Cloning and characterization of a human orthologue of testis-specific succinyl CoA: 3-oxo acid CoA transferase (Scot-t) cDNA

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Succinyl CoA: 3-oxo acid CoA transferase (scot; EC 2.8.3.5) is a key enzyme for metabolism of ketone bodies. Previously, we have cloned a testis-specific succinyl CoA: 3-oxo acid CoA transferase (scot-t) cDNA from a subtracted cDNA library of mouse testis and demonstrated its expression to be specific to late haploid male germ cells. In this study, the human orthologue of mouse scot-t was cloned and characterized. The entire coding region of the mRNA and the deduced amino acid sequence of human Scot-t (h-Scot-t) showed 75.4 and 75.8% identity with mouse scot-t respectively. The mRNA was exclusively expressed in the testis, and the protein was localized to the midpiece of ejaculated spermatozoa where mitochondria exist. We showed that the h-Scot-t gene was intronless by using a polymerase chain reaction technique and that a non-functional pseudogene, 98% similar to h-Scot-t, was also located on the human genome (1p33-34.3). Furthermore, the genomic structure of the actual h-Scot-t transcription unit was found to be located in an intron (1p34.1–35.3) of the bone morphogenetic protein 8 (BMP8) gene. In conclusion, we have demonstrated that h-Scot-t is a single intronless gene specifically expressed in the testis.

Key words: intronless/OXCT/Scot/spermatozoa/testis

Introduction

Ketone bodies are produced in the liver and are exported to peripheral tissues for use as an energy source (Mitchell et al., 1995). Succinyl CoA: 3-oxo acid CoA transferase (OXCT/SCOT) is a key enzyme for energy metabolism of ketone bodies (Mitchell et al., 1995). OXCT is localized in the mitochondria of several tissues and catalyses the formation of acetoacetyl CoA by transfer of a CoA moiety from succinyl CoA to 3-oxo acid, which is further broken down to two acetyl CoA molecules capable of entering the tricarboxylic acid cycle (Solomon and Jencks, 1969; Williamson et al., 1971; Fukao et al., 1997). Scot cDNA have been cloned from pig and human heart (Lin and Bridger, 1992; Kassovska-Bratinova et al., 1996).

Previously, we have cloned a novel gene encoding a haploid germ cell-specific scot, named scot-t, from a subtracted cDNA library of mouse testis (Koga et al., 2000). By Northern blot, Western blot and immunohistochemical analyses, the expression of mouse scot-t was detected in the testis, especially in late spermatids, but not in the other somatic tissues. The nucleotide sequence of mouse scot-t was 63.4 and 62.7% identical to heart Scot in pig and human heart, respectively. The deduced amino acid sequence was 68.0 and 67.4% identical. Residues 1–39 at the NH₂ terminus of scot-t constitute a putative signal sequence that may target the mitochondria. Indeed, immunofluorescent staining has demonstrated the mitochondrial localization of Scot-t protein in fixed spermatozoa from cauda epididymidis (Koga et al., 2000). The amino acid sequence of mouse Scot-t has a glutamate residue (amino acid residue: 341) corresponding to glutamate 344, known to be conserved in all CoA transferases including Scot. These results suggest that the gene product of scot-t plays a role in energy metabolism and may indicate the existence of a novel metabolic system utilizing ketone bodies as an energy source for sperm mobility.

Several investigators have reported the importance of mitochondrial enzymes for energy metabolism in sperm mobility and function (Pascual et al., 1996; Yeung et al., 1996; Ruiz-Pesini et al., 1998). In the present study, we isolated the human orthologue of the mouse scot-t and identified the structure of the genomic DNA, as the gene product may also play an important role in normal male fertilization.

Materials and methods

Cloning of the human cDNA

An oligo-dT-primed cDNA library of human testis constructed in the pAP3neo vector (Tanaka et al., 1997) was screened with a 32P-
Characterization of a testis-specific Scot cDNA

A human testis cDNA library was screened with mouse scot-t cDNA as a probe. Eight independently isolated and overlapping cDNA clones, together containing ~1.8 kb, were sequenced. The complete nucleotide sequence, corresponding to the longest cDNA among these positive clones, and its deduced amino acid sequence are shown in Figure 1A and B (DDBJ accession no. AB050193). The human scot-t cDNA (h-Scot-t) consisted of 1749 nucleotides with an open reading frame of 1551 nucleotides encoding 517 putative amino acid residues. The sequence ATTAAA located 17 nucleotides upstream of the poly(A) tract, deviated one nucleotide from the most common consensus polyadenylation signal AATAAA (Figure 1A) and was the same as that of mouse scot-t and scot-t2 (Tanaka, Direct submission to DDBJ, 2000). This A-to-T substitution of the consensus sequence is well tolerated and is the most frequent variant of the polyadenylation signal (Sheets et al., 1990; Wahle and Keller, 1992).

The entire coding region of h-Scot-t cDNA and the deduced amino acid sequence showed 75.4 and 75.8% identity with mouse scot-t (DDBJ accession no. AB022180 and AB049996), respectively and also had 68.8 and 74.6% identity with human OXCT/SCOT (Kassovska-Bratinova et al., 1996) (DDBJ accession no. U62961), respectively (Figure 1). The hydrophilic bridge region, amino acids 271–308 at 95 °C, and 90 s at 72 °C. Aliquots of the amplified products were subjected to 1.0% agarose gel electrophoresis and stained with 0.5 µg/ml concentration of ethidium bromide. Amplified products were visualized with UV light. Each PCR product was recovered after gel electrophoresis, and purified on Suprec PCR spin column (Takara). The DNA sequences were determined using the same PCR primers.

Results

Isolation and sequencing of human Scot-t cDNA

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Expression of human Scot-t cDNA

We examined the expression of the human Scot-t gene in various organs by Northern blot analysis with the full-length h-Scot-t cDNA as a probe. As shown in Figure 2, the h-Scot-t gene was expressed as a 1.8 kb mRNA in the testis, but not in the other organs we examined. Western blotting with anti-mouse scot-t rabbit antiserum (Koga et al., 2000) showed one positive signal with a molecular weight of 50 kDa in the extract of tests and spermatozoa (Figure 3).
Figure 1.
Figure 1. Comparison of the nucleotide sequence and the deduced amino acid sequence of mouse and human succinyl CoA: 3-oxo acid CoA transferase. (A) Alignments of human Scot-t (h-Scot-t), human Scot-t pseudo genomic DNA (h pseudo genome), mouse Scot-t1 (m-Scot-t1), and mouse Scot-t2 (m-Scot-t2). Asterisks under sequences indicate nucleotides identical to those in h-Scot-t. Gaps introduced to maximize the alignment are represented by dashes. The ATG for initiation of protein synthesis, the stop codon, and the 18+1 bp deletion in human Scot-t pseudo genomic DNA are shadowed. The primers (A1, A2, B1 and B2) used to identify the genomic DNA and cDNA sequence are underlined. The double underline indicates the potential polyadenylation signal. DDBJ/EMBL/GenBank accession nos. are given in parentheses. (B) Comparison of amino acid sequences of human SCOT-t (h-SCOT-t), OXCT/SCOT (hum OXCT), mouse Scot-t1 (m-Scot-t1), and mouse Scot-t2 (m-Scot-t2). Asterisks in hum OXCT and m-Scot-t1 indicate the same amino acids as in h-SCOT-t. Gaps introduced to maximize the alignment are represented by dashes. The N terminal 39 residues (shadowed) are thought to compose the mitochondrial targeting sequence. The C terminal amino acid sequence was prepared for the anti-Scot-t antiserum (shadowed). Hydrophilic bridge regions (amino acid residues 271–284) and the active-site glutamate (E) are indicated by open boxes. Asterisks in the m-Scot-t2 amino acid sequence indicate the same residue as in m-Scot-t1. The DDBJ/EMBL/GenBank accession nos. are shown in parentheses.
Figure 2. Northern blot analysis of human Scot-t mRNA. The human multiple tissue blot was hybridized with $^{32}$P-labelled full-length human Scot-t cDNA. The same membranes were rehybridized with $^{32}$P-labelled β-actin cDNA as a control. The numbers in the left margin indicate size markers, and the arrow at the right margin indicates the position of Scot-t hybridization signal (1.8 kb).

Figure 3. Expression of human SCOT-t protein. (A) Western blot analysis of recombinant protein in Escherichia coli. Extracts of E.coli. transformed with pET30a (1) and pET-Scot-t (2) were loaded. The pET-Scot-t plasmid contained h-Scot-t cDNA at a multi-cloning site of pET30a for the expression of a full-length h-SCOT-t protein fused with six histidine residues. (B) Western blot analysis of mouse testis (1), human testis (2) and human spermatozoa (3) using polyclonal antiserum against Scot-t protein. Approximately 50 µg of protein was loaded in each lane. Numbers in the left margin indicate molecular weights of marker proteins.

staining. Positive staining was restricted to the midpiece of the sperm flagellum containing a large amount of mitochondria (Figure 4).

**Genomic localization and construction of h-Scot-t**

On searching the DDBJ, EMBL, Swiss-Prot, GenBank, and PIR data banks for a human genomic clone having sequence homology with h-Scot-t cDNA, we found two independent genomic clones (accession no. AL033527 and AL033526) which mapped to 1p34.1-35.3 and 1p33-34.3 respectively. One clone (AL033527) contained the complete h-Scot-t open reading frame (ORF) as an intronless gene inside the intron of the bone morphogenetic protein 8 (BMP8) gene (Ellington, Direct submission to EMBL/GenBank/DDBJ, 2000). An homologous DNA fragment of ~19 kb was located in the genomic clone AL033526 (1p33-34.3). The h-Scot-t was localized at the centre of the homologous 19 kbp DNA fragments, but it was deleted of 19 nucleotides at the ORF in AL033526 (Figures 1 and 5). To confirm the genomic arrangement of the h-Scot-t gene, PCR with human genomic DNA was performed using two sets of oligonucleotide pairs as a template encompassing both the 5’ and 3’ ends of the h-Scot-t ORF (A1-2, Figure 5). The size of the PCR product coincided with that of the h-Scot-t cDNA used as a template (Figure 5A), suggesting that the h-Scot-t gene lacks an intron. When the PCR products were subcloned and sequenced, two distinct types of clones were found. One type should have the same sequence as h-Scot-t cDNA and the other, had a deletion of nucleotides 745 to 762, and also 778. Although the h-Scot-t cDNA we cloned was only one of eight independently isolated clones, two forms of cDNA may exist. We next examined whether two mRNA types were expressed in human testis by PCR analysis using the primer pair B1 and B2, which amplified a 91 bp h-Scot-t fragment including nucleotides 745 to 778 (Figure 5). When we used genomic DNA as a template, we detected two PCR amplified-DNA fragments, of 91 and 72 bp, by acrylamide gel electrophoresis. On using a cDNA library as a template, only one band was detected (Figure 5B). These results indicate that the h-Scot-t gene is intronless, and that a nonfunctional pseudogene deleted of 19 nucleotides exists in the human genome. The sequence of the h-Scot-t-like pseudogene has been deposited in the DDBJ/EMBL/GenBank data bank under accession no. AB052900.
Characterization of a testis-specific Scot cDNA

Discussion

In the present study, we screened a human testicular cDNA library with mouse scot-t cDNA as a probe and isolated human Scot-t (h-Scot-t) cDNA consisting of 1749 nucleotides and a single open reading frame coding for 517 amino acids. Although the germ cell-specific Scot-t genes isolated in human and mouse (Koga et al., 2000) have high similarity with the somatic cell-type OXCT/SCOT gene (Kassovska-Bratinova et al., 1996), there are some differences. The major difference in sequence was at the NH₂ terminus where the mitochondrial leader sequence is located. The human Scot-t protein retains most of the common features of the mitochondrial leader sequence, such as a predominance of dibasic residues and no acidic amino acids (Kassovska-Bratinova et al., 1996; Mihara, 2000). Immunohistochemical analysis demonstrated that human Scot-t localized to the midpiece of spermatozoon where mitochondria exist. The tetrapeptide, SENG, is also conserved at position 340–343 of human Scot-t, where the CoA-binding residue glutamate is located, suggesting that this enzyme has CoA transferase activity (Rochet and Bridger, 1994). In contrast, residues 285–297 of human Scot-t are poorly conserved among other CoA transferases, e.g. pig scot, the scot-like C. elegans peptide, bacterial CoA transferase (Lin and Bridger, 1992; Kassovska-Bratinova et al., 1996), and also mouse scot-t (Koga et al., 2000).

Comparison of the scot-t cDNA and genomic sequence demonstrated that h-Scot-t is intronless and a pseudogene exists in the human genome. A homology search of the BLAST database with the human genomic sequence, revealed that both h-Scot-t gene and the h-Scot-t-like pseudogene are located on chromosome 1 (1p33-35.4). On the other hand, human OXCT/SCOT spans more than 100 kb and contains 17 exons, on chromosome 5 (5p13) (Fukao et al., 2000). The fact that the h-Scot-t gene lacks an intron and the presence of the h-Scot-t-like pseudogene indicates that h-Scot-t was made by a retrotransposon, a class of genes created by reverse transcription of a mature mRNA and insertion of the DNA copy into the genome (Vanin, 1985; Valentin et al., 2000). Recently, we have cloned two independent scot-t from a mouse testis cDNA library (AB022180 and AB049996). The mouse scot-t1 and scot-t2, so named by us, differ by just 25 nucleotides in the open reading frame and by just three amino acids (Figure 1). In the human, the pseudogene of h-Scot-t has five different nucleotides as well as a deletion of 19 bp relative to h-Scot-t. In mouse, both scot-t1 and scot-t2 should be functional in spermatozoa, whereas one of the h-Scot-t would change to be a non-functional pseudogene via a 19 bp deletion in human. Furthermore, the genomic structure of the h-Scot-t transcription unit was found to be located in an intron (1p34.1–35.3) of the bone morphogenetic protein 8 (BMP8) gene (Oakaynak et al., 1992). A homologous DNA fragment of ~19 kb containing h-Scot-t was located in the genomic clones of both AL033527

Figure 4. Localization of human SCOT-t in spermatozoa. Immunofluorescence microscopic image of spermatozoon stained with anti-Scot-t antibody. The arrowhead indicates the midpiece of a spermatozoon positively stained with FITC-labelled antibody. (A) UV light. (B) Visible light. (C) UV light. (D) Schematic representation of spermatozoa. Bar = 50 µm.
Figure 5. Analysis of the h-Scot-t genome by polymerase chain reaction (PCR). (A) PCR was performed with A1 and A2 primers. Amplified PCR products were analysed by 1% agarose gel electrophoresis. Lane 1, size marker DNA; lane 2, human Scot-t cDNA; lane 3, genomic DNA used as a template. The band in lane 3 was isolated from gel, subcloned and sequenced. (B) PCR was performed with B1 and B2 primers. Human Scot-t cDNA (lane 1), genomic DNA (lane 2), or a testicular cDNA library (lane 3) was used as a template. Amplified PCR products were analysed by 10% polyacrylamide gel electrophoresis. Each band in lanes 2 and 3 was isolated from gel and sequenced directly. (C) Human Scot-t cDNA and the positions of designed primers are represented schematically. Sequences of primers were described in Materials and methods and in Figure 1. (D) Schematic representation of the human Scot-t genome and flanking region. Open boxes indicate the human Scot-t gene. Shadowed boxes indicate exons of the BMP8 gene. Arrows indicate the direction of mRNA transcription for each gene. The OXCT/SCOT gene in the hypothetical protogenome gave rise to an ancestor gene which was then duplicated. One version was conserved and gave rise to the true gene and the other was changed to a pseudogene through deletion and mutation. The numbers of 56.3 and 60.3 Mb indicate physical distance from a telomere of chromosome 1 [Human Genome Reconstruction Projects (HGREP) http://hgrep.ims.u-tokyo.ac.jp/cgi-bin/HTG tool/view.cgi?layer=top].
(1p34.1–35.3) and AL033526 (1p33-34.3). Although these two 19 kb DNA fragments also included some of the BMP8 exons (Figure 5D), neither the h-Scot-t nor the BMP8 gene on chromosome 1p33-34.3 (AL033526) is functional. The region homologous to h-Scot-t has a deletion of 19 nucleotides and does not seem to be transcribed and some of the BMP8 exons are not included in this chromosome. These results suggested that the 19 kbp DNA fragment containing h-Scot-t in chromosome 1p34.1–35.3 (AL033527) would have been translocated to a locus in chromosome 1p33-34.3 (AL033526) and changed to a pseudogene (Figure 5D).

In a previous study in the mouse, BMP8, a member of the transforming growth factor β superfamily, was disrupted by gene targeting (Zhao et al., 1996). The disrupted genomic region of BMP8 exon 3 and 4 included an intron where scot-t was located. The authors have reported that the BMP8 gene reflected maintenance of spermatogenesis by phenotype in gene-targeted mouse. Two scot-t genes are expressed in mouse, nevertheless, a part of the phenotype of the BMP8 gene-targeted mouse may be due to disruption of one scot-t gene.

The region upstream of the initiation codon of h-Scot-t does not contain a TATA box, or cAMP-responsive promoter elements, but is GC-rich and has many CpG islands which may be the motif of regulation for haploid germ cell-specific expression. The design of the DNA sequence is similar to the promoter region of the haploid germ cell-specific gene haspin, whose transcription unit is located in an intron of the integrin αE gene, as an intronless gene (Tanaka et al., 2001). An ~19 kb region including the h-Scot-t transcription unit and putative promoter region rich in GC with many CpG islands also has high homology with the region of the pseudogene on chromosome 1p33-35.4. Although both the 5′- and 3′-flanking sequences of the h-Scot-t coding region should have high homology with the pseudogene, they differ in the way gene expression is regulated. As only h-Scot-t and not the pseudogene was transcribed, the 19 bp of h-Scot-t absent in the pseudogene may affect the promoter activity or stability of transcribed products.

We have demonstrated that the expression of h-Scot-t is limited to the testis, as was shown in the mouse. Some of the germ cell-specific isozymes involved in energy metabolism were also expressed at specific stages of germ cell differentiation and the physiological roles of these isozymes still remain to be elucidated (Eddy and O’Brien, 1998; Hecht, 1998). Recently, several cases of ketoacidosis caused by a deficiency of OXCT/SCOT due to a nonsense mutation in the OXCT/SCOT gene have been reported (Kassovska-Bratinova et al., 1996). Loss of SCOT-t activity due to mutation in the Scot-t gene may also cause male infertility.

In conclusion, we have isolated a human orthologue of mouse scot-t expressed specifically in the testis and have demonstrated that h-Scot-t is a single and intronless gene. The physiological role of h-SCOT-t in the energy metabolism of spermatozoa would be of interest to human reproductive biology.

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