

# Localization of the human FSH receptor to chromosome 2 p21 using a genomic probe comprising exon 10

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## ABSTRACT

Screening of a human genomic library with a cDNA probe corresponding to the transmembrane domain of the FSH receptor (FSHR) resulted in the identification of a positive clone with a DNA insert of approximately 17.5 kb. Part of the clone encoded exon 10 of the FSHR gene. Sequence analysis of this exon revealed an open reading frame corresponding to base positions 855–2085 of the FSHR cDNA, thereby coding for 410 amino acids. Exon 10 was found to comprise the seven transmembrane domains, the C-terminal intracellular domain and a fragment of 81 amino acids belonging to the

extracellular N-terminal domain of the FSHR. The exon/intron boundary is in phase 2 and the amino acid which resides in this junction is isoleucine. The genomic clone was used to map the chromosomal localization of the human FSHR gene. *In situ* hybridization experiments allowed the allocation of the human gene to chromosome 2 p21. As this position is identical to that of the human LH receptor gene, these two receptor genes may have evolved from a common ancestor.

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## INTRODUCTION

Follicle-stimulating hormone (FSH) is a heterodimeric glycoprotein which is synthesized in the pituitary gland. Like other dimeric glycoprotein hormones, luteinizing hormone (LH)/choriogonadotrophin and thyroid-stimulating hormone (TSH), it consists of a common  $\alpha$  subunit and a  $\beta$  subunit which bestows biological specificity (Pierce & Parsons, 1981; Ryan *et al.* 1988). FSH is essential for normal reproductive functions, e.g. initiation and maintenance of spermatogenesis in the male and regulation of follicular maturation in the female.

The biochemical effects of FSH are mediated by its interaction with specific receptors expressed by Sertoli cells of the seminiferous tubules in the testis or by granulosa cells in the ovary. Upon hormone binding, the receptor activates the guanine nucleotide-binding protein  $G_s$  which stimulates the enzyme adenylyl cyclase as an intracellular effector system (Johnson & Dhanasedaran, 1989). The glycoprotein hormone receptors belong to the superfamily of G protein-coupled receptors which

are characterized by the common structural feature of seven transmembrane domains. They differ structurally from other G protein-coupled receptors, however, in that they contain a large extracellular domain in the N-terminal part of the polypeptide which is required for interaction with complex glycoprotein hormones (McFarland *et al.* 1989; Parmentier *et al.* 1989; Sprengel *et al.* 1990).

Recent studies on the genomic organization of the glycoprotein hormone receptors revealed that the region of the receptors comprising the seven transmembrane domains and the intracellular C-terminal tail is encoded by one large exon, whereas the extracellular domain is encoded by nine exons for the rat FSH receptor (FSHR) (Heckert *et al.* 1992) and human TSH receptor (TSHR) (Gross *et al.* 1991) and ten exons in the case of the rat LH receptor (LHR) (Koo *et al.* 1991). *In situ* chromosomal hybridization experiments have shown that the human LHR gene can be mapped to chromosome 2 p21 (Rousseau-Merck *et al.* 1990a) and that the human TSHR gene can be localized on chromosome 14 q31 (Rousseau-Merck *et al.* 1990b).

Determination of the chromosomal localization of the FSHR gene is an important step in the systematic investigation of the possible role played by the FSHR in cases of male idiopathic infertility. We therefore screened a genomic library to obtain a large clone of the FSHR gene which could then be used to identify the chromosomal localization of the gene.

## MATERIALS AND METHODS

### Screening of a human genomic library and sequencing of inserts

The DNA probe used to screen a genomic library corresponded to nucleotides 1019–2179 of the human FSHR sequence (Kelton *et al.* 1992) and was generated according to the protocol described previously (Gromoll *et al.* 1992). The probe was radioactively labelled using the random primer technique and [ $\alpha$ - $^{32}$ P]dCTP (Amersham, Braunschweig, Germany) in the presence of unlabelled dATP, dTTP and dGTP to a specific activity of  $1 \times 10^8$  c.p.m./ $\mu$ g template DNA (Sambrook *et al.* 1989).

A human genomic EMBL3 library was screened for the transmembrane domain of the FSHR. Using *E. coli* C600 *hflA* as host cells and an approximate density of 30 000 plaque-forming units per 150 mm plate we screened approximately 500 000 plaque-forming units with the DNA probe described above. Two replicate membrane filters were obtained from each plate and treated further as described elsewhere (Levy *et al.* 1992). The phage DNA was finally fixed to the nylon membranes by baking at 80 °C for 2 h. Replicate filters with amplified recombinant phage DNA were prehybridized for 3 h at 65 °C in a solution containing 5  $\times$  Denhardt's solution (1  $\times$  Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% BSA), 6  $\times$  SSC (1  $\times$  SSC is 150 mM NaCl and 15 mM sodium citrate), 0.5% SDS, 100  $\mu$ g denatured salmon sperm DNA/ml and 0.3 M sodium phosphate buffer, pH 7.0. Hybridization was carried out for 16 h at 65 °C in the same solution with  $1 \times 10^6$  c.p.m. labelled DNA probe/ml. The filters were rinsed three times for 30 min at 60 °C with 2  $\times$  SSC, 0.5% SDS followed by a final wash for 30 min at 65 °C with 0.2  $\times$  SSC, 0.5% SDS. The filters were dried and

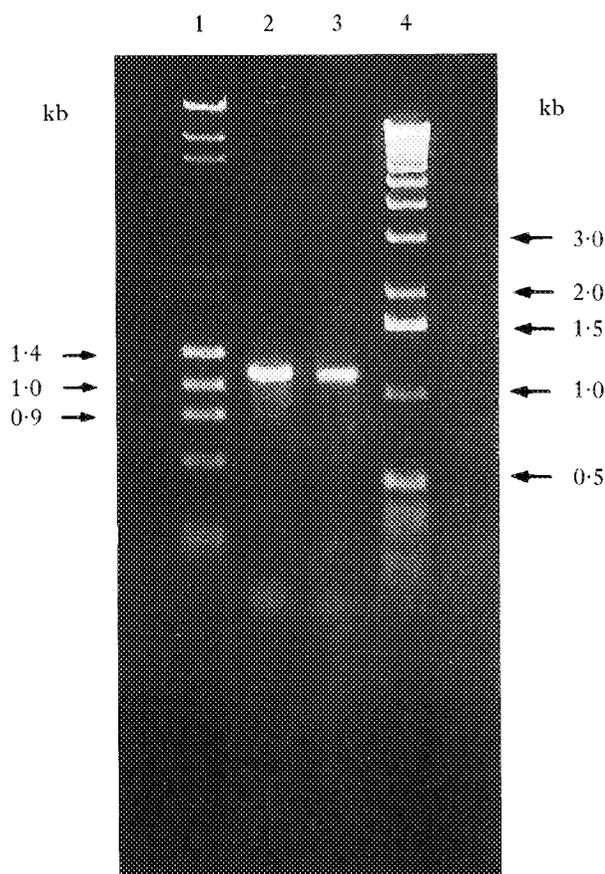


FIGURE 1. PCR amplification of the transmembrane domain of the human FSHR. Lane 1: DNA markers; lane 2: DNA of clone 7.II.B; lane 3: genomic DNA; lane 4: DNA markers. The amplified products were subjected to 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

exposed to Kodak X-Omat AR film with two intensifying screens at -70 °C for 1 day.

One positive clone (7.II.B) was isolated, the plaque was purified and phage DNA was prepared according to common protocols (Sambrook *et al.* 1989). The genomic insert was removed from the EMBL3 vector by digestion with the restriction endonuclease *Sal*I. The DNA fragment was then further digested with *Ssp*I and inserted into pBluescript SK(-) (Stratagene, Heidelberg, Germany) by blunt end ligation. Both strands of the subcloned DNA insert were directly sequenced by the dideoxy chain-termination method of Sanger *et*

FIGURE 2. Nucleotide sequence and deduced amino acid sequence of exon 10 of the human FSHR. The seven transmembrane domains are boxed (TM I–TM VII). Intronic sequences at the 5' end of exon 10 are written in lower case. Oligonucleotides used for the PCR amplification are underlined.

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← 2.0

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tctcaggaagaaactcatcatttctaccctgcacaaagacag 855

856 TCTGAGCTTCATCCAATTTGCAACAAATCTATTTTAAGGCAAGAAGTTGATTATATGACT  
S E L H P I C N K S I L R Q E V D Y M T

916 CAGACTAGGGGTCAGAGATCCTCTCTGGCAGAAGACAATGAGTCCAGCTACAGCAGAGGA  
Q T R G Q R S S L A E D N E S S Y S R G

976 TTTGACATGACGTACACTGAGTTTGACTATGACTTATGCAATGAAGTGGTTGACGTGACC  
F D M T Y T E F D Y D L C N E V V D V T

1036 TGCTCCCCTAAGCCAGATGCATTC AACCCATGTGAAGATATCATGGGGTACAACATCCTC  
C S P K P D A F N P C E D I M G Y N I L

1096 AGAGTCTCTGATATGGTTTATCAGCATCCTGGCCATCACTGGGAACATCATAGTGCTAGTG TM I  
R V L I W F I S I L A I T G N I I V L V

1156 ATCCTA ACTACCAGCCAATATAAACTCACAGTCCCCAGGTTTCCTTATGTGCAACCTGGCC TM II  
I L T T S Q Y K L T V P R F L M C N L A

1216 TTTGCTGATCTCTGCATTGGAATCTACCTGCTGCTCATTGCATCAGTTGATATCCATACC  
F A D L C I G I Y L L L I A S V D I H T

1276 AAGAGCCAATATCACAACTATGCCATTGACTGGCAAACCTGGGGCAGGCTGTGATGCTGCT TM III  
K S Q Y H N Y A I D W Q T G A G C D A A

1336 GGCTTTTTCACTGTCTTTGCCAGTGAGCTGTCACTTACACTCTGACAGCTATCACCTTG  
G F F T V F A S E L S V Y T L T A I T L

1396 GAAAGATGGCATAACCATCACGCATGCCATGCAGCTGGACTGCAAGGTGCAGCTCCGCCAT  
E R W H T I T H A M Q L D C K V Q L R H

1456 GCTGCCAGTGTCAATGGTGATGGGCTGGATTTTGGCTTTGTCAGCTGCCCTCTTTCCCATC TM IV  
A A S V M V M G W I F A F A A A L F P I

1516 TTGGCATCAGCAGCTACATGAAGGTGAGCATCTGCCTGCCATGGATATTGACAGCCCT  
F G I S S Y M K V S I C L P M D I D S P

1576 TTGTCACAGCTGTATGTATGTCCCTCCTTGTGCTCAATGTCCTGGCCCTTGTGGTCACT TM V  
L S Q L Y V M S L L V L N V L A F V V I

1636 TGTGGCTGCTATATCCACATCTACCTCACAGTGCAGAACCCCAACATCGTGTCTCTCTCT  
C G C Y I H I Y L T V R N P N I V S S S

1696 AGTGACACCAGGATCGCCAAGCGCATGGCCATGCTCATCTTCACTGACTTCTCTGCATG TM VI  
S D T R I A K R M A M L I F T D F L C M

1756 GCACCCATTTCTTTCTTTGCCATTTCTGCCTCCCTCAAGGTGCCCTCATCACTGTGTCC  
A P I S F F A I S A S L K V P L I T V S

1816 AAAGCAAAGATTCTGCTGGTTCTGTTTCAACCCATCAACTCCTGTGCCAACCCCTTCTCTC TM VII  
K A K I L L V L F H P I N S C A N P F L

1876 TATGCCATCTTTACCAAAAACCTTTCGCAGAGATTTCTTCATTCTGCTGAGCAAGTGTGGC  
Y A I F T K N F R R D F F I L L S K C G

1936 TGCTATGAAATGCAAGCCAAATTTATAGGACAGAAACTTCATCCACTGTCCACAACACC  
C Y E M Q A Q I Y R T E T S S T V H N T

1996 CATCCAAGGAATGGCCACTGCTCTTACGCTCCCAGAGTCACCAGTGGTTCCACTTACATA  
H P R N G H C S S A P R V T S G S T Y I

2056 CTTGTCCCTCTAAGTCATTTAGCCCAAACATAAACAATGTGAAAATGTATCTGAGTA  
L V P L S H L A Q N END

2116 TTGAATGATAAATTCAGTCTTGCCTTTGAAGGGTATGTCACAAGGAGCTGACAGTGCTT  
2166 CTACACATTTTCATCTAATTTAATATT

*al.* (1977), using alkali-denatured double-stranded plasmid DNA as template and applying the primer walk technique as described by Gudermann *et al.* (1992). Exon 10 and an additional 300 bp at the 5' end of this exon were sequenced.

### PCR amplification

PCRs were performed using oligonucleotide F2 as a forward primer (corresponding to nucleotides 1019–1038 of the human FSHR cDNA plus an external EcoRI restriction site and two additional nucleotides, TC) and R2 as a reverse primer (corresponding to nucleotides 2160–2179 of the human FSHR cDNA plus an external KpnI restriction site and two additional nucleotides, TC). The reaction mixtures were incubated using a Hybaid temperature cycler for 30 cycles of 94 °C for 50 s, 65 °C for 1 min and 72 °C for 1.5 min, followed by a final extension step at 72 °C for 10 min. PCR products were size-fractionated on 1% agarose gels and visualized by ethidium bromide staining.

Human blood leucocyte DNA was isolated using the phenol extraction method according to standard protocols (Sambrook *et al.* 1989). Written consent was obtained from patients to perform experiments on DNA extracted from human blood cells.

### In situ hybridization

Human metaphase spreads were prepared from 5-bromodeoxyuridine (BrdU)-synchronized cultures (Speit, 1984; Fan *et al.* 1990). Briefly, cells were incubated with methotrexate for 17 h at 37 °C. Blood leukocytes were collected by centrifugation, resuspended in fetal calf serum (FCS)-free medium and washed twice with FCS-free medium. The block was released by adding BrdU (30 µg/ml, final concentration).

DNA of the positive phage clone (7.II.B) was labelled with biotin-11-dUTP (Sigma, Deisenhofen, Germany) in a standard nick translation reaction. Phage DNA (80 ng) was coprecipitated with 5 µg human C<sub>0</sub>t<sub>1</sub> DNA (BRL, Eggenstein, Germany) and 10 µg salmon sperm DNA, resuspended in 10 µl hybridization solution (50% formamide, 2 × SSC, 10% dextran sulphate) and denatured for 5 min at 76 °C. Preannealing was allowed for 30 min at 37 °C. The probe was added to previously denatured chromosome preparations (denatured in 70% formamide, 2 × SSC for 2 min at 80 °C, then dehydrated through a series of increasing ethanol concentrations). A coverslip was added and sealed with rubber cement. Hybridization was allowed overnight at 37 °C. The coverslip was removed and the slides were washed in 50%

formamide, 2 × SSC at 42 °C (3 × 5 min), followed by three washes in 0.1 × SSC at 60 °C. A blocking step was included (3% BSA in 4 × SSC for 30 min at 37 °C).

The biotinylated probe signals were detected with avidin conjugated to fluorescein (Vector Laboratories, Burlingame, CA, U.S.A.), followed by three washes in 4 × SSC, 0.1% Tween 20 at 37 °C (5 min each). The chromosomes were counterstained in 4,6-diamidino-2-phenylindole (DAPI; 150 ng/µl) and mounted in antifade (DABCO; Sigma). Images were recorded with a Zeiss Axiophot epifluorescence microscope coupled to a cooled CCD (charge-coupled device) camera (PM 512; Photometrics, Tucson, AZ, U.S.A.). The sequentially acquired gray scale images were converted to a tint scale and merged using computer software as described previously (Ried *et al.* 1992). Photographs were taken directly from the computer screen.

### RESULTS

Screening of a human genomic EMBL3 library using a cDNA probe covering the transmembrane domain of the human FSHR resulted in the identification of one genomic clone (7.II.B) containing a DNA insert of approximately 17.5 kb. The clone was further characterized by PCR. Using the same oligonucleotides that were originally applied to amplify the cDNA of the transmembrane domain of the human FSHR (Gromoll *et al.* 1992), a DNA fragment of 1.1 kb could be amplified from the genomic clone 7.II.B. This fragment was identical in size to the amplified cDNA fragment obtained previously (Gromoll *et al.* 1992). Furthermore, amplification of genomic DNA using the same PCR primers also resulted in a DNA fragment of 1.1 kb (Fig. 1). As the various PCRs displayed the same fragment size for the amplified product, it could be concluded that no intronic sequences were present within the region of genomic DNA that was analysed. Therefore, the genomic clone was likely to contain exon 10 of the human FSHR gene. According to the genomic organization of the rat FSHR (Heckert *et al.* 1992), this is the only exon that can give rise to a fragment of more than 1 kb when amplified by PCR.

Clone 7.II.B was characterized further by sequence analysis. An open reading frame from nucleotide 855 to nucleotide 2085 (positions correspond to the published cDNA sequence by Kelton *et al.* 1992) encoding 410 amino acids could be detected (Fig. 2). The sequence was found to be identical to the published cDNA sequence of

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FIGURE 3. (a) Human metaphase spread after hybridization with the genomic clone specific for exon 10 of the human FSHR. The chromosomal signal on 2 p21 is indicated by the arrows. (b) Idiogram of the G banded chromosome 2 with the map position of the FSHR clone denoted by a bar.

Kelton *et al.* (1992). Exon 10 of the human FSHR encodes the C-terminal half of the receptor which includes the seven transmembrane domains, the entire C-terminal cytoplasmic domain and an extracellular N-terminal segment of 81 amino acids. The codon of the amino acid at the intron/exon boundary is interrupted between the second and third nucleotides. This finding is consistent with an intron phase 2 which has been described for all of the introns in the rat FSHR gene (Heckert *et al.* 1992). It has been demonstrated for both gonadotrophin receptors that the amino acid which resides at each intron/exon boundary is either a leucine or an isoleucine (Heckert *et al.* 1992). In the case of exon 10 of the human FSHR gene, the exon/intron splicing site interrupts the codon for isoleucine.

The chromosomal localization of the FSHR gene was determined by fluorescence *in situ* hybridization on chromosomes from synchronized peripheral blood lymphocytes. In order to assign the mapping position to cytogenetically defined bands, DAPI staining was used, which produces a G banding pattern. Fifteen randomly selected metaphases were evaluated. The signal could be observed in 85% of the metaphases on both chromatids at chromosomal mapping position 2 p21 (Fig. 3).

## DISCUSSION

The structure of exon 10 of the human FSHR gene (Fig. 2) shows striking similarities to exon 10 of the rat FSHR gene. The region close to the intron/exon junction (nucleotide positions 850–860) is highly conserved between the two species. As is the case for all glycoprotein hormone receptor genes known so far, an intron phase 2 can be observed at the exon/intron boundary of exon 10 and the amino acid at the exon/intron junction is isoleucine (see Heckert *et al.* 1992). The extracellular part of exon 10 consists of 81 amino acids; 80 amino acids are present in the rat. This region is the least conserved when compared with FSHRs of the monkey (Gromoll *et al.* 1993), the pig (Yarney *et al.* 1993) and the rat (Sprengel *et al.* 1990), thereby reflecting a certain degree of species specificity. In addition, this region might play a role in the determination of binding and receptor activation characteristics of glycoprotein hormone receptors because it is unique for the FSHR when compared with the LHR and TSHR (Segaloff & Ascoli, 1993).

While this work was in progress, Rousseau-Merck *et al.* (1993) localized the FSHR gene to chromosome 2 p21–16. The authors used two different cDNA probes mainly covering the extra-

cellular domain and parts of the transmembrane domain. Due to the high background of their radioactive hybridization procedure only a diagram showing the regional distribution of silver grains on chromosome 2 was presented. The FSHR gene was localized to the region p21–16 of chromosome 2, whereas we localized it solely to chromosome 2 p21. However, localization on the distal part of chromosome band 2 p16 cannot be ruled out entirely, due to the limits of resolution of fluorescence *in situ* hybridization mapping. Our localization result was confirmed using a gene mapping approach described by Lichter *et al.* (1990). The fractional length values determined for the FSHR gene were 0.16–0.18. These values are consistent with the assignment of the probe to band 2 p21 or the distal region of 2 p16. Signals at different chromosomal locations were not detected, indicating the specificity of the hybridization.

Apart from the FSHR gene, the CAD (carbamoyl phosphate synthetase 2, aspartate transcarbamylase and dihydroorotase) gene coding for a trifunctional protein involved in pyrimidine biosynthesis, the non-erythrocytic  $\beta$ -spectrin gene and the cardiac sarcolemmal  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger gene have been assigned to chromosome 2 p21 (Human Gene Mapping Catalogue (1991). *Cytogenetics and Cell Genetics* 58, 149). So far only a few chromosomal rearrangements, including band 2 p21, have been reported (Mitelman *et al.* 1991). To our knowledge, none of them is involved in the broad spectrum of fertility disorders.

Based on the structure of exon 10 of the human FSHR as described above, it is reasonable to assume that the human receptor has the same overall genomic organization as the rat FSHR: nine exons which encode the extracellular domain and possess an exon size ranging from 72 to 186 nucleotides, and a tenth exon coding for the entire transmembrane domain. The similarities in sequence, the intron/exon structure and the intron phasing of the gonadotrophin receptor genes indicate that these genes may have evolved by duplication of a common ancestral gene (Patthy, 1987). This hypothesis could also serve to explain the identical chromosomal localization of the LHR and FSHR genes, whereas the TSHR gene might have been shifted to chromosome 14 q31 by gene translocation in the course of the evolutionary process.

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