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IDENTIFICATION OF A NOVEL MEMBER OF THE NUCLEAR RECEPTOR SUPERFAMILY WHICH IS CLOSELY RELATED TO REV-ERBA

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SUMMARY: Degenerate PCR primers were designed based upon conserved regions within the DNA and ligand binding domains of several nuclear receptors. PCR was performed using these primers starting with total brain cDNA. Several members of the nuclear receptor superfamily were identified, one of which was novel, but showing a high degree of homology to the previously known orphan receptor Rev-ErbA. This new orphan receptor, Rev-ErbAß, was further characterised, and a cDNA with an intact open reading frame was isolated from a rat brain cDNA library. Northern blot analysis shows two different sizes of mRNA, 4 and 5.5 kb, respectively, in all tissues analysed. A more detailed study of this orphan was then performed using in situ hybridisation, showing a high expression of the new orphan receptor in particular in the cerebellum, the dentate gyrus of the hippocampus and pituitary gland of adult rats.

INTRODUCTION: The superfamily of nuclear receptors has continued to grow over the past years to encompass more than 40 members from various species as of today (1). The vast majority of these novel receptors are "orphans", for which a function and/or a ligand is still unknown. Some investigators have proposed that many orphans are expressed in the central nervous system (2). We are interested in the regulation of P450 isoforms in the brain (3), and since nuclear receptors appear to be important in P450 control (4) we decided to examine the complexity of nuclear receptors in the brain. A comparison of the amino acid sequence from 20 different nuclear receptors reveals regions exhibiting a high degree of homology. In addition to the highly conserved residues within the DNA-binding region there are some short stretches in the so-called Ti-domain that are well conserved, in particular within the RAR/T_3R subfamily of receptors. We have used PCR primers for two of these conserved regions to perform PCR on cDNA from total rat brain.

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In the present study we report the isolation of a novel orphan receptor using this method. We have furthermore isolated a nearly full length cDNA for the new receptor from a cDNA library and investigated its tissue distribution by means of Northern blot analysis and in situ hybridisation.

MATERIALS AND METHODS

PCR: Two sets of oligonucleotides were synthesised (Fig. 1). Commercially available cDNA from total rat brain (Clontech) was subjected to PCR using approximately 2 ng of cDNA as starting material. 100 pmoles of each oligonucleotide set was used in a total volume of 100µl. The first 3 cycles of the PCR were run using 1 min at 94°, 2 min at 40°, 2 min at 72°, followed by 35 cycles using 1 min at 94°, 2 min at 60° and 2 min at 72°. After a final elongation step of 10 min at 72°, an aliquot was analysed on gel. The remaining product was phenol extracted and ethanol precipitated. After digestion with SalI and NotI, the product was subcloned into Bluescript vector. The resulting clones were sequenced using T3 and T7 primers (Stratagene) and cycle sequencing with fluorescent terminators (Applied Biosystems).

Library screening: $5x10^5$ pfu of an oligo-dT-primed λZAP-library from rat brain (Stratagene) were plated and screened under high stringency conditions with the whole insert of one of the two PCR clones representing a novel orphan receptor. One positive clone was isolated and the cDNA was subcloned by in vivo excision as described by the manufacturer. The clone was then restriction mapped and sequenced, using a series of internal oligonucleotide primers, by cycle sequencing with fluorescent terminators.

Northern blot: A rat Multiple Tissue Northern Blot (Clontech) containing 2 µg of poly-A-RNA per lane was hybridised with the same probe as was used in the library screening and washed under high stringency conditions. The blot was then exposed for 5 days with two intensifying screens.

In situ hybridisation: Three Sprague-Dawley rats were decapitated after which their brains and various peripheral organs were excised and frozen on a block of dry ice. Coronal, sagittal

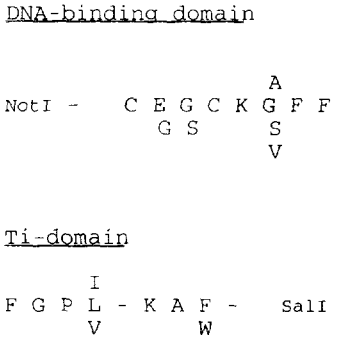

Figure 1.
Schematic view of the sequences used for PCR amplification of members of the nuclear receptor superfamily. When more than one amino acid is represented at one position in the sequence the respective oligos were synthesised separately. To facilitate cloning of the PCR products, the recognition sequences of two infrequently cutting enzymes, NotI and SalI, were added to the oligonucleotides.

and horizontal sections representing different parts of the brain and sections of the peripheral organs were then cut with a Microm HM500 cryostat and thawed onto ProbeOn (Fischer Scientific, Pennsylvania, USA) glasses. Two oligonucleotide probes directed against nucleotides 544-591 and 937-984 of the Rev-ErbAß gene, were synthesised (Institute of Biotechnology, University of Kuopio, Finland). The probes exhibited less than 60% homology with any other known gene, when compared against the known sequences in the GenBank database. The oligonucleotides were labelled with [α-^{33}P]-dATP (NEN, Boston, Ma, USA) using terminal deoxynucleotidyltrans-ferase (Amersham Int., Buckinghamshire, UK) as previously described (5). The sections were briefly air dried and hybridised at 42° C for 18 h with 10 x 10^6 cpm/ml of the probe in a mixture containing 4 x SSC (1 x SSC=0.15 M NaCl, 0.015 M sodium citrate), 50% formamide, 1 x Denhardt's solution (0.02% polyvinyl-pyrrolidone, 0.02% bovine serum albumin and 0.02% Ficoll), 1% sarkosyl, 0.02 M phosphate buffer (pH 7.0), 10% dextran sulphate, 500 µg/ml heat denatured salmon sperm DNA and 200 mM dithiothreitol. After hybridisation, the sections were rinsed 4 times at 55°C in 1 x SSC for 15 min at a time and subsequently let to cool for 1 h at room temperature. The sections were then dipped in distilled water, dehydrated with 60 and 90% ethanol and air dried. Thereafter the sections were covered with Amersham ß-max autoradiography film (Amersham Int.) The autoradiography films were developed using LX24 developer and AL4 fixative (Kodak, Rochester, NY, USA).

Controls included the use of a hybridisation mixture containing 100 molar excess of unlabelled probe, which abolished all signal, and the use of several non-related probes, which gave different results to those seen with the probes directed against the Rev-ErbAß mRNA. Furthermore, the two different Rev-ErbAß probes gave identical results, ensuring the specificity of the hybridisations.

RESULTS AND DISCUSSION

PCR using degenerate primers to the DNA-binding domain and to the Ti-domain gave an array of fragments ranging in size from 400 to more than 1500 base pairs (data not shown). The fragments were subcloned, and 39 of the resulting clones were partially sequenced. More than 50% of these clones turned out to represent either NGFI-B (6) or the rat form of Nurr1 (2), which is probably an isoform of NGFI-B. Two of the clones, however, show about 55 % overall homology to the Rev-ErbA orphan receptor (7). To further characterise this novel orphan, a nearly full-length clone was isolated from a rat brain cDNA library. The length of the clone is 3645 bp, and it was sequenced completely (Fig. 2). The clone contains an open reading frame of 533 amino acids followed by a long 3' -nontranslated region ended by a poly-A-signal and a poly-A-tail. There are three in-frame methionines in the amino-terminal part of the receptor. The sequence surrounding the first of these methionines is, for 3 out of 5 positions, identical to the Kozak consensus for translational start sites (8). As there are no stop codons in the sequence preceding this position the possibility exists that the new orphan may have a longer amino-terminal domain than present in the clone we have isolated.

When comparing our sequence to those of other members of the nuclear receptor superfamily it seems likely that we have isolated an isoform of the Rev-ErbA receptor, since

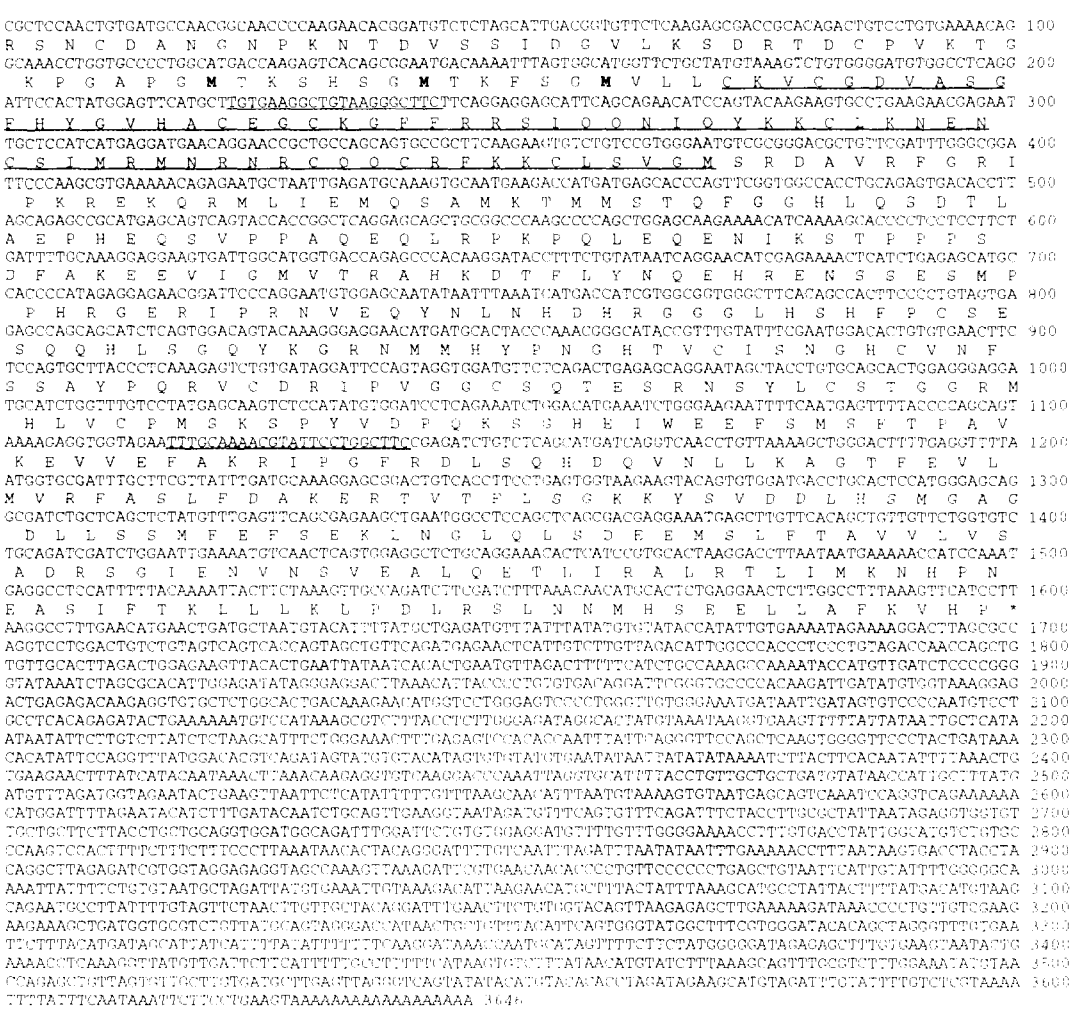

Figure 2.
Nucleotide sequence and deduced amino acid sequence of the Rev-ErbAß cDNA clone. The DNA-binding domain and the positions of the two oligonucleotides used in the cloning are underlined. Bold typing indicates the three potential translational starting methionines in the amino-terminal domain.

our receptor is almost 100% identical to Rev-ErbA in its DNA-binding domain (Fig. 3). The part of the carboxy-terminal domain that is claimed to be responsible for dimerization and ligand binding (1) also shows a high degree of conservation, about 72%. The other parts of our new receptor show little or no homology to Rev-ErbA . Taken together, these sequence comparisons lead us to suggest the name Rev-ErbAß for the new receptor and, consequently, Rev-ErbAα would be an appropriate name for the previously isolated Rev-ErbA variant.

Interestingly, Rev-ErbAß shows 78% homology in the DNA-binding domain to E75A, one of the orphan receptors from *Drosophila* that are induced by ecdysone (9) (Fig. 4). Also

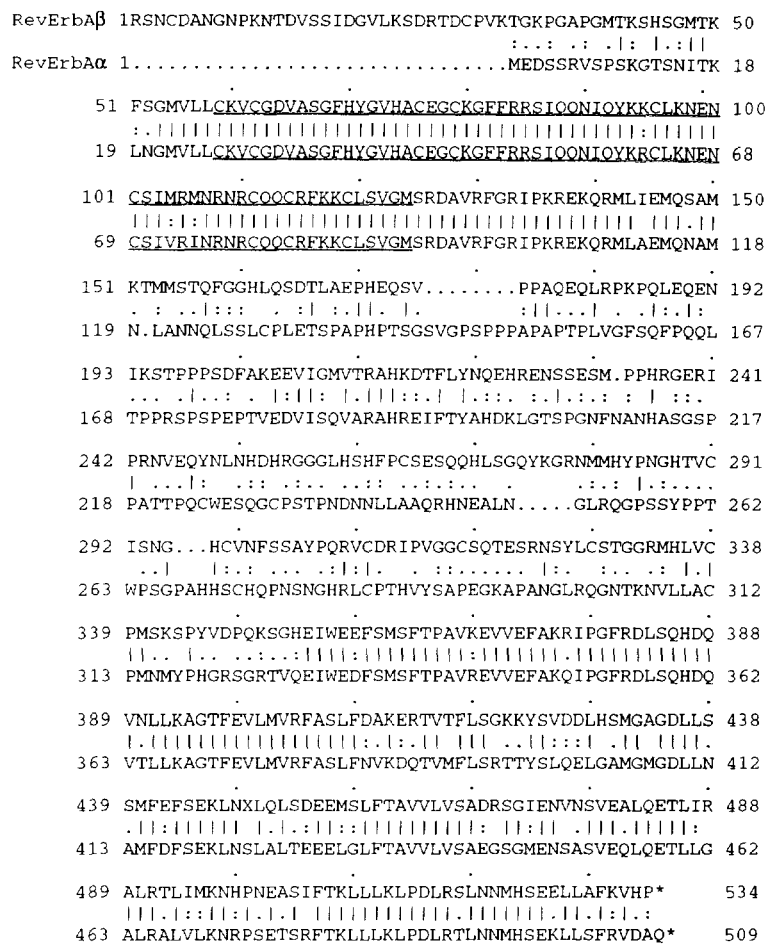

Figure 3.
Alignment of the rat Rev-ErbAβ amino acid sequence to that of rat Rev-ErbAα. Alignment was made using the program GAP of the UW GCG package. Vertical lines indicate identity, and one or two dots represent conservative amino acid replacements. The DNA-binding domain is underlined.

rat PPARα (10) shows a relatively high degree of homology in the DNA-binding domain. Since there are even further examples of receptors that show higher homology to Rev-ErbAβ than the thyroid hormone receptors, it seems justified to state that neither Rev-ErbAβ nor Rev-ErbAα are thyroid hormone receptors in the classical sense.

Northern blots were carried out using RNA from different tissues. Under high stringency conditions, two bands are visible, one strong band with a size corresponding to about 5.5 kb, and a weaker band of 4 kb (Fig. 5). Both these bands are visible in all tissues examined, with the highest expression in skeletal muscle, kidney and heart. Only very weak expression is seen in testis.

% AMINO ACID IDENTITY TO RevErbAβ

	A/B	DBD	HINGE	TI-DM
rRevErbAα	18	96	30	72
dE75A	22	78	28	38
rPPARα	18	68	16	35
mRARα	22	64	18	33
dDHR3	22	62	17	29
rNGFI-B	27	61	15	28
hERR2	24	61	15	25
hERR1	19	61	14	28
dEcR	24	58	19	33
rT3Rα1	18	55	19	33

Figure 4.
Comparison of the Rev-ErbAß sequence to those of members of the nuclear receptor superfamily. Alignment was made using the programs FASTA and GAP of the UW GCG package. The scores for the 10 receptors that show highest homology in the DBD are shown. When several isoforms exist only the score for one of these is shown. The division between "hinge" domain and Ti-DM domain is from Laudet et al. (1).

It has been shown for many members of the nuclear receptor superfamily that start of transcription may occur from several different promoters giving alternate 5' untranslated regions and/or amino-terminal domains (11,12). Our attempts to isolate the 5' end of both these transcripts have so far been unsuccessful.

To learn more about the expression of Rev-ErbAß in different parts of the brain and to further refine the tissue distribution studies we have performed in situ hybridisation. Using two oligonucleotide probes corresponding to unique regions of Rev-ErbAß within its hinge region. The most intense expression of Rev-ErbAß in the brain was seen in the granular cells of the cerebellar cortex (Fig. 6a), and in the dentate gyrus and areas CA1-CA4 of the hippocampal formation (Fig. 6b). Rev-ErbAß mRNA was seen in smaller amounts also in other brain areas. Of the other organs examined Rev-ErbAß mRNA was expressed in a high amount in the anterior and intermediate lobes of the pituitary. In addition, moderate amounts of Rev-ErbAß mRNA were seen in the liver, testis, symphatetic and sensory ganglia, the thyroid and parathyroid glands, the adrenal cortex, and in the intestinal epithelium (data not shown). Small amounts of Rev-ErbAß mRNA were also seen in the spleen and thymus.

It is interesting to note, that this tissue distribution is somewhat different to that of RevErbAα, which also seems to be rather ubiquitously expressed, but with its highest expression in muscle, brain and adipose tissue.

The Rev-ErbAα orphan receptor, to which our novel orphan seems to be a close relative, was first isolated by means of its unique genomic localisation, on the non-coding strand of the exon that gives rise to the α2 form of the thyroid hormone receptor. This thyroid

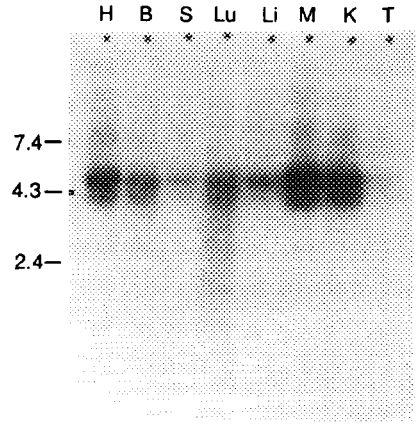

Figure 5.
Northern blot analysis of Rev-ErbAß tissue distribution. The RNAs used are from left to right: H- heart; B- brain; S- spleen; Lu- lung; Li- liver; M- skeletal muscle, K- kidney; T- testis. The sizes given are in kilobase pairs.

hormone receptor form does not bind hormone (13), due to replacement of the carboxy-terminal part of the ligand binding domain with a sequence that shows no homology to the corresponding part of other receptors. No ligand has so far been identified for Rev-ErbAα and thus its physiological role has for a long time been a puzzle. Recently, however, it has been shown to play a role in adipocyte differentiation (15). Two different reports have defined the response element of Rev-ErbAα as either a thyroid hormone response element (14), or a unique 11 bp sequence, to which it is claimed to bind as a monomer (16).

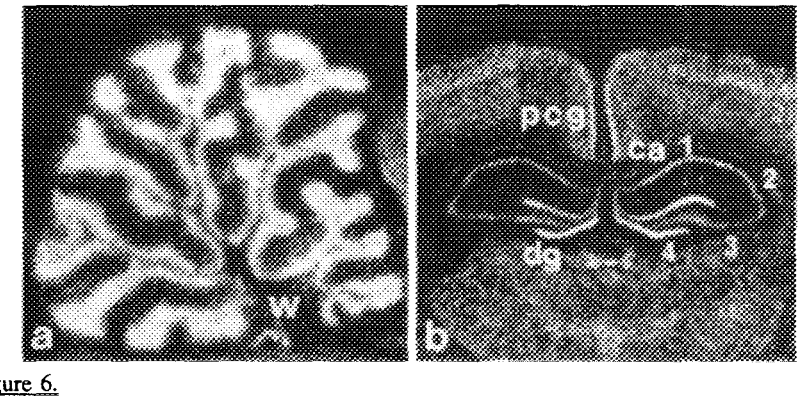

Figure 6.
Film radioautographs demonstrating Rev-ErbAß mRNA in the cerebellar cortex (a), and in the dentate gyrus (dg) and areas CA1-CA4 of the hippocampus (b). Rev-ErbAß mRNA can also be seen in the post cingulate cortex (pcg). No radioactive signal can be observed in the white matter (W) of the cerebellum.

Our finding, that at least one isoform of Rev-ErbAα exists, which is highly expressed in many different tissues, supports the notion that these two receptors have important biological roles in their own right, not just by representing an interesting genetic phenomenon, as was originally suggested for Rev-ErbAα.

During the preparation of this manuscript, a sequence (17) was submitted to the GenBank data base showing 88% overall homology to Rev-ErbAß at the amino acid level, probably representing the human form of Rev-ErbAß.

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