Cloning cDNA Coding for a Putative Calcitonin Gene-Related Peptide Receptor Submitted

Joel Cummings

Carroll College, Helena, MT

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Cloning cDNA Coding for a Putative Calcitonin Gene-Related Peptide Receptor

Submitted in partial fulfillment of the requirements for graduation with honors to the Department of Biology and Chemistry at Carroll College, Helena, Montana

Joel Cummings

April 7, 1995
Signature Page

This thesis for honors recognition has been approved for the
Department of Biology and Chemistry by:

Dr. John Addis, Director

Dr. John Christenson, Reader

Joan Stottlemyer, Reader

April 7, 1995
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ABSTRACT

Calcitonin gene-related peptide (CGRP) has been shown to interact specifically with at least two types of receptors. Cloning of either receptor would be a major breakthrough since this knowledge could be applied to the development of drugs to augment or antagonize CGRP's actions in the body.

With this goal in mind, Fisher and Chatterjee used an approach applying cDNA library screening combined with polymerase chain reaction (PCR) to obtain a partial cDNA sequence corresponding to a gene that could code for the CGRP receptor. A new project to find the entire sequence involved screening a human liver cDNA library using a putative CGRP receptor gene-specific probe. PCR was used as a means of verifying the presence of the correct cDNA. The cDNA was prepared for amplification and subsequent sequencing by in vivo excision.

The DNA sequence that was finally obtained apparently was not the same sequence as the putative CGRP receptor DNA and contained no homology to any known gene.
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INTRODUCTION

Calcitonin gene-related peptide (CGRP) is a 37-amino-acid peptide that is the result of tissue-specific alternative RNA splicing of the calcitonin gene (1). Although much is known about CGRP and its interaction with its receptor, the CGRP receptor has yet to be cloned and sequenced. Based on the assumption that the CGRP receptor is a member of the Group III family of G protein-coupled receptors, Fisher and Chatterjee (personal communication) at the University of Iowa College of Medicine began a project to search for cDNA homologous to the DNA which encodes for the calcitonin receptor. Using an approach based on polymerase chain reaction (PCR), Chatterjee was able to obtain an approximately 600 base-pair sequence of cDNA. This cDNA coded for a region of a peptide showing 60% homology in amino acid sequence to the calcitonin receptor. He reasoned that this cDNA was a likely candidate for a sequence contained in the CGRP receptor gene.

The present study continuing the work of Chatterjee is a new project to isolate the full-length putative CGRP receptor cDNA. Putative CGRP receptor cDNA was found by an approach using bacteriophage cDNA library screening. The synthetic DNA probe for this screening procedure was based upon the partial sequence obtained by Chatterjee. PCR using putative CGRP receptor-specific primers was used as a means of confirming that the proper DNA sequence was contained in plaques picked from this library.
LITERATURE REVIEW

Calcitonin gene-related peptide (CGRP) has become a research topic of great interest because of CGRP’s involvement in many biological processes. For example, immunocytochemical studies have shown that CGRP has a widespread distribution in the central nervous system. Consequently, CGRP is thought to serve as a neurotransmitter or neuromodulator (3). Some of CGRP’s actions in the central nervous system include control of body temperature (4), control of motor activity (4), and a role in psychobehavioral function (5). CGRP in the peripheral nervous system has been shown to inhibit gastric acid secretion in the stomach via vagal pathways (6), cause vasodilation of splanchnic blood vessels of the digestive tract through its release from primary afferent neurons (7), and participate in the action of nonadrenergic, noncholinergic nerves present in the digestive system (8). In the cardiovascular system, CGRP has been shown to be a potent vasodilator with positive chronotropic and inotropic effects on atrial tissues (CGRP increases the rate and force of contraction) (9). These effects on the cardiovascular system seem to be due to the circulation of CGRP in the plasma (10).

Although CGRP has widespread functions, CGRP is a recently discovered molecule. The production of CGRP mRNA was first noted during spontaneous and permanent switching of serially transplanted rat medullary thyroid carcinoma lines from "high" to "low" calcitonin production (11). Researchers later noted that multiple mRNA’s are generated from the calcitonin gene as a result of tissue-specific
alternative splicing of RNA produced by the calcitonin gene (12). The calcitonin gene consists of six exons. Splicing of the first four exons generates calcitonin mRNA. The first three exons are spliced to the fifth and sixth exons generating mRNA encoding for a 15,000-dalton primary translation product which is proteolytically cleaved to form the mature 37-amino-acid neuropeptide known as \(\alpha\)-CGRP, a C-terminal pentapeptide, and an 81-amino-acid N-terminal peptide (12). Calcitonin expression is seen primarily in the C cells of the thyroid while \(\alpha\)-CGRP is expressed in various areas of the central nervous system and peripheral nervous system (14).

More recently, a gene was discovered coding for mRNA that is translated into a similar peptide known as \(\beta\)-CGRP (13). This gene is known to be different from the calcitonin/\(\alpha\)-CGRP gene because it appears to contain no sequences with close homology to the calcitonin-coding exon present in the calcitonin/\(\alpha\)-CGRP gene (15). Although \(\alpha\)- and \(\beta\)-CGRP are products of different genes, they differ from one another by only three amino acids (16). The exact reason for this biological diversity has not yet been determined. Further complicating this issue is that \(\alpha\)- and \(\beta\)-CGRP have similar distributions throughout the body (15).

Since the two forms of CGRP vary little in amino acid sequence, their structures are essentially identical. The N-terminal of the CGRP is in the form of a loop created by a disulfide bond found between amino acids 2 and 7. The presence of an \(\alpha\)-helix was first predicted by studies using analogs of CGRP produced by amino acid substitution creating varying lengths of idealized amphiphilic \(\alpha\)-helices (17). Since the analog with \(\alpha\)-helix from amino acids 8 to 18
had the highest affinity for liver receptors and stimulated adenylate cyclase better than other analogs, it was concluded that this region of CGRP contained α-helix. This prediction was later confirmed by studies using nuclear magnetic resonance (H NMR) (18). Following the approximately three turns of α-helix, the CGRP molecule appears to be predominantly random coil. However, there is evidence that there is a turn-type conformation between residues 19 and 21 (18).

CGRP is believed to have two, possibly more, types of receptors. The presence of two types of receptors is demonstrated by the sensitivity to antagonism or agonism by human CGRP (hCGRP) fragments. For example, CGRP1 receptors, those most prevalent in atrial tissues, are sensitive to the antagonistic action of a hCGRP fragment of amino acids 12 through 37 while the CGRP2 receptors, those most prevalent in rat vas deferens, are not. Furthermore, CGRP2 receptors are sensitive to the agonistic actions of a full length linear analog, produced by disrupting the 2-7 disulfide bond with an acetamidomethyl moiety while CGRP1 receptors are not (19).

Studies using varying sized fragments of CGRP have also contributed to our knowledge of how CGRP interacts with its receptor. The affinity of CGRP for its receptor is independent of the N-terminal portion containing the 2-7 disulfide bridge. N-terminal fragments made of amino acids 1-7, 1-8, 2-7 or 2-8 containing the disulfide bond show very little affinity for the CGRP receptor. On the other hand, a C-terminal fragment made of amino acids 12 to 37 had a relatively high affinity for the receptor. Similarly, a linear analog lacking the disulfide bond had an even higher potency (19).
Although the disulfide bond is not important for the binding of CGRP to its receptor, this bond is highly important for the biological function of CGRP. When the bond is disrupted, as in a linear analog of CGRP, biological activity such as chronotropic effects in atria are diminished to almost zero in type 1 and type 2 receptors (19).

Interestingly, CGRP will interact with receptors specific for other ligands. Similarly, other ligands will bind to CGRP specific receptors. The first example of this phenomenon was seen in the interaction of CGRP and calcitonin with each other's receptors. In the kidney, rat CGRP has been shown to bind to high affinity receptors for calcitonin approximately as well as human calcitonin binds (20). Similar results are seen in studies using LLC-PK1 kidney cells, D+Rc kidney cells and D+Sc kidney cells (21). Conversely, calcitonin will bind to high affinity binding sites for rat CGRP found in the spinal column although with about 1000 times less affinity than rat CGRP binds (21). The fact that this cross reactivity occurs is not surprising considering the similarity of structure of CGRP and calcitonin. Both have amino terminal disulfide bridges and carboxy terminal amide groups. Also, calcitonin and CGRP are similar in overall size (21). Another example of cross reactivity is that seen between CGRP and amylin. In membrane receptors in rat liver, amylin will fully displace specifically-bound radiolabelled CGRP (22). Thus, CGRP and amylin seem to bind to the same receptor. Studies on the kinetics of amylin and CGRP binding to rat hepatocytes support this conclusion (22). These pharmacological similarities along with structural similarities place these three
peptides in a small family known as the calcitonin family of peptides (23).

The crossreactivity of calcitonin with CGRP receptors would seem to indicate that the as yet unisolated CGRP receptor is a member of the Group III family of G protein-coupled receptors. This family includes receptors for parathyroid hormone, secretin, vasoactive intestinal peptide, and calcitonin (2). These receptors, like other G protein-coupled receptors, have N-terminal potential N-linked glycosylation sites and cysteine residues in the second and third extracellular domains. These extracellular cysteines possibly form a disulfide bond for ligand binding (24). Although the Group III family of G protein-coupled receptors has these glycosylation sites and cysteine residues in common with other G protein-coupled receptors, the transmembrane domains of this family differ significantly from the membrane-spanning domains of the other members of the G protein-coupled superfamily (25).

Similarities among the members of the Group III family of G protein-coupled receptors are seen in the comparison between the parathyroid hormone receptor and the renal calcitonin receptor. These two receptors consist of seven transmembrane domains, an N-terminal extracellular domain and a C-terminal intracellular domain. These receptors have 17 of 18 amino acids in common in the carboxy-portion of the seventh membrane-spanning domain and its adjacent extracellular region (26). Two of four potential glycosylation sites are conserved in the calcitonin receptor. Additionally, seven of eight functionally important, extracellular
cysteines are conserved, five of which reside proximal to the first membrane-spanning domain (27).

Another interesting feature of this family of G protein-coupled receptors is that its members are able to couple to both adenylate cyclase and phospholipase C. For example, LLC-PK1 cells have a single calcitonin receptor that can enhance cAMP production and activate protein kinase C by coupling the calcitonin receptor to different G proteins during various stages of the cell cycle (28). Activation of protein kinase C may occur via receptor coupling to a G protein. This coupling leads to the activation of phospholipase C, resulting in increases in diacylglycerol and inositol-1,4,5, triphosphate. These events in turn cause the release of calcium from intracellular stores. Activation of adenylate cyclase causes the conversion of ATP to cAMP. Calcium and cAMP can then act as second messengers causing intracellular changes.

Although much is known about CGRP and its interaction with its receptor, the DNA encoding for the receptor has never been cloned and sequenced. Having the sequence would be of great benefit because increased knowledge of CGRP's interaction could lead to new types of drug therapy. For example, drugs could be developed which interact with receptors in the heart. This interaction could either augment or antagonize CGRP's action. Researchers hope this therapeutic intervention would decrease a person's risk of heart disease. It was for this reason that Fisher and Chatterjee began a project to clone cDNA coding for the CGRP receptor. First, Chatterjee extracted total mRNA from various rat tissues. Next, first-strand cDNA was synthesized using avian myoblastic viral
(AMV) reverse transcriptase. On the assumption that the CGRP is a member of the Group III family of G protein-coupled receptors, Chatterjee employed a PCR method using degenerate primers (primers which amplify cDNA encoding for all members of this family rather than just one specific cDNA). The conserved amino acid sequences within the 3rd and 7th transmembrane domains of the cloned vasoactive intestinal peptide (VIP), secretin, parathyroid hormone (PTH) and calcitonin (CT) receptors served as the basis for synthesis of three degenerate primers in the initial PCR. The amino acid sequences within the 3rd and 7th transmembrane domains of the vasoactive intestinal peptide, secretin, calcitonin, and the parathyroid hormone receptors are shown below and the underlined regions in the calcitonin receptor indicate amino acid sequences selected for oligonucleotide synthesis:

<table>
<thead>
<tr>
<th>3rd transmembrane domain</th>
<th>7th transmembrane domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIP</td>
<td>AAVVFFGYCVMANFFWLLVEGLYL</td>
</tr>
<tr>
<td>SECRETIN</td>
<td>LVMIFFQYCIMANYAWLLVEGLYL</td>
</tr>
<tr>
<td>PTH</td>
<td>VAVTVFLYFLTTNYYWILVEGLYL</td>
</tr>
<tr>
<td>CT</td>
<td>VLHFFHQVMSCNYRWMLCEGVYL</td>
</tr>
</tbody>
</table>

The primer designated by the sequence WMLCEGVYL is a forward primer called TM3. The two reverse primers corresponding to the sequences FOGFFVA and CFCNHEVOG are known as 21UP and 26UP, respectively. These three degenerate primers allowed for two successive rounds of PCR for 30 cycles each. In the first round of PCR, a primer combination of TM3 and 26UP was used. The first round PCR product was used as the template for the second round
which had primer combinations TM3 and 21UP. The final PCR mixture was subjected to agarose gel electrophoresis and a single band of approximately 600 base pairs was observed. A product of this size was expected since there are approximately 600 base pairs between primers TM3 and 21UP.

The PCR product, which should have been a mixture of cDNA for all members of the Group III family of G protein-coupled receptors, was ligated into a vector. The cDNA containing vectors were then used to transform *Escherichia coli* cells so that each cell contained a single vector. These cells were plated on agarose and incubated until colonies formed. Approximately 20 individual colonies were picked and amplified in LB medium.

Following extraction of the cDNA containing vectors, the cDNA was sequenced. Of the 20 sequences obtained, most coded for the calcitonin, vasoactive intestinal peptide, secretin, or parathyroid hormone receptors. However, one sequence obtained (Fig. 1) was not for any of these receptors and appeared to be a novel sequence encoding for a protein with 60% homology to the calcitonin receptor. Chatterjee hypothesized that this cDNA encoded for the CGRP receptor (personal communication).
MATERIALS AND METHODS

Preparation and screening of the human liver cDNA library

In order to obtain a full-length cDNA containing the sequence Chatterjee had determined to code for a prospective CGRP receptor, an approach using DNA library screening was employed as described in Current Protocols in Molecular Biology (29). A human liver cDNA library carried in the Uni-Zap XR bacteriophage lambda vector (Stratagene) was needed as the source of the CGRP receptor cDNA coding for the sequence from start to stop codon. In order to plate the library for screening, the cDNA library, which had a titer of 4.5 X 10¹⁰ phage particles per ml, was diluted 1:1000 in SM buffer (11.6% NaCl/4.0% MgSO₄/1 M Tris-Cl pH 7.5/0.01% gelatin). Ten µl of 10⁻⁴ diluted recombinant phage were combined with 20 µl of XL-1 Blue Escherichia coli host cells and 200 µl of SM buffer. Following combination of host cells and phage, the mixture was then incubated at 37°C for 10 min. Next, 8 ml of 0.7% agarose, previously equilibrated to 50°C, were mixed with the combined host cells and phage. Agarose, host cells, and phage were then poured onto 150-mm culture plates containing 50 ml of previously poured LB agar (10% Tryptone/5% yeast extract/5% NaCl/1% 1 N NaOH/12% agar). This procedure was repeated for five more plates producing six plates total. After these plates were poured, they were incubated at 37°C for 8 hr.

Following the 8-hr incubation, plaques from the agar plates were transferred to nitrocellulose membranes. First, the nitrocellulose
membranes were placed on the plates for approximately 2 min. Three asymmetric holes were punched into the membranes so that proper orientation could be determined later. The filters were then dried on the benchtop for 10 min at room temperature. Next, the filters were treated to denature the attached DNA. They were first placed, DNA side up, on Whatman 3MM paper saturated with 0.2 M NaOH /1.5 M NaCl for 2 min. The NaOH was neutralized by transferring the filters to another sheet of Whatman paper saturated with 0.4 M Tris-Cl, pH 7.6 / 2X SSC (3M NaCl/0.3 M Na3citrate-2H2O/pH 7.0) for 2 min and then transferred to 3 MM paper saturated with 2 X SSC for 2 min. Finally, the filters were dried in a vacuum oven for 100 min at 80° C.

Once the nitrocellulose membranes were dry, they were used for hybridization. First, the filters were prehybridized in approximately 30 ml of prehybridization solution I (72 ml Formamide/36 ml 20X SSC/3 ml 1 M Tris-Cl, pH 7.6/1.5 ml 100X Denhardt's solution/6.75 ml water/30 ml 50% dextran sulfate/0.75 ml 20% SDS) at 42° C overnight in a rotating incubator.

Preparation of synthetic DNA probe

While the membranes were being prehybridized, a probe specific for the prospective CGRP receptor was prepared. This probe was produced by a method using PCR. Putative CGRP receptor DNA from a human brain cDNA library was amplified using a degenerate forward primer and putative CGRP receptor specific reverse primers: TM3 forward, 26UP reverse for the first round and TM3 forward, 21 UP reverse for the second (Fig. 1). This PCR combination produced an
approximately 600 base-pair length of DNA which would anneal only to DNA with the putative CGRP receptor sequence obtained by Chatterjee.

Following the amplification of the DNA by PCR, the PCR product was precipitated. First, 50 µl of 7.5 M ammonium acetate and 450 µl of ethanol were added to 100 µl of PCR product. This mixture was placed in a methanol-dry ice bath for 1 hr and centrifuged for 20 min at 4°C. Then supernatant was drawn off and discarded. Next the pellet was rinsed with 80% ethanol, dried, and resuspended in 20 µl of double-distilled water.

The resuspended DNA was labelled using a Boehringer Mannheim nick translation kit. Two µl of the suspended PCR product were added to 6 µl of dNTP’s, 4 µl of 10X buffer provided with the kit, 5 µl of alpha 32P CTP (10 mCi/ml: 250µCi), 19 µl of double-distilled water and 4 µl of DNA-polymerase I mixed with DNase I in 50% (v/v) glycerol. The reaction was allowed to proceed at 15°C for 35 min and was terminated by adding 2 µl of 0.5 M EDTA and heating at 65°C for 10 min.

The labelled probe was precipitated by adding 2 µl of salmon sperm DNA (10mg/ml), 4.8 µl of 3M sodium acetate (pH 5.5) and 100 µl of 100% ethanol. This mixture was incubated in an ethanol-dry ice bath for 30 min. Following incubation, the DNA was centrifuged at 2000 X g for 15 min at 4°C. The supernatant was drawn off and saved for scintillation counting. The pellet was rinsed with 200 µl of 70% ethanol, subsequently dried, and resuspended in 100 µl of TE buffer (10mM Tris-HCl, pH 7.4/1mM EDTA, pH 8.0). Scintillation counting of the pellet and supernatant indicated a 33.8%
incorporation of alpha $^{32}$P-CTP. After prehybridization, 50 µl of this probe were added to the hybridization bags containing prehybridization solution I and filters. The filters were hybridized for 20 hr at 42°C in a rotating incubator.

Following hybridization, filters were washed under low stringency conditions of four 15-min washes in 2X SSC, 0.1% SDS at room temperature. The filters were next placed on X-ray film and exposed for three days at -80°C using an intensifying screen.

Plaques containing the cDNA sequence for the putative CGRP receptor were then removed along with the surrounding agar using the wide end of a sterile pasteur pipette and placed in 1 ml of SM buffer and 50 µl of chloroform.

**PCR to confirm presence of putative CGRP receptor cDNA**

In order to confirm that the cored out plaques contained the desired cDNA, two rounds of PCR using the TM3 degenerate forward primer and putative CGRP receptor gene-specific reverse primers were used. The routine PCR mixture contained 50 mM Tris-HCl, pH 8.3/1.5 mM MgCl$_2$/200 µM dNTPs/0.5 µl of *Taq* DNA polymerase (Perkin-Elmer Cetus), and 20 pmol of both reverse and forward primers. The standard PCR conditions involved initial heating at 94°C for 5 min, holding at 80°C during the addition of *Taq* polymerase, and 30 cycles of denaturation at 94°C for 2 min, primer annealing at 50°C for 2 min and primer extension at 72°C for 3 min.

Figure 1 shows the amino acid sequence of the suspected CGRP receptor obtained by Chatterjee. This amino acid sequence indicates the location and direction of the various primers used in this
project. For the first round, the TM3 forward primer and the VEEAIKGE reverse primer were used. Following the first round, the amplified DNA was diluted 1:100 with double-distilled water in order to remove non-specific DNA. For the second round, the TM3 forward primer and the ARSLYYY reverse primer were used.

**Plaque purification**

After the presence of the desired cDNA was confirmed by PCR, several plaques were chosen for plaque purification. Plating and screening were done according the same protocol as was used for the original cDNA library. However, with the plaque purification step, the vector with the cDNA source came from the agar containing positive plaques. Assuming that each plaque contained $10^6$ to $10^7$ phage particles, the DNA was diluted 20X, 200X and 2000X in SM buffer.

As before, the plaque-purified plates were screened using nitrocellulose and placed on X-ray film. Positive plaques were picked and confirmed to be true positives by use of two rounds of PCR using putative CGRP receptor gene-specific reverse primers. First-round primers were the TM3 forward primer and the VEEAIKGE reverse primer. Following the 1:100 dilution in double-distilled water, second-round primers were TM3 and ARSLYY.

**In vivo excision**

In order to prepare the cDNA for sequencing, Stratagene’s\(^1\) in vivo excision protocol was used to rescue the pBluescript SK- phagemid

\(^{1}\) Technical Services: 11099 North Torrey Pines Road, La Jolla, CA 92037
from the lambda zap vector. The in vivo excision process results in the formation of a circularized phagemid containing the cDNA insert. This process relies on proteins encoded by a helper phage known as the R408 helper phage. These proteins normally recognize the R408 bacteriophage origin of replication which has been subcloned into the Uni-Zap XR vector. The Uni-Zap XR vector was made accessible to the R408 proteins by coinfecting 200 μl of OD$_{600}$=1.0 XL-1 Blue *E. coli* in 10mM MgSO$_4$ with 200 μl of Uni-Zap phage stock from the plaque purification step and 1 μl of R408 helper phage diluted 1:25 in SM buffer. A negative control was made by infecting XL-1 Blue cells with only R408 helper phage and no recombinant Uni-Zap XR phage. The phage, *E. coli* mixture was then incubated 15 min at 37° C to allow entry of the phage. Following the 15-min incubation, 5 ml of LB medium were added. These mixtures were then incubated at 37° C for 3 hr while shaking at 300 rpm.

During this 3-hr incubation, "helper" proteins expressed by the R408 phage recognize the initiator DNA of the origin of replication that is within the Uni-Zap XR vector. These proteins then nick one of the two DNA strands. New DNA synthesis begins duplication of the plus strand DNA that lies downstream (3') of the nicking site. Synthesis continues until a terminator signal is reached downstream from the intitiator site. The single, newly-synthesized DNA molecule is then circularized by a gene II product of the R408 helper phage. This circularized DNA will contain all DNA that was between the initiator and the terminator sequences in the Uni-Zap XR vector. More specifically, this DNA includes the pBluescript SK- phagemid that has been engineered into the Uni-Zap vector and any cDNA insert
present within the Uni-Zap vector. Following circularization, the pBluescript SK- phagemid containing the inserted DNA is packaged for secretion from the cell due to signals that are contained within the terminator origin DNA sequence. After the 3 hr of incubation, secretion of the newly made phagemids into the medium is complete.

In order to remove *E. coli* host cells used for the in vivo excision, the 5 ml of LB medium was heated to 70°C for 20 min and subsequently centrifuged for 5 min at approximately 2000 X g at 4°C. Supernatant containing the pBluescript phagemid with its inserted cDNA was decanted into a sterile tube. The pellet of *E. coli* was discarded.

In order to produce a double-stranded phagemid from the new single-stranded pBluescript SK- phagemid, 20 µl of 1:1000 diluted in LB phagemid stock supernatant were combined with 200 µl of OD600=1.4 XL-1 Blue host cells. Tubes were incubated at 37°C for 15 min to allow transformation. Next, 100 µl of the mixture were plated on LB-Ampicillin plates (50 µg/ml Ampicillin per ml of LB agar) and incubated overnight at 37°C. Since the phagemid contains an Ampicillin-resistance gene, only those bacteria that had been successfully transformed would grow.

After the plates had incubated overnight, single colonies containing double-stranded pBluescript and the cDNA insert were picked and used to inoculate 5 ml of LB / 50 µg per ml Ampicillin media. The 5 ml LB was incubated overnight at 37°C and shaken at 300 rpm.
Isolation of pBluescript Sk- phagemid by minipreparation

A minipreparation was done in order to isolate the phagemid and inserted cDNA. One and one half ml of the overnight LB culture were placed in a microfuge tube and centrifuged at 12,000 X g for 1 min. Supernatant was removed by aspiration and discarded. The bacterial pellet was resuspended in 100 μl of ice-cold lysis buffer (100 mg per ml RNase A/50mM Tris-Cl/10mM EDTA, pH 8.0) and incubated for 5 min at room temperature. Two hundred μl of freshly prepared 0.2 N NaOH/1%SDS were added and mixed by inversion. This mixture was incubated on ice for 5 min. Next, 150 μl of ice cold 3 M potassium acetate (pH 4.8) were added, mixed by inversion, and incubated on ice for 5 min. Following this incubation, tubes were centrifuged for 30 min at 12,000 X g. The supernatant was transferred to fresh tubes and 450 μl of TE-saturated phenol / chloroform were added. This mixture was vortexed for 1 min and centrifuged at 12,000 X g for 2 min. The upper aqueous phase was transferred to a new tube. The lower phase was again treated with TE-saturated phenol / chloroform, vortexed and centrifuged as before. The combined aqueous phases of the chloroform extraction were added to 400 μl of 24:1 chloroform / isoamyl alcohol, vortexed for 1 min and centrifuged for 2 min at 12,000 X g. The upper aqueous phase was transferred to a fresh tube and the lower phase was again treated with chloroform / isoamyl alcohol, vortexed and centrifuged as before. The combined aqueous phases were added to 2.5 volumes of 95% ethanol. Precipitation of phagemid DNA was allowed to proceed overnight at -20° C. Following the overnight treatment at -20° C,
the ethanol was centrifuged at 12,000 X g for 15 min at 4°C. The supernatant was drawn off by aspiration and discarded. The DNA pellet was dried and resuspended in 50 µl of 5mM Tris-HCl, pH 7.4/1mM EDTA.

**PCR to confirm presence of putative CGRP receptor cDNA**

As was done with plaques containing the Uni-Zap XR with inserted cDNA, phagemids of the in vivo excision were confirmed to contain the proper cDNA by using two rounds of PCR with insert-specific primers. The first round of PCR used a TM3 forward primer and a VEEAIKGE reverse primer. The second round of PCR used TM3 forward primers and the ARSLYY reverse primer.

**Transforming DH5 alpha cells with miniprep DNA**

Following confirmation by PCR, the phagemid DNA was transformed into DH5 alpha host cells in order to obtain sufficient DNA by a midiprep procedure for sequencing. Two µl of β-mercaptoethanol were added to 50 µl of host cells. Next, 5 µl of phagemid DNA isolated by miniprep were added to the host cells. This mixture was placed on ice for 30 min and subsequently placed at 42°C for 2 min followed by treatment on ice for another 2 min. Four hundred fifty µl of SOC medium were then added. This culture was shaken for 1 hr at 300 rpm and 37°C. Finally, the mixture of transformed cells was transferred to 100 ml of LB-Ampicillin medium (50 µg Ampicillin per ml of LB medium). This LB-Ampicillin medium was incubated overnight at 37°C while shaking at 300 rpm.
Isolation of pBluescript Sk- phagemid by midipreparation

In order to isolate phagemid DNA again, a Qiagen midiprep plasmid purification protocol was used. First, the DH5 alpha cells were pelleted by centrifugation at 6000 X g for 15 min at 4°C. Supernatant was drawn off and discarded, and the bacterial pellet was resuspended in 4 ml of P1 buffer (100 μg per ml RNase A/50mM Tris-HCl/ 10mM EDTA, pH 8.0). Next, 4 ml of P2 buffer (200mM NaOH/1% SDS) were added and gently mixed. This mixture was incubated at room temperature for 5 min. Following the 5 min incubation, 4 ml of P3 buffer (3 M potassium acetate, pH 5.5) were added. This mixture was incubated for 15 min on ice and centrifuged for 30 min at 4°C and 30,000 X g. Following centrifugation, the supernatant was placed on a QIAGEN-tip 100 column which had previously been equilibrated with 4 ml of QBT buffer (750 mM NaCl/50mM MOPS/15% ethanol/0.15% Triton X-100/pH 7.0). The column was washed two times with 10 ml of QC buffer (1.0M NaCl/50mM MOPS/15% ethanol/pH 7.0). DNA was eluted from the column by washing with 5 ml QF buffer (1.25 M NaCl/50mM Tris-HCl/15% ethanol/pH 8.5). DNA was precipitated by adding 0.7 volumes of room-temperature isopropanol and centrifuging at 15,000 X g for 30 min at 4°C. The DNA pellet was then washed with 5 ml of cold 70% ethanol, air dried, and redissolved in 50 μl of 5 mM Tris-HCl, pH 7.4/1 mM EDTA.

Once again, two rounds of PCR were used to confirm the presence of the desired cDNA insert. For the first round, TM3 forward and
Poly T with T3 reverse primer were used. For the second round, TM3 forward and ARSLYY reverse primers were used.

The isolated phagemid DNA was sent to the University of Iowa College of Medicine CORE facility to be sequenced.
RESULTS

The initial library screening of the first agar plates indicated that there were many plaques in the human liver cDNA library which appeared to contain the putative CGRP receptor cDNA (Fig. 2). Fifty-three plaques were chosen to be further processed based on several criteria: (1) Plaques chosen showed darkening of the autoradiogram associated with radioactive probe hybridization; (2) The darkened areas of the autoradiogram were not perfectly symmetrical but had "tails" indicating that they were true positives and not nonspecific hybridization; (3) Plaques chosen were reasonably well isolated from negative plaques to minimize the risk of removing nonspecific cDNA.

Results of the PCR using TM3 forward, VEEAIKGE reverse in the first round and TM3, ARSLYY in the second round confirmed that the desired DNA was contained in 26 of the plaques (Fig. 3). Since the first round of amplification is followed by a 1:100 dilution and a second round of amplification, the likelihood of nonspecific gene amplification is very small. Consequently, the PCR results strongly indicated the presence of putative CGRP receptor cDNA.

The screening of the plates used for plaque purification had many strong positives (Fig. 4). Plaques were again chosen according to the three criteria discussed above.

PCR using TM3, VEEAIKGE first-round primer combination and TM3, ARSLYY second-round primer combination indicated that all chosen plaques but three contained the desired cDNA insert (Fig. 5).
Based on these results, 10 plaques were chosen to be used for the in vivo excision. The PCR done following the miniprep using a TM3, VEEAIKGE primer combination in the first round and TM3, ARSLYY in the second showed that there were eight phagemids containing the proper cDNA (Fig. 6).

Following the in vivo excision, two of the putative CGRP cDNA-containing phagemids were chosen to transform the DH5 alpha cells for the midiprep. Following the isolation of phagemid DNA the PCR using TM3, poly T with T3 and TM3, ARSLYY indicated that the cDNA isolated was not the desired product (Fig. 7).

Although the results of the PCR were negative, the phagemid obtained was sequenced (Fig. 8).
DISCUSSION

Since CGRP receptors are known to be present in the liver, it was not surprising to find plaques containing the putative CGRP receptor cDNA in the human liver cDNA library.

The PCR using CGRP-specific reverse primers showed that of the 53 positives chosen 26 were in fact true positives. The expected product of approximately 150 base pairs, the number of base pairs from the 5' end of the forward primer site to the 3' end of the reverse primer site, was obtained from the plaques which were true positives. Apparently, the putative CGRP receptor cDNA was relatively abundant since there were approximately 26 confirmed positive plaques out of approximately 2730 plaques total. Those plaques that turned out to be negative by PCR were probably non-CGRP-receptor DNA-containing plaques. In all probability, these negative plaques were closely neighboring a positive on the autoradiogram. Consequently, negative plaques could have been removed instead of the adjacent positive plaque due to the difficulty of correlating the autoradiogram to plaques in the agar plate.

Following confirmation by PCR, bacteriophage for plaque purification were chosen based on intensity of hybridization indicated by the autoradiogram and by intensity of PCR amplification. This step was done in order to increase the likelihood of picking a plaque containing only putative CGRP receptor cDNA and not any cDNA from a nonspecific neighboring plaque. By simple visual examination, it was apparent that the plates containing
plaque-purified bacteriophage had a greater percentage of positive plaques. In fact, very few of the plaques on the agar plates were negative on the autoradiogram.

The same primer combinations used in the first library screening PCR showed that all but three of the chosen plaques had the expected 150 base-pair product. This result was expected because of the higher percentage of positive plaques on the autoradiogram and a greater distance between each of the plaques due to an increased dilution of the plated phage. These two factors decreased the difficulty of correlating the autoradiogram to positive plaques on the agar plates. Consequently, this step greatly increased the likelihood of picking a positive plaque rather than a neighboring negative plaque. The plaques picked would contain little if any contaminating cDNA and would contain a quite pure form of CGRP-specific cDNA.

The PCR used to confirm the miniprep DNA showed the expected 150 base-pair product in nine of the 10 plaques that had undergone the in vivo excision process. Consequently, this PCR demonstrated that the in vivo excision was a success. Phagemids containing prospective CGRP-receptor cDNA were present and could be greatly amplified by replication in DH5 alpha bacterial host cells.

Following the midiprep, essentially a large-scale miniprep, phagemid DNA was shown to be present by running the DNA through an agarose gel. This cDNA was verified by PCR using a new reverse primer, poly T with T3, instead of the usual VEEAIKGE. Surprisingly, this PCR showed that the desired cDNA was most likely not present in the phagemid. Perhaps the poly T with T3 primer was not working
properly. This is rather unlikely since the cDNA was in a relatively pure form and the poly A sequence which this primer recognizes is present in the cDNA. Another possible explanation could be that the transformation of DH5 alpha cells was not successful. However, the presence of vector sequences was necessary for the sequencing reaction. Apparently, the cDNA isolated by midiprep contained such sequences since a sequence was obtained.

Even though the isolated phagemids probably did not contain the prospective CGRP-receptor DNA, the isolated phagemid DNA was sequenced by the University of Iowa College of Medicine CORE facility. This sequence was checked against all other sequences in a gene bank. The sequence contained no homology to any known gene.
Figure 1. Sequence of putative CGRP receptor. Diagram shows the sequence obtained by Chatterjee (top sequence) and the sequence of the calcitonin receptor (bottom sequence). Arrows indicate the position of primers used in Chatterjee’s study and in this study. X’s indicate amino acids found in one sequence but not the other.
Figure 2. Autoradiogram from first library screening. The library screening of the first agar plates produced autoradiograms which indicated many positive plaques.
Figure 3. PCR to confirm the first autoradiogram results. Results of the PCR using TM3 forward, VEEAIKGE reverse in the first round and TM3, ARSLYY in the second round. Note that the product is approximately 150 base-pairs, the number of base-pairs from the 5' end of the forward primer site to the 3' end of the reverse primer site.
Figure 4. Autoradiogram from the plaque purification. The plaque purification autoradiogram indicates that there is an increased percentage of positive plaques. Note the presence of tails on some of the plaques indicating specific hybridization.
Figure 5. PCR to confirm the second autoradiogram. PCR using TM3, VEEAIAKGE first round primer combination and TM3, ARSLLYY second round primer combination confirmed the presence of putative CGRP receptor cDNA.
Figure 6. PCR to confirm cDNA isolated by miniprep. The PCR done following the miniprep using TM3, VEEAIKGE primer combination and TM3, ARSLYY primer combination showed that there were nine phagemids containing the proper cDNA.
Figure 7. PCR to confirm cDNA isolated by midiprep. Following the isolation of phagemid DNA, the PCR using TM3, poly T with T3 and TM3, ARSLYY indicated that the cDNA isolated was not the desired sequence.
Figure 8. Sequence of DNA isolated by midiprep.
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