**Cig30 and Pitx3 Genes Are Arranged in a Partially Overlapping Tail-to-Tail Array Resulting in Complementary Transcripts**

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The mouse Cig30 gene codes for a 30-kDa membrane glycoprotein, which appears to have a role in the recruitment of brown adipose tissue. To elucidate the structure of the Cig30 gene, we have isolated a λ phage genomic DNA clone containing the entire mouse gene and found that Cig30 consists of four exons that are spread over 4 kilobase pairs of genomic sequence. Using a fluorescence in situ hybridization assay and interspecific backcross panel mapping, we have localized the Cig30 locus to the distal region of mouse chromosome 19, between the Tlx1 and Ins1 loci. Sequencing of the corresponding λ clone to completion revealed that the insert contained yet another gene in the opposite orientation. It turned out to be the newly identified homeobox gene Pitx3. Interestingly, the genes are very tightly linked, so that the 3' ends of their transcripts are complementary. Thus, our results provide evidence for bidirectional transcription of a several hundred base pair-long DNA region as a result of the extremely tight linkage between Cig30 and Pitx3.

The membrane glycoprotein encoded by the mouse Cig30 gene is homologous to several polypeptides of yeast and nematode origin (1). Despite the amount of biochemical data pertaining to the function of this novel gene family, conclusive evidence elucidating its physiological role is still lacking. The present knowledge comes almost exclusively from studies of three paralogous yeast genes of this family. Two of these yeast genes, known as APA1/SRE1/ELO3, have been implicated in a complex pleiotropic phenotype suggesting a defect in a plasma membrane and cytoskeleton (2). In accord with this view, it has recently been proposed that both APA1/SRE1/ELO3 and APA1/SRE1/ELO3 are involved in fatty acid chain elongation resulting in very long chain fatty acids of up to 26 carbon atoms (3), whereas the third yeast parologue, the APA1/SRE1/ELO3 gene, was found to catalyze the elongation of shorter fatty acid species, primarily between C-14 and C-16 (4).

In a search for genes selectively induced during the recruitment process in brown adipose tissue, Cig30 was identified as the first mammalian member of this gene family (1). Under thermo-neutral conditions, Cig30 is weakly expressed in brown fat, liver, and skin. Following a 3-day cold exposure, Cig30 expression in brown fat is strongly and selectively induced more than 200 times. Highly elevated Cig30 mRNA levels are also found in two other conditions of brown fat recruitment, i.e. during perinatal development and after cafeteria diet. Although the effect of cold on Cig30 expression can be largely mimicked by norepinephrine infusion in vivo, our studies on primary cultures of brown adipocytes have revealed that a dexamethasone stimulus is also required for full Cig30 expression, implying that a synergistic action of catecholamines and glucocorticoids is necessary for maximal Cig30 stimulation.

To study the regulation of Cig30 in greater detail, and in pursuit of Cig30 gene disruption by homologous recombination, we isolated a 14-kb genomic clone from the mouse 129 strain liver genomic DNA library and clarified the genomic structure of the Cig30 gene. Unexpectedly, we found that Cig30 is tightly linked to Pitx3, a homeobox gene and established that the transcription units of both genes overlap.

**EXPERIMENTAL PROCEDURES**

**Mouse Cig30 Genomic Cloning—**Genomic clones of Cig30 were isolated by plaque hybridization of a commercial mouse 129 strain liver genomic DNA library in the Lambda FIX II vector (Stratagene catalog no. 946308) with a 32P-labeled probe corresponding to 1.2 kb from the 5' end of the Cig30 cDNA (GenBank accession no. U97107). Hybridization was carried out overnight at 45 °C in 50% formamide, 5× SSC, 5× Denhardt's solution, 50 mM sodium phosphate, pH 6.5, 0.5% SDS, and 100 μg/ml degraded herring sperm DNA. The membranes were first washed twice for 15 min at room temperature in 2× SSC, 0.1% SDS, and a high stringency wash was then performed at 55 °C in 0.1× SSC, 0.1% SDS for 15–30 min. Screening of approximately 1.5 × 106 plaques yielded two positive recombinants, which were isolated in both additional rounds of plaque purification. Phage DNA was prepared on a large scale by the polyethylene glycol precipitation method as described previously (5). Restriction fragments generated by various combinations of restriction enzymes were analyzed by Southern blotting, and the presence of the 3' end of the Cig30 gene was checked by hybridization with a 3' untranslated region probe (0.8 kb from the 3' end of the Cig30 cDNA).

**DNA Sequencing and Sequence Analysis—**Using recombinant phage DNA as a template, the genomic insert was sequenced by the primer walking strategy. Sequencing was performed with an ABI 373A automatic DNA sequencer (Applied Biosystems) on reactions prepared by the dye-termination method, using the ABI Prisms dye terminator cycle sequencing ready reaction kit (Perkin-Elmer). The sequence information was compiled and analyzed using the University of Wisconsin Genetics Computer Group software (6). The complete nucleotide se-

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The abbreviations used are: kb, kilobase (pair); ORF, open reading frame; bp, base pair(s); nt, nucleotide(s); RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; EST, expressed sequence tag(s).
sequence of the 13,869-bp genomic fragment has been deposited in GenBank (accession no. AF054504).

Fluorescence in Situ Hybridization—Fluorescence in situ hybridization was performed as described previously (7). The probe, consisting of the entire 14-kb fragment of mouse Cig30 genomic DNA in the phage vector, was labeled with biotin-16-UTP by nick translation using the BioNick Kit (Life Technologies, Inc.). 100 ng of labeled DNA was coprecipitated in the presence of 20 μg of Cot1 DNA (Life Technologies, Inc.), denatured at 70 °C for 10 min, and pre-annealed at 37 °C for 10 min. An overnight hybridization was then carried out on metaphase chromosomes from the mouse SV 22CD cell line (8). The signal was detected using fluorescein isothiocyanate-conjugated avidin (Vector Laboratories). Fluorescence images were captured using a high performance cooled charge-coupled device C4880 camera (Hamamatsu Laboratories). Fluorescence images were captured using a high performance cooled charge-coupled device C4880 camera (Hamamatsu Laboratories) interfaced to a PC 486DX33 with a Matrox 640 card. All digital image acquisition, processing, and analysis functions were accomplished using an Alcatel software package (Fluogene).

Interspecific Mouse Backcross Mapping—Chromosomal linkage of Cig30 was determined using the interspecific backcross DNA panel mapping resource of The Jackson Laboratory that were generated by mating C57BL/6J × SPRET/Ei females and SPRET/Ei males (9). A total of 94 mice were used to map Cig30 by Southern blot analysis of a polymorphic BsmHI restriction fragment, the size of which was 8.2 and 7.5 kb in C57BL/6J and Mus spretus mice, respectively. The probe was a Cig30 ORF cDNA fragment (see below) labeled with [32P]dCTP as described elsewhere (10). Hybridization and washing conditions have been described elsewhere (11). Chromosome linkage and recombination distances were calculated by MapManager.

RNA Isolation, Northern Blotting, and DNA Probes—Total RNA was isolated using the ULTRASPECK™ RNA isolation system (BIOTECH) from the skin and liver of a 6-week-old NMRI male mouse kept at room temperature and from the brown adipose tissue of another sibling male mouse that was cold-exposed at 4 °C for 5 days. Northern blotting and hybridization was performed as described previously (1).

The DNA probes used were the following: the Cig30 ORF probe (a 871-bp fragment corresponding to nt 162–1056 in the Cig30 cDNA), the Cig30 3′ probe (a 294-bp PstI-Stul fragment corresponding to nt 10646–10939 in the genomic clone), and the Pitx3 probe (a 897-bp PstI-PstI fragment corresponding to nt 11443–12340 in the genomic clone). The probes were labeled with α-32PdCTP using a random primed DNA labeling kit (Roche Molecular Biochemicals).

3′ RACE and PCR Primers—Amplification of the 3′ cDNA ends was performed with mouse liver Marathon-Ready cDNA and the Advantage KlenTaq polymerase mix (both from CLONTECH) following the supplier’s recommendations. Five μl of cDNA template were amplified using the Cig30-specific primer (5′-CTTCTTGGAAACGGGATAGGGGAGTC-3′, nt 10973–10998 in the genomic clone) and the AP1 primer in touch-down PCR, which was carried out for a total of 40 cycles (5 cycles of 94 °C for 30 s, 72 °C for 2 min, 5 cycles of 94 °C for 30 s, 70 °C for 2 min; 30 cycles of 94 °C for 30 s, 68 °C for 2 min). For nested PCR, 10 pmol of the forward primer (5′-GTTGACCAAGGGTGAATTT-3′, nt 11011–11028) was used together with 10 pmol of the reverse primer (5′-TGAAAGGGGTAGCAGGC-3′, nt 11177–11160) on 2 μl of the 3′ RACE product without further purification. PCR was performed with KlenTaq polymerase for 30 cycles (94 °C for 30 s, 68 °C for 1 min, 72 °C for 1 min, and the PCR products were electrophoresed in a 2% agarose gel in 0.5× TBE and photographed.

DNA Extraction and Southern Blotting—Genomic DNA was prepared from mouse tail or human lymphocytes by the simplified mammalian DNA isolation procedure published by Laird et al. (12). Tail biopsies were collected from 3-week-old mice of the C57BL/6J strain and used directly for DNA isolation. For human DNA preparation, cell nuclei were first purified from the blood of a normal Caucasian female donor by the following procedure. 40 ml of blood was mixed with 60 ml of buffer A (0.32 M sucrose, 5% Triton X-100, 5 mM EDTA, 10 mM Tris-HCl, pH 7.6) and centrifuged for 15 min at 3000 rpm in a tabletop centrifuge. The pellet was resuspended in 40 ml of buffer B (0.32 M sucrose, 5 mM EDTA, 10 mM Tris-HCl, pH 7.6) and centrifuged at 3000 rpm for 10 min. The washing step was repeated once with 20 ml of buffer B. The final pellet was resuspended in 400 μl of buffer B and frozen at −80 °C.

Digestive DNA was separated on an 0.8% agarose gel and transferred to a Hybond N+ membrane (Amersham Pharmacia Biotech) according to standard procedures (5). Hybridization and washing conditions were identical to those used for library screening, except that the second wash was carried out under low-stringency conditions (15 min at 40 °C in 0.5× SSC, 0.1% SDS). The radiolabel was detected by exposing to DuPont Cronex x-ray films with an intensifying screen at −80 °C.

### RESULTS

**Isolation of Mouse Cig30 Genomic Clones**—A mouse genomic library was screened using Cig30 cDNA (1) as a probe. The screening yielded two independent λ clones, which were characterized by restriction analysis and Southern blotting. Following digestion of the phage DNA with SalI and XbaI, the size of the two inserts was estimated to be 14 and 15 kb, respectively. Subsequent hybridization with probes specific for the 5′ and 3′ ends of the Cig30 cDNA indicated that both genomic clones contained the entire Cig30 gene (not shown). In the 14-kb genomic clone, the Cig30 gene appeared to be conveniently located in the middle of the insert, and we determined its nucleotide sequence by primer walking strategy directly from the phage DNA.

**Organization of the Cig30 Gene**—Sequencing revealed that the Cig30 gene consists of four exons, which span 4 kb of genomic DNA (Table I and Fig. 3A). The exons range in size from 132 to 1319 bp and the size of introns range from 222 to 1132 bp. The splice junctions of the three intervening introns conform to the 5′-GTAG-3′ rule (13), matching almost perfectly the broader consensus for the donor site (GTRAGT) and for the acceptor site (YYNYAG) (14). The first two introns are phase I, interrupting codons between the second and third nucleotide of the coding triplet, whereas intron 3 is phase I. The translation initiation codon (ATG) is located in the first exon, and the protein coding sequence stretches over all four exons, the major part being in exon 4.

The Cig30 gene encodes a 30-kDa membrane glycoprotein with at least five putative transmembrane domains (1). The exon/intron structure of Cig30 does not, however, correlate with the transmembrane organization of the CIG30 protein.

**Mapping of Mouse Cig30 to Chromosome 19**—The chromosomal location of Cig30 in the mouse was determined by fluorescence in situ hybridization analysis of mouse SV 22CD metaphase chromosomes using the 14-kb λ genomic clone as a probe. In the karyotype of the SV 22CD cell line, all chromosomes are fused in pairs, except for chromosome 19 and the slightly larger X chromosome (8). This chromosomal abnormal-
ity was accidentally of great benefit in the chromosomal determination, because the twin spot signals were observed specifically on a single unfused chromosome pair, which was readily identified as chromosome 19 (Fig. 1).

To further refine the localization of the Cig30 gene, we employed interspecific backcross analysis using progeny derived from matings of ((C57BL/6J x SPRET/Ei)F1 x SPRET/Ei), known as Jackson BSS (9). Restriction fragment length polymorphism between C57BL/6J and M. spretus in the Cig30 locus was revealed by Southern blot hybridization, using the coding region of the Cig30 cDNA as a probe. The 7.5-kb BamHI M. spretus restriction fragment length polymorphism was used to follow the segregation of the Cig30 locus in backcross mice. The mapping results indicated linkage with markers in the distal region of mouse chromosome 19. Cig30 segregated with markers D19Mit3 and D19Mit4, placing Cig30 between the Tlx1 (T-cell leukemia, homeobox 1) and Ins1 (insulin 1) genes (Fig. 2).

Cig30 Is Tightly Linked to Pitx3—We analyzed the entire 13,869-bp contiguous sequence carrying the Cig30 gene with the Testcode program (6) and found several regions downstream of the last Cig30 exon with a high likelihood of protein coding capacity (not shown). A BLAST search revealed that these regions correspond in fact to the Pitx3 gene (Fig. 3). Pitx3 is a homeobox-containing transcription factor gene belonging to the Rieg/Pitx homeobox gene family (15), which has been implicated in anterior segment mesenchymal dysgenesis and congenital cataracts in humans (16). The Pitx3 gene consists of four exons; their exon-intron junctions have been characterized (15), but the genomic sequence has not been published. Our 4.3-kb genomic clone covers, however, the last three exons and two introns of the Pitx3 gene. Sequence identity between the published Pitx3 cDNA sequence (GenBank accession no. AF005772) and the sequence we have determined from our genomic clone is 99.68% over a total region of 1251 nucleotides. Of the four mismatches, two are in the protein coding region, and both of them are silent. Extending the earlier published information on the Pitx3 gene structure, our data allowed the determination of the exact size of the second and third introns to 182 and 377 bp, respectively.

3' Ends of the Long Cig30 and Pitx3 mRNA Species Overlap—Cig30 and Pitx3 are oriented in a tail-to-tail manner in a strikingly tight linkage. In fact, their published cDNA sequences (1, 15) appear to be complementary over the last 10 nucleotides. As we had earlier noted lack of a polyadenylation signal consensus at the 3' end of the Cig30 cDNA and the presence of several Cig30 mRNA species on Northern blots (1), we decided to establish the major cleavage site of Cig30 transcript. When total RNA from mouse skin, liver, and brown adipose tissue was probed with a Cig30 ORF probe, we consistently detected three Cig30 mRNA species, designated I, II, and III (Fig. 3). For Pitx3 localization, see Fig. 3 legend.

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sequences corresponding to 3' ends of randomly cloned Cig30 transcripts. We found five cDNA fragments that are schematically aligned in Fig. 3B. Three of the five cloned 3' ends (GenBank accession no. AA798121, AA798103, and AA562425) conform with cleavage immediately downstream of the polyadenylation signal (Fig. 5A). Notably, in addition to the sequence published by us (GenBank accession no. U97107), the two other EST cDNA clones (GenBank accession no. AA241332 and AA062140) indicate cleavage events at various points several hundred base pairs downstream. These transcripts constitute an overlap with the fourth Pitx3 exon in an antiparallel manner (Fig. 5B), ranging from 10 up to about 300 bp (Fig. 5C). We found no EST clone indicative of a longer Pitx3 transcript than the published Pitx3 cDNA sequence (GenBank accession no. AF005772). This sequence contains a perfect polyadenylation signal consensus (Fig. 5B) and apparently corresponds to a full-length transcript. In contrast, none of the long Cig30 transcripts (Fig. 5, B and C) possess a polyadenylation signal upstream of the cleavage site, suggesting that their polyadenylation is controlled by some other signal or occurs more or less randomly. Consistent with that view, we obtained no predominant PCR product in a 3' RACE experiment performed on mouse liver cDNA using the specific primer downstream of the Cig30 cleavage site at 10951. However, following a second round of PCR with two nested PCR primers (corresponding to nt 11011–11028 and 11177–11160), we amplified the anticipated 167-bp product, confirming that in liver such Cig30 transcripts exist that overlap Pitx3 by at least 238 bp (Fig. 6). In a parallel reaction, the same pair of primers failed to amplify the product directly from mouse liver cDNA, implying that the product obtained by nested PCR was specific for Cig30 and not for Pitx3 transcription. Moreover, the Pitx3 probe (Fig. 3C) gave no signal after Northern hybridization to the blots in Fig. 4 (not shown), indicating that (i) Pitx3 is not expressed in brown fat, liver, or skin in adult mice and that (ii) very few, if any, Cig30 transcripts exist that would overlap with Pitx3 by more than 500 bp.

Detection of Human CIG30—No human EST sequence has as yet been reported that is clearly orthologous to the mouse Cig30 cDNA. Thus, we asked whether there is a nucleotide sequence in the human genome that would specifically hybridize with mouse Cig30 cDNA. As shown in Fig. 7, following digestion with BamHI, EcoRI, HindIII, and XbaI, the mouse Cig30 ORF probe not only strongly hybridized to mouse genomic fragments (which were of the predicted size) but also specifically hybridized to human genomic DNA, indicating the presence of a homologous CIG30 sequence. In both cases, a simple pattern of hybridization was seen, suggesting that Cig30 is a single-copy gene in both the mouse and human genomes.

![Cig30 and Pitx3 Overlap](image)

**DISCUSSION**

Cig30 is the first mammalian member of a gene family that has been suggested to be involved in the elongation of very long chain fatty acids. We report here its genomic structure and chromosomal location. Our mapping results indicate that Cig30 is located in the distal region of mouse chromosome 19, which links with D19Mit4. Semina et al. (15) obtained an identical result using the same BSS panel to follow Pitx3 polymorphism. Furthermore, in addition to the BSS panel, they also used the (C57BL/6J × M. spreptus)F1 × C57BL/6J panel from The Jackson Laboratory and identified two crossover events (in 94 animals) between Pitx3 and D19Mit4. By combining the data from both crosses, Semina et al. suggest that Pitx3 is located 1.06 ± 0.75 centimorgans proximal of D19Mit4. In the mouse genome data base map, Pitx3 has been accordingly assigned to 46.5 centimorgans from the proximal end of chromosome 19. The genetic distance between Cig30 and Pitx3 is minimal, and Cig30 should therefore be assigned to the same
FIG. 5. Nucleotide sequence alignment of EST clones with genomic DNA (GenBank accession no. AF054504). A, 3’ end cDNA sequences corresponding to mRNA species cleaved immediately downstream of the polyadenylation signal (AATAAA) in Cig30, B and C, cDNA sequences corresponding to overlapping transcripts. Nucleotide positions that differ from the Cig30 sequence are in lowercase letters (Pitx3 cDNA GenBank accession no. is U97107).

chromosomal location. It cannot be concluded from our data whether Cig30 is proximal or distal to Pitx3.

The distal part of mouse chromosome 19 shares a region of homology with human chromosome 10q. Human paralogues of several mouse genes linked with Cig30, such as HOX11, CYP17, or COL17A1, map to chromosome 10q24, suggesting that human CIG30 could reside in the same region. In good agreement, Semina et al. (16) mapped human PITX3 to 10q25. Several human disorders of unknown genetic etiology have been mapped to the long arm of chromosome 10. Among these are urofacial syndrome (17), endometrial carcinomas (18), juvenile intestinal polyposis (19), infantile onset spinocerebellar ataxia (20), corneal dystrophy of Bowman layer (21), and partial epilepsy (22). However, none of these syndromes is easily reconcilable with the pattern of Cig30 expression observed in the mouse model (brown fat, liver, skin) nor with the tentative biochemical role of the CIG30 protein (biosynthesis of complex lipids). Obviously, further progress in this area must await the cloning of the human CIG30 gene. This should be facilitated by our finding that the mouse Cig30 cDNA hybridizes to a unique nucleotide sequence in the human genome.

It is generally accepted that adjacent mammalian genes are normally separated by tens of thousands of kilobases. Although gene clusters are occasionally found in vertebrates, they typically consist of homologous genes that probably arose by gene duplication (such as globin genes or homeobox genes), or they involve genes that are functionally related (e.g. histone genes). Here we show that two apparently unrelated mouse genes occupy as little as 8–9 kb totally (depending on the size of the first Pitx3 intron). Moreover, this study indicates that the 3’ end of Cig30 overlaps with the 3’ end of Pitx3. Similar arrays are uncommon in eukaryotes (23–25).

Clearly, overlapping genes in opposite orientations generate transcripts that could form RNA duplexes, if present in the same cell. Although both Cig30 and Pitx3 genes exhibit rather restricted tissue expression patterns, Pitx3 appears to be expressed primarily during embryonal development. In day 10 postcoitum mouse embryos, Pitx3 mRNA is found in the lens placode and the forming lens pit and then, later, throughout all stages of lens development. Beginning with day 12 postcoitum, Pitx3 mRNA has been also detected in the midbrain region, tongue, incisor primordia, condensing mesenchyme around the sternum and vertebrae and in the head muscles (16). Cig30 expression is confined to the brown fat, liver, and skin in adult mice, but EST clones AA241332 and AA062140 isolated from total fetuses of 12.5 and 19.5 days postcoitum, respectively, indicate that Cig30 is turned on already during embryonal development. Interestingly, these EST clones correspond to the long Cig30 mRNA species that overlaps with Pitx3. Thus, the possibility cannot be excluded that both genes are temporally and spatially co-expressed, which would imply that some Cig30 transcripts could interact with Pitx3 mRNA. Such an interaction, however, is unlikely to have any regulatory role, considering the small amount of transcripts involved. Nevertheless, the tight linkage between Cig30 and Pitx3 provides a new

FIG. 6. PCR analysis of 3’ Cig30 cDNA ends in mouse liver. Lane 1, 3’ RACE product obtained with a Cig30-specific primer corresponding to nt 10973–10998; lane 2, nested PCR performed on 2 μl of the 3’ RACE product; lane 3, PCR performed directly on mouse liver cDNA using the same primer combination as for nested PCR. M1, 1 Kb Plus DNA ladder molecular weight marker (Life Technologies, Inc.); M2, DNA molecular weight marker XIII (Roche Molecular Biochemicals).

FIG. 7. Detection of the Cig30 gene in mouse and human genomic DNA. Southern blot analysis was performed with 10 μg/lane of either mouse or human genomic DNA digested with restriction enzymes indicated as follows: B, BamHII; E, EcoRI; H, HindIII; X, XhoI. The blot was hybridized to the Cig30 ORF probe and washed as described under “Experimental Procedures.”
example of closely associated genes in vertebrates, and it will
be of interest in evolutionary terms to investigate whether this
presumably fortuitous gene association is conserved in other
mammalian species.

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