CLONING AND PARTIAL SEQUENCING OF A PUTATIVE G-PROTEIN COUPLED M4 MUSCARINIC RECEPTOR FROM THE DOGFISH SHARK (SQUALUS ACANTHIAS) USING NESTED PCR.

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Using novel PCR-based molecular biological techniques, we have isolated, cloned and partially sequenced a new fragment of a putative G-protein coupled receptor from the dogfish shark (Squalus acanthias). This sequence shows strong homology (>85%) to the rat m4 muscarinic acetylcholine receptor.

Recent molecular analysis has revealed that receptors and ion channels belong to distinct protein superfamilies (Bonner, Science, 237,527.1987; Wilks, A.F. Technique, 1,66.1989). The G-protein (GTP-binding) coupled receptors, such as muscarinic and adrenergic receptors, are characterized by seven transmembrane domains, while the ligand-gated ion channel receptors, e.g. GABA and nicotinic receptors, possess only four transmembrane domains. The family of atrial natriuretic peptide (ANP) like receptors are characterized by a single transmembrane domain.

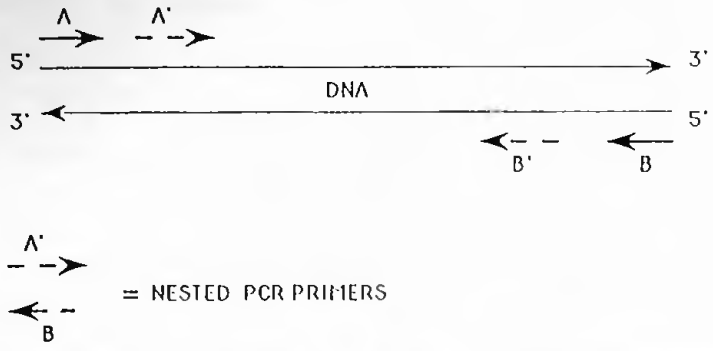
To accomplish the cloning of receptors, we have applied the technique of the polymerase chain reaction (PCR)(Saiki, R.K., et al. Science, 239,487.1988) in a novel fashion Others have designed highly redundant synthetic primers and used them to generate many (>80) products. Because of the inherent primer redundancy, much time is required to sift through the non-specific products. Libert et al (Science, 244,569.1989) analyzed over 80 clones in attempting to isolate the thyrotropin receptor. Only five (6.25%) clones fulfilled the desired rhodopsin-superfamily structure. A recent review article suggested sequencing of up to 50 clones to obtain the desired DNA target.(Wilks, A.F. Technique, 1,66.1989). We have successfully circumvented such large-scale cloning and DNA sequencing by utilizing the increased specificity of "nested"PCR. This technique overcomes in part the problem of non-specific amplification from a complex template.

By performing a computer analysis (Clustal) of the known protein and/or DNA sequences of the currently described G-coupled proteins, highly conserved stretches were used as molecular "probes" to isolate new receptors that share this sequence.

Nested PCR, illustrated in the top panel of figure 1 below, uses two sets of primers to increase specificity by reducing the complexity of the template DNA to be amplified (Mullis et al.Methods Enzymol., 155, 335.1987). This technique decreases the problem of non-specific amplification from a complex template. The first step is low-specificity PCR using an outer pair of primers (A and B). Gel electrophoresis of the resulting PCR products from several species are shown in figure 1, bottom panel A. The second step uses an aliquot of the first-round PCR as the template for an internal "nested"primer pair (A' and B').

Figure 1 bottom panel B, illustrates that the resultant PCR products show amplification and increased specificity of putative receptor genes (homologous to muscarinic sub-family) in the several species. These bands were excised and purified prior to cloning. The PCR product was (blunt-end) cloned into the plasmid vector pBluescript KSII$^+$, and sequenced by a modified alkaline-lysis double-stranded protocol.

NESTED PCR



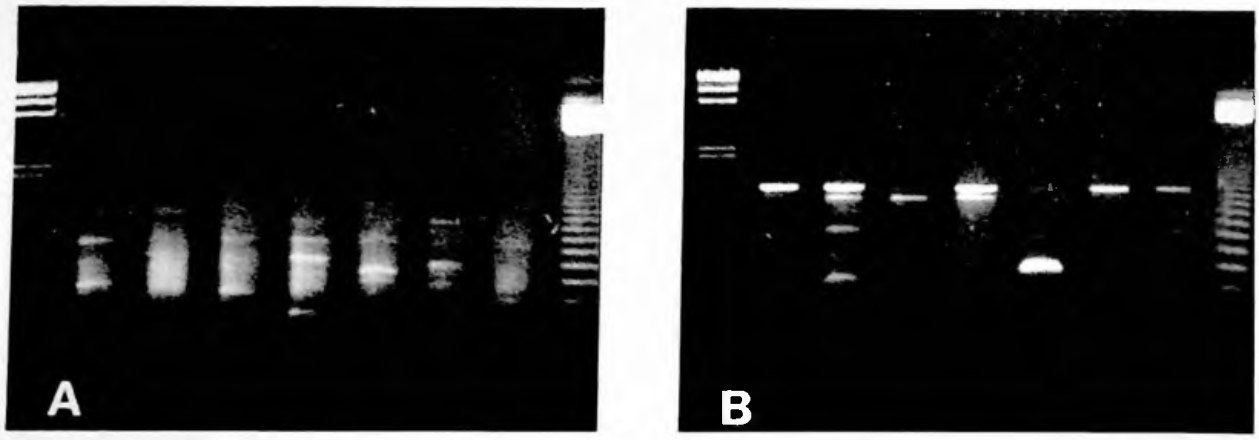


Figure 1. Top Panel: Model of nested PCR primers. Lower Panel: (Panel A) Low specificity PCR using an outer pair of primers (A&B) for muscarinic receptors against genomic DNA from several species. (Panel B): PCR using an aliquot of the first round PCR product as a template for an internal "nested" primer pair (A' and B'). Species are (from left to right in Panels A&B) human, hamster, shark, rat, mouse, cow and sheep.

The DNA sequence read from the cloned shark fragment was analyzed against the EMBL database using the Fasta program on a Vax-VMS mainframe computer. The most significant match shown as shown in figure 2 was with the rat m4 muscarinic acetylcholine receptor. The portions in boxes represent the primer sequences.

Homology between Shark (Squalus acanthias) genomic DNA and Rat m4 muscarinic acetylcholine receptor

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                  C  A  D  L  I  I  G  V  F  S  M  H  L  Y  T  V  Y  I  I  K  G  Y  W
Shark genomic DNA TGTGCGGATCTGATTATCGGAGTCTTTTCTATGAACTTGTATACAGTCTACATTATTAAAGGCTACTGG
                  X:::::::::: :: :: :: : :: ::::::::: : :: ::  : :: :: :: ::  ::::::::
Rat M4 receptor   TGTGCGGATCTCATCATAGGGGCATTCTCTATGAACCTCTACACTTTGTATATCATCAAGGCTACTGG
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Figure 2.

We are currently analyzing DNA sequences generated using these techniques for several species, including shark (Squalus acanthias), human, Syrian hamster, rat, mouse, cow, and sheep genomic DNA. We will then use these sequences to screen genomic libraries to obtain full-length receptor products.