Molecular Characterization of a Functional cDNA Encoding the Serotonin 1c Receptor

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Neurons that release serotonin as a neurotransmitter project to most regions of the central and peripheral nervous system and mediate diverse neural functions. The physiological effects of serotonin are initiated by the activation of multiple, distinct receptor subtypes. Cloning in RNA expression vectors was combined with a sensitive electrophysiological assay in *Xenopus* oocytes in order to isolate a functional cDNA clone encoding the 5HTlc serotonin receptor. Injection of RNA transcribed in vitro from this clone into *Xenopus* oocytes elicits serotonin sensitivity. Mouse fibroblasts transformed with this clone bind serotonin agonists and antagonists and exhibit an increase in intracellular Ca^{2+} concentrations in response to serotonin. The sequence of the 5HTlc receptor reveals that it belongs to the family of G protein–coupled receptors, which are thought to traverse the cytoplasmic membrane seven times. Moreover, in situ hybridization and RNA blot analysis indicate that the 5HTlc receptor is expressed in neurons in many regions of the central nervous system and suggest that this subclass of receptor may mediate many of the central actions of serotonin.

SEROTONIN, 5-HYDROXYTRYPTAMINE (5-HT), IS A BIOGENIC amine that functions as a neurotransmitter (*1*), a hormone (*2*), and a mitogen (*3*). Serotonin-containing neurons project to most regions of the mammalian central nervous system and mediate diverse neural functions. In the spinal cord, for example, serotonin is involved in the inhibitory control of sensory input and in the facilitation of motor output (*4*). In the cortex, transmission at serotonergic synapses contributes to affective and perceptual states, and these synapses represent a major site of action of psychotropic drugs (*5*).

Serotonin exerts its physiological effects by binding to cell surface receptors. Several serotonin receptor subtypes (including 5HT 1a, 1b, 1c, 5HT2, and 5HT3) have been defined on the basis of their pharmacological properties (*6*). Individual receptor subtypes reveal characteristic ligand-binding profiles and couple to different intracellular second messenger signaling systems. The 5HT1a and 5HT1b receptors regulate adenylate cyclase activity (*7*), whereas the 5HT1c and 5HT2 receptors regulate the production of phospholipase C–generated second messengers (*8*). The activation of these second messenger pathways by serotonin modulates the excitable properties of both central and peripheral neurons (*9*). However, the structural basis for the distinct ligand-binding properties of the multiple serotonin receptors is not known.

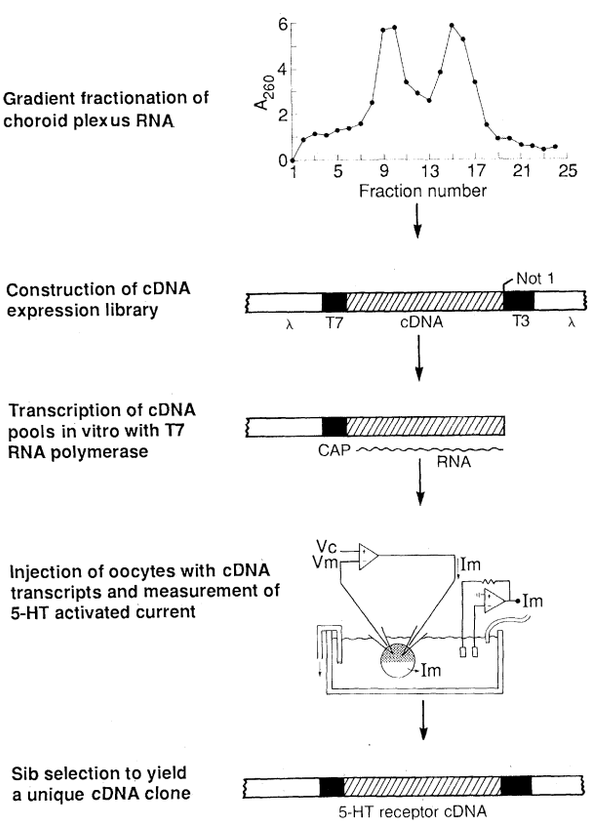

Fig. 1. Cloning strategy for isolation of a functional 5HT1c cDNA clone. Choroid plexus total RNA was fractionated by sucrose gradient sedimentation for enrichment for serotonin receptor mRNA. Fraction 1 corresponds to the top of the gradient (smaller RNA's), and fraction 25 to the bottom (larger RNA's). Positive fractions 19 and 20 (stippled bar) were identified by voltage-clamp recording of injected oocytes. RNA from these fractions was used to construct a cDNA library in the bacteriophage expression vector λZAP in which cDNA inserts (hatched rectangle) are flanked by promoters for T3 and T7 RNA polymerases (black boxes). DNA derived from pools of clones was digested with the restriction endonuclease Not I, cleaving the DNA at the position shown. These truncated DNA templates were transcribed in vitro with T7 RNA polymerase in the presence of the cap precursor GpppG to produce functional RNA copies of the cDNA inserts. *Xenopus* oocytes were injected with this RNA, cultured for 3 days, and assayed for sensitivity to serotonin by voltage-clamp recording of 5HT-activated currents. A pool of cDNA clones giving a positive response was identified and progressively subdivided into smaller pools (sib selection) until a single positive clone was obtained.

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The expression of functional receptors in *Xenopus* oocytes has provided a sensitive assay for detection of mRNA encoding serotonin receptors, in particular the 5HT1c receptor, that couple via inositol phospholipid signaling systems (*10*). Lubbert *et al.* have isolated a cDNA clone encoding the carboxyl-terminal portion of the 5HT1c receptor by hybrid depletion of choroid plexus mRNA coupled with oocyte expression (*11*). We have combined cloning in RNA expression vectors with an electrophysiological assay in oocytes to isolate and characterize the expression of a functional cDNA clone encoding the entire 5HT1c receptor.

Experimental strategy. The cloning of most neurotransmitter receptors has required the purification of receptor, the determination of partial protein sequence, and the synthesis of oligonucleotide probes with which to obtain cDNA or genomic clones. However, the serotonin receptors have not been purified, and antibodies to the receptors have not been generated. We have therefore designed a cDNA expression system that permits identification of functional cDNA clones encoding serotonin receptors in the absence of protein sequence information. A similar approach has been used to isolate a cDNA encoding the bovine neuropeptide substance K receptor (*12*).

Our cloning strategy (Fig. 1) is based on quantitative considerations of the following findings. High levels of serotonin 5HT1c receptors (10^5 per cell) are expressed in the choroid plexus (*13*), a nonneuronal cell type in the central nervous system associated with the production of cerebrospinal fluid. *Xenopus* oocytes injected with choroid plexus mRNA exhibit a serotonin-evoked inward current. Application of serotonin appears to liberate inositol phosphates that raise intracellular Ca^{2+}, leading to the opening of Ca^{2+}-dependent chloride channels (*10*). Patch clamp recording has demonstrated that the conductance of a single serotonin-activated chloride channel is about 3 pS (*14*). Thus, the opening of 10^6 chloride channels will result in the generation of a readily detectable current of 100 nA. If we assume that the translational efficiency of the oocyte approximates that of a plexus cell, then injection of RNA from ten plexus cells, or as little as 2 pg of poly(A)$^+$ RNA, should lead to the expression of 10^6 serotonin receptors on the oocyte surface. Parenthetically, the amplification of signaling usually associated with G protein–coupled receptors (*15*) suggests that considerably fewer than 10^6 receptors need be occupied to open 10^6 chloride channels. To determine experimentally the sensitivity of the oocyte expression assay, we injected oocytes with serial dilutions of poly(A)$^+$ RNA isolated (*16*) from the rat choroid plexus. Injection of as little as 5 pg of poly(A)$^+$ RNA (or 5 fg of pure receptor mRNA, if we assume 0.1 percent abundance) was sufficient to generate an inward current of about 100 nA (Fig. 2). Since it is possible to inject up to 50 ng of RNA into an oocyte, it should be possible to detect a cDNA clone encoding the 5HT1c receptor, even if present at an exceedingly low frequency in a cDNA expression library.

A cDNA library was therefore constructed from choroid plexus mRNA in λZAP, a vector permitting the in vitro transcription of cDNA inserts. RNA transcribed from populations of cDNA clones were screened for their ability to induce serotonin-evoked currents in *Xenopus* oocytes. A single clone encoding a functional serotonin 5HT1c receptor was purified from a large population of clones by procedures of sib selection (Fig. 1).

Expression of serotonin receptor in oocytes. A cDNA expression library was constructed in the vector λZAP from a size-fractionated mRNA population. Total RNA isolated from the choroid plexus was fractionated by sucrose density sedimentation (*17*), and individual fractions were assayed for serotonin receptor mRNA by voltage clamp analysis of oocytes (*18*). Consistent with reported data (*19*), injection of RNA in the size range 5 to 7 kb resulted in a serotonin-evoked inward current in oocytes (Fig. 1). RNA in this fraction was used to construct a cDNA library in the cloning vector λZAP (*17*). This vector contains bacteriophage T7 and T3 promoters flanking the cDNA insertion site that permit the in vitro synthesis of RNA transcripts in either direction. A size-enriched library consisting of 1.2×10^6 independent clones was generated. Five pools of 10^5 independent clones were amplified, DNA was purified and transcribed with T7 polymerase (*20*), and RNA copies of the pooled cDNA's were microinjected into oocytes.

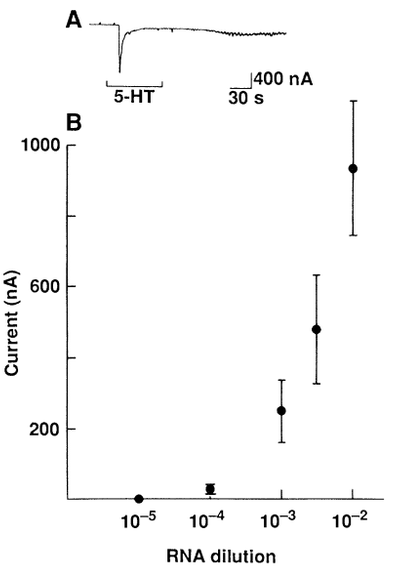

Fig. 2. Expression of functional serotonin receptors in *Xenopus* oocytes after injection of choroid plexus poly(A)$^+$ RNA. (**A**) Voltage clamp recording from a *Xenopus* oocyte injected with 0.5 ng of poly(A)$^+$ RNA (10^{-2} dilution) isolated from rat choroid plexus. The oocyte membrane potential was maintained at −50 mV, and serotonin ($10^{-6}M$) was applied by superfusion for the duration indicated. (**B**) Current responses evoked by application of serotonin ($10^{-6}M$) in oocytes injected with progressive dilutions of rat choroid plexus poly(A)$^+$ RNA. The RNA stock was at an initial concentration of 1 ng/nl. Each oocyte was injected with 50 nl of diluted RNA, incubated for 3 days, and voltage-clamped at −50 mV (*18*). Plots indicate mean and standard error of serotonin-evoked currents obtained from two to six oocytes.

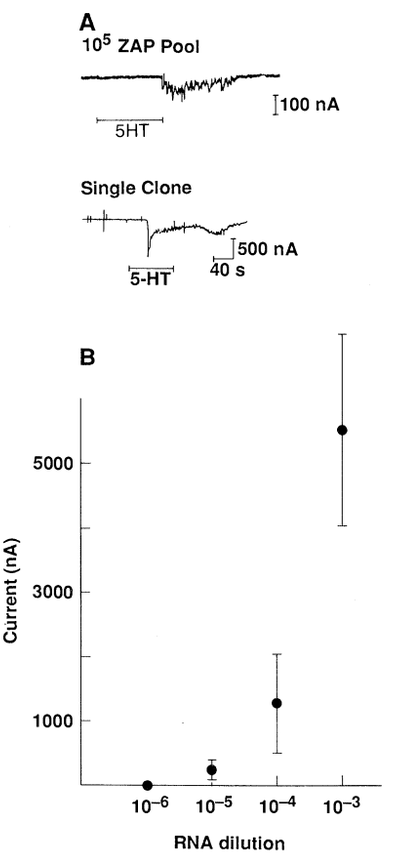

Fig. 3. Serotonin sensitivity of *Xenopus* oocytes injected with RNA transcribed in vitro from λZAP cDNA clones. (**A**) Inward current evoked by serotonin ($10^{-6}M$) in oocytes injected with RNA transcribed from a pool of 10^5 λZAP cDNA clones (approximately 100 ng) (top trace) and from a single λZAP cDNA clone (pSR-1c) at 10^{-4} dilution (5 pg) (bottom trace). Both oocytes were held at −50 mV. (**B**) Serotonin-evoked currents after serial dilution of RNA transcribed in vitro from the λZAP cDNA clone pSR-1c. The undiluted RNA was at a concentration of 1 ng/nl. Each oocyte was injected with 50 nl of diluted RNA. Cells were incubated for 3 days and voltage-clamped at −50 mV. Each point represents the mean and standard error of serotonin-evoked currents obtained from three to seven oocytes.

RNA transcribed from one of the five pools resulted in the generation of a 20- to 50-nA inward current (Fig. 3A). This pool of 10^5 clones was further subdivided into pools of 20,000, 2,000, 200, 20, and finally individual clones. At each stage, at least one pool of RNA generated serotonin-responsive currents. The magnitude of the serotonin-evoked current normalized per microgram of injected RNA increased as the sib selection proceeded.

Following this protocol, we isolated a single phage that was shown to encode an RNA that elicited serotonin sensitivity when injected into oocytes (Fig. 3A). The cDNA insert from this λZAP phage was subcloned with the flanking T7 and T3 promoters by rescue excision to generate the plasmid pSR-1c. Serial dilutions of RNA transcribed in vitro from pSR-1c demonstrated that injection of 0.5 pg of RNA was sufficient to generate a serotonin-evoked current of about 200 nA (Fig. 3B). Pharmacological analysis of injected oocytes revealed that this cDNA encodes a serotonin receptor with properties similar to those of the 5HT1c subtype (*21*). Superfusion of oocytes with 1 μ*M* mianserin blocked the current evoked by 10 n*M* 5HT in injected oocytes, whereas 1 μ*M* spiperone produced only a small decrease in the serotonin-evoked current. The relative potency of these antagonists is therefore similar when assayed against 5HT1c receptors present in the choroid plexus or in oocytes injected with pSR-1c–derived RNA.

The nucleotide sequence and deduced protein sequence of serotonin receptor 1C. The nucleotide sequence and deduced protein sequence of the 5HT1c receptor are shown in Fig. 4. One long open reading frame was identified within this sequence which extends from an ATG at nucleotide position 688 to a termination codon at residue 2068. pSR-1c encodes a protein 460 amino acids in length with a relative molecular size of 51,899 kD. Stop codons are

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-687 GGCGCTCTGGTGCTCACTGAGGAAGCTTCCTTAGGTGTACCGATCTTAATGATTGAGCC
-628 CTTGGAGCAGCAAGATTGTTAATCTTGGTTGCTCCTTTGGCCTGTCTATCCCTTACCTT
-569 CCTATTACATATGAACTTTTCTTCGTTCTGCACATCGATTGTCGTCGGCGTCGTGGAGA
-510 TCGTCGTGGTGCTCCGGTGGTGGTCTTCGTCCGCTTAGAATAGTGTAGTTAGTTAGGGG
-451 CCTTCAAAGAAGAAAGAAGAAGCGATTGGCGCGGAGAGATGCTGGAGGTGTCAGTTTCT
-392 ATGCTAGAGTAGGGTAGTGAAACAATCCCCAGCCAAACCTTTCCGGGGGGGCGCAGGTTG
-333 CCCACAGGAGGTCGACTTGCCGGCGCTGTCCTTCGCGCCGAGCTCCCTCCATCCTTCTT
-274 TCCGTCTGCTGAGACGCAAGGTTGCGGCGCGCACGCTGAGCAGCGCACTGACTGCCGCG
-214 GGCTCCGCTGGGCGATTGCAGCCGAGTCCGTTTCTCGTCTAGCTGCCGCCGCGGCGACC
-155 TGCCTGGTCTTCCTCCCGGACGCTAGCGGGTTGTCAACTATTACCTGCAAGCATAGGCC
 -96 AACGAACACCTTCTTTCCAAATTAATTGGAATGAAACAATTCTGTTAACTTCCTAATTC
 -37 TCAGTTTGAAACTCTGGTTGCTTAAGCCTGAAGCAATC

   1 ATG GTG AAC CTT GGC AAC GCG GTG CGC TCG CTC CTG ATG CAC CTA
     Met Val Asn Leu Gly Asn Ala Val Arg Ser Leu Leu Met His Leu

  46 ATC GGC CTA TTG GTT TGG CAA TTC GAT ATT TCC ATA AGT CCA GTA
     Ile Gly Leu Leu Val Trp Gln Phe Asp Ile Ser Ile Ser Pro Val

  91 GCA GCT ATA GTA ACT GAC ACT TTT AAT TCC TCC GAT GGT GGA CGC
     Ala Ala Ile Val Thr Asp Thr Phe Asn Ser Ser Asp Gly Gly Arg

 136 TTG TTT CAA TTC CCG GAC GGG GTA CAA AAC TGG CCA GCA CTT TCA
     Leu Phe Gln Phe Pro Asp Gly Val Gln Asn Trp Pro Ala Leu Ser

 181 ATC GTC GTG ATT ATA ATC ATG ACA ATA GGG GGC AAC ATT CTT GTT
     Ile Val Val Ile Ile Ile Met Thr Ile Gly Gly Asn Ile Leu Val

 226 ATC ATG GCA GTA AGC ATG GAG AAG AAA CTG CAC AAT GCA ACC AAT
     Ile Met Ala Val Ser Met Glu Lys Lys Leu His Asn Ala Thr Asn

 271 TAC TTC TTA ATG TCC CTA GCC ATT GCT GAT ATG CTG GTG GGA CTA
     Tyr Phe Leu Met Ser Leu Ala Ile Ala Asp Met Leu Val Gly Leu

 316 CTT GTC ATG CCC CTG TCC CTG CTT GCT ATT CTT TAT GAT TAT GTC
     Leu Val Met Pro Leu Ser Leu Leu Ala Ile Leu Tyr Asp Tyr Val

 361 TGG CCT TTA CCT AGA TAT TTG TGC CCC GTC TGG ATT TCA CTA GAT
     Trp Pro Leu Pro Arg Tyr Leu Cys Pro Val Trp Ile Ser Leu Asp

 406 GTG CTA TTT TCA ACT GCG TCC ATC ATG CAC CTC TGC GCC ATA TCG
     Val Leu Phe Ser Thr Ala Ser Ile Met His Leu Cys Ala Ile Ser

 451 CTG GAC CGG TAT GTA GCA ATA CGT AAT CCT ATT GAG CAT AGC CGG
     Leu Asp Arg Tyr Val Ala Ile Arg Asn Pro Ile Glu His Ser Arg

 496 TTC AAT TCG CGG ACT AAG GCC ATC ATG AAG ATT GCC ATC GTT TGG
     Phe Asn Ser Arg Thr Lys Ala Ile Met Lys Ile Ala Ile Val Trp

 541 GCA ATA TCA ATA GGA GTT TCA GTT CCT ATC CCT GTG ATT GGA CTG
     Ala Ile Ser Ile Gly Val Ser Val Pro Ile Pro Val Ile Gly Leu

 586 AGG GAC GAA AGC AAA GTG TTC GTG AAT AAC ACC ACG TGC GTG CTC
     Arg Asp Glu Ser Lys Val Phe Val Asn Asn Thr Thr Cys Val Leu

 631 AAT GAC CCC AAC TTC GTT CTC ATC GGG TCC TTC GTG GCA TTC TTC
     Asn Asp Pro Asn Phe Val Leu Ile Gly Ser Phe Val Ala Phe Phe

 676 ATC CCG TTG ACG ATT ATG GTG ATC ACC TAC TTC TTA ACG ATC TAC
     Ile Pro Leu Thr Ile Met Val Ile Thr Tyr Phe Leu Thr Ile Tyr

 721 GTC CTG CGC CGT CAA ACT CTG ATG TTA CTT CGA GGT CAC ACC GAG
     Val Leu Arg Arg Gln Thr Leu Met Leu Leu Arg Gly His Thr Glu

 766 GAG GAA CTG GCT AAT ATG AGC CTG AAC TTT CTG AAC TGC TGC TGC
     Glu Glu Leu Ala Asn Met Ser Leu Asn Phe Leu Asn Cys Cys Cys

 811 AAG AAG AAT GGT GGT GAG GAA GAG AAC GCT CCG AAC CCT AAT CCA
     Lys Lys Asn Gly Gly Glu Glu Glu Asn Ala Pro Asn Pro Asn Pro

 856 GAT CAG AAA CCA CGT CGA AAG AAG AAA GAA AAG CGT CCC AGA GGC
     Asp Gln Lys Pro Arg Arg Lys Lys Lys Glu Lys Arg Pro Arg Gly

 901 ACC ATG CAA GCT ATC AAC AAC GAA AAG AAA GCT TCC AAA GTC CTT
     Thr Met Gln Ala Ile Asn Asn Glu Lys Lys Ala Ser Lys Val Leu

 946 GGC ATT GTA TTC TTT GTG TTT CTG ATC ATG TGG TGC CCG TTT TTC
     Gly Ile Val Phe Phe Val Phe Leu Ile Met Trp Cys Pro Phe Phe

 991 ATC ACC AAT ATC CTG TCG GTT CTT TGT GGG AAG GCC TGT AAC CAA
     Ile Thr Asn Ile Leu Ser Val Leu Cys Gly Lys Ala Cys Asn Gln

1036 AAG CTA ATG GAG AAG CTT CTC AAT GTG TTT GTG TGG ATT GGC TAT
     Lys Leu Met Glu Lys Leu Leu Asn Val Phe Val Trp Ile Gly Tyr

1081 GTG TGT TCA GGC ATC AAT CCT CTG GTG TAC ACT CTC TTT AAT AAA
     Val Cys Ser Gly Ile Asn Pro Leu Val Tyr Thr Leu Phe Asn Lys

1126 ATT TAC CGA AGG GCT TTC TCT AAA TAT TTG CGC TGC GAT TAT AAG
     Ile Tyr Arg Arg Ala Phe Ser Lys Tyr Leu Arg Cys Asp Tyr Lys

1171 CCA GAC AAA AAG CCT CCT GTT CGA CAG ATT CCT AGG GTT GCT GCC
     Pro Asp Lys Lys Pro Pro Val Arg Gln Ile Pro Arg Val Ala Ala

1216 ACT GCT TTG TCT GGG AGG GAG CTC AAT GTT AAC ATT TAT CGG CAT
     Thr Ala Leu Ser Gly Arg Glu Leu Asn Val Asn Ile Tyr Arg His

1261 ACC AAT GAA CGT GTG GCT AGG AAA GCT AAT GAC CCT GAG CCT GGC
     Thr Asn Glu Arg Val Ala Arg Lys Ala Asn Asp Pro Glu Pro Gly

1306 ATA GAG ATG CAG GTG GAG AAC TTA GAG CTG CCA GTC AAC CCC TCT
     Ile Glu Met Gln Val Glu Asn Leu Glu Leu Pro Val Asn Pro Ser

1351 AAT GTG GTC AGC GAG AGG ATT AGT AGT GTG TAA
     Asn Val Val Ser Glu Arg Ile Ser Ser Val ---

1384 GCGAAGAGCAGCGCAGACTTCCTACAGGAAAGTTCCTGTAGGAAAGTCCTCCCCACCCC
1443 CCGTGATTTTCCTGTGAATCATAACTAATGTAAATATTGCTGTGTGACAAGACAGTGTT
1502 TTTATAAATAGCTTTGCAACCCTGTACTTTACATCATGCGTTAATAGTGAGATTCGGG
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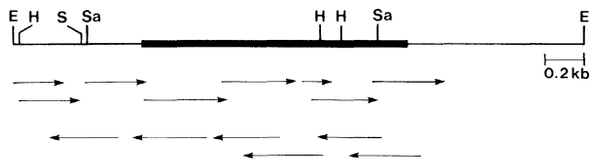

Fig. 4. Nucleotide sequence and predicted amino acid sequence of the rat 5HT1c receptor. The sequencing protocol is shown diagramatically. Sequences were determined for individual restriction fragments after subcloning into M13 vectors or with synthetic oligonucleotides as internal primers. The coding region is indicated by the heavy bar. Restriction sites are shown as indicated (E, Eco RI; H, Hind III; S, Sal I; and Sa, Sac I). Numbers in the left-hand margin indicate nucleotide position. DNA sequence was determined by the chain termination method of Sanger *et al.* (*41*).

found in all three frames at frequent intervals upstream of the ATG, and downstream of the termination site. Interestingly, the coding sequence is preceded by a long 5′ untranslated region with minimum length of 700 bp. The functional 5HT1c receptor cDNA we have cloned is only 3 kb, whereas the mRNA containing this sequence is more than 5 kb. It is likely therefore that the cDNA clone does not extend to the 3′ terminus of the mRNA, but terminates artificially in the 3′-untranslated region.

The 5HT1c receptor shares numerous sequence and structural properties with the family of receptor molecules that has been predicted to span the lipid bilayer seven times (Fig. 5). This family includes rhodopsin and related opsins (*22*), the α- and β-adrenergic receptors (*23*), the muscarinic cholinergic receptors (*24*), the substance K neuropeptide receptor (*12*), the yeast mating factor receptors (*25*), and the oncogene c-*mas* (*26*). Each of these receptors is thought to transduce extracellular signals by interaction with guanine nucleotide-binding (G) proteins (*23, 27*). On the basis of structural homologies with rhodopsin, it is likely that the amino terminus of the 5HTlc receptor is located on the extracellular side of the plasma membrane, with the carboxyl terminus located intracellularly. In this scheme, three extracellular loops alternate with three intracellular loops to link the seven transmembrane domains.

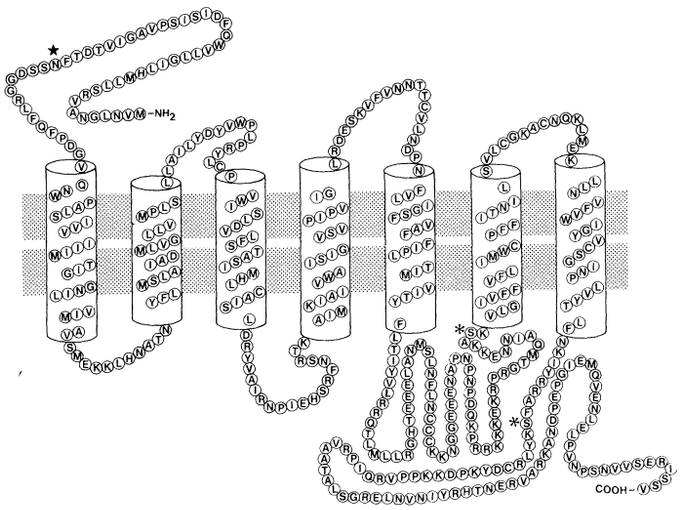

Fig. 5. A model for the transmembrane topology of the rat 5HT1c receptor. The receptor is shown as containing seven hydrophobic transmembrane regions. By analogy with the structure of rhodopsin, the amino terminus is located on the extracellular face of the lipid bilayer and the carboxyl terminus is located on the cytoplasmic side. Asterisks indicate serine residues that represent potential phosphorylation sites. The star indicates an asparagine residue in the amino terminal region that is a potential *N*-glycosylation site.

Although the amino-terminal 65 amino acids of the 5HT1c receptor are thought to reside on the extracellular face of the membrane, the protein lacks a definable signal sequence. A potential *N*-linked glycosylation site is present at position 39. The receptor contains seven hydrophobic stretches (20 to 30 amino acids) that represent potential transmembrane domains. These domains constitute the regions of maximal sequence similarity with the other transmitter receptors of this class. The 5HT1c receptor shares 25 percent sequence identity with the β_2-adrenergic receptor, and 20 percent identity with the muscarinic and substance K receptors (Fig. 6).

A conserved aspartate residue is present within the second putative transmembrane domain and, by analogy with the β_2-adrenergic receptor, this residue may participate in ligand binding. Mutations in this conserved aspartate in the β_2-adrenergic receptor reduce agonist binding (*28*). In addition, amino acid residues in this domain can be cross-linked to affinity ligands of the β_2-adrenergic receptor (*27*). These data, taken together with the observation that all ligands capable of associating with this family of receptors have amino groups, suggest that aspartate participates in stabilizing the ligand in the plane of the membrane.

The third cytoplasmic loop, thought to interact with the different G proteins (*29*), is of widely varying length in different receptors. No sequence similarities within this domain are apparent among receptors that couple to common signaling systems. In the 5HT1c receptor, this loop consists of 77 amino acids, many of which are basic, a feature characteristic of other receptors in this family.

Both the carboxyl-terminal cytoplasmic domain and the third cytoplasmic loop contain sequences that may serve as substrates for

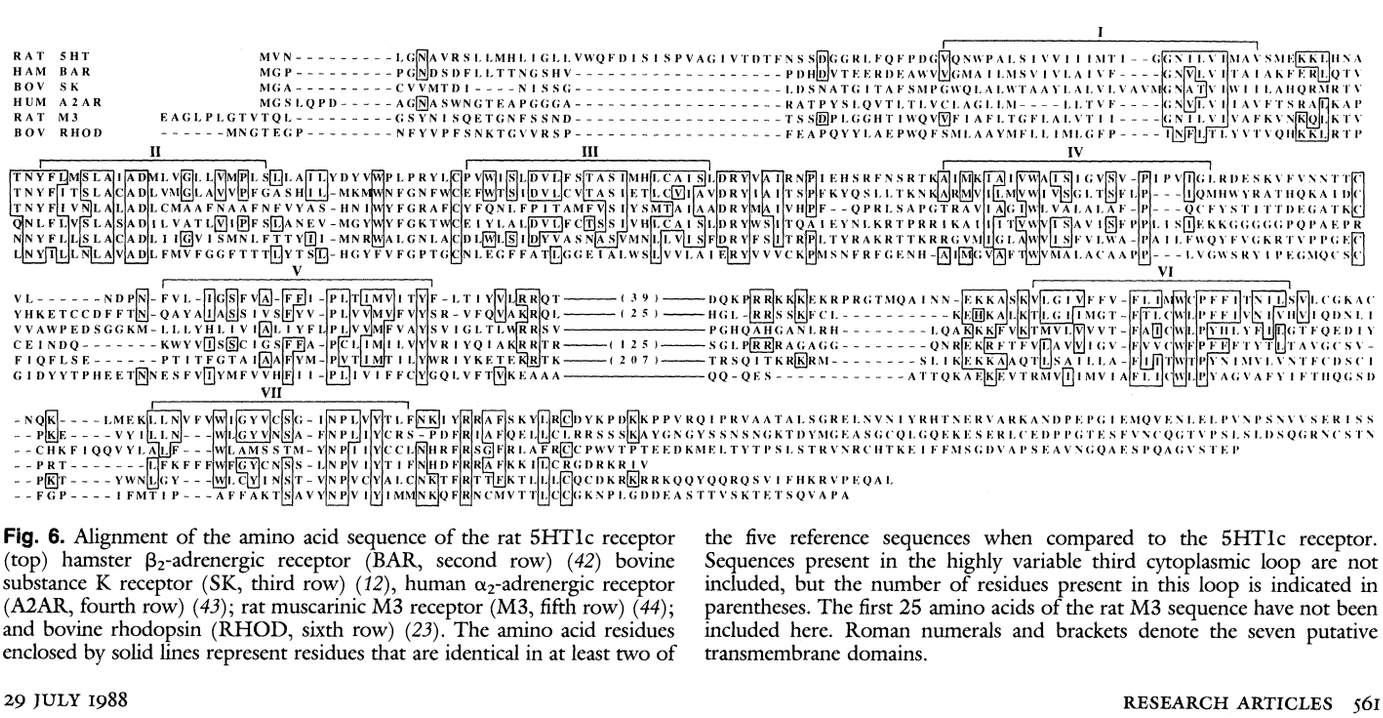

Fig. 6. Alignment of the amino acid sequence of the rat 5HT1c receptor (top) hamster β_2-adrenergic receptor (BAR, second row) (*42*) bovine substance K receptor (SK, third row) (*12*), human α_2-adrenergic receptor (A2AR, fourth row) (*43*); rat muscarinic M3 receptor (M3, fifth row) (*44*); and bovine rhodopsin (RHOD, sixth row) (*23*). The amino acid residues enclosed by solid lines represent residues that are identical in at least two of the five reference sequences when compared to the 5HT1c receptor. Sequences present in the highly variable third cytoplasmic loop are not included, but the number of residues present in this loop is indicated in parentheses. The first 25 amino acids of the rat M3 sequence have not been included here. Roman numerals and brackets denote the seven putative transmembrane domains.

phosphorylation by cyclic AMP–dependent protein kinase or protein kinase C. In addition, there are four serine residues in the carboxyl-terminal 12 amino acids which, by analogy with rhodopsin and the adrenergic receptors, may represent additional phosphorylation sites. These potential sites of phosphorylation may play a role in regulating the activity of the receptor molecule (*30*).

5HT1c receptor expression in mammalian fibroblasts. The deduced protein sequence of pSR-1c indicates that we have cloned a new member of the gene family encoding G protein–coupled neurotransmitter receptors. To establish further that this clone encodes a serotonin receptor, we have demonstrated that the introduction of this cDNA into mammalian fibroblasts renders these cells responsive to serotonin. The 3-kb Eco RI cDNA insert from pSR-1c was cloned into the expression vector pMV7 (*31*). This vector contains a murine leukemia virus long terminal repeat which serves as a promoter for expression of the serotonin cDNA as well as an independent expression cassette encoding neomycin phosphotransferase. Transformed NIH 3T3 cells resistant to neomycin were isolated and a single clone expressing significant levels of receptor mRNA (SR 3T3) was identified by RNA blot analysis.

Membranes prepared from SR 3T3 cells exhibited high affinity binding sites for ligands that interact with the 5HT1c receptor on choroid plexus cells (*32*). The SR 3T3 cells expressed 10^3 to 10^4 high affinity binding sites per cell for ^{125}I-labeled lysergic acid diethylamide (^{125}I-LSD), whereas no specific high affinity sites were detected on the parental NIH 3T3 cell line. Moreover, the relative ability of specific antagonists to inhibit ^{125}I-LSD binding to SR 3T3 cells paralleled their potency on choroid plexus cells (Fig. 7) (*21*). Mianserin (IC_{50} = 20 n*M*) was about two orders of magnitude more effective in displacing ^{125}I-LSD binding than was spiperone (IC_{50} = 2 μ*M*). Binding of ^{125}I-LSD was also displaced by serotonin with an IC_{50} value of 20 n*M*. The expression of specific high affinity binding sites for 5HT1c-selective ligands on transformed 3T3 cells provides independent confirmation that pSR-1c encodes a serotonin 1c receptor.

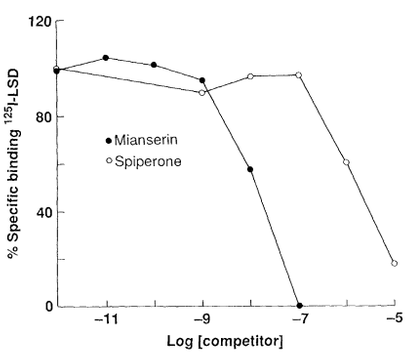

Fig. 7. Pharmacological properties of the 5HT1c receptor expressed in transfected mouse fibroblasts as determined by binding of ^{125}I-LSD. Membranes were prepared from a fibroblast cell line (SR 3T3) transformed with an expression vector (pMV7) containing the pSR-1c cDNA insert. These membranes were incubated with ^{125}I-LSD (1 n*M* final concentration) either alone or in the presence of the competitor drugs mianserin or spiperone at the concentrations shown. Nonspecific binding of ^{125}I-LSD was determined with an equivalent amount of membranes prepared from untransformed NIH 3T3 cells. This binding was not displaceable by 10^{-7}*M* mianserin and it represented 45 percent of the total ligand bound.

To ascertain whether the binding of serotonin activates intracellular signaling pathways in transformed fibroblasts, SR 3T3 cells were loaded with the Ca^{2+}-sensitive dye indo-1 and analyzed in a fluorescence-activated cell sorter (*33*). Indo-1 undergoes a characteristic and quantitative shift in its fluorescence emission spectrum as a function of Ca^{2+} concentration and serves as a quantitative measure of intracellular free Ca^{2+} (*34*). The SR 3T3 cells and the control NIH 3T3 cells loaded with indo-1 were exposed to serotonin immediately before fluorescence analysis to reduce the possibility of desensitization that may result from prolonged exposure of cells to agonist. Ninety-five percent of the SR 3T3 cells loaded with indo-1 showed a marked increase in intracellular Ca^{2+} when exposed to serotonin, whereas control, untransfected NIH 3T3 cells did not respond to serotonin (Fig. 8). These experiments indicate that the introduction of pSR-1c cDNA in mammalian fibroblasts leads to the expression of functional serotonin receptors. Thus, the 5HT1c receptor is capable of triggering the same transduction machinery regardless of the cell type in which it is expressed.

Expression of 5HT1c serotonin receptor in the nervous system. RNA blot analysis and in situ hybridization (*35*) were performed to examine the expression of 5HT1c receptor mRNA in different brain regions and in peripheral tissues. In situ hybridization revealed a heavy grain density associated with epithelial cells of the choroid plexus in the third, fourth, and lateral ventricles. Strikingly, hybridization was not restricted to cells of the choroid plexus but appeared in numerous neuronal cell groups throughout the central nervous system. In the micrograph shown, labeled neurons are observed in the lateral habenula, whereas neurons in medial habenula were not labeled (Fig. 9). Higher magnification of this region revealed the presence of silver grains in neuronal perikarya. The 5HT1c mRNA was also observed in neurons in cortical structures and in a variety of subcortical brain regions (*36*).

Although it is possible that the in situ analysis detects mRNA species other than that of the 5HT1c receptor, the distribution of 5HT1c receptor mRNA determined by in situ hybridization is supported by RNA blot analysis (Fig. 10). An intense 5.2-kb RNA was observed in choroid plexus, and in other regions of the brain, including the basal ganglia, hypothalamus, hippocampus, pons medulla, and spinal cord. Longer exposure reveals the presence of a small amount of receptor mRNA in the olfactory bulb. Receptor RNA was not detected in the cerebellum or in liver, kidney, intestine, heart, and lung. Titration studies with purified pSR-1c RNA indicate that the 5HT1c receptor RNA comprises about 0.02 percent of the choroid plexus message population. The relative abundance of this RNA in other regions of the brain is at least ten times lower. In situ hybridization, however, indicated that some neurons express receptor mRNA levels comparable to those in choroid plexus cells. These findings demonstrate that the 5HT1c receptor is not restricted to epithelial cells of the choroid plexus, but is expressed in numerous discrete neuronal cell groups in many

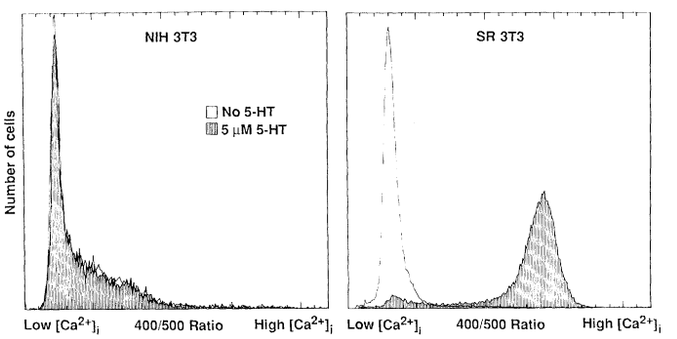

Fig. 8. Activation of 5HT1c receptors in transformed fibroblasts elevates intracellular Ca^{2+} concentration. Untransformed cells (NIH 3T3) or transformed cells expressing pSR-1c cDNA (SR 3T3) were loaded with the Ca^{2+}-sensitive dye indo-1. Changes in the level of intracellular free-Ca^{2+} following exposure to 5 μ*M* serotonin (hatched peak) were monitored with a flow cytometer to measure the ratio of emissions at 400 and 500 nm. In each case, the resting intracellular Ca^{2+} concentration in the absence of serotonin is also shown (open peak). For NIH 3T3 cells, the two peaks are coincident. For SR 3T3 cells, 95 percent of the population showed an elevation in intracellular Ca^{2+} in the presence of serotonin. The mean resting Ca^{2+} concentration in NIH 3T3 and SR 3T3 cells was similar.

regions of the rat brain and spinal cord. This distribution of 5HT1c receptors suggests that this receptor subtype may mediate many of the central actions of serotonin.

Diversity of receptor subtypes. We have cloned and characterized a functional cDNA encoding the 5HT1c subclass of serotonin receptor. RNA transcribed from this cDNA clone (pSR-1c) confers serotonin sensitivity to *Xenopus* oocytes. The expression of pSR-1c in mouse fibroblasts results in the appearance of high affinity serotonin-binding sites on the cell surface. Exposure of these transformants to serotonin increases intracellular Ca^{2+} levels. Moreover, abundant expression of 5HT1c receptor mRNA is observed in subsets of neurons throughout the brain, suggesting that the 5HT1c receptor plays an important role in central neurotransmission.

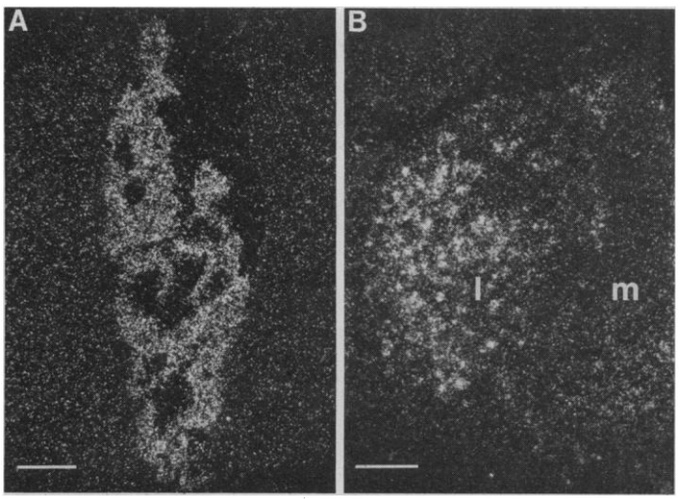

Fig. 9. Localization of 5HT1c receptor mRNA in rat brain by in situ hybridization. ^{35}S-Labeled antisense RNA probes were prepared by in vitro transcription of pSR1c cDNA, and hybridized with cryostat sections (15 μm) of adult rat brain tissue fixed with 4 percent paraformaldehyde (*36*). (**A**) Intense hybridization to epithelial cells of the choroid plexus located in the lateral ventricle. Adjacent cortical neural tissue exhibits low or background hybridization signals. (**B**) RNA hybridization to neuronal cell bodies located in the lateral habenula nucleus (*1*), but not to neurons in the medial habenula (m) or in adjacent ventral regions of the midbrain. Sense RNA probes revealed no specific hybridization. Scale bars = 100 μm.

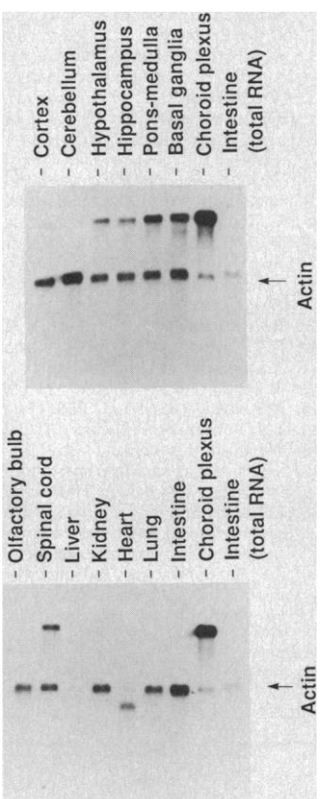

Fig. 10. RNA blot analysis of the tissue distribution of 5HT1c receptor mRNA. Poly(A)$^+$ RNA (2 to 20 μg) prepared from different brain regions and peripheral tissues (*16*) was subjected to electrophoresis through an 0.8 percent agarose-formaldehyde gel, blotted onto GeneScreen (New England Nuclear), and hybridized with a ^{32}P-labeled probe prepared from the 3-kb Eco RI cDNA insert from pSR-1c. Tissue regions are indicated above each lane. Filters were again hybridized with a ^{32}P-labeled human α-actin probe to assess relative amounts of RNA in each lane. The 5HT1c mRNA was judged to be approximately 5.2 kb, with 18*S* and 28*S* ribosomal RNA as standards.

The procedure used to isolate the functional 5HT1c receptor cDNA has combined cloning in RNA expression vectors with a sensitive electrophysiological assay for serotonin receptor function in *Xenopus* oocytes. Similar procedures have been used to isolate the cDNA encoding the receptor for the neuropeptide substance K (*12*), and the Na^+-glucose cotransporter (*37*). In our experiments, the injection of as little as 5 pg of choroid plexus poly(A)$^+$ RNA (1 fg of receptor mRNA) into oocytes was adequate to generate serotonin-evoked membrane currents. The sensitivity of the oocyte expression assay presumably results from the signal amplification associated with the coupling of these receptors to second messenger systems. In the oocyte, this assay is at present limited to receptors that activate second messenger systems, in particular phospholipase C, which elevate intracellular calcium levels. This cloning strategy may therefore be generally applicable to the isolation of genes encoding neurotransmitter and growth factor receptors that initiate a similar signal amplification, even if their mRNA's are present in exceedingly small amounts in the total RNA population.

Pharmacological studies, and more recently gene cloning have established that multiple receptor subtypes exist for most, if not all, neurotransmitters. The existence of multiple receptor subtypes provides one mechanism by which a single neurotransmitter can elicit distinct cellular responses. The variation in cellular response can be achieved by the association of individual receptor subtypes with different G proteins and different signaling systems. Further flexibility is provided by the ability of distinct receptors for the same ligand to activate or inhibit the same second messenger system. For example, among the adrenergic receptors, β_1 and β_2 receptors activate adenylate cyclase, α_2 receptors inhibit adenylate cyclase, and α_1 receptors activate phospholipase C pathways (*28*).

A similar array of cellular responses can be elicited by serotonin in cells bearing different receptor subtypes. Both 5HT1c and 5HT2 receptors stimulate the phospholipase C–mediated production of inositol phosphates, whereas 5HT1a and 5HT1b receptors may regulate adenylate cyclase activity (*7*) or couple to G proteins that directly activate ion channels (*9*). The diverse neural actions of serotonin are thought to be mediated by activation of these distinct receptor subtypes. For example, the hallucinatory and perceptual disturbances associated with administration of LSD and other psychedelic serotonin analogs (*38*) are probably elicited by activation of cortical 5HT2 receptors (*39*). In contrast, the inflammatory and pain-producing effects of serotonin are mediated via 5HT3 receptors on peripheral sensory endings (*40*). The ability to express 5HT receptors in new cellular environments devoid of other receptor subtypes should permit the characterization of transduction systems associated with these specific receptors. We anticipate that the cloning of additional receptor subtypes will help to elucidate the mechanisms of action of serotonin in the nervous system.

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17. Total RNA (250 μg), extracted from choroid plexus tissue obtained from 12 adult rats, was fractionated on a 5 to 25 percent (w/v) sucrose gradient [O. Mayuhas and R. P. Perry, *Cell* **16**, 139 (1979)]. Positive fractions from the sucrose gradient were combined, and the RNA was concentrated by ethanol precipitation. The first strand cDNA was synthesized with oligo(dT)$_{12-18}$ as a primer [J. P. Kraus *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2047 (1986)]. The second strand was synthesized according to H. Okayama and P. Berg [*Mol. Cell Biol.* **2**, 161 (1982)]. The double-stranded cDNA was methylated with Eco RI methylase, and the ends were flushed with T4 DNA polymerase. Phosphorylated Eco RI linkers (Pharmacia) were ligated to the cDNA, and excess linkers were cleaved off with Eco RI endonuclease [T. V. Huynh, R. A. Young, R. W. Davis, in *DNA Cloning*, I. D. M. Glover, Ed. (RL Press, Oxford, 1985), vol. 1, p. 49]. The cDNA was separated from free linkers by chromatography on Ultragel AcA 34 (LKB, Inc.) (C. J. Watson and J. F. Jackson, in *ibid.*, p 79). Complementary DNA's were inserted into the Eco RI site of λZAP (Stratagene, San Diego, CA). Recombinant bacteriophage were propagated with the bacterial strain BB4 (Stratagene). Bluescript plasmids were rescued from ZAP clones by M13 excision (Stratagene).
18. Oocytes were surgically removed from frogs and immediately dissected into groups of 10 to 20 cells. Individual oocytes were obtained by enzymatic dissociation in collagenase [2 mg/ml in Barths medium containing 2 percent Ficoll (w/v), for 2 hours at room temperature with constant agitation], and oocytes were stored at 18°C in Barths medium containing 2 percent Ficoll, penicillin, and streptomycin. Cells were typically injected with 50 to 100 nl of sample. For voltage-clamp recordings, oocytes were placed in a continuous-flow chamber perfused with frog Ringer solution. All drugs were introduced through this system by switching the perfusion line inlet. The oocytes were voltage-clamped at −50 mV with the use of a two-electrode voltage clamp (Axoclamp 2A) having a virtual ground and remote switchable headstage. Membrane currents were recorded through the virtual ground and filtered at 10 Hz. Responses were either digitized at 5 Hz and stored on a PDP 11/73 microcomputer or recorded on a Gould chart recorder. Electrodes were 2 to 5 megohm resistance and were filled with 3*M* KCl.
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20. The cDNA library was amplified in pools of 20,000 clones. Bacteriophage were eluted from each plate (150 mm) and kept as separate stocks. The majority of each phage stock was used to prepare DNA by a standard protocol [T. Maniatis, E. F. Fritch, J. Sambrook, *Molecular Cloning* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982)]. When, in the course of sib selection, the cDNA pool size was reduced to fewer than 2000 clones, phage stocks were reamplified in order to obtain sufficient amounts of DNA for transcription. For the initial screen, DNA derived from five pools of 20,000 clones was combined so as to represent 10^5 clones per sample. Prior to transcription, approximately 20 μg of each DNA sample was digested to completion with the restriction endonuclease Not I, thereby cleaving each recombinant molecule downstream from the T7 promoter and after the cDNA insert. This was followed by digestion with proteinase K (100 μg/ml, 37°C, 30 min) and subsequent phenol-chloroform-isoamyl alcohol extractions. DNA was recovered by ethanol precipitation and dissolved in 5 μl of TE. Transcription reactions were carried out in 50- or 100-μl volumes and contained 20 μg of DNA template, 40 m*M* tris-HCl (*p*H 7.9), 7 m*M* $MgCl_2$, 10 m*M* DTT, 2 m*M* spermidine, 10 m*M* NaCl, BSA at 25 μg/ml, human placental RNAse inhibitor at 2000 unit/ml, 0.5 m*M* ATP, CTP, and UTP, 0.2 m*M* GTP, 1 m*M* GpppG, and 70 units of T7 RNA polymerase. Samples were incubated at 40°C for 1 to 2 hours. Reaction mixtures were extracted with phenol-chloroform, nucleic acid was recovered by ethanol precipitation, and the pellet was dissolved in a small volume of water, usually 5 μl, for microinjection into oocytes. Typically, between 3 and 10 μg of RNA was synthesized in these reactions, making the ratio of RNA to cDNA roughly 5.
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