, although AChR-related mRNA sequences have been conclusively demonstrated (1, 9).

In insects, nicotinic AChRs are believed to be concentrated in the central nervous system (CNS). Alpha-bungarotoxin-binding components with pharmacological specificities similar to that of vertebrate AChR have been identified from the CNS of *Drosophila melanogaster* and other insect species (2, 25, 26, 28). Furthermore, the *Drosophila* alpha-bungarotoxin-binding component is localized to the synaptic regions of the CNS (25, 28), and electrophysiological experiments have demonstrated acetylcholine-induced currents in dispersed neurons from the third-instar larval CNS (34). In locusts, the correlation between the toxin-binding component and AChR has been more completely investigated. The putative AChR, purified on the basis of alpha-bungarotoxin binding, reacts with antibody raised against *Torpedo* AChR, and antibody raised against the locust protein reacts specifically with the neuropil regions of the CNS (3). Thus, there is a good correlation between the insect toxin-binding component and the presumptive AChR. A more precise relationship has not been elucidated primarily owing to an incomplete biochemical characterization of the putative insect AChR.

To define the nature of the *Drosophila* AChR, we undertook the cloning and characterization of gene sequences related to cloned vertebrate AChR cDNAs. Comparison of our cloned *Drosophila* genomic sequences and recently isolated cDNA sequences (12) with vertebrate AChR sequences established that insects possess AChR-related genes. Additionally, we report here that transcripts from the cloned AChR-related gene are localized to the CNS throughout *Drosophila* development.

MATERIALS AND METHODS

Isolation and analysis of *Drosophila* AChR subunit-related clones. A lambda bacteriophage library of *Drosophila* genomic DNA (18) was screened under low-stringency hybridization conditions with probes prepared from the *Torpedo* AChR alpha- and gamma-subunit cDNAs (24). The alpha probe hybridized with highly repeated *Drosophila* sequences, making it impossible to use this probe for the library screen. Only clones identified by hybridization with the gamma probe are presented in this report. The precise hybridization conditions have been described before (32). Individual *Eco*RI segments from the genomic clones were subcloned into plasmid vectors for mapping of restriction endonuclease cleavage sites and subsequently into bacteriophage M13mp series vectors (19) for sequencing by the method of Sanger et al. (27). Restriction endonuclease-cleaved genomic or cloned DNAs were electrophoresed through 1% agarose gels and transferred to nitrocellulose by the method of Southern (29).

Polytene chromosome hybridizations. Polytene chromo-

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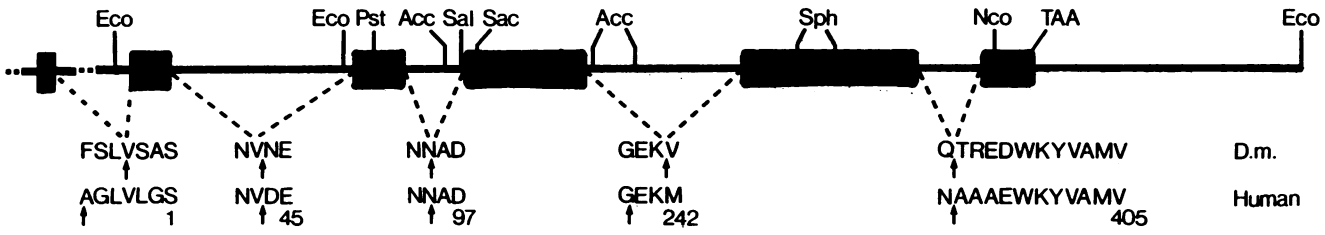

FIG. 1. Restriction endonuclease map of the cloned genomic AChR subunit-related segment. Amino acid-coding regions are indicated by filled rectangles, while intron regions are indicated by the solid lines connecting them. The region of the genomic clone encoding the signal peptide was located by hybridization with a synthetic oligonucleotide complementary to the signal peptide sequence identified from an AChR subunit-related cDNA (12). The restriction map of the intervening DNA has not been determined. It is not known whether introns are present in the genomic sequences beyond the indicated termination codon, TAA. Below the restriction map are the amino acid sequences (in the single-letter code) that surround the splice sites in the *Drosophila* AChR subunit-related gene as determined in this study and the homologous splice sites in the human AChR alpha-subunit gene (22). Numbers below the human sequence indicate the position of amino acids in the mature human protein (22). Vertical arrows indicate the positions of the intron sequences. Exact positions of the intron sequences are shown in Fig. 2. D.m., *D. melanogaster*.

some squashes were prepared from salivary glands dissected from late-third-instar larvae and hybridized with biotinylated DNA probes (17). To prepare the hybridization probe, the 3.2-kilobase (kb) *Eco*RI segment shown in Fig. 1 was gel purified and nick translated in the presence of biotinylated dUTP (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Procedures for hybridization and detection of the biotinylated probe specified by William Engels and supplied by the manufacturer of the probe detection kit (Bethesda Research Laboratories) were followed.

RNA extraction and hybridization. Extraction of RNA from the various developmental stages and specific tissues was performed as described before (32). Staging of embryos and larvae was accomplished by a 30-min precollection followed by a 1-h collection. Staging of pupae and adults was accomplished by collecting white prepupae. RNA gel electrophoresis, blotting, and hybridization procedures were performed by the methods of Gregory et al. (10).

RNA hybridization probes. RNA probes were prepared by in vitro transcription of an AChR subunit cDNA segment subcloned into a plasmid vector containing a bacteriophage T7 promoter (31) (the Bluescribe vector from Stratagene, Inc.). The cDNA clone was isolated from an adult head cDNA library (14) by hybridization with an AChR64B probe. The cDNA segment is approximately 2 kb in length and begins at nucleotide 500 of the published cDNA sequence (12). ^{3}H-labeled or ^{35}S-labeled antisense (or sense) RNA probes were used for in situ hybridization experiments, and ^{32}P-labeled antisense RNA probes were used for RNA blot hybridizations. DNA templates were removed by brief DNase treatment, and probes were phenol-chloroform extracted and ethanol precipitated. The probes were hydrolyzed to approximately 150 bases before in situ hybridization (6).

Tissue preparation and in situ hybridization methods. Animals were collected at the indicated stages of development (determined as described above) and processed for cryostat sectioning generally following the procedures of Hafen et al. (11) and Mitchison and Sedat (20). Pupae were dissected from their pupal cases before being embedded. Adults were taken within minutes of eclosion before hardening of their cuticle could take place. Embedded tissue was sectioned in a cryostat at −20 to −23°C. Poly-L-lysine-coated slides (6) were used to pick up sections measuring 8 to 10 μm.

For in situ hybridizations, the basic method of Cox et al. (6) with slight modifications (13, 15) was used. Autoradiography was as described by Pardue and Gall (23). The slides were lightly stained with Giemsa stain before examination by light microscopy.

RESULTS

Isolation of *Drosophila* genomic AChR-related sequences. A cDNA segment representing the gamma subunit of the *Torpedo* AChR (24) was used to probe a *D. melanogaster* genomic library under low-stringency hybridization conditions (32). Two clones representing overlapping genomic segments were isolated, each of which contained an identical 3.2-kb *Eco*RI segment that was complementary to the *Torpedo* probe. No other sequences within these genomic clones hybridized with the *Torpedo* probe. The restriction maps of the 3.2-kb genomic *Eco*RI segment and an adjacent 0.8-kb *Eco*RI segment are shown in Fig. 1. The nucleotide and deduced amino acid sequences of presumptive exons from the AChR-related gene are shown in Fig. 2. Exon locations were determined by comparison of the genomic sequence with the sequence of a *Drosophila* AChR-related cDNA sequence (12) (see below). The deduced amino acid sequence was most closely related to alpha and beta subunits of vertebrate AChR subunits rather than to gamma subunits. Specifically, the overall homology levels were 42% with rat neural alpha subunit (1), 39% with *Torpedo* alpha subunit (30), 39% with human alpha subunit (22), and 36% with *Torpedo* beta subunit (30). However, if the comparison was restricted to the regions of each of the proteins presumed to encode membrane-spanning regions M1 through M3, the homology levels were 64% with rat neural alpha subunit, 52% with *Torpedo* alpha subunit, 55% with human alpha subunit, and 45% with *Torpedo* beta subunit. Within the same region, the homology of the human alpha subunit with the rat neural alpha subunit is approximately 72%. The amino acid sequence deduced from the genomic clone was compared with the National Biomedical Research Foundation protein database and found to have homology only with vertebrate AChR subunit sequences.

Excluding non-amino acid-coding regions, the nucleotide sequence shown in Fig. 2 differs from the sequence of a *Drosophila* AChR-related cDNA isolated from an adult head cDNA library (12) in 10 positions, only 3 of which result in changes in the deduced amino acid sequence. The amino acid positions that differ with respect to the cDNA are isoleucine instead of valine at position 49, aspartic acid instead of glutamic acid at position 366, and valine instead of leucine at position 367. Because the genomic clone and the

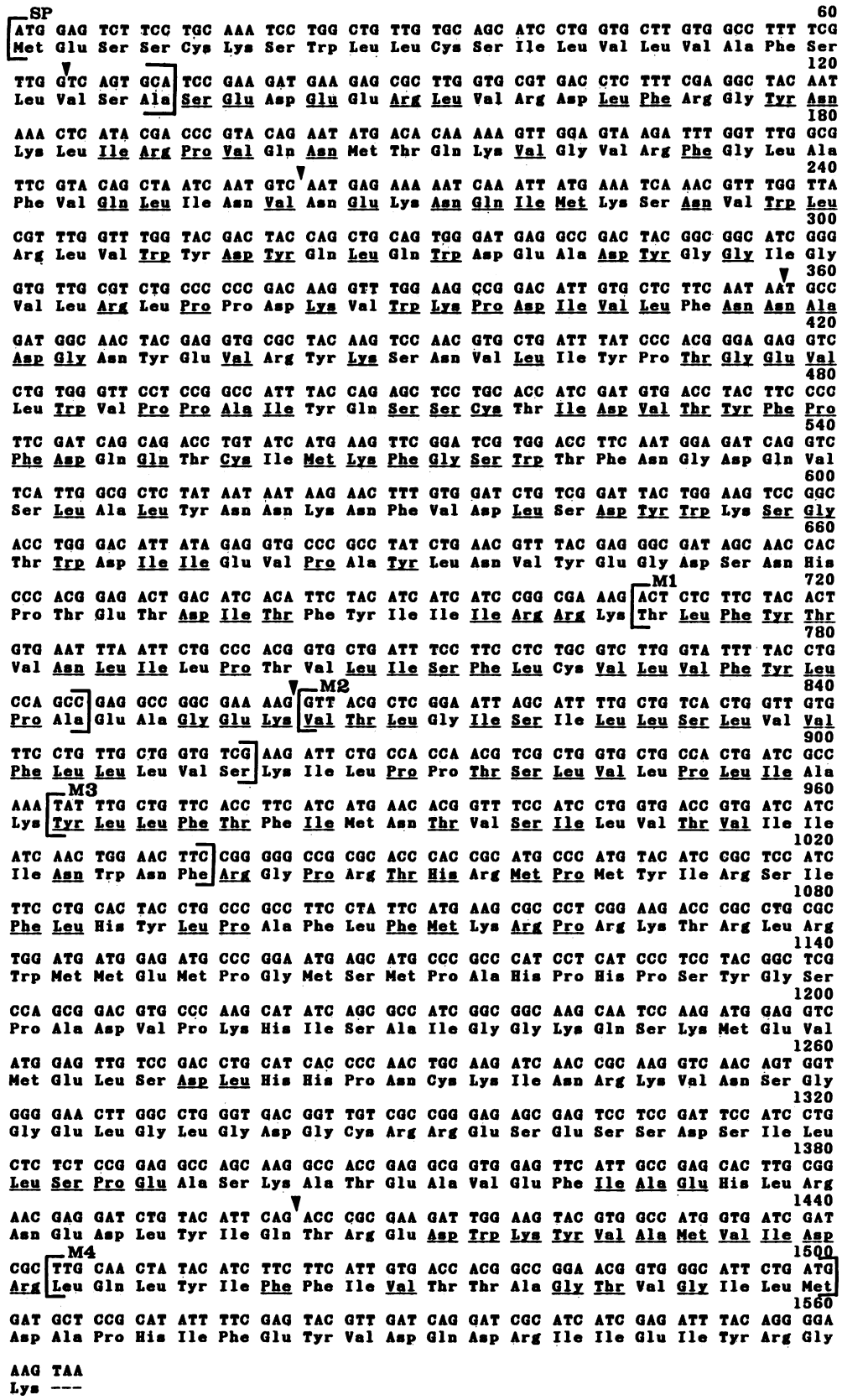

FIG. 2. Nucleotide and deduced amino acid sequences of presumptive exons from the AChR subunit-related gene. Positions of amino acids conserved relative to the rat neural alpha subunit (1) are underlined. Brackets enclose the portions of the sequence that could encode signal peptide (SP) or membrane-spanning domains (M1 to M4). These regions were identified on the basis of hydropathy analysis and by analogy to the structure of the *Torpedo* subunits. Arrowheads indicate the positions of intron sequences as determined by the point of divergence between the genomic sequence and cDNA sequences from *D. melanogaster* (12) and vertebrate cDNA sequences (30) and by the presence of consensus splice donor and acceptor sequences within the introns (21).

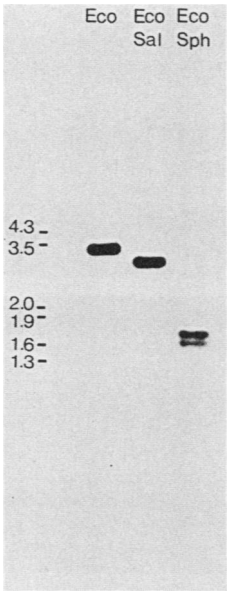

FIG. 3. Organization of the genomic AChR-related subunit segment. *Drosophila* embryonic DNA was digested with the indicated restriction endonucleases, electrophoresed through a 1% agarose gel, and transferred to nitrocellulose by the Southern procedure (29). Size determinations were made relative to plasmid DNA digested with the same restriction endonucleases and to lambda phage DNA digested with *Eco*RI and *Hin*dIII. The sizes of relevant size standards from the lambda phage DNA digest are indicated (kilobases).

cDNA clone were both derived from *D. melanogaster* Canton S, they could represent variant forms of the same gene.

Examination of the positions of intron sequences also suggested that vertebrate AChR genes and the *Drosophila* AChR-related gene had a common ancestor. There are five presumptive introns within the amino acid-coding region (Fig. 1 and 2) identified by the point of divergence of the genomic and cDNA sequences (12) and by the presence of consensus splice donor and acceptor sequences (21). Two of these introns occupy identical positions with respect to the human alpha-subunit gene (22): those between the Val-43 and Asn-44 codons and between nucleotides 1 and 2 of the Asn-95 codon. The other three introns are present in regions of the gene that encode structurally analogous protein domains: between the signal peptide and the mature protein and before each of the putative M2 and M4 membrane-spanning domains. The structure of one of the exons, the one encoding amino acids 44 to 95, corresponds exactly with that found in a cDNA clone presumably arising from unspliced mRNA (12).

With regard to the subunit identity of this *Drosophila* AChR-related gene, the presence or absence of adjacent cysteine residues at positions equivalent to positions 192 and 193 in the *Torpedo* alpha sequence is of potential importance. All known alpha subunits contain this pair of cysteine residues (5, 30). Adjacent cysteine residues were not found in these positions in the published sequence of an adult head cDNA clone (12) nor in the homologous positions in the genomic sequence.

To investigate the genomic organization of the AChR-related sequences, we did Southern blot hybridization experiments (Fig. 3). Cleavage of genomic DNA with *Eco*RI yielded a segment identical in size to the cloned 3.2-kb *Eco*RI segment. The restriction endonucleases *Sal*I and *Sph*I which cleaved the cloned 3.2-kb *Eco*RI segment also cleaved the homologous genomic segment, yielding hybridizing fragments identical in size to those in the cloned gene. Thus, the organization of the genomic AChR subunit-related sequences is reflected in the cloned genomic segment.

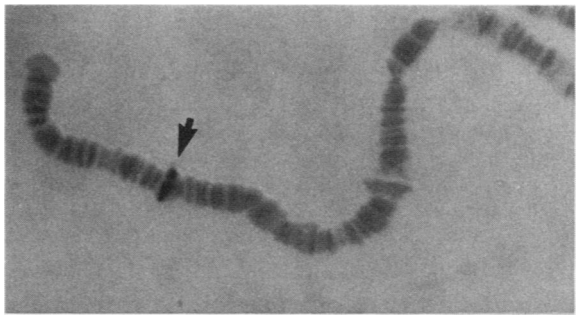

FIG. 4. Cytological location of the AChR subunit-related sequences. Chromosome squashes were prepared from third-instar larval salivary glands and hybridized with a biotinylated probe prepared from the genomic AChR subunit-related clone as described in Materials and Methods. The only site of hybridization was on chromosome arm 3L within position 64B (indicated by the arrow) (4).

Chromosomal location of AChR subunit-related gene. The chromosomal location of the cloned AChR subunit-related sequences was determined by in situ hybridization to salivary gland polytene chromosomes (Fig. 4). Hybridization was detected at a single site within the polytene chromosomal region, 64B (4). The AChR-related gene represented by the cloned segment shown represented in Fig. 1 will be referred to hereafter as the AChR64B gene.

Temporal expression of AChR64B transcripts. Hybridization experiments were done on RNA prepared from various

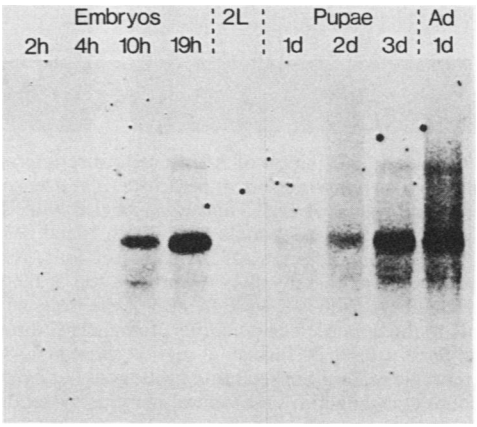

FIG. 5. Temporal expression of AChR64B RNA. RNA was extracted from the indicated stages of development and electrophoresed through a 1% agarose-formaldehyde gel. Each lane contains 30 μg of total animal RNA. The hybridization probe was a ^{32}P-labeled antisense RNA transcribed from a cDNA clone. Hybridization conditions and the origin of the cDNA clone are detailed in Materials and Methods. Size determination of the AChR64B transcript was made relative to the sizes of *Drosophila* actin mRNA (8) and an RNA marker ladder (purchased from Bethesda Research Laboratories). Hybridization of RNA selected on oligod(T)-cellulose revealed only the 3-kb RNA species (data not shown). d, Day; Ad, adult; 2L, second instar larva.

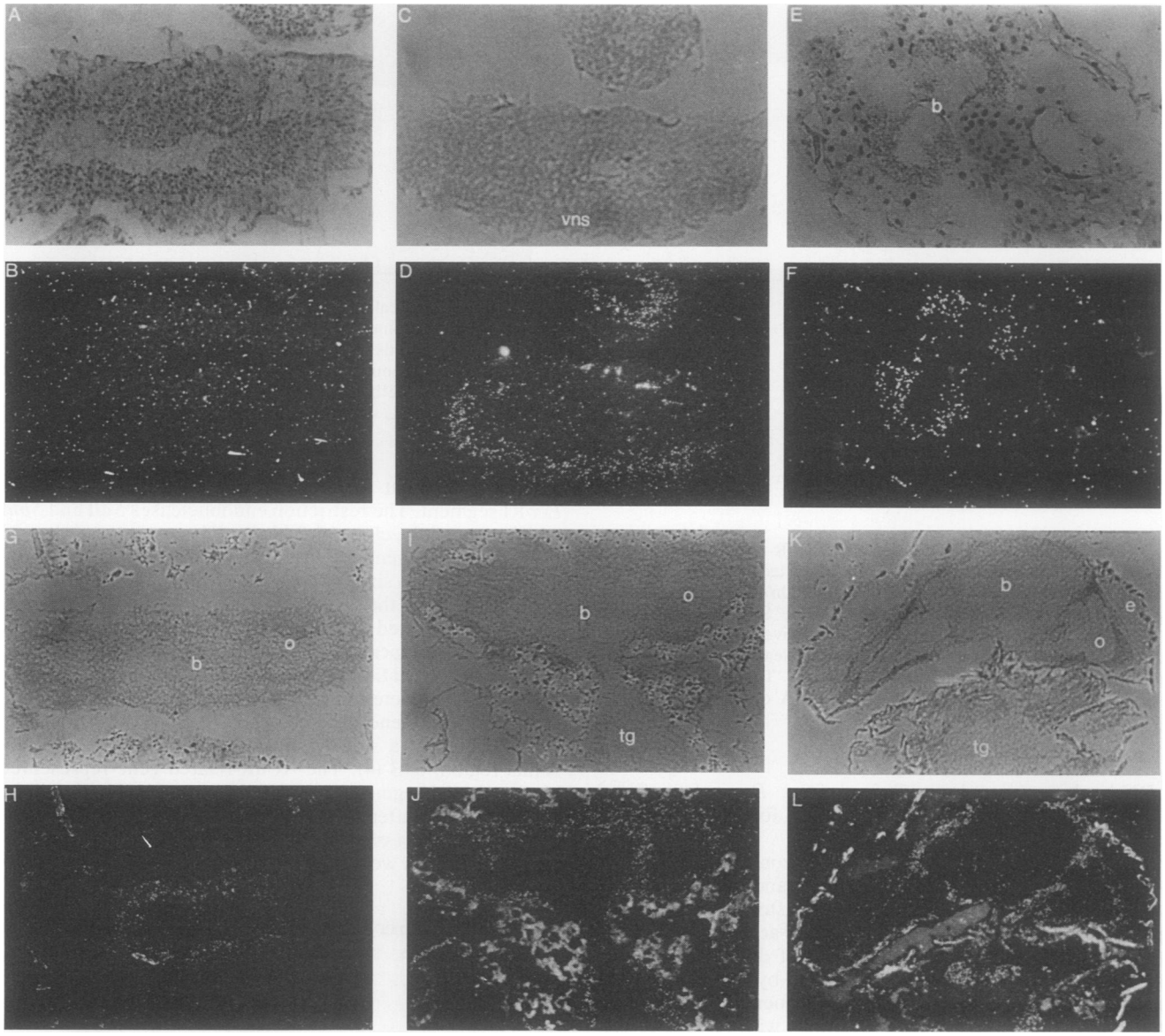

FIG. 6. Spatial distribution of AChR64B transcripts in the CNS. Cryostat sections prepared from animals at the stages of development indicated below were hybridized with AChR64B antisense RNA probes. Antisense probes were synthesized from the adult head cDNA clone described in the legend to Fig. 5 and were labeled with ^{3}H for all hybridizations except the 7-h-old embryos in panels A and B for which a ^{35}S-labeled probe was used. Panels A, C, E, G, I, and K are photomicrographs of sections under bright-field illumination. Panels B, D, F, H, J, and L are photomicrographs of the same sections, respectively, under dark-field illumination. (A and B) Parasagittal section through a 7-h-old embryo. The dorsal side is toward the top of the page and anterior is to the right. (C and D) Parasagittal section through a 12-h-old embryo. The dorsal side is toward the top of the page and anterior is to the left. (E and F) Horizontal section through a second-instar larva. Anterior is to the upper left-hand corner. In panels G through L, anterior is toward the top of the page. (G and H) Horizontal section through a 24-h-old pupa. (I and J) Horizontal section through a 48-h-old pupa. (K and L) Horizontal section through a newly eclosed adult head and anterior thoracic region. Sense-strand probes did not hybridize above background levels in any case. Abbreviations: b, brain; e, eye; o, optic lobe; tg, thoracic ganglion; vns, ventral nervous system.

stages of development to define the expression pattern of AChR64B sequences (Fig. 5). During early embryogenesis, as exemplified by the 2-h RNA lane, AChR64B transcripts were not detected. AChR64B transcripts were detected at low and variable levels in 4-h-old embryo RNA. A single major 3-kb AChR64B transcript was detected in RNA prepared from 10-h-old embryos and at significantly increased levels in RNA prepared from 19-h-old embryos. The AChR64B RNA species was also detected at low levels in RNA prepared from second-instar larvae and pupae 1 day postpupariation. During the next two days of metamorphosis and the first day of adulthood, increased levels of the 3-kb transcript were observed. These results confirm and extend the previous report of AChR subunit transcripts in late embryos and 3-day-old pupae (12).

Tissue localization of AChR64B transcripts. If the AChR64B transcript encodes a subunit of a functional nicotinic AChR, then cells of the CNS should contain transcripts from the gene. Therefore, cryostat sections prepared from animals at various stages of development were hybridized

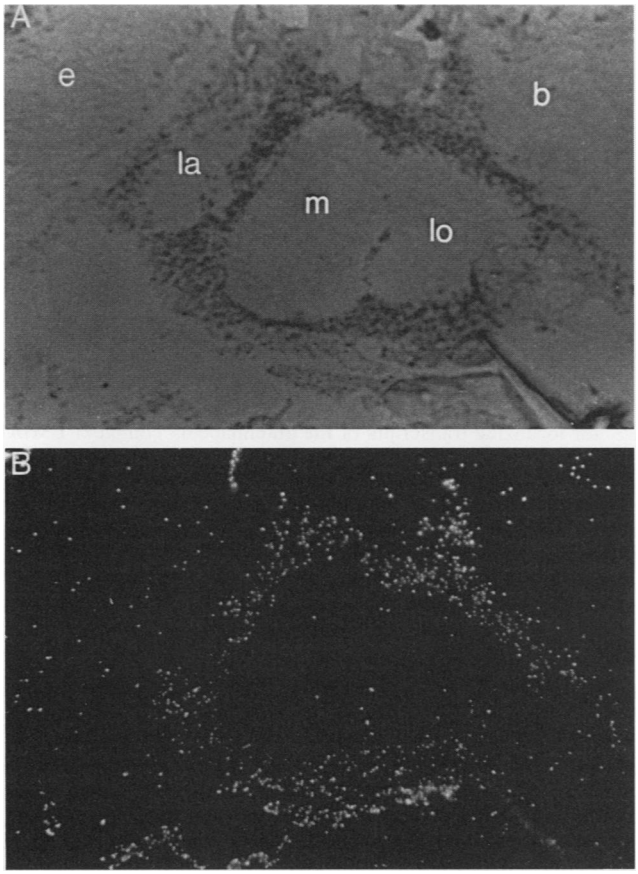

FIG. 7. Spatial distribution of AChR64B transcripts in the optic lobe of a newly eclosed adult. (A and B) Bright-field and dark-field illumination, respectively, of a horizontal section through the left side of the head of a newly eclosed adult. This section shows a portion of the eye, the optic lobe, and part of the brain. Sense-strand probes did not hybridize above background levels in any case. Abbreviations: b, brain; e, eye; la, lamina; lo, lobula; m, medulla.

with AChR64B antisense RNA probes (Fig. 6). In agreement with the RNA blot hybridization analysis, AChR64B transcripts were not detected by in situ hybridization to sections of early embryos. An example of such a hybridization to a 7-h-old embryo is shown in Fig. 6 (A and B). By approximately 12 h of embryonic development, AChR64B transcripts were detected in the developing CNS (Fig. 6C and D). At this stage of embryonic development, the CNS can be recognized by the well-differentiated ventral cord and the subesophageal and supraesophageal ganglia (16). No hybridization of the AChR64B probe outside of these CNS regions was detected in sections of embryos at any stage of development.

In second-instar larvae, AChR64B transcripts were also localized to the CNS. Hybridization of the AChR64B probe to the cellular cortex of the brain lobes is shown in Fig. 6E and F. AChR64B transcripts were also detected in the ventral nervous system (data not shown) but were not detected in any other tissues of second-instar larvae.

In contrast to the majority of larval cells, which undergo histolysis during metamorphosis, cells of the larval CNS largely survive and form a portion of the adult CNS. During metamorphosis, major structural rearrangements of the larval brain occur owing to morphogenesis of the optic lobe. Within the first 24 h after pupation, the basic structure of the optic lobe is formed from progenitor cells originally located in the proliferative centers of the larval brain hemispheres (33). Continued differentiation of these cells during metamorphosis results in the formation of the mature adult optic lobe. At 24 h postpupariation, AChR64B transcripts were detected in the cellular cortex of the midbrain but not in the cells of the developing optic lobe (Fig. 6G and H). However, by 48 h postpupariation, the levels of AChR64B transcripts in the cellular cortex of the optic lobe were similar to those in the cortical regions of the midbrain (Fig. 6I and J).

During the latter stages of metamorphosis, a major change in brain morphology is the increase in the size of the neuropil at the expense of the cortical regions. Thus, the adult midbrain and optic lobes are composed of thin cortical regions surrounding extensive neuropil regions (16). These cortical regions of the newly eclosed adult contains AChR64B transcripts (Fig. 6K and L). A more detailed view of the distribution of AChR64B transcripts in the optic lobe of a newly eclosed adult is shown in Fig. 7. Here it can be seen that the cortical regions surrounding the lobula and medulla contain uniform levels of AChR64B transcripts, while the cellular cortex of the lamina contains relatively low levels of AChR64B transcripts.

DISCUSSION

The experimental results described in this report provided direct evidence that a *Drosophila* gene structurally related to AChR is expressed in the CNS. Within the synaptic regions of the adult brain, the presumptive AChR is localized to the neuropil where the extensions of neurons are concentrated (16). Thus, the expected site of AChR gene transcription would be the cell bodies from which the neurons project. We showed here that AChR64B transcripts are localized to the cell bodies of the midbrain, of the optic lobe, and of the thoracic ganglia as well. We also observed that the cortical region of the lamina seems to contain lower levels of AChR64B transcripts. This observation is in agreement with previous reports that laminal neurons contain less toxin-binding component than the other brain regions (25, 28). Thus, there is a one-to-one correspondence between the brain regions that express the AChR64B gene and the brain regions that contain the previously characterized alpha-bungarotoxin-binding component. Furthermore, we presented experimental results that suggest a temporal relationship between the development of the CNS and induction of AChR64B gene expression. We showed that there is a delay in the accumulation of AChR64B transcripts in the cellular cortex of the optic lobe after its morphogenesis. Our findings on the timing of AChR64B gene expression during establishment of the embryonic CNS correlate well with this general pattern. The earliest events in neurogenesis occur soon after gastrulation (16), but it is not until several hours later that expression of AChR64B transcripts can be detected by RNA blot hybridization or by in situ hybridization. Taken together, these results suggest that induction of AChR subunit gene expression is a relatively late event in the formation of *Drosophila* cholinergic neurons. This conclusion is in agreement with studies in *Manduca sexta* in which the formation of an alpha-bungarotoxin-binding component (presumed to be the AChR) lags behind the synthesis of other components of the cholinergic system in antennal neurons during metamorphosis (26).

Direct evidence that the AChR64B-encoded protein represents a subunit of a nicotinic CNS AChR rather than another related protein also expressed in the CNS is pres-

ently not available. However, the overall sequence homology and the positional conservation of introns in the *Drosophila* gene relative to vertebrate AChR genes strongly suggest that the AChR64B gene has a common ancestor with known vertebrate AChR genes. It is not possible to conclude definitively which subunit of vertebrate AChR the AChR64B gene corresponds to on the basis of the present sequence data. However, the AChR64B sequence and head cDNA sequence are most closely related to alpha- and beta-subunit sequences although they were isolated by hybridization with a gamma-subunit probe. The failure by two different laboratories to isolate subunit sequences with preferential gamma homology suggests that *D. melanogaster* does not possess a gamma-subunit homolog. It is clear that the proteins encoded by the AChR64B sequence and the head cDNA sequence (12) are virtually identical and would lack the cysteine 194 and 195 residues thought to be important for the alpha-bungarotoxin-binding property of AChR. Thus, it seems likely that there exists at least one other *Drosophila* AChR-related subunit, one presumably encoding the well-characterized toxin-binding component. If such a subunit exists, then the AChR64B gene could represent a beta subunit or a variant alpha subunit. Further information on the primary sequence of additional *Drosophila* AChR-related proteins will be required to elucidate the subunit structure of the insect AChR.

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