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Cloning of a *Xenopus laevis* muscarinic receptor encoded by an intronless gene

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Abstract The *Xenopus laevis* oocyte has endogenous sites that bind muscarinic agonists, which have been pharmacologically characterized as M3 and/or M1 receptor subtypes. In order to define the molecular identity of the receptor protein we have analyzed a *Xenopus* oocyte cDNA library and cloned a 2.9 kb cDNA fragment encoding a muscarinic receptor (xMR). The deduced amino acid sequence reveals a protein of 484 residues with an apparent molecular weight of 54,188 Da. Amino acid comparison with previously cloned mammalian muscarinic receptors showed a 78% identity with the human m4 subtype, presenting at the same time clustered differences within the amino-terminal region and third intracellular loop. Genomic Southern analysis displayed the presence of one main gene belonging to this subtype, and the PCR analysis revealed an intronless gene.

Key words: G protein-coupled receptor; cDNA cloning; *Xenopus laevis* oocyte

1. Introduction

The oocyte maturation process in *Xenopus laevis* is an important step in preparing this germinal cell for fertilization. The oocyte has two signal transducing systems regulated by G proteins that affect this process; the adenylyl cyclase (AC) and the phospholipase C systems (PLC) [1–6]. Progesterone inhibits the AC system, triggering maturation by lowering cytosolic cAMP levels [7–9]. Acetylcholine (Ach), through an endogenous muscarinic receptor, activates the PLC system, increasing intracelular levels of inositol trisphosphate (IP3) and calcium (Ca^{2+}) [10–12].

Electrophysiological studies have shown that Ach promotes a complex membrane depolarization by the opening of Ca^{2+}-sensitive Cl^- channels [6,13,14]. Similar responses have been found when exogenous mammalian muscarinic and 5HT1c receptors are expressed in the oocyte [15,16]. These receptors couple to endogenous G proteins of the G_o/G_q subtype [17,18] and activate a PLC of the β subtype [19].

Pharmacological studies carried out to identify the nature of the endogenous receptor, led to the proposal that *Xenopus laevis* oocyte has two different muscarinic receptors in the plasma membrane, mainly M3 and to a lesser extent the M1 subtype [10,20–22]; the molecular identity of this receptor, however, remains obscure. As a first step in the understanding of the molecular nature and function of this G protein-coupled receptor, here we report the cloning, sequencing and characterization of a cDNA encoding a muscarinic receptor from *Xenopus laevis* oocyte.

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The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank with accesion number X65865.

2. Materials and methods

2.1. Isolation and sequencing of oocyte xMR cDNA clone

A stage VI *Xenopus laevis* oocyte cDNA library [23] (kindly donated by Dr. D. Melton, USA) was screened with an oligonucleotide corresponding to a highly conserved amino acid region present in all five mammalian muscarinic receptors. The primer was 5′-CTCAAGACGGTCAACAACTACTTCCTGCTGAGCCTGGCCTGCGCTGACCTCATCATCGGC-3′, corresponding to the amino acid sequence NH_2-LQTVNNYFLFSLACADLIIG-COOH. The oligonucleotide was labelled in its 5′ end with polynucleotide kinase and [γ-^{32}P]ATP, and probed to 300,000 independent lysis plaques. Hybridizations were done at 45°C in a buffer containing 1 × SSC, 5 × Denhardt's, 10 mM sodium phosphate buffer, pH 6.7, 1% SDS and 100 μg/ml of herring sperm DNA. Washings were done between 45°C and 55°C in a buffer containing 0.1 × SSC and 0.1% SDS. One positive phage clone was further purified through 3 rounds of consecutive screenings and the cDNA subcloned into the *Eco*RI site of pTZ18R(+) vector (Pharmacia). The DNA sequencing was performed on both strands by the dideoxy chain termination technique using Sequenase version 2.0 (US Biochem.), [α-^{35}S]dATP and successive synthetic oligonucleotides [24].

2.2. PCR amplification of Xenopus genomic DNA

PCR amplification was carried out using *Taq* DNA polymerase (Promega Co.) under the following conditions: 30 cycles of denaturation at 94°C, 1 min; annealing at 52°C, 1 min; and extension at 72°C, 1 min.

2.3. Southern blot analysis

Genomic DNA was restriction digested, blotted onto Nytran nylon membranes and hybridized as described [25], with a specific ^{32}P-labeled probe. The DNA probe was the purified product of PCR amplification of a region of the xMR gene prepared using a specific pair of primers.

3. Results

3.1. Cloning of a cDNA encoding the complete open reading frame (ORF) of a Xenopus laevis oocyte muscarinic receptor

Fig. 1 shows the nucleotide and amino acid deduced sequence of the xMR cDNA. The cDNA sequence is 2,860 nucleotides long and predicts an ORF encoding a 484 amino acid protein with a calculated molecular weight of 54,188 Da. The presence of a complete ORF in the cDNA was demonstrated

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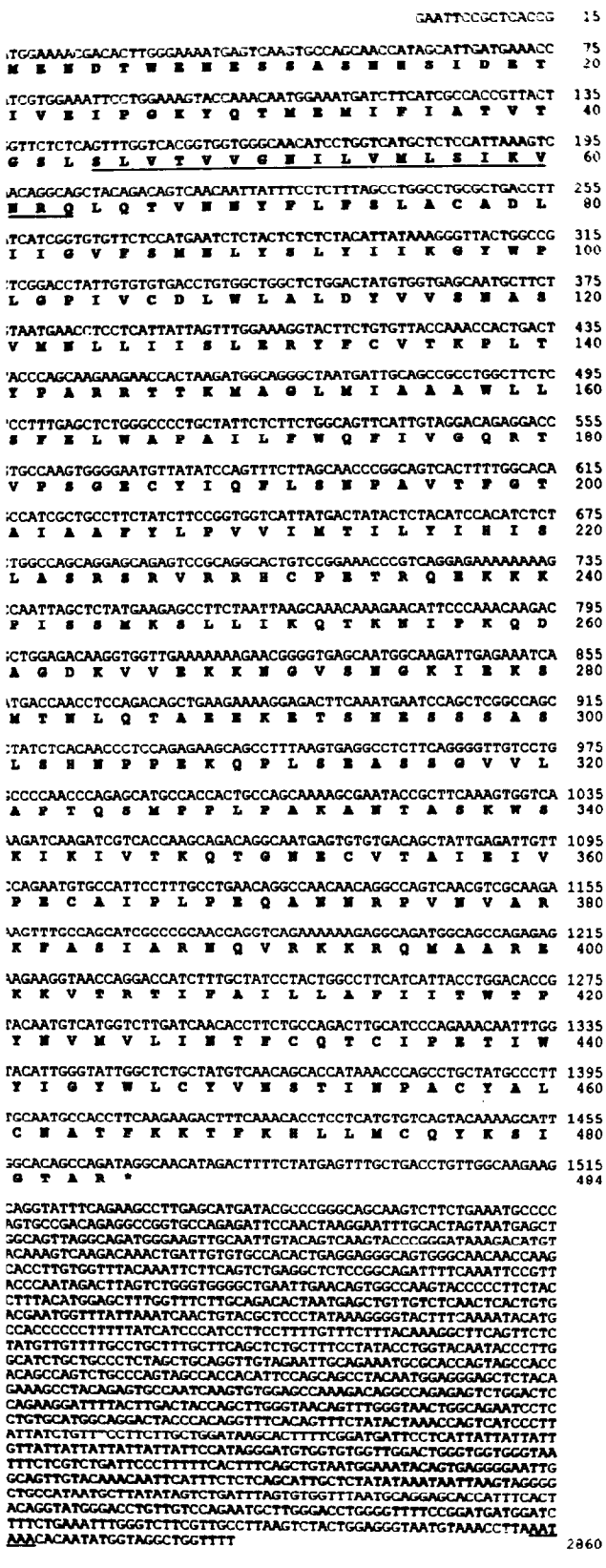

Fig. 1. Nucleotide and deduced amino acid sequence of the *Xenopus laevis* oocyte cDNA clone. The solid bar below the amino acid sequence indicates the DNA region recognized by the oligonucleotide probe deduced from mammalian muscarinic receptor DNA sequences m1–m5 (see section 2). The solid bar below the 3′ non-coding nucleotide region indicates the polyadenylation signal. Numbers at the right of the sequences indicate the nucleotide and amino acid position along the cDNA. The single-letter amino acid code is used.

←

by in vitro transcription and translation of the gene, showing a protein product of 54,000 Da (data not shown). The long 3′ unstranslated region contains one polyadenylation signal at its end (bases 2833–2838) but no poly(A) tail was found.

3.2. Analysis of the xMR predicted amino acid sequence

All G protein-coupled receptors (GPCR) have been shown to possess seven hydrophobic transmembrane domains [26], and this feature has been used to classify our sequence as a GPCR, through hydropathy analysis. (data not shown). Fig. 2 shows an alignment of the xMR protein sequence with other known human muscarinic species, m1–m5 [27]. The highest identity appears with m4 (78%), and to a lesser extent with the remaining sequences (between 37 and 54%).

3.3. Structure of the xMR genomic gene by Southern and PCR analysis

Xenopus genomic DNA was subjected to PCR amplification with 9 different oligonucleotides in order to determine the size of the xMR gene. Fig. 3A shows a diagram of the xMR cDNA and the localization of the primers utilized to amplify different regions of the gene. Fig. 3B shows the electrophoretic analysis of the PCR products. As can be seen, all PCR products correspond to the expected sizes of an intronless gene. In order to analyze the genomic profile of the xMR gene, a specific probe, described in section 2, was utilized in a Southern blot analysis. *Xenopus* genomic DNA was digested with *Hha*I or double digested with *Hha*I and *Pvu*II. As shown in Fig. 4A the xMR cDNA has two *Hha*I sites, located at positions 247 and 2,161 and two *Pvu*II sites at positions 874 and 2,566. Thus in an intronless gene the expected hybridizing band size for the single digestion is 1,914 bp and two bands of 1,287 bp and 627 bp for the double digestion. The results shown in Fig. 4B agree with the restriction analysis since the DNA hybridization pattern obtained after the Southern blotting shows a major band close to 1,900 bp (*Hha*I single digestion) and two bands close to 1,300 bp and 600 bp (*Hha*I/*Pvu*II double digestion). From these two bands the one having 627 bp shows a stronger hybridization than the 1,287 bp fragment. The different strength in the hybridization pattern is perfectly explained by the localization of the probe with respect to the size of the complementary regions that hybridize with the probe (see Fig. 4A).

4. Discussion

The molecular identity of the muscarinic receptor involved in acetylcholine transducction signal in *Xenopus* oocyte is not yet characterized. Electrophysiological and pharmacological studies have implicated the M3 and M1 type receptors in this action. In mammalian cells these receptors are coupled to the phospholipase C_β, increasing its activity [28]. In oocytes acetylcholine stimulates the IP3 pathway [19] or inhibit the adenylyl cyclase [32]. For a better understanding of the physiological role of this receptor we considered it important to characterize it at the molecular level and decided to clone its gene from

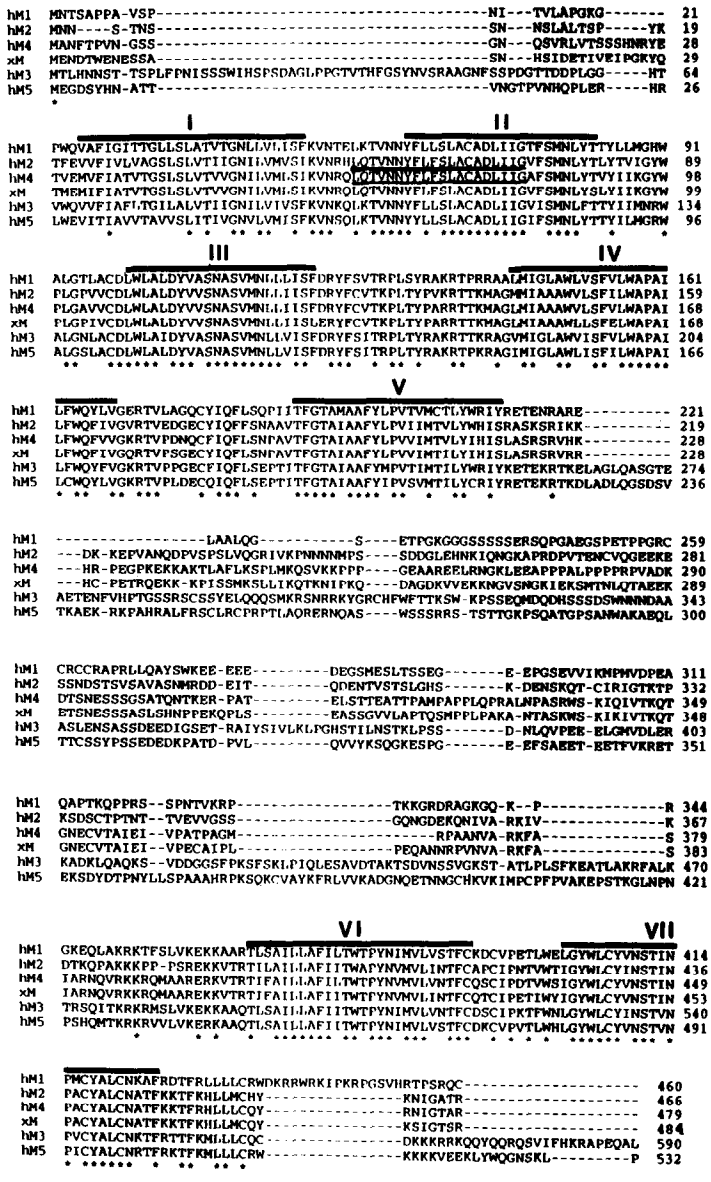

Fig. 2. Comparison of the deduced amino acid sequence of the *Xenopus* muscarinic receptor (xMR) with the five human muscarinic receptors, hm1–hm5 [27]. Identical amino acids along the sequences are indicated by * symbols and the putative transmembrane domains are indicated as I–VII and solid bars.

a *Xenopus laevis* oocyte cDNA library. The deduced amino acid sequence from the cloned cDNA revealed an ORF encoding a protein of 458 residues (Fig. 1) with a deduced molecular weight of 54,188 Da The in vitro transcription and translation of the cloned xMR revealed a product of 54,000 Da, which is in agreement with the cDNA ORF. Hydrophathy analysis of the sequence revealed the presence of seven transmembrane domains (Fig. 2, roman numbers I–VII), indicating that the oocyte protein belongs to the G protein-coupled receptor family. Surprisingly, the deduced primary structure did not show the highest homology with the m3 or m1 subtypes (37–54%), but with the human m4 receptor (78%) (Fig. 2). Interestingly, the *Xenopus* receptor sequence has important amino acid differences clustered mainly at the amino-terminal region and third intracellular loop. The m4 oocyte receptor could be negatively regulating the adenylyl cyclase activity, as it does in mammalian cells [28], or it could be positively regulating the phospholipase C activity, as previously shown by pharmacological studies [21,22]. Further genetic manipulation and expression of this gene should be done in order to clarify this issue.

Finally at the DNA level we have established through Southern analysis and PCR amplification the presence of one intronless gene for a *Xenopus* muscarinic receptor (Figs. 3 and 4). This feature is shared by other muscarinic receptors [29], and considering the strength of the major hybridizing band in comparison to other very faint ones (Fig. 4B) we can conclude that in *Xenopus* there is only one muscarinic receptor gene of this type detectable under the described hybridization conditions.

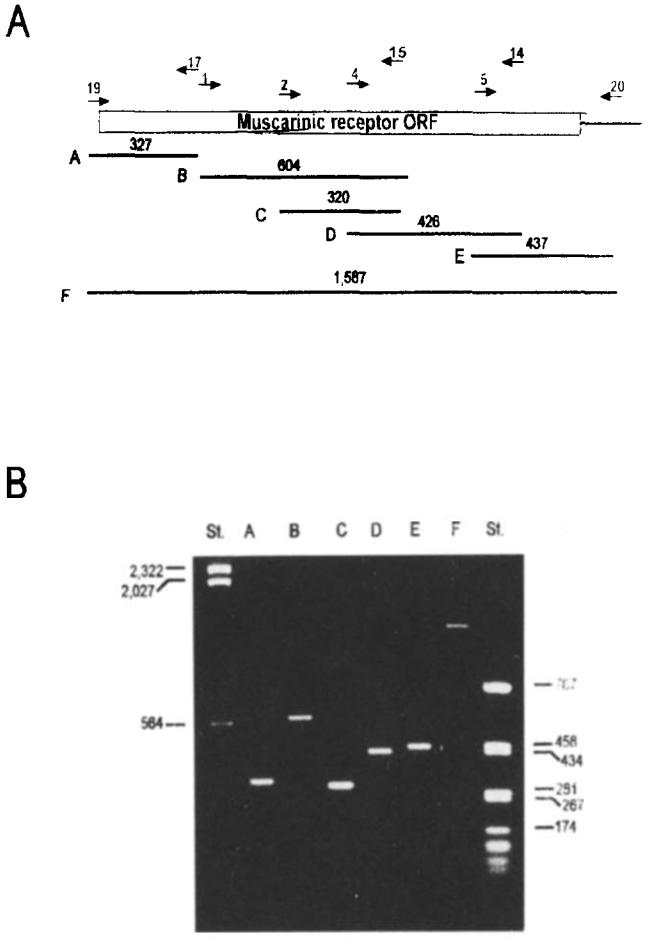

Fig. 3. PCR amplification of *Xenopus laevis* genomic DNA. (A) The diagram shows the *Xenopus* cDNA structure. The box indicates the open reading frame and thin black lines of the 5′ and 3′ non-coding regions. The arrows indicate the oligonucleotide primers used during the PCR amplification. The thick black bars, preceded by capital letters, indicate the amplified DNA fragments, and the numbers below the bars show the expected size of each fragment. (B) Electrophoretic analysis of PCR products. Numbers at the left and at the right indicate the size of DNA standards. Capital letters at the top indicate the PCR amplified products as shown in A.

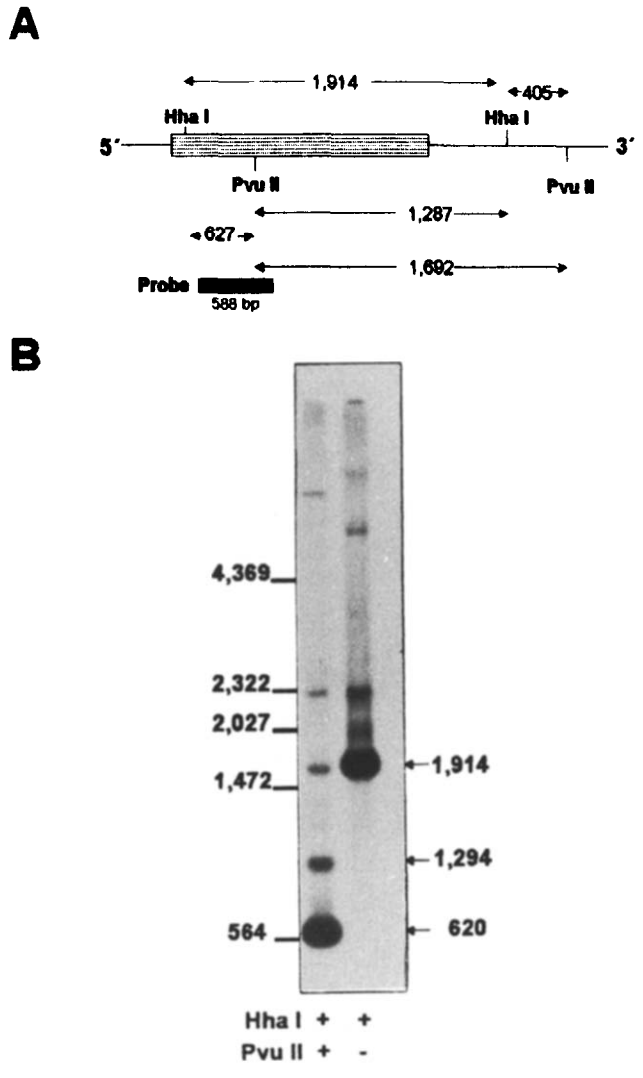

Fig. 4. Southern analysis of *Xenopus* genomic DNA. (A) The diagram shows the *Xenopus* cDNA structure and the *Hha*I and *Pvu*II restriction sites (vertical bars). The grey box indicates the open reading frame and the flanking thin black lines the 5′ and 3′ non-coding regions. The size of the DNA fragments digested by each restriction enzyme are indicated. The thick black bar and the number below it indicate the position at which the probe hybridized and the size of the probe, respectively. (B) Electrophoretic analysis of genomic DNA digested with *Hha*I and *Hha*I plus *Pvu*II. Transferred DNA was probed with the PCR amplified fragment shown in A.

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