cDNA cloning of a serotonin 5-HT_{1C} receptor by electrophysiological assays of mRNA-injected Xenopus oocytes

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Hermann Lübbert*, Beth J. Hoffman†, Terry P. Snutch*, Terry Van Dyke*, Arnold J. Levine†, Paul R. Hartig*, Henry A. Lester*, and Norman Davidson*

*Church Chemical Laboratories, 164-30, and Division of Biology, California Institute of Technology, Pasadena, CA 91125; †Department of Biology and Health Sciences, The Johns Hopkins University, School of Hygiene and Public Health, Baltimore, MD 21205-2167; and ‡Department of Molecular Biology, Princeton University, Princeton, NJ 08544

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ABSTRACT We describe a strategy for the cloning of neurotransmitter-receptor and ion-channel cDNAs that is based on electrophysiological assays of mRNA-injected Xenopus oocytes. This procedure circumvents the purification of these membrane proteins, which is hindered by their low abundance and their hydrophobic nature. It involves methods for RNA fractionation by high-resolution gel electrophoresis, directional cDNA cloning in a single-stranded vector, and screening of the cDNA library by voltage-clamp measurements of currents induced by serotonin in mRNA-injected oocytes. The applicability of our approach is demonstrated by the isolation of a serotonin receptor cDNA clone from a mouse choroid plexus papilloma. The clone was identified by hybrid-depletion and hybrid-selection procedures. The receptor expressed in oocytes injected with hybrid-selected RNA is fully functional, indicating that it is composed of a single subunit encoded by a 5-kilobase RNA. The pharmacology of the hybrid-selected receptor confirms that we have successfully cloned a serotonin 5-HT_{1C} receptor cDNA.

The neurotransmitter serotonin displays diverse psychological and physiological effects in nervous tissues and in smooth muscle (1). Serotonin receptors are targets for many medications used in the treatment of psychiatric diseases and of disorders related to smooth muscle tonus. Pharmacological and electrophysiological studies indicate that there are at least five kinds of serotonin binding sites in the rat and mouse central nervous system. Following the original classification into 5-HT_{1} and 5-HT_{2} receptors (2), four subtypes of the 5-HT_{1} binding sites, denoted 1A, 1B, 1C, and 1D, have been characterized by pharmacological criteria in rat and mouse brain (3–7). In addition, there are at least three 5-HT_{3} (M) receptor types located in the peripheral nervous system (8).

Xenopus oocytes have been used to study neurotransmitter receptors and ion channels expressed on the oocyte surface after injection of mRNA isolated from electrically excitable tissues. Only one type of serotonin binding site, the 5-HT_{1C} receptor, induces measurable conductances in Xenopus oocytes injected with RNA isolated from rat or mouse brain (9). The receptor couples to an oocyte guanine nucleotide binding protein (G protein) and stimulates a Ca-activated Cl-channel, presumably via phosphatidylinositol hydrolysis (10, 11). A single 5- to 6-kilobase (kb) size class of poly(A)^+ RNA isolated from rat brain is sufficient to generate the serotonin response in the oocytes (9). The mRNA coding for the 5-HT_{1C} subtype is especially enriched in choroid plexus (9). The epithelial cell layer of this tissue possesses the highest known density of 5-HT_{1C} receptors (5, 6).

Earlier we proposed the use of the oocyte expression system for cloning ion channel or neurotransmitter receptor cDNAs in those cases for which the conventional approach through protein purification is not readily applicable (12). Here, we report techniques developed for the enrichment of RNAs coding for low-abundance receptors or ion channels, followed by the construction of libraries containing cDNA directionally inserted in single-stranded vectors. A method is presented for screening cDNA libraries by hybrid depletion using voltage-clamp measurements on oocytes. We have applied this approach to the serotonin 5-HT_{1C} receptor which has not yet been purified and for which antibodies or protein sequence data are not available. We used RNA isolated from choroid plexus tumors that develop spontaneously in transgenic mice (strain SV11) carrying copies of the simian virus 40 early region (13, 14). A cDNA library was constructed from tumor RNA enriched for the 5-HT_{1C} receptor RNA by size fractionation using gel electrophoresis. A screening procedure based on electrophysiological tests of mRNA-injected Xenopus oocytes was then used for the isolation of a cDNA clone for the 5-HT_{1C} receptor.

MATERIALS AND METHODS

mRNA Isolation and Size Fractionation. Choroid plexus tumors were dissected from SV11 mice (14), quick frozen in liquid nitrogen, and stored at –80°C. The frozen tumors were homogenized in 6 M guanidine hydrochloride/0.2 M NaOAc, pH 5.1/100 mM 2-mercaptoethanol, and RNA was isolated by a modification of the procedure of Chirgwin et al. (15). For the size fractionation, 20 μg of poly(A)^+ RNA was separated on a vertical 0.8% agarose gel (Bio-Rad, ultrapure) as described (16); however, some modifications were necessary to achieve good resolution of biologically active, high molecular weight RNA. The gel contained 5 mM CH_{3}HgO\textsubscript{O} (AESAR, Johnson & Matthey, Seabrook, NH) and was buffered with 0.1 M boric acid/5 mM Na_{2}B\textsubscript{4}O\textsubscript{7}/0.3 mM EDTA. The RNA was disaggregated in 80% recrystallized formamide for 2 min at 65°C, cooled on ice, and then one-third volume of a solution containing 50 mM CH\textsubscript{3}HgO\textsubscript{O}, 4X gel buffer, 40% (wt/vol) glycerol, and 0.05% bromophenol blue was added before loading (slot size: 0.4 cm × 4 cm). Preelectrophoresis (30 min) and electrophoresis were performed at 5–6 V/cm at 4°C. RNA was electroeluted from gel slices into NH\textsubscript{4}OAc/sucrose step gradients in a modification of the procedure described by Saha et al. (17). Then it was recovered from the high-salt boundary and precipitated with ethanol several times. Analysis of the fractionated RNA by electrophoresis through an agarose gel containing formalde-
hyde, blotting to nitrocellulose, and hybridization with a poly(dT) probe were performed as described (9).

**Xenopus Oocyte Injection and Electrophysiology.** Detailed protocols for the preparation and injection of the oocytes and the electrophysiology have been described (9).

**cDNA Library Construction.** For the cDNA synthesis, about 100 ng of highly enriched, size-selected RNA was heated to 65°C for 2 min, mixed with 0.85 μg of phosphorylated oligonucleotide primer (pGCCAGGTCGACTCTAGTTTITTTTTTT) and 8.5 units of reverse transcriptase (Raven 2, Amersham), and incubated for 1 hr at 42°C (10-μl reaction volume) using the buffer conditions described (18). The reaction was stopped by addition of 3 μl of 0.25 M EDTA, 4 μl of H₂O, and 3 μl of 1 M NaOH. After RNA hydrolysis for 30 min at 60°C, 1 μl of 1 M HCl was added, and the cDNA was size-selected and separated from unincorporated primer and free nucleotides by two passes over a 1-ml Sepharose CL-4B (Sigma) column equilibrated in 30 mM NaOH/2 mM EDTA to keep the single-stranded cDNA extended. The excluded volume of the second passage was concentrated by extraction with 2-butanol and the cDNA was precipitated with ethanol, washed twice with 75% ethanol, and dried lightly. For the subsequent G-tailing (18), the cDNA was incubated for 1 hr at 37°C in 0.2 M sodium cacodylate, pH 6.9/1 mM CoCl₂/0.9 mM dGTP/500 units of terminal transferase (Pharmacia) per ml. The reaction was stopped by adjusting the mixture to 25 mM EDTA and heating for 10 min at 70°C.

The vector pUC119 (19) was digested with Kpn I, C-tailed in 0.2 M sodium cacodylate, pH 6.9/1 mM CoCl₂/0.9 mM dCTP, 1000 units of terminal transferase per ml for 90 sec at 15°C, and digested with Pst I. The small fragment was removed by gel filtration on a 5-ml Bio-Rad A-50m column. The excluded volume from this column was extracted with phenol/CHCl₃ and precipitated with ethanol twice.

The G-tailed cDNA was twice precipitated together with 1 μg of this vector as carrier and dissolved in 50 μl of annealing buffer (0.01 M Tris-HCl, pH 8/1 mM EDTA/0.15 M NaCl) containing 0.5 μg of a phosphorylated oligonucleotide (pTCTAGAGTCGACTCTAGTCGCA) complementary to the first-strand primer. The mixture was heated to 70°C for 5 min and then incubated sequentially for 20 min at 58°C, 15 min at 48°C, and 30 min at 37°C. Afterwards, the DNA was ligated in 50 μl with 1500 units of T4 DNA ligase (New England Biolabs) under standard conditions for 15 hr at 15°C. The mixture was then twice extracted with phenol/CHCl₃, concentrated by extraction with 2-butanol and precipitated with ethanol. The gap-filling reaction was performed for 5 hr at 15°C and for 1 hr at 22°C in a 50-μl volume with 24 units of DNA polymerase I (Amersham) and 10 units of Escherichia coli DNA ligase (New England Biolabs) as described (18). The DNA was diluted 1:20 with H₂O and transformed into E. coli R4 (20) by a modification of the Hanahan procedure (21).

**Preparation of Single-Stranded DNA.** A logarithmically growing culture of bacteria carrying the pUC119 plasmid (OD₅₀₀ < 0.1) was infected with 2-10 plaque-forming units per ml M13K07 (19). About 1 hr after infection, the culture was adjusted to contain 70 μg of kanamycin per ml. The phage was harvested 6-12 hr later, and single-stranded DNA was isolated as described for phage M13 (22).

**Hybrid Depletion.** To achieve optimal hybridization kinetics, the single-stranded DNA was linearized as follows. A 22-base oligonucleotide complementary to the EcoRI cleavage site in the polylinker was annealed to the single-stranded DNA, which was then digested with EcoRI. The linear single-stranded DNA was hybridized at 65°C with 20 μg of RNA in 50 μl of reaction mixture containing 1 M NaCl, 50 mM sodium phosphate (pH 6.5), 2 mM EDTA, and 15 μg of poly(dA) (Sigma). Pools of 20 clones were tested at a total DNA concentration of 1 mg/ml. After hybridization, the nucleic acid components were separated by centrifugation on a density gradient (128 g of CsCl and 24 g of guanidine hydrochloride added to 100 ml of 20 mM NaOAc, pH 5.2/1 mM EDTA) in a VTI 65.2 rotor at 55,000 rpm and 16°C for 2 hr. The RNA was purified by three precipitations with ethanol and dissolved in 10 μl of H₂O for oocyte injections.

**Hybrid Selection.** Nitrocellulose filters (Schleicher & Schuell, 9 mm) were loaded with 30 μg of linearized single-stranded DNA as described (23). Prehybridizations were performed for 12 hr at 45°C in 200 μl containing 70% formamide, 0.4 M NaCl, 50 mM sodium phosphate (pH 6.5), 1 mM EDTA, 25 μg of poly(dA), 25 μg of poly(rC) (Sigma), and 0.4 mg of yeast RNA (Sigma, type III) per ml. For hybridizations, 100 μl of the same buffer was used, but the yeast RNA was replaced by 30 μg of total RNA isolated from choroid plexus tumors, and the incubation was continued at 45°C for 4.5 hr. Unhybridized RNA was then precipitated twice with ethanol. The filters were incubated for an additional 15 min at 45°C in the same buffer containing poly(dA) and poly(rC) but no mRNA. Afterwards, they were rinsed quickly and washed three times for 3 min at 55°C in 0.15 M NaCl/0.0015 M sodium citrate, pH 7/0.1% NaDodSO₄. Elution of hybridized RNA was performed as described (23). For the injections, unhybridized RNA was dissolved in 20 μl of H₂O, and eluted RNA, in 5 μl.

**RESULTS**

**RNA Fractionation.** The typical two-component C1-currents induced by serotonin in Xenopus oocytes injected with RNA isolated from mouse brain and choroid plexus tumors are shown in Fig. 1 A and B, respectively. Tumors of the epithelial cell layer in choroid plexi from SV11 mice (14) have a 5-HT₁C binding site density of 6.6 pmol/mg of microsomal protein (24). This is about twice as high as that in rat choroid plexus and several-fold higher than in larger animals, which could provide greater amounts of tissue (6). Based on the site density, we estimate that even in the choroid plexus tumors there is only 1 serotonin binding site per 60,000 protein molecules, an abundance that may be reflected on the RNA level. Therefore, the receptor RNA should be highly enriched.

![FIG. 1. Serotonin-evoked C1-currents in Xenopus oocytes.](image-url)
prior to construction of a library in order to reduce the number of clones that have to be tested.

Twenty micrograms of tumor poly(A)* RNA was enriched for the serotonin receptor RNA by high-resolution gel fractionation, which allows more accurate sizing and better enrichment of biologically active RNA than does velocity sedimentation. A single RNA fraction of 4.8–5.2 kb was sufficient for generating the serotonin response in injected oocytes (Fig. 2), as found previously with RNA isolated from rat brain (9). We recovered about 100 ng of RNA in the active fraction.

Selection Procedure. The specific requirements for the identification of a positive clone would determine the strategy for constructing an appropriate cDNA library. Hybrid depletion can be used even if a receptor or channel is composed of several subunits, while hybrid selection can only be used for single-subunit proteins. Since in our hands membrane filter hybridizations are slower and less efficient, we wished to hybridize RNA to its cDNA in solution and then to separate RNA from DNA and from DNA:RNA hybrids. This separation was best accomplished by using a CsCl gradient containing guanidine hydrochloride to reduce the density of nucleic acids (25). The gradient was adjusted so that RNA banded close to the bottom of the tube while DNA floated at the top. Banding of RNA caused significantly less degradation than did pelleting (our unpublished results). Under appropriate conditions, an RNA should hybridize completely to its cDNA clone and band in the middle of the gradient as a DNA:RNA hybrid, leaving the RNA band depleted of this specific RNA. If a cDNA clone coding for a channel or receptor were present in the hybridization pool, then injection of the depleted RNA from the gradient should result in a reduced electrical response in the oocytes. To test this idea, a 750-bp cDNA of the voltage-gated Na-channel cloned in phage M13 (23) was hybridized for 30 min with RNA at a DNA concentration of 50 μg/ml, and the nucleic acid components were separated in the density gradient. In the oocytes, the voltage-clamp currents generated by injection of the hybrid-depleted RNA were about 10% of those induced by the control RNA hybridized to M13 vector DNA only (data not shown).

Generation of a cDNA Library. We wanted to construct a cDNA library in a way that would facilitate the selection procedure described above. The vector pUC119 allows the formation of single-stranded DNA after infection with the M13K07 helper phage (19). The directional cDNA cloning strategy (Fig. 3) is designed such that the single-stranded plasmids contain cDNA inserts in the antisense orientation. These inserts can be hybridized to their complementary RNAs without complications from DNA-DNA reassociation. This method permits a high cloning efficiency and a high frequency of inserts because excess vector can be used with minute amounts of cDNA (26). The cDNA can be size-selected before directional insertion into the vector. In addition, the length of cDNA inserts is not compromised by the presence of any particular restriction site within the insert. Using this approach, we obtained about 35,000 individual colonies from 100 ng of highly size-selected 5-kb RNA.

Identification of a 5-HT1C Receptor cDNA Clone. To reduce the number of clones to be considered, the library was prescreened by a subtractive hybridization with 32P-labeled kidney cDNA. This tissue was chosen for the functional and structural similarity of renal tubule epithelium with choroid plexus epithelium. Injection of kidney poly(A)* RNA did not trigger a serotonin response in oocytes. Clones that hybridized with the kidney cDNA (about 25% of the library) were excluded from further screening. To minimize differences in the representation of individual clones, single-stranded DNAs were prepared from groups of 10 clones and 2 groups were pooled for testing. They were then hybridized to total choroid plexus tumor RNA, and those testing positive were rescreened and eventually subdivided until an individual positive clone was identified. From 1200 colonies tested, we have isolated 1 clone that tested positive in several independent hybrid-depletion experiments. In the experiment shown in Table 1, experiment A, RNA bands were recovered from density gradients after hybridization with individual clones. For clone D9, the serotonin response in oocytes injected with this hybrid-depleted RNA was <20% of that seen with the other clones. When the RNA-DNA hybrids recovered from the middle of the same gradient were injected, the serotonin response was at least 10-fold greater for clone D9 than for clones E1, E3, or E4 (Table 1, experiment B). Addition of excess poly(rC) to the hybridization mix had no influence on this result, indicating that it is not caused by nonspecific binding of the dG homopolymer present in the single-stranded DNA (data not shown). For the experiment shown in Table 1, total RNA from choroid plexus tumors was used because of the relatively high abundance of 5-HT1C receptor RNA in this tissue and the resulting large serotonin-induced conductances in the oocytes. However, we knew of no other electrophysiological response triggered by choroid plexus RNA to serve as an internal control (9). Therefore, we performed a similar experiment with poly(A)* RNA isolated from the cortices and midbrains of 14-day-old C57BL mice (Table 2, experiment A). RNA that was hybrid-depleted with clone D9 generated greatly decreased serotonin responses in the oocytes, while the kainate response and the voltage-activated sodium current were unaffected. Again, addition of poly(rC) to the hybridization mix had no influence on this result (data not shown).

In addition to the hybrid selection shown in Table 1, experiment B, we used the standard procedure for hybrid selection with DNA bound to nitrocellulose filters. The rather stringent hybridization and washing conditions were chosen to decrease nonspecific binding and thus to avoid a back-
ground response in the oocytes. The experiment shows that the RNA coding for the serotonin receptor hybridized selectively with clone D9 (Table 2, experiment B, hybridized). For the other clones tested, the RNA remained in the hybridization solution (not hybridized). Taking the results of these hybrid-depletion and hybrid-selection experiments together, we conclude that we have identified a cDNA clone that hybridizes with serotonin receptor mRNA.

**Characterization of the Clone.** The serotonin-induced conductances in Xenopus oocytes injected with rat brain RNA display 5-HT<sub>1C</sub> pharmacology (9). In addition, 5-HT<sub>1C</sub> is the only identified serotonin receptor type in choroid plexus tumors (24). To confirm the subtype of the hybrid-selected receptor, we performed a simple pharmacological characterization. The response waveforms in oocytes injected with RNA that was hybrid-selected with clone D9 resemble those of oocytes injected with RNA isolated from mouse brain or choroid plexus tumor RNA (experiment B).

**Results.**

### Table 1. Identification of a serotonin receptor clone

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Voltage-clamp currents in oocytes after injection of RNA tested with individual clones, nA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E1</td>
</tr>
<tr>
<td>A</td>
<td>581 ± 63</td>
</tr>
<tr>
<td>B</td>
<td>22.5 ± 18</td>
</tr>
</tbody>
</table>

Single-stranded DNA (500 µg/ml) from several individual clones was hybridized with 20 µg of tumor RNA. RNAs (experiment A) and DNA-RNA hybrids (experiment B) were separated on density gradients and injected into oocytes. Responses to 20 nM (experiment A) or 50 nM (experiment B) serotonin were recorded. Values are means ± SEM from several independent experiments.

### Table 2. Hybrid depletion of mouse brain (experiment A) and hybrid selection of tumor RNA (experiment B)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>E1</th>
<th>D9</th>
<th>D10</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Na current</td>
<td>567 ± 95</td>
<td>575 ± 86</td>
</tr>
<tr>
<td></td>
<td>Kainate (0.1 mM)</td>
<td>300 ± 45</td>
<td>283 ± 29</td>
</tr>
<tr>
<td></td>
<td>Serotonin (20 nM)</td>
<td>1014 ± 103</td>
<td>80 ± 24</td>
</tr>
<tr>
<td>B</td>
<td>Hybridized</td>
<td>0</td>
<td>495 ± 78</td>
</tr>
<tr>
<td></td>
<td>Not hybridized</td>
<td>1750 ± 238</td>
<td>469 ± 66</td>
</tr>
</tbody>
</table>

In experiment A, 25 µg of mouse cortex and midbrain poly(A)<sup>+</sup> RNA was hybridized with single-stranded cDNA (250 µg/ml) from individual clones for 75 min, followed by density gradient separation. Hybrid-depleted RNAs were injected into oocytes, and voltage-clamp currents were measured. In experiment B, choroid plexus tumor RNA was hybrid-selected with DNA from cDNA clones. RNAs eluted from the filters (hybridized) and RNAs remaining in the hybridization solution (not hybridized) were injected into oocytes, and the responses to 1 µM (hybridized) or 50 nM serotonin (not hybridized) were recorded. Values in both experiments A and B are the means ± SEM of two independent experiments with several oocytes each.

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**Fig. 3.** Directional cDNA cloning strategy. For details, see Materials and Methods and Results.
from choroid plexus tumors (Fig. 1). A response to 0.1 μM serotonin was greatly reduced by 0.25 μM mianserin and only slightly affected by 5 μM spiperone (data not shown), corresponding to apparent Kᵢ values of <80 nM for mianserin and >1700 nM for spiperone (27). Together with the nanomolar affinity for serotonin, this indicates that, indeed, we have cloned a cDNA for the 5-HT₁C receptor type (9). The clone contains a 1.9-kb cDNA and, as determined by RNA blot analysis, hybridizes to a 5-kb RNA species in RNAs isolated from choroid plexus tumors or mouse brain (data not shown).

**DISCUSSION**

We have developed a strategy for cloning neurotransmitter-receptor and ion-channel cDNAs even though they are expressed at low abundances. This approach is designed for those cases in which generally used tools for molecular cloning such as antibodies or oligonucleotide probes based on protein sequence data are not available. It involves high-resolution RNA fractionation, directional cDNA library construction, and library screening using the oocyte electrophysiological assay. In the design of these methods, our emphasis has been on the maintenance of the biological activity of very large RNAs. Clone isolation by hybrid arrest, using the density gradient separation procedure, is effective even if several subunits are necessary for receptor or ion-channel function. This method uses the advantages of solution hybridization and circumvents the uncertainties involved in procedures such as antisense RNA or DNA injections that do not appear to block the translation consistently for every RNA (our unpublished results).

We have applied our approach to the cloning of a serotonin 5-HT₁C receptor cDNA. With the isolated clone, serotonin receptor mRNA can be specifically removed from the RNA band in a density gradient and relocated to the position of DNA-RNA hybrids. Furthermore, the cloned cDNA bound to nitrocellulose hybridizes specifically with the receptor mRNA. The identity of the receptor type was confirmed by a pharmacological characterization of the receptor expressed in oocytes injected with hybrid-selected RNA. This RNA encoded a fully functional receptor, which indicates that only one receptor subunit is necessary for function, similar to the muscarinic M1 and the β-adrenergic receptors. These receptors are also coupled to G proteins (28, 29). The clone hybridizes with only one RNA band on a RNA blot and gives a simple pattern on a Southern blot consistent with a single gene (data not shown), indicating that indeed we have cloned a portion of the gene coding for the serotonin 5-HT₁C receptor.

Although the existence of several serotonin receptor types in the brain and in peripheral tissues is well documented (2–8), no 5-HT receptor type has been purified yet. Our partial-length cDNA insert should enable us to obtain a full-length clone for the 5-HT₁C receptor and possibly to isolate clones for other 5-HT receptor types by cross-hybridization. The isolation and analyses of 5-HT receptor clones should contribute to understanding the structure and function as well as the observed diversity of serotonin receptors.

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