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Cloning and characterization of a *Drosophila* serotonin receptor that activates adenylate cyclase

(guanine nucleotide-binding protein/5HT1A receptor/cyclic AMP/glycosaminoglycan/circadian rhythm)

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ABSTRACT **Using a strategy based on nucleotide sequence homology between genes encoding receptors that interact with guanine nucleotide-binding proteins, we have isolated *Drosophila* genomic and cDNA clones encoding a functional serotonin receptor (5HT-dro receptor). This protein is expressed predominantly in *Drosophila* heads and exhibits highest homology with the human 5HT1A receptor. The predicted structure of the 5HT-dro receptor reveals two unusual features: (*i*) eight putative transmembrane domains instead of the expected seven and (*ii*) a Gly-Ser repeat that is a potential glycosaminoglycan attachment site. When stably introduced into mouse NIH 3T3 cells, the 5HT-dro receptor activates adenylate cyclase in response to serotonin and is inhibited by serotonin receptor antagonists such as dihydroergocryptine. The 5HT-dro receptor or closely related receptors might be responsible for the serotonin-sensitive cyclase that has been suggested to play a role in learning and modulation of circadian rhythm in a number of invertebrate systems.**

Serotonin (5-hydroxytryptamine) is a neurotransmitter found in both vertebrates and invertebrates that plays a role in various physiological mechanisms, including sleep, appetite, pain perception, learning, and the control of cyclic events (for a review see ref. 1).

We decided to study the role of serotonin in *Drosophila melanogaster* in order to have access to the powerful genetic techniques that are available for this species. In flies, genetic evidence suggests a role of serotonin in learning processes (2). For example, the *ddc* mutants, which lack the enzyme dopamine decarboxylase and therefore do not synthesize serotonin and dopamine, exhibit altered learning abilities (3). Biochemical and pharmacological studies performed in other insect systems have also suggested a role for serotonin in physiological mechanisms such as salivary gland secretion and the control of circadian rhythms (for a review see ref. 4). The ability of a single neurotransmitter to mediate a wide range of effects is related to the existence of different types of receptors that are coupled to distinct signal-transduction pathways. The recent isolation of three mammalian serotonin receptors has revealed that they belong to the large family of transmembrane proteins that interact with guanine nucleotide-binding proteins (G proteins) (5–7). These G-protein-coupled receptors, which include the muscarinic acetylcholine receptors, the adrenergic receptors, and the opsins, share a predicted seven-transmembrane-domain structure with highly conserved amino acid sequences, especially within certain transmembrane regions (8). The interaction between receptors and various G proteins leads to the activation of different second-messenger pathways. For example, some serotonin receptors activate phospholipase C while others activate or inhibit adenylate cyclase (9).

In *Drosophila* and in other insects, pharmacological studies have suggested the existence of different types of serotonin receptors, some of which activate adenylate cyclase (10–15). In order to study the function of these receptors, we decided to clone the corresponding genes. Using a strategy based on nucleotide sequence homologies between genes encoding G-protein-coupled receptors, we isolated a *Drosophila* cDNA clone that encodes a functional serotonin receptor (5HT-dro receptor).¶ This protein is a member of the G-protein-coupled receptor family that exhibits highest sequence homology with the human 5HT1A receptor. Northern hybridization analysis revealed that the 5HT-dro mRNA is expressed predominantly in *Drosophila* heads. Furthermore, we expressed the 5HT-dro receptor in mouse NIH 3T3 cells and demonstrated that it is able to activate adenylate cyclase in response to serotonin and that this activity can be blocked by serotonin receptor antagonists.

MATERIALS AND METHODS

Isolation and Sequence of Genomic and cDNA Clones. A genomic library from the wild-type Canton-S strain of *D. melanogaster*, constructed in the λ phage EMBL4, was probed with two degenerate oligonucleotides corresponding to consensus sequences found in transmembrane domains VI [5′-TT(C/T)(A/G)(C/T)(C/G)(C/A/G)TCTGCTGGCTGC-CCTTCTTC-3′] and VII [5′-TGG(T/C/A)T(G/T)GGCTA (T/C)G(T/C)CAA(T/C)(A/T)(G/C)-3′]. Oligonucleotides were labeled at the 5′ end with polynucleotide kinase. Hybridizations (40°C, 5× SSC/5× Denhardt's solution/20 mM sodium phosphate buffer, pH 6.5/1% SDS containing tRNA at 100 μg/ml; 1× SSC is 150 mM NaCl/15 mM sodium citrate, pH 7; 1× Denhardt's solution is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin) and washings (40°C, 2× SSC/1% SDS) were performed in non-stringent conditions.

The cDNA library (a gift from Lily Jan, University of California, San Francisco) was prepared from heads of *Drosophila* from the wild-type Oregon-R strain. [The library contains both random-primed and oligo(dT)-primed cDNAs and was prepared from RNAs larger than 3 kilobases (kb)]. This library was probed with a 5′-end-labeled oligonucleotide (64 nucleotides long, positions 1397–1460; Fig. 1) at high stringency (42°C, 40% formamide/5× SSC/1× Denhardt's solution/20 mM sodium phosphate buffer, pH 6.5, containing tRNA at 100 μg/ml). Sequencing of the cDNA and the genomic clone was performed by the dideoxy technique using

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Abbreviation: G protein, guanine nucleotide-binding protein.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M55533).

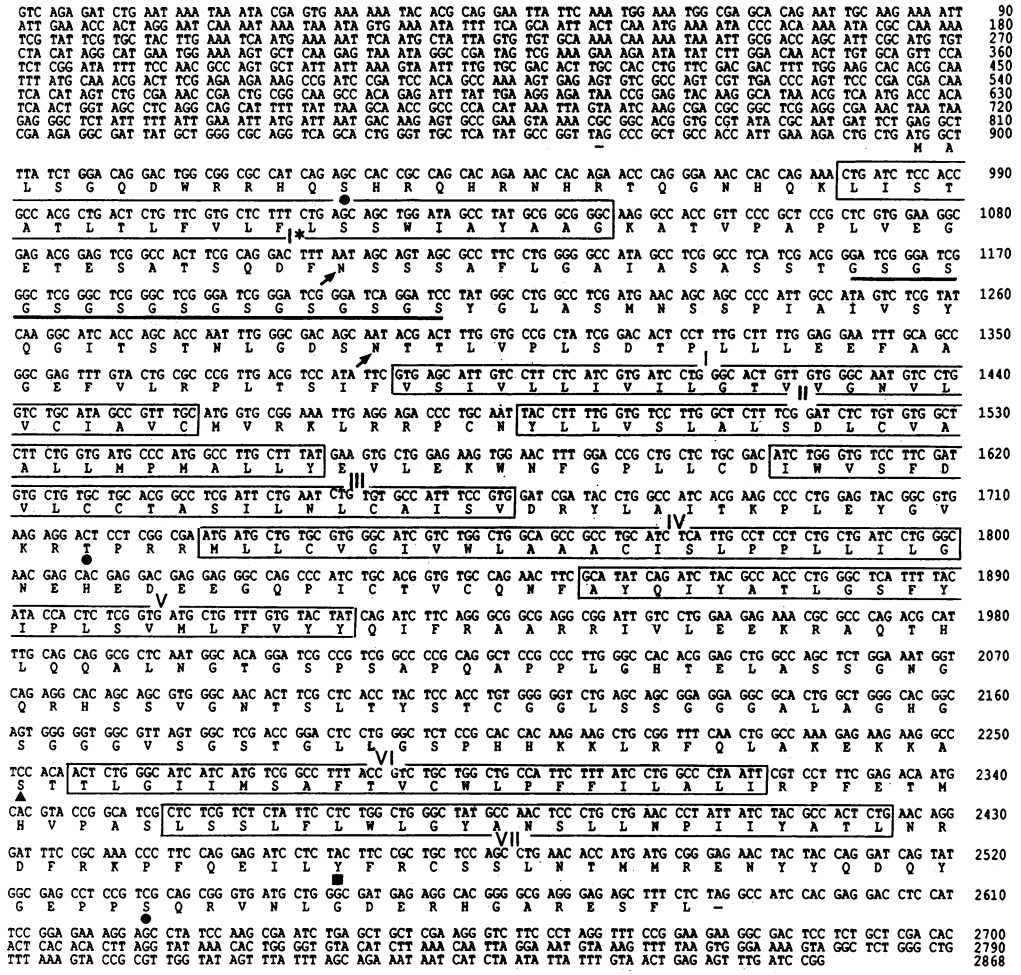

FIG. 1. Nucleotide sequence of the 5HT-dro receptor cDNA and predicted amino acid sequence. The seven putative transmembrane domains found in all G-protein-coupled receptors are boxed and numbered (I–VII). I* represents an additional putative transmembrane domain. Arrows indicate sites of potential N-linked glycosylation. Circles, triangles, and squares correspond to consensus sites for protein kinase C, protein kinase A, and tyrosine kinase, respectively. The Gly-Ser repeat is underlined.

successive synthetic oligonucleotides. The 4-kilobase-pair (kbp) cDNA was sequenced on both strands (Fig. 1) with the exception of 1100 bp of 3′ untranslated sequence. The 3.2-kbp *Eco*RI genomic fragment was sequenced on both strands from one *Eco*RI site (nucleotide 2868 in Fig. 1) to the position corresponding to the 5′ end of the cDNA (nucleotide 1 in Fig. 1).

Construction of Cell Lines Expressing the 5HT-dro mRNA. A 4-kbp *Eco*RI fragment corresponding to the longest cDNA was inserted into the *Eco*RI site of expression vector pSG5 (16). The resulting recombinant plasmid, pEB, was introduced into mouse NIH 3T3 cells by calcium phosphate-mediated transfection, together with the recombinant pRSVneo, which encodes resistance to the neomycin analog G418. Transformed clones were selected in the presence of G418 at 0.5 mg/ml. Isolated foci were amplified and total RNA was prepared and analyzed for expression of 5HT-dro mRNA. Two cell lines were selected (NpEB2 and NpEB3) that expressed high levels of 5HT-dro mRNA as measured by Northern blot analysis.

Northern Blot Analysis. Heads and bodies of adult *Drosophila* were separated by freeze–fracture. Poly(A)$^+$ mRNA was prepared, fractionated in 1% agarose/2.2 M formaldehyde gel, and transferred to a nitrocellulose filter. DNA probes were ^{32}P-labeled by random priming and hybridized to filters at high stringency (42°C, 50% formamide/5× SSC/1× Denhardt's solution/20 mM sodium phosphate buffer, pH 6.5/0.1% SDS containing tRNA at 100 μg/ml). Washings were performed at high stringency (60°C, 0.1× SSC/0.1% SDS).

cAMP Assays. All drugs were obtained from Sigma. Cells were seeded into six-well plates at ≈3 × 10^5 cells per well, washed once with phosphate-buffered saline, and incubated for 25 min at 37°C with 100 μM 3-isobutyl-1-methylxanthine (an inhibitor of cAMP phosphodiesterase) and test agents in phosphate-buffered saline. The reaction was stopped by aspiration of the medium, followed by the addition of 1 ml of ice-cold ethanol. After 2 hr at room temperature, the ethanol was collected and lyophilized. The pellet was reconstituted and cAMP was quantitated with a radioimmunoassay kit (NEN; NEK-033). The basal level of cAMP observed in the absence of serotonin was about the same in all cell lines (≈300 pmol/mg of protein). Forskolin typically yielded a 12- to 15-fold increase in cAMP.

RESULTS

Isolation of *Drosophila* Genomic and cDNA Clones Encoding a Protein with Sequence Homology to the Human 5HT1A Receptor. Sequence comparisons of receptors for biogenic monoamines (epinephrine, dopamine, and serotonin) reveal that these receptors constitute a subfamily among the larger family of G-protein-coupled receptors. The highest sequence homology is found in the putative transmembrane domains VI and VII (8). We therefore constructed two series of degenerate oligonucleotides corresponding to consensus sequences found in these two domains. A *Drosophila* genomic library was probed with both series of oligonucleotides at low stringency. We obtained one genomic clone that hybridized strongly with both oligonucleotides. Southern blot analysis indicated that this genomic clone contained a 3.2-kb *Eco*RI fragment that hybridized with both oligonucleotides. This fragment was therefore subcloned and sequenced. The nucleotide sequence revealed one long open reading frame encoding a protein of 564 amino acids that exhibited a striking homology to G-protein-coupled receptors.

To ensure that we had cloned the entire coding region, we isolated corresponding cDNAs. A random-primed *Drosophila* head cDNA library was probed with an oligonucleotide corresponding to domain I of the predicted protein. We obtained three overlapping cDNAs that had a common 5′ end and variable 3′ ends. The longest cDNA, 4 kbp, was sequenced (Fig. 1), with the exception of ≈1100 bp of 3′ untranslated sequence. The cDNA sequence contained a single open reading frame encoding a predicted protein of 564 amino acids. This sequence was identical to that of the genomic clone, indicating that the coding region of this gene does not contain introns, a feature that is common among genes encoding G-protein-coupled receptors (8).

Comparison of the predicted amino acid sequence of the 5HT-dro receptor with two protein data banks [Swiss Prot (April 2, 1990) and the Protein Identification Resource of the National Biomedical Research Foundation (April 2, 1990)] revealed significant homologies with all G-protein-coupled receptors. These homologies were found over each putative transmembrane domain and at their borders but not in the amino and carboxyl-terminal tails or in the long third cytoplasmic loop (all of which are quite variable in length in this receptor family). The percent of homology between the 5HT-dro receptor and the other members of this family was therefore calculated over the whole protein with the exception of these three regions (domains represented in Fig. 2). The highest scores of homology were for the human 5HT1A serotonin receptor (49%; ref. 7), a *Drosophila* octopamine receptor (44%; ref. 17), the human β_1-adrenergic receptor and the rat D_2 dopamine receptor (40%; refs. 18 and 19), the human α_2-adrenergic receptor and the hamster β_2-adrenergic receptor (38%; refs. 20 and 21), and the rat 5HT1C serotonin receptor (34%; ref. 5). From these scores it appears that the 5HT-dro receptor is closer to the human 5HT1A receptor than to any other member of that receptor family.

Predicted Structure of the 5HT-dro Receptor Reveals Eight Putative Transmembrane Domains and a Potential Glycosaminoglycan Attachment Site. In contrast with the predicted seven-transmembrane-domain structure of all known G-protein-coupled receptors, hydropathy analysis of the 5HT-dro receptor sequence revealed the existence of eight hydrophobic domains that are long enough to span the cytoplasmic membrane (boxes I*–VII in Fig. 1). Domains I–VII are homologous to the seven putative transmembrane domains of G-protein-coupled receptors (Fig. 2), whereas domain I*, which is located near the amino-terminal tail of the protein, could be either an additional transmembrane domain or an unusually long cleavable signal sequence.

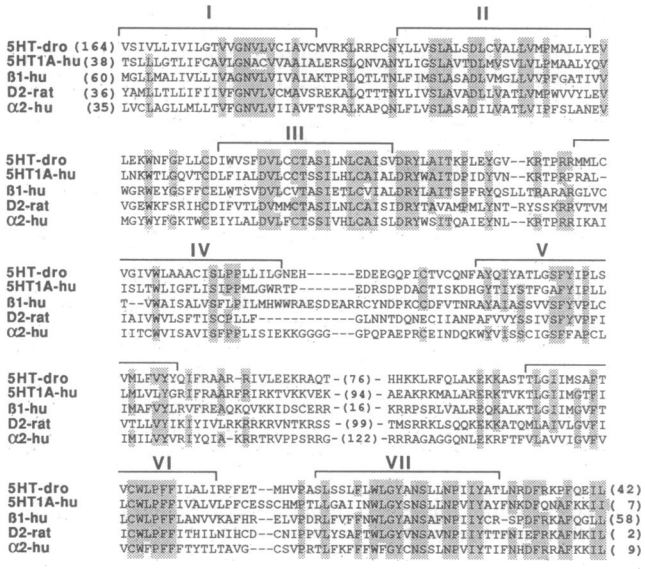

FIG. 2. Amino acid similarity between the 5HT-dro receptor and other G-protein-coupled receptors. 5HT1A-hu, β1-hu, D2-rat, and α2-hu correspond respectively to the human 5HT1A receptor, the human β_1-adrenergic receptor, the rat D_2 dopamine receptor, and the human α_2-adrenergic receptor. Putative transmembrane domains (I–VII) are indicated by brackets. Numbers in parentheses correspond to the number of amino acids that are not represented.

Sequence comparisons revealed a second unusual feature in the amino acid sequence of the first putative extracellular domain of the 5HT-dro receptor. This sequence exhibits homology to a rat yolk sac tumor chondroitin sulfate proteoglycan (22); to a *Drosophila* clock gene, period (*per*) (23, 24); and to a *Neurospora* clock gene, frequency (*frq*) (25). The region of homology is a Ser-Gly motif that is repeated 10 times in the 5HT-dro receptor and 24 times in the rat chondroitin sulfate proteoglycan. The homologous sequence in the *per* protein is a Thr-Gly repeat, and in the *frq* protein it is a region that contains both Ser-Gly and Thr-Gly dipeptides. Ser-Gly and Thr-Gly repeats are putative attachment sites for glycosaminoglycans such as chondroitin sulfate, keratan sulfate, or heparan sulfate (26, 27). The first extracellular domain of the 5HT-dro receptor might therefore be linked to glycosaminoglycans.

Serotonin Activates Adenylate Cyclase in NIH 3T3 Cells Expressing the 5HT-dro Receptor. To determine whether the 5HT-dro cDNA encoded a functional receptor, this cDNA was cloned into the expression vector pSG5, which contains a simian virus 40 promoter and β-globin splice and polyadenylylation sequences (16). The resulting recombinant, pEB, was then introduced into mouse NIH 3T3 cells together with a recombinant, pRSVneo, encoding resistance to G418, by calcium phosphate cotransfection. G418-resistant colonies were isolated, amplified, and analyzed for expression of mRNA encoding the 5HT-dro receptor. We selected two cell lines (NpEB2 and NpEB3) that expressed a high level of 5HT-dro mRNA, as well as a control cell line, Np, that had been transfected with the pSG5 expression vector alone.

The effects of various concentrations of neurotransmitters on the production of cAMP by NpEB2 and Np cells were analyzed as described in *Materials and Methods*. At 1 μM, epinephrine, dopamine, octopamine, or tyramine had no effect on cAMP levels in either NpEB2 cells or Np cells (Fig. 3*A*). In contrast, 1 μM serotonin provoked a 5-fold increase in cAMP in NpEB2 cells while it had no effect on control Np cells (Fig. 3*A*). This serotonin-induced increase in cAMP was concentration-dependent and saturable. The concentration of serotonin required for half-maximal stimulation (EC_{50}) was 60 nM (Fig. 3*B*). These results demonstrate that the 5HT-dro cDNA encodes a functional serotonin receptor. The effect of serotonin was not limited to the particular cell line NpEB2, as a similar increase in cAMP was obtained with an independent cell line (NpEB3) expressing a high level of receptor mRNA (data not shown). Serotonin had no effect on inositolphospholipid hydrolysis in NpEB2 cells (data not shown).

To compare the pharmacological characteristics of the 5HT-dro receptor with those of other known serotonin receptors, we studied the effects of various agonists and antagonists on serotonin-induced increases in cAMP in NpEB2 cells. Two serotonin analogs, 2-methyl-5-hydroxytryptamine and 5-methoxytryptamine, and a nonselective 5HT1 agonist, (+)-lysergic acid diethylamide (LSD), had weaker agonist activity than serotonin, the concentrations required for half-maximal stimulation being 0.6, 0.8, and 1.5 μM, respectively. In contrast, 8-hydroxy-1-(*N*,*N*-dipropyl) aminotetralin, a potent 5HT1A agonist, did not elicit an increase in cAMP, indicating that the 5HT-dro receptor has a different pharmacological profile than the 5HT1A receptor (for a review see ref. 9).

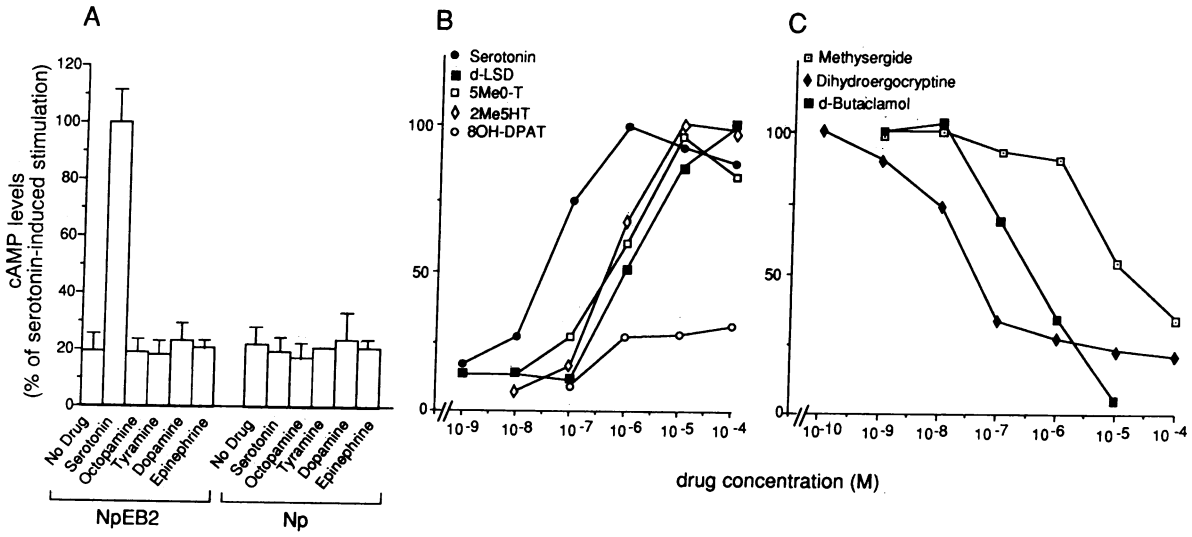

FIG. 3. Serotonin-induced increase in cAMP in cells expressing the 5HT-dro receptor: effects of agonists and antagonists. cAMP levels are expressed as a percentage of the value obtained with 1 μM serotonin (100%). cAMP levels presented are the means of at least four independent experiments performed in duplicate. Standard deviations in *B* and *C* were <20% of the presented values. (*A*) Effect of 1 μM serotonin, (±)-octopamine, tyramine, dopamine, or (+)-epinephrine on NIH 3T3 cells expressing the 5HT-dro receptor mRNA (NpEB2 cells) and mock-transfected NIH 3T3 cells (Np cells). (*B*) Effect of various concentrations of serotonin, (+)-lysergic acid diethylamide (d-LSD), 2-methyl-5-hydroxytryptamine (2Me5HT), 5-methoxytryptamine (5MeO-T), or 8-hydroxy-1-(*N*,*N*-dipropyl)aminotetralin (8OH-DPAT) on NIH 3T3 cells expressing the 5HT-dro receptor (NpEB2 cells). (*C*) Effect of antagonists on serotonin-induced increase in cAMP. NpEB2 cells were exposed to 1 μM serotonin together with various concentrations of methysergide, dihydroergocryptine, or (+)-butaclamol.

Antagonists were assayed at variable concentrations in the presence of a constant amount of serotonin, 1 μM, which corresponds to the lowest concentration of serotonin required to elicit a maximal stimulation of cAMP levels. Fig. 3*C* shows the effect of two ergot alkaloids, dihydroergocryptine and methysergide, and of the neuroleptic (+)-butaclamol, which are antagonists of certain serotonin receptors (9). The concentrations required to elicit half-maximal inhibition of serotonin activity were 0.02 μM for dihydroergocryptine, 6 μM for methysergide, and 0.3 μM for (+)-butaclamol.

We also investigated the binding of several radioligands to membranes of NIH 3T3 cells expressing the 5HT-dro receptor (NpEB2 cell line). No specific binding could be detected with [^{3}H]serotonin. In contrast, ^{125}I-labeled LSD displayed a high affinity for membranes of NpEB2 cells, whereas no specific binding to membranes of control NIH 3T3 cells was observed. The calculated equilibrium dissociation constant (K_d) of ^{125}I-LSD was 640 pM and its B_{max} was 28 fmol of receptor per mg of membrane protein (data not shown). A serotonin receptor with a similar K_d for ^{125}I-LSD was reported in the nervous system of the marine snail *Aplysia* (28).

Tissue Localization of the 5HT-dro Receptor. Northern hybridization analysis of poly(A)$^+$ RNA from adult *Drosophila* heads and bodies revealed a single mRNA species, 5.5 kb in length, detected only in heads (Fig. 4).

DISCUSSION

We have isolated a *Drosophila* cDNA encoding a functional serotonin receptor that, upon introduction into mouse NIH 3T3 cells, activates adenylate cyclase. This activation was dependent on the concentration of serotonin, saturable, and blocked by dihydroergocryptine, an antagonist of several mammalian serotonin receptors. Other known or putative *Drosophila* neurotransmitters, including dopamine, epinephrine, octopamine, and tyramine, did not elicit a response in transfected cells. Together these results demonstrate that the 5HT-dro receptor is a serotonin receptor that can activate adenylate cyclase in mammalian cells.

A serotonin-sensitive adenylate cyclase has been reported in head membranes of *Drosophila*. This serotonin-evoked stimulation of adenylate cyclase was most efficiently inhibited by dihydroergocryptine (13). The 5HT-dro receptor might therefore be responsible for this activity.

That the 5HT-dro receptor is able to activate adenylate cyclase in NIH 3T3 cells suggests that it is able to couple with a G protein expressed in these cells. This G protein is probably G_s, which has been shown to stimulate adenylate cyclase and which is expressed in many cell lines. As regards the *Drosophila* G protein, with which the 5HT-dro receptor presumably interacts *in vivo*, a good candidate is a recently cloned G protein that is homologous to G_s (29). The successful coupling we observed between a *Drosophila* receptor and a mammalian G protein suggests that second-messenger machineries are remarkably conserved throughout evolution. Nevertheless it is possible that the 5HT-dro receptor functions differently in flies than in mouse cells and activates different second-messenger pathways.

The 5HT-dro receptor contains a Gly-Ser repeat, which is a putative glycosaminoglycan attachment site (26). Consistent with the role of proteoglycans in adhesion processes (30), a possible function of glycosaminoglycans, in the case of the 5HT-dro receptor, would be to localize this protein in a specialized compartment of the cell, next to particular com-

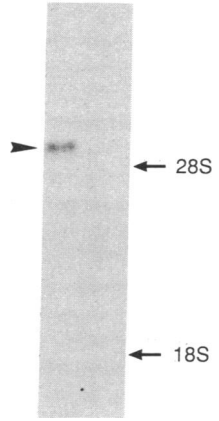

FIG. 4. Distribution of mRNA encoding the 5HT-dro receptor. Northern blot analysis of poly-(A)$^+$ mRNA (10 μg per lane) from adult *Drosophila* heads (lane 1) and bodies (lane 2). Arrows denote positions in the gel of mouse 28S and 18S ribosomal RNAs. The arrowhead corresponds to the 5.5-kb mRNA detected in heads. The probe used was the ^{32}P-labeled *Eco*RI genomic fragment. This experiment was performed twice with independent mRNA preparations.

ponents of the extracellular matrix. Such a localization is observed at the neuromuscular junction, where extracellular matrix proteins are involved in the clustering of the acetylcholine receptors (30).

In molluscs and in insects, serotonin has been suggested to play a role in learning processes and in biological rhythms. In particular, in the marine snail *Aplysia*, a serotonin-sensitive adenylate cyclase has been shown to be involved in a simple form of learning (31) and in the modulation of circadian rhythms (32, 33). The 5HT-dro receptor or closely related receptors might therefore regulate such physiological events. The identification of the 5HT-dro receptor gene, combined with the powerful genetic techniques available in *Drosophila*, should permit a genetic dissection of the physiological role of this receptor.

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