

Cloning and sequence analysis of brain cDNA encoding a *Xenopus* D₂ dopamine receptor

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Received 28 January 1991

A D₂ dopamine receptor pharmacologically different from the mammalian D₂ receptor has previously been characterized in the amphibian *Xenopus laevis*. Here we report the cloning of a *Xenopus* D₂ receptor which revealed about 75% amino acid sequence identity with its mammalian counterpart and the presence of an additional 33 amino acid sequence in the 3rd cytoplasmic loop instead of the additional 29 residues in the large form of the mammalian D₂ receptor. All 7 predicted transmembrane domains are highly conserved between the *Xenopus* and mammalian D₂ receptors, as are the 1st and 2nd intracellular loop, the 1st and 3rd extracellular loop and the carboxy-terminal portion of the receptors. The amino-terminal portion, the 2nd extracellular loop and the middle portion of the 3rd intracellular loop of these receptors, however, differ considerably. Knowledge of the locations of these regions of conservation and divergence within the D₂ receptors of *Xenopus* and mammals will help to delineate portions of the receptor molecule that are functionally important. Interestingly, the 5'-untranslated region of the *Xenopus* D₂ receptor mRNA contains 4 small open reading frames which may affect translational efficiency.

Dopamine receptor; cDNA sequence; Background adaptation; *Xenopus* brain

1. INTRODUCTION

The neurotransmitter dopamine regulates the activity of a number of vertebrate neural and endocrine cells. It is thought that disturbances of dopamine concentrations in the brain cause several neurological diseases [1,2]. Dopamine signalling is transduced by 2 pharmacologically different dopamine receptors, the D₁ receptor and the D₂ receptor [3,4]. Recent cloning of a rat D₂ dopamine receptor cDNA showed that this receptor belongs to the family of G-protein-coupled receptors [5]. The structures of the members of this family are characterized by the presence of a core of seven membrane-spanning domains which display significant homology between the family members [6]. Further characterization of D₂ receptor cDNAs and polymerase chain reaction (PCR) analysis of D₂ receptor expression revealed in human, bovine and rat the presence of a 2nd, larger form of the D₂ receptor [7-12]. This large form differs from the first-cloned rat D₂ receptor by an additional stretch of 29 amino acid residues located in the predicted 3rd cytoplasmic loop between transmembrane domains V and VI. The occurrence of the 2 molecular forms of the D₂ receptor is the result of an alternative splicing event, the functional significance of which is not clear.

In amphibians the dopamine receptor is likely to be involved in the regulation of the melanotrope cells of the intermediate pituitary gland during background adaptation of the animal [13]. Melanotrope cells of animals on a black background release α -melanocyte-stimulating hormone (α -MSH) which stimulates dispersion of pigment granules in skin melanophores [14]. In the amphibian *Xenopus laevis* dopaminergic neurons originating from hypothalamic nuclei innervate the melanotrope cells [15,16] and, in animals on a white background, dopamine receptor activation leads to an inhibition of the biosynthetic and secretory activity of the melanotrope cells [17]. This results in an inhibition of α -MSH release and thus in a bleaching of the animal. Specific D₂ dopamine receptor agonists and antagonists have been used to characterize the dopamine receptor present on the membrane of the *Xenopus* melanotrope cells as a D₂-type of dopamine receptor which appears to be pharmacologically somewhat different from the mammalian D₂ receptor [13]. The question arises whether the observed pharmacological differences are due to differences between the structures of the *Xenopus* and mammalian D₂ receptors. As a first step towards a resolution of this question, we used a rat D₂ dopamine receptor cDNA as a probe to isolate a cDNA clone encoding a *Xenopus* D₂ dopamine receptor.

2. MATERIALS AND METHODS

To determine the hybridization conditions for library screening,

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Southern blot analysis of *Xenopus* blood cell genomic DNA was initially performed. On the basis of the results of this analysis a *Xenopus* brain cDNA library in the vector λ gt11 (a generous gift of Drs K. Richter and G. Krell, Salzburg, Austria) was screened with replica nitrocellulose filters at 57°C in 6×SSC (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% SDS, 40 mM sodium phosphate buffer (pH 7.0), 3× Denhardt's solution, 0.1% sodium pyrophosphate, 1 mM EDTA, 100 µg/ml salmon sperm DNA and 8% dextran sulphate. Washing of filters was performed at room temperature and at 57°C in 2×SSC, 0.1% SDS, 0.1% sodium pyrophosphate and 1 mM EDTA. Hybridization probes were a 1.3 kb *KpnI*-*BglII* fragment of rat D₂ receptor cDNA clone pGRB-2 and a 0.55 kb *KpnI*-*PvuII* fragment of pGRB-2 (pGRB-2 is a nearly full-length cDNA clone of 2.4 kb generously provided by Dr O. Civelli, Portland, Oregon, USA) [5]. Probes were labelled with ³²P by nick translation or random priming according to standard procedures [18]. Phage plaques hybridizing with both probes were purified, λDNA was prepared and of the appropriate clones the cDNA was inserted into *EcoRI*-digested pUC18 DNA. DNA sequencing of both strands and with overlapping M13 mp18 and mp19 subclones was performed with the dideoxy chain termination method [19].

3. RESULTS AND DISCUSSION

3.1. The *Xenopus* D₂ dopamine receptor

A *Xenopus* brain cDNA library (1.5×10⁶ clones) was screened under low-stringency hybridization conditions using as a probe a 1.3 kb DNA fragment of rat D₂ dopamine receptor cDNA clone pGRB-2 [5] which covers the nearly complete protein-coding region. This screening gave a total of 140 hybridization-positive signals, 10 of which represented strong positives. Upon rescreening with a 0.55 kb DNA fragment of pGRB-2 encoding the amino-terminal region of the rat D₂ receptor, *Xenopus* brain cDNA clone pHR1XD2 (length of insert 2.1 kb) was selected for further analysis. Fig. 1 shows the nucleotide sequence of pHR1XD2 cDNA which comprises 2068 bp. The longest open-reading frame (ORF) codes for a 442 amino acid protein (relative molecular mass *M_r* 49680). This protein lacks a signal sequence and it contains 7 putative transmembrane regions, a structural characteristic of G-protein-coupled receptors [20]. From the high degree of identity between the amino acid sequence of the pHR1XD2 protein and mammalian D₂ dopamine receptor structures (Fig. 2) we conclude that pHR1XD2 cDNA encodes a *Xenopus* D₂ dopamine receptor. This receptor corresponds to the large form of the D₂ dopamine receptor relative to the first-cloned rat D₂ receptor. Interestingly, the *Xenopus* D₂ receptor contains an additional 33 amino acid sequence in the predicted intracellular domain between transmembrane regions V and VI, whereas in the large form of the mammalian D₂ receptor only an additional 29 amino acids are present in this region. The occurrence of the 2 molecular forms of the mammalian D₂ receptors appears to be the result of alternative splicing, involving an exon of 87 bp in the human, bovine and rat genes [7–12]. In the *Xenopus* D₂ receptor gene a somewhat larger exon of 99 bp is expected to be involved. The fact that the region in the

Xenopus D₂ receptor mRNA which corresponds to the mammalian splice site (GTG/ATTCTT, nucleotides 1279–1287, Fig. 1) does not conform very well to the consensus sequence for splice donor sites (AG/GT^AAGT) [21] might explain why in *Xenopus* the exon–intron splicing event did not occur after 87 bp in this exon. The corresponding splice region within the mammalian D₂ receptor gene sequence (ATG/GTAAGT) [7–9] conforms well to the consensus sequence.

3.2. The 5'-untranslated mRNA region

An unusual feature of the *Xenopus* D₂ receptor mRNA concerns the presence of an exceptionally long 5'-noncoding segment with 4 small ORFs, 3 of which are in-frame with the D₂ receptor-coding sequence (Fig. 1). The existence of these small ORFs means that the ORF encoding the *Xenopus* D₂ receptor protein starts at the 5th AUG codon. This is remarkable because translation of most eukaryotic mRNAs starts at the 1st AUG [22]. Whether the small ORFs are translated and, if so, what the functional role of the resulting peptides might be, remains to be established. It is of interest that the region which contains the most-downstream small ORF (TATGGCTTGAAGAGCT, nucleotides 456–471, Fig. 1) is identical between *Xenopus*, human and rat D₂ receptor mRNAs, whereas the remainder of the 5'-untranslated region is highly divergent between the 3 species (this region is not reported for the bovine receptor). Unfortunately, a full comparison between the *Xenopus* 5'-untranslated segment and the corresponding mammalian regions is not possible since all previously reported D₂ receptor cDNAs contain only relatively short 5'-noncoding regions. Only 5–10% of vertebrate mRNAs have upstream AUG codons; this set of mRNAs is apparently nonrandom in that many mRNAs encoded by protooncogenes, growth-control genes and receptor genes belong to it [23–25]. The role of long 5'-untranslated sequences with upstream AUG codons has not been clearly established in higher eukaryotes. In yeast, however, upstream AUGs repress the efficiency of translation initiation of the long downstream ORF, and thus a function for these sequences at the level of regulation of translation has been suggested [26,27]. The presence of the small upstream ORFs in the *Xenopus* D₂ receptor mRNA would seem to necessitate that the process of translation reinitiation must occur for D₂ receptor translation. The AUG codon of the 3rd small ORF lies in an excellent context for initiating translation (ACCAUGG, nucleotides 355–361, Fig. 1; consensus for strong eukaryotic translation initiation sequence is ^ANNAUGG, whereby N is mostly a C) [22]. The 3rd AUG will thus very likely function as a ribosome start site. Moreover, the AUG start codon of the D₂ receptor is in a rather unfavorable context (UUCAUGG). Therefore, the only reason why

ribosomes can still reach the initiation site of D₂ receptor translation for reading the real message seems to be that terminator codons occur in-frame with the AUG triplets of the small ORFs and that these terminators are upstream from the D₂ receptor AUG start codon. Apparently, the D₂ receptor starting AUG presents itself within a reasonably short distance such that

ribosomes remain attached to the mRNA to start up again. As an alternative to the above, the mRNA region containing the small ORFs could be spliced out in the mature functional D₂ receptor mRNA. If this turns out to be the case, we must have cloned an incompletely spliced RNA. Upstream AUG-containing mRNAs are often produced in several forms due to alternative splic-

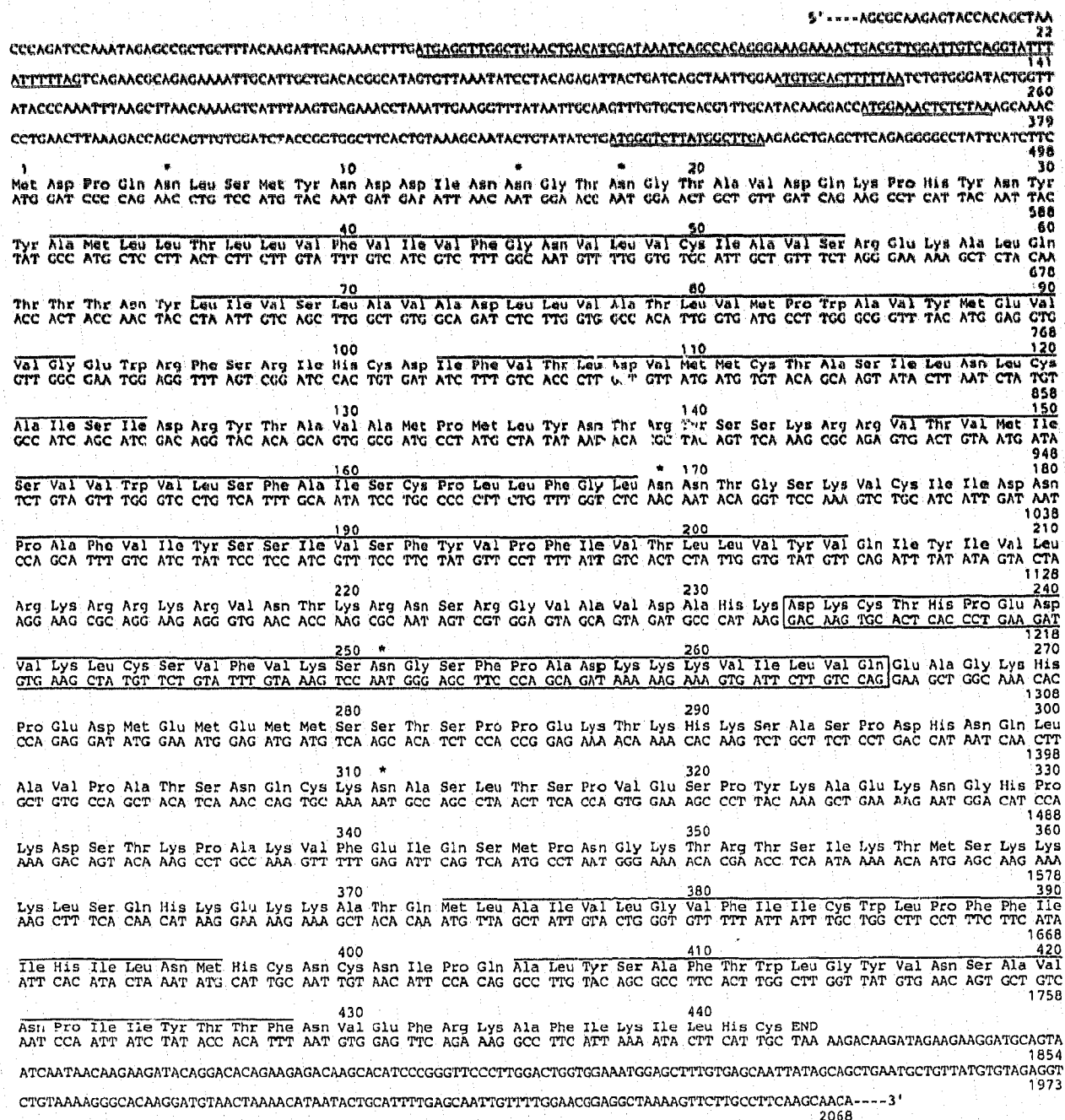


Fig. 1. Nucleotide sequence and deduced amino acid sequence of brain cDNA encoding a *Xenopus* D₂ dopamine receptor. The 4 small open-reading frames in the 5'-untranslated mRNA region are underlined. Predicted transmembrane domains are overlined. The 99-bp/33-amino acid insertion sequence is boxed; the small isoform of the mammalian receptor lacks this sequence [7-12]. Asterisks indicate potential N-linked glycosylation sites [33].

ing [23], and it has been suggested that in proto-oncogene expression upstream AUG codons may be lost during the chromosomal rearrangements that accompany activation of some oncogenes [24]. In this respect it is interesting to note that a 5'-noncoding splice variant of the human D₂ receptor mRNA has recently been described [28]. Whether this finding implies that a critical regulatory step in D₂ receptor gene expression concerns the processing of stable, untranslatable pre-mRNA to functional D₂ receptor mRNA or, alternatively, whether the translational machinery is forced to deal with the upstream AUG triplets, has to await further experimentation.

3.3. Comparison between *Xenopus* and mammalian D₂ dopamine receptors

The overall degree of amino acid sequence identity between the *Xenopus* and (the large forms of the) human, bovine and rat D₂ receptors is 75%, 75% and 74%, respectively, while on the nucleotide sequence level in the protein-coding regions the identity is 67%, 68% and 65%, respectively. The evolutionary span between these species is about 350 million years. No significant nucleotide sequence identity was found between the 5'-untranslated nor the 3'-untranslated regions of the D₂ receptor mRNAs of the 4 species, except for the short sequence in the 5'-untranslated region (see above). All predicted transmembrane domains are highly conserved as are the regions between

domains I and II (first intracellular loop), II and III (first extracellular loop), III and IV (second intracellular loop), VI and VII (third extracellular loop), and the carboxy-terminal portion of the receptors. In contrast, the amino-terminal region as well as the 2nd extracellular loop between domains IV and V, and most of the 3rd cytoplasmic loop between domains V and VI display a low degree of sequence identity. Despite the low homology between the amino-terminal portions of the D₂ receptors, the 3 potential sites for N-linked glycosylation located in this region are present in all 4 species. In the 2nd extracellular loop and the 3rd intracellular loop, however, not all potential glycosylation sites are conserved (Fig. 2). Interestingly, the most conserved regions within the 3rd cytoplasmic loop, namely those flanking transmembrane domains V and VI, are believed to be involved in binding of the receptor to a G-protein, possibly in conjunction with the well-conserved 2nd cytoplasmic loop [29]. Except for the amino- and carboxy-terminal ends, most of the third cytoplasmic loop, including the region containing the site of deletion/insertion of the 33 amino acid residues (29 residues in the mammalian receptors), constitutes one of the least conserved regions of the D₂ receptor. This low degree of identity is pertinent to the question of whether the alternative splicing event is of functional significance (cf. [8]). Aspartates 80, 108 and 114 (numbering for mammalian receptors) and the regions surrounding these residues in transmembrane



Fig. 2. Alignment of the amino acid sequences of *Xenopus*, human and (the small isoform of the) rat D₂ dopamine receptors. The one-letter amino acid notation is used. Gaps (—) have been introduced to achieve maximum identity. Residues identical among the receptor structures of the three species are boxed as are the residues identical between the *Xenopus* and human receptors in the insertion sequence. Predicted transmembrane domains I to VII are overlined. Asterisks indicate potential N-linked glycosylation sites [33]. The dot indicates a potential cAMP-dependent protein kinase phosphorylation site [34]. Triangles denote the ends of the insertion sequence of the mammalian receptor.

domains II and III are identical among the 4 species, which is in line with their proposed important role in binding of the cationic amine to the receptor [30]. Similarly, several serine and threonine residues in the 3rd cytoplasmic loop are well conserved; these residues could serve as sites for phosphorylation and they might therefore be involved in the regulation of D₂ receptor functioning [31]. The conservation of all cysteine residues might reflect the important role for these amino acids in establishing the tertiary structure of the D₂ receptor [32]. On the basis of our comparative analysis we conclude that the observed patterns of conservation and divergence of regions within the D₂ receptor protein will help to delineate the portions of the receptor molecule which are important for proper receptor functioning.

In *Xenopus* it appears that, through D₂ receptor triggering, dopamine regulates the activity of the melanotrope cells of the intermediate pituitary gland during the process of background adaptation of the animal [13]. Preliminary results of PCR analysis using primers directed against portions of the 3rd and 6th transmembrane domains of the *Xenopus* D₂ receptor have revealed that, as in mammals [5,8], the D₂ receptor is expressed in the *Xenopus* intermediate pituitary. Structure-function studies are in progress to examine the relationship between the structural differences described here and the previously reported [13] different pharmacological characteristics of the *Xenopus* and mammalian D₂ receptors.

Acknowledgements: This work was supported by the Netherlands Organization for Scientific Research (NWO). We thank Dr O. Civelli for providing rat D₂ receptor cDNA clone pGRB-2 and Dr B.G. Jenks for critical reading of the manuscript.

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