Structural Organization and Chromosomal Assignment of the Human obese Gene*

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The obese (ob) gene has been identified through a positional cloning approach; the mutation of this gene causes marked hereditary obesity and diabetes mellitus in mice. We report here the isolation and characterization of the human ob gene. Southern blot analysis demonstrated a single copy of the ob gene in the human genome. The human ob gene spanned ~20 kilobases (kb) and contained three exons separated by two introns. The first intron, ~10.6 kb in size, occurred in the 5'-untranslated region, 29 base pair (bp) upstream of the ATG start codon. The second intron of 2.3 kb in size was located at glutamine +49. By rapid amplification of 5'-cDNA ends, the transcription initiation sites were mapped 54–57 bp upstream of the ATG start codon. The 172-bp 5'-flanking region of the human ob gene contained a TATA box-like sequence and several cis-acting regulatory elements (three copies of GC boxes, an AP-2-binding site, and a CCAAT/enhancer-binding protein-regulatory elements (three copies of GC boxes, an AP-2-binding site, and a CCAAT/enhancer-binding protein-binding site). By the fluorescence in situ hybridization technique, the ob gene was assigned to human chromosome 7q31.3. This study should establish the genetic basis for ob gene research in humans, thereby leading to the better understanding of the molecular mechanisms underlying the ob gene.

The ob gene has been found in human obesity (5). Expression of the ob gene is markedly augmented in adipose tissue in several rodent models of genetic obesity (C57BL/6) and Zucker fatty (fa/fa) rats (2) and Zucker fatty (fa/fa) rats (8) and in rodent models of acquired obesity obtained by pure overfeeding of normal rats or by ventromedial lesion to rat hypothalamus (7). The augmentation of ob gene expression in adipose tissue is also region-specific (4, 7, 8). Furthermore, ob gene expression is also increased in human obesity in proportion to disease severity (5). These observations suggest the pathophysiologic roles of the ob gene in the development of obesity. Indeed, nonsense mutation of the ob gene has been proven to be the obesity-causing mutation in C57BL/6J ob/ob mice (2). On the other hand, no such mutation of the ob gene has been found in human obesity (5).

To understand the pathophysiologic roles of the ob gene in humans, it is important to elucidate the structural organization of the human ob gene. Furthermore, molecular characterization of the ob gene from any species has not so far been reported. We report here the isolation and structural organization of the human ob gene. Using the fluorescence in situ hybridization technique, we also determined the chromosomal assignment of the human ob gene.

EXPERIMENTAL PROCEDURES

Genomic Southern Blot Analysis—Human genomic DNA extracted from blood leukocytes was digested with restriction endonucleases SacI, EcoRI, KpnI, SphI, and NcoI; electrophoresed on a 0.7% agarose gel (5 μg/ml); and transferred onto a Biodyne A nylon membrane (Pall, Glen Cove, NY) (9). The membrane was prehybridized at 42 °C in a solution containing 50 mM sodium phosphate buffer (pH 7.0), 5 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate (pH 7.0)), 0.1% SDS. The blot was used to expose an x-ray film with an intensifying screen for 1 week.

Genomic Library Screening—A human genomic DNA library derived from leukocyte DNA in λEMBL3 (CLONTECH, Mountain View, CA) was screened with the 32P-labeled human ob cDNA fragment (3) as a probe. The membrane was washed three times at 55 °C in 0.1 × SSC and 0.1% SDS. The blot was used to expose an x-ray film with an intensifying screen for 1 week.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) D63708, D63709, and D63710.

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Fig. 1. Southern blot analysis of human genomic DNA. Samples of human genomic DNA (~5 μg/ lane) digested with SacI, EcoRI, Kpnl, SplII, and Ncol were analyzed by 0.7% agarose gel electrophoresis, blotted, and hybridized with the 32P-labeled human ob cDNA probe (3). The HindIII fragments of a DNA were used as size markers.

RESULTS

Genomic Southern Blot Analysis—Southern blot analysis of human genomic DNA with the human ob cDNA probe identified a single hybridizing band upon digestion with restriction endonucleases EcoRI, Kpnl, and SplII (3.7, 18, and 4.3 kb in size, respectively). On the other hand, digestion with Sad and Ncol gave two hybridizing bands of 7.2 and 3.8 kb in size and of 5.8 and 5.1 kb in size, respectively (Fig. 1).

Isolation and Characterization of the Human Ob Genomic Fragments—To isolate the human ob gene, ~6 × 105 recombinants from a human genomic DNA library in λEMBL3 were screened with the 32P-labeled human ob cDNA probe (3). A single positive clone (λOB1-8) harbored an ~14-kb human ob genomic fragment, which contained the 5.3-kb downstream half of the first intron and the second and third exons of the human ob gene (Fig. 2). The 5.3-kb genomic fragment (fragment 1) was amplified by PCR and contained the first exon (~29 bp) and the 5.3-kb upstream half of the first intron (Fig. 2). To obtain the 5′-flanking region of the human ob gene, ~5 × 105 clones from a second human genomic DNA library in λEMBL3 were screened with 32P-labeled fragment 1. Six positive clones were identified and plaque-purified. DNA from one clone (λOB3-1) harbored an ~16-kb genomic DNA fragment that contained the 5.0-kb 5′-flanking region of the human ob gene (Fig. 2).

Structural Organization of the Human ob Gene—Fig. 3 shows the nucleotide and deduced amino acid sequences of the human ob gene. The exon/intron borders were determined by comparison of the nucleotide sequences of the human ob gene with those of human ob cDNA (3). The human ob gene spanned ~20 kb and was organized into three exons separated by two introns. Splicing donor and acceptor consensus sequences (16) were located at the putative exon/intron borders. The first intron was ~10.6 kb in size and occurred in the 5′-untranslated region, 29 bp upstream of the ATG start codon. The second intron, ~2.3 kb in size, was located at glutamine +49. The

The abbreviations used are: PCR, polymerase chain reaction; kb, kilobase(s); bp, base pair(s); RT, reverse transcription; RACE, rapid amplification of cDNA ends; C/EBP, CCAAT/enhancer-binding protein.

0.1% SDS twice at 60 °C and in 0.2 × SSC and 0.1% SDS three times at 60 °C. Appropriate restriction fragments were subcloned into the phBlueScript vector (Stratagene Inc., La Jolla, CA) for further analysis.

Polymerase Chain Reaction—PCR2 was used to obtain the genomic fragment that contains the first exon and the upstream half of the first intron of the human ob gene (fragment 1) (see Fig. 2). Using a Model 381A DNA synthesizer (Applied Biosystems Inc., Foster City, CA), two oligonucleotide primers (sense, 5′-TAGGATCCCGAAGCCACAGGT-3′; antisense, 5′-CTACTTGGGACAGTGGAGTTTGTC-3′) were synthesized based upon the nucleotide sequences of human ob cDNA (3) and the first intron of the human ob gene, respectively (see Fig. 2). Using human genomic DNA as template, PCR was performed with a TaKaRa Shuzo LA PCR kit. The reaction profile was as follows: denaturation at 98 °C for 20 s and annealing and extension at 68 °C for 3 min for 30 cycles. The amplified DNA fragment of 5.3 kb in size was subcloned into the pGEM-T vector (Promega, Madison, WI) for sequencing.

Tissue Preparation and RNA Extraction—Human adipose tissue was obtained at the time of operation from the subcutaneous abdominal fat pad of a 58-year-old female patient with gastric cancer. Tissues were frozen in liquid nitrogen and stored at –70 °C until use. Total RNA extraction was carried out as described (3, 4, 8).

Reverse Transcription-PCR—RT-PCR was performed to determine the presence or absence of any introns in the 3′-untranslated region of the human ob gene. Approximately 10 μg of total RNA from human adipose tissue was reverse-transcribed by random hexamer priming using Superscript Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). The single-stranded cDNA was subjected to PCR as described (11). The human ob cDNA-specific PCR primers were generated using a Model 381A DNA synthesizer. Amplified DNA fragments were subcloned into the pGEM-T vector for sequencing. The presence or absence of any introns in the 3′-untranslated region was determined by comparison of the nucleotide sequences of the cloned human ob genomic fragment with those of the RT-PCR products that cover the entire 3′-untranslated region of the human ob gene.

Rapid Amplification of 5′-cDNA Ends (5′-RACE)—The 5′-RACE experiment was performed essentially as described (4) using the 5′-AmpliFINDER RACE kit (CLONTECH). Approximately 50 μg of total RNA from human adipose tissue was reverse-transcribed by a human ob cDNA-specific antisense primer (5′-ATGGGGTGAGCCCAAGCATAT-3′). The single-stranded cDNA was ligated to the AmpliFINDER anchor and amplified by PCR using the AmpliFINDER anchor primer and a second upstream human ob cDNA-specific antisense primer (5′-TGTGGATGCGGCAACAGTT-3′). A single fragment of ~200 bp in size was obtained, which was subcloned into the pGEM-T vector for sequencing.

Rapid Amplification of 3′-cDNA Ends (3′-RACE)—The 3′-RACE experiment was carried out as described (9) to determine the 3′-end of the human ob gene. Approximately 10 μg of total RNA from human adipose tissue was reverse-transcribed by adaptor oligo(dT)15 priming (5′-GGGATCCGGATCCCTGAGTTGTGGTTTTTTTTTTTTTT-3′) using Superscript Moloney murine leukemia virus reverse transcriptase. After synthesis of the second strand cDNA by a 5′-gene-specific primer (5′-GGCCGAAAGATGAGATTCT-3′), PCR was carried out using the primer and the adaptor oligonucleotide (without 13 dT nucleotides on the 3′-end) (7). An aliquot of the reaction was further subjected to PCR using a downstream 5′-gene-specific primer (5′-TAGGATCCCGAAGCCACAGGT-3′) and the adaptor primer. The 3′-RACE product was analyzed by a 1.5% agarose gel, and amplified DNA was subcloned into the pGEM-T vector for sequencing.

DNA Sequencing—Nucleotide sequences were determined by the dideoxy chain termination method (12) using Sequenase version 2.0 (U. S. Biochemical Corp.) and a DyeDeoxy terminator Cycle sequencing kit (Applied Biosystems Inc.). Sequence-specific primers were synthesized using a Model 381A DNA synthesizer. All DNA sequences were confirmed by reading both DNA strands.

Fluorescence In Situ Hybridization—Metaphase spreads were prepared from phytohemagglutinin-stimulated lymphocyte culture by a thymidine synchronization, 5-bromoodeoxyuridine release technique for the delineation of G-bands. Before hybridization in situ, chromosomes were stained in Hoechst 33258 and irradiated with UV (13). The BamHI/Sall-digested fragments of the isolated genomic clone (λOB1-8) (see Fig. 2) were labeled with biotin-16-dUTP (Boehringer Mannheim GmbH, Mannheim, Germany) by nick translation. Hybridization sig...
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**Fig. 2.** Schematic representation of the structure of the human ob gene and cDNA. a, the genomic clones (λOB3-1, λOB1-8, and fragment 1) and the BamHI (B) and XhoI (X) restriction sites. b, the sequencing strategy. Arrows denote the extent of sequence obtained. c, the structure of the human ob gene. Exons are boxed and numbered. The coding region is depicted by a closed box. d, the structure of human ob cDNA. The coding region is depicted by a closed box.

third exon contained the downstream coding region and the 3'-untranslated region of the human ob gene. Since complete nucleotide sequences of the 3'-untranslated region of the human ob cDNA have not yet been reported, nucleotide sequences of the third exon were determined by sequencing the 3'-RACE product and the corresponding genomic regions. Comparisons of the nucleotide sequences of the human ob genomic regions with those of the 3'-RACE/RT-PCR products that cover the entire 3'-untranslated region revealed the absence of any introns in the 3'-untranslated region of the human ob gene (data not shown).

Determination of the Transcription Initiation Sites of the Human ob Gene—To determine the transcription initiation sites of the human ob gene, the 5'-RACE experiment was carried out. To exclude the nucleotide misincorporation during the PCR amplification, a total of 10 clones were sequenced. Sequence analysis identified the transcription initiation sites 54–57 bp upstream of the ATG start codon (G at position –57, three clones; T at position –56, one clone; A at position –55, one clone; G at position –54, five clones) (Fig. 3). The 5'-end of the cloned human ob cDNA (3) was located 44–47 bp downstream of the transcription initiation sites (Fig. 3). The 5'-ends of mouse and rat ob cDNAs have been located 57 and 60 bp upstream of the ATG start codon, respectively (2, 4, 17). Although there is a high nucleotide sequence similarity in the 5'-untranslated region between mouse and rat ob cDNAs (93%), nucleotide sequences of the 5'-untranslated region of the human ob gene were less homologous to those of mouse and rat ob cDNAs (51 and 47%, respectively).

Analysis of the 5'-Flanking Region of the Human ob Gene—The 172-bp 5'-flanking region of the human ob gene sequenced in this study contained a TATA box-like sequence (TATATAWAW, W = A/T; positions –87 to –81) (16) 27–30 bp upstream of the transcription initiation sites (Fig. 4). A computer search of the 5'-flanking region for cis-acting regulatory elements also revealed the presence of three copies of GC boxes (GGCGCGG) (18) at positions –79 to –74, –155 to –150, and –160 to –155; a binding site for CCAAT/enhancer-binding protein (C/EBP) (TKNGYAAK, K = G/T, N = A/C/G/T, and Y = C/T) (19) at positions –111 to –103; an E box (CANNNG, N = A/C/G/T) (20) at positions –114 to –109; and an AP-2 binding site (CCCAGGCC) (21) at positions –199 to –192.

Analysis of the 3'-Flanking Region of the Human ob Gene—Molecular cloning studies of mouse, rat, and human ob cDNAs (2–7) have revealed no polyadenylation sites for their mRNAs, and the 3'-end of ob cDNA from any species has never been elucidated. To determine the 3'-end of human ob cDNA, the 3'-RACE experiment was carried out. Using total RNA from human adipose tissue, a single band of ~130 bp in size was obtained. Sequence analysis of the 3'-RACE products revealed that the cytosine nucleotide at position +4183 is followed by the poly(A) stretch (data not shown), suggesting that the cytosine nucleotide at position +4183 is the 3'-end of human ob cDNA or the 3'-end of the third exon of the human ob gene (Fig. 3). The overall size of the three exons (4240 bp) and the potential poly(A) stretch (usually ~200 bp) is consistent with that of human ob mRNA (~4.5 kb) as revealed by Northern blot analysis (3). No typical polyadenylation signal (AAATAAA) (22) was found near the putative poly(A) addition site. Nucleotide sequences of the 3'-untranslated region of the human ob gene were ~50% homologous to those of mouse ob cDNA (2). In the 3'-flanking region of the human ob gene, there was a characteristic CT-rich sequence at positions +4417 to +4538 (Fig. 3).

Chromosomal Assignment of the Human ob Gene—The chromosomal localization of the human ob gene was determined by the fluorescence in situ hybridization technique (Fig. 5). A total of 50 metaphase cells were examined. Of these, nine cells (14%) exhibited twin-spot signals on both homologous 7q31.3 chromosomes, and the other 17 cells (34%) had twin-spot signals on one 7q31.3 chromosome and a single spot on another 7q31.3 chromosome. Such specific accumulation of the signals could not be detected on any other chromosomes. These results indicate that the human ob gene is localized on chromosome 7q31.3.
**DISCUSSION**

In this study, we succeeded in the isolation and characterization of the human ob gene. Southern blot analysis of human genomic DNA identified a single hybridizing band upon digestion with EcoRI, KpnI, or SphI and two hybridizing bands upon digestion with SacI or NcoI (Fig. 1). These results are consistent with the restriction endonuclease map showing that a single site of SacI and NcoI is observed in the second intron of the genomic region that covers the ob cDNA sequence used as a probe, while EcoRI, KpnI, and SphI sites are not present (data not shown). These results indicate that the ob gene is present as a single-copy gene in the human genome. Using the mouse ob cDNA fragment as a probe, Zhang et al. (2) identified, by Southern blot analysis of human genomic DNA, a single hybridizing band of approximately 11 kb in size upon digestion with EcoRI. Differences in the size of the hybridizing band observed may represent the restriction fragment length polymorphisms between the human genomic DNAs used.

This study demonstrates that the human ob gene is encoded by a single-copy gene in the human genome.
posed of three exons separated by two introns. The first intron occurred in the 5'-untranslated region, and the coding region was separated by a single intron at glutamine +49. It has been demonstrated that in mice and humans, two different cDNAs encode the 166/167-amino acid ob proteins, which differ in the presence or absence of glutamine +49 (2, 3). On close inspection, there are one donor and two acceptor sites (18) around the junction region (Fig. 3). Furthermore, we have also observed that there is an internal alternative splice site (23) at glutamine +49 of the mouse ob gene. These observations suggest that the two ob proteins in mice and humans are generated by the alternative mRNA splicing mechanism.

The 5'-flanking region of the human ob gene contained a TATA box-like sequence and several cis-acting regulatory elements (three copies of GC boxes, a C/EBP-binding site, an E box, and an AP-2-binding site). The C/EBP transcription factor has been implicated in the coordinate transcriptional activation of adipocyte-specific genes during the course of adipocyte differentiation (24, 25). We and others (26) have observed that ob gene expression is induced in stromal-vascular cells or 3T3-F442A preadipocytes during the course of adipocyte development and/or maturation, although no significant amount of ob mRNA is present in undifferentiated cells. Therefore, the C/EBP-binding site in the 5'-flanking region of the human ob gene might be involved in the transcriptional activation of the ob gene during adipocyte differentiation. Further studies are needed to elucidate the functional significance of these cis-acting regulatory elements.

We demonstrated by the fluorescence in situ hybridization technique that the ob gene is mapped on human chromosome 7q31.3. Of particular note is that the cystic fibrosis transmembrane conductance regulator gene has been assigned to human chromosome 7q31.3 (27). It has been demonstrated that the ob gene is localized on the proximal region of mouse chromosome 6 (2, 28). The mouse chromosomal region on which the ob gene is located is part of a known segment with genes that are conserved between mice and humans and is syntenic to human chromosome 7q (29). This study has provided direct evidence that the ob gene is a member of the conserved syntenic group in mice and humans and further helps gene mapping in both species.

In conclusion, we succeeded in the isolation and characterization of the human ob gene. Using the fluorescence in situ hybridization technique, we also determined the chromosomal assignment of the human ob gene. This study helps to establish the genetic basis of ob gene research in humans, thereby leading to a better understanding of the physiologic and pathophysiologic implications of the ob gene.

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