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Human Mitochondrial Enoyl-CoA Hydratase Gene (ECHS1): Structural Organization and Assignment to Chromosome 10q26.2–q26.3

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The second step in mitochondrial fatty acid β-oxidation is catalyzed by short chain enoyl-CoA hydratase (ECHS1; EC 4.2.1.17). Inherited disorders of this pathway of energy metabolism present clinical and laboratory features resembling sudden infant death syndrome and Reye-like syndrome. To investigate the role of ECHS1 further, the gene structure was analyzed and its chromosomal locus determined. A fragment of rat liver ECHS1 cDNA was employed for isolation and characterization of two overlapping genomic clones encompassing the entire human ECHS1 gene. The gene, approximately 11 kb, is composed of eight exons, with exons I and VIII containing the 5′- and 3′-untranslated regions, respectively. Two major transcription start sites, located 62 and 63 bp upstream of the translation initiation codon, were mapped by primer extension analysis. The immediate 5′-flanking region of the ECHS1 gene is GC-rich and contains several copies of the SP1 binding motive but no typical TATA or CAAT boxes are apparent. *Alu* repeat elements have been identified within the region −1052/−770 relative to the cap site and in intron 7. The human ECHS1 gene locus was assigned to chromosome 10q26.2–q26.3 by fluorescence *in situ* hybridization. © 1997 Academic Press

INTRODUCTION

Short chain enoyl-CoA hydratase (ECHS1, EC 4.2.1.17) catalyzes the hydration of 2-*trans*-enoyl-CoA intermediates to L-3-hydroxy-acyl-CoAs during mitochondrial β-oxidation. The 2-*trans*-enoyl-CoA substrates of ECHS1 either are produced during degradation of saturated fatty acids in the acyl-CoA dehydrogenase reaction or arise as intermediates in the course of unsaturated fatty acid oxidation by the 3,2-*trans*-enoyl-CoA isomerase reaction in which 3-*cis*- and 3-*trans*-intermediates are isomerized to 2-*trans*-enoyl-CoAs.

ECHS1 has been purified from ox liver (Hass and Hill, 1969), rat liver (Furuta *et al.*, 1980), and pig heart (Fong and Schulz, 1977). Rat liver (Minami-Ishii *et al.*, 1989) and human liver (Kanazawa *et al.*, 1993) ECHS1 cDNAs have been cloned and sequenced. Site-directed mutagenesis of rat ECHS1 cDNA suggests glu-164 as an amino acid of the active site (Müller-Newen *et al.*, 1995).

Mitochondrial fatty acid β-oxidation is an important pathway providing energy, especially under physiological conditions in which glucose as the primary source of energy is reduced. Inherited disorders of mitochondrial fatty acid β-oxidation form a relatively new class of metabolic diseases associated with high mortality and morbidity rates in affected infants and younger children. Metabolic decompensation linked to an increased dependence on fat as primary metabolic fuel is a common feature of fatty acid oxidation disorders and is also described in sudden infant death syndrome and Reye-like syndrome (Hale and Bennett, 1992). More than 12 different defects have been described in humans until now. There are strong indications for additional, yet unrecognized genetic disorders of mitochondrial fatty acid oxidation (Coates and Tanaka, 1992).

Mitochondrial ECHS1 belongs to the low homology hydratase/isomerase enzyme superfamily exhibiting significant sequence homologies to different monofunctional and polyfunctional enzymes with 2-enoyl-CoA hydratase and 3,2-*trans*-enoyl-CoA isomerase (DCI) activity from mitochondria, peroxisomes, or bacteria (Müller-Newen *et al.*, 1995). The ECHS1 and DCI activities of the polyfunctional enzymes are located in the respective N-terminal portions. A common evolutionary origin is suggested for these structurally and functionally related enzymes.

The present work describes the structural organization and chromosomal localization of the human ECHS1 gene. Transcription start sites and 1.5 kb of

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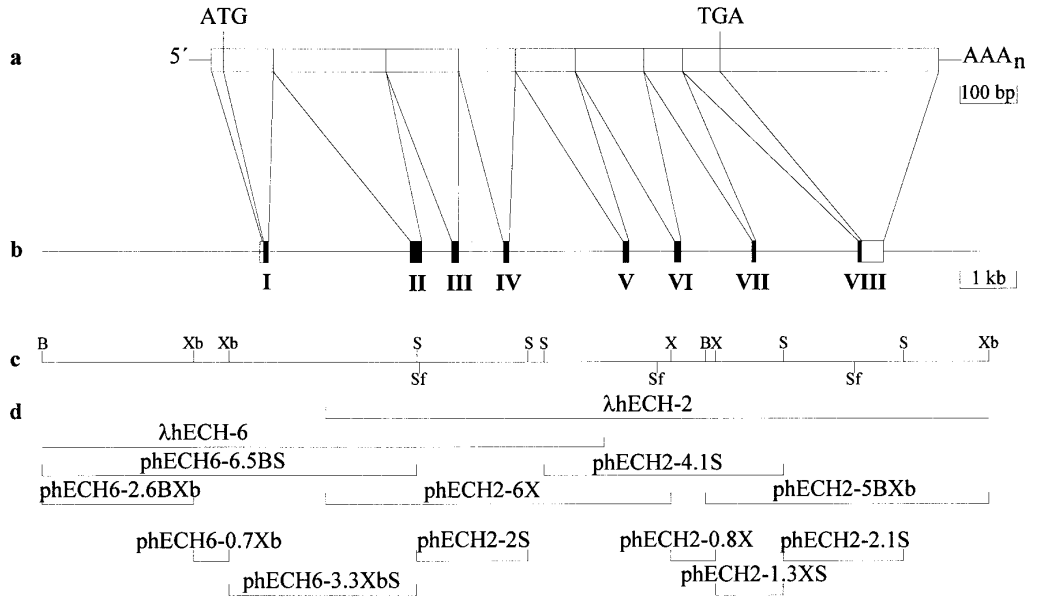

FIG. 1. Structural organization of the human ECHS1 gene. (**a**) Human ECHS1 mRNA. (**b**) Arrangement of the exons, numbered I–VIII, with the coding regions, represented by filled boxes, and the 5′- and 3′-untranslated regions of exons I and VIII, respectively, indicated by open boxes. (**c**) Cleavage sites of the restriction endonucleases *Bam*HI (B), *Sfi* I (Sf), *Sac*I (S), *Xho*I (X), and *Xba*I (Xb) used for characterization and subcloning. (**d**) The locations of the two overlapping genomic phage clones λhECH-2 and λhECH-6 and the subclones used for sequence analysis.

the 5′-flanking sequence were determined. These data provide the tools to investigate the molecular basis of fatty acid oxidation defects and to study the regulation of ECHS1 expression. Comparison of the gene structures of ECHS1 and the previously characterized DCI (Janßen *et al.*, 1994) may support understanding of the evolutionary relationship of the enzymes.

MATERIALS AND METHODS

Isolation of genomic ECHS1 clones. A human placental genomic DNA library in EMBL-3 SP6/T7 (Product No. HL1067j; Clontech) was screened with a ^{32}P-labeled (Feinberg and Vogelstein, 1983) 630-bp *Nco*I fragment derived from the previously cloned rat ECHS1 cDNA (Müller-Newen *et al.*, 1995). Screening of 5×10^5 phage plaques yielded seven positive clones (λhECH-1 to 7) (Maniatis *et al.*, 1982). Their genomic inserts were analyzed by Southern blot hybridization analysis with the 5′-end-labeled oligonucleotides HydG-5′s, 5′-ATGGCCGCCCTGCGTGTCCTGCTGTCCTGC-3′, and HydG-3′as, 5′-TCACTGGTCTTTGAAGTTGGCCT-3′, as well as the 630-bp *Nco*I fragment. The inserts of phage clones λhECH-2 (≈18 kb) and λhECH-6 (≈15 kb) were overlapping and covered the entire ECHS1 gene.

Restriction analysis and subcloning of phage DNA. For further analysis the genomic inserts of λhECH-2 and λhECH-6 were partially mapped by single and double restriction digestion with *Bam*HI, *Sac*I, *Sfi* I, *Xba*I, and *Xho*I, followed by Southern blot hybridization either with cDNA fragments or with appropriate oligonucleotides as probes. Sequence analysis of genomic fragments containing exons and the 5′-flanking region cloned into pBluescript SK(+) (Stratagene) was performed by the chain-termination method (Sanger *et al.*, 1977) using the T7-sequencing kit (Pharmacia, Uppsala/Sweden) with M13 primers and gene-specific oligonucleotides as internal primers. If not completely sequenced, intron sizes were determined

TABLE 1

Exon/Intron Sizes and Sequences at Splice Junctions of the Human ECHS1 Gene

Exon	Size (bp)	Exon/intron junction sequences: 5′-Donor site	Exon/intron junction sequences: 3′-Acceptor site	Intron	Size (bp)	Amino acids interrupted
I	⩾151	GCC TCG G gtgagt	cttgctccag GT GCT AAC	1	≈2500	Gly^{30}
II	198	TTT GCA G gtacgc	ctcctgctag CT GGA GCT	2	527	Ala^{96}
III	128	GGC TAT GCC gtgagt	tgttttacag TTT GGC GGG	3	779	Ala^{138}/Phe^{139}
IV	100	ATC CCA G gtaaag	ctccctgcag GT GCG GGC	4	≈2000	Gly^{172}
V	105	CAA GCA G gtatgg	tgtgtttcag GT CTT GTC	5	790	Gly^{207}
VI	120	AAT GCA G gtaggg	tgtttttcag CT TTT GAA	6	1250	Ala^{247}
VII	68	TTT GCC ACT gtgagt	ttcctgccag GAT GAC CGG	7	≈1800	Thr^{269}/Asp^{270}
VIII	⩾448	3′-UTR				
Consensus splice signal		NN NAG gt agt (a, g)	ccccccc c n ag G (ttttttt t)			

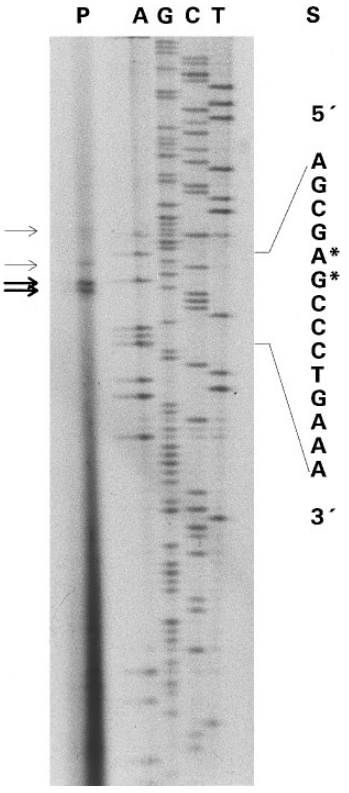

FIG. 2. Determination of the transcription initiation sites for the human ECHS1 gene. For primer extension analysis 40 μg of human liver total RNA was hybridized with the 5′-end-labeled oligonucleotide HydG-5′as and reverse transcribed as described under Materials and Methods. Primer extension products (lane **P,** marked by arrows) were analyzed on a denaturing, 5% polyacrylamide gel together with a sequence ladder (lanes **A, G, C,** and **T**) obtained by dideoxy-sequencing the 5′-flanking region (subclone phECH6-3.3XbS) with primer HydG-UTRas. The two nucleotides identified as major transcription start sites are indicated by asterisks (lane **S**).

by polymerase chain reaction (PCR) of the appropriate genomic subclones.

Primer extension. Transcription start sites were determined by primer extension analysis following the standard procedure (McKnight and Kingsbury, 1982). In brief, the 30-mer antisense oligonucleotide HydG-5′as, 5′-GCAGGACAGCAGGACACGCAGGGCGGCCAT-3′, complementary to the first 30 nucleotides of the coding sequence of exon I, was 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. After annealing to 40 μg of human liver total RNA (Chomczynski and Sacchi, 1987), the reverse transcription was performed with Moloney murine leukemia virus RNaseH$^-$ reverse transcriptase (GIBCO-BRL). Extension products were separated on a 7 *M* urea, 5% polyacrylamide gel together with a sequence ladder obtained by sequencing the 5′-flanking region (subclone phECH6-3.3XbS) with the 17-mer antisense primer HydG-UTRas, 5′-GCAGGACAGCAGGACAC-3′.

Chromosomal assignment of human ECHS1. DNAs isolated from a mapping panel of somatic cell hybrids (NIGMS human/rodent somatic cell hybrid panel 2) and, as control, from human, mouse, and Chinese hamster total DNA (1 μg DNA template) were subjected to amplification using the primer pair HydC-1 (sense), 5′-TGGTACCAAGGCTACCATCTATCCTTGTGC-3′, and HydC-2 (antisense), 5′-GTCGCATGCCTCTGCCATCACAGAGGCGCA-3′, corresponding to genomic sequences of introns 1 and 2, respectively. Template DNAs and primer were denatured in the presence of 4 m*M* $MgCl_2$ and standard concentrations of dNTP at 94°C for 4 min and then subjected to 25 cycles each at 94°C for 1 min, 68°C for 1 min, 72°C for 1.5 min, and a final extension step at 72°C for 10 min. The reaction products were electrophoresed on a 2% agarose gel with *Msp*I-digested pBR322 as marker.

Fluorescence in situ chromosomal hybridization. Human metaphase cells were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes. The ECHS1 probe was the human genomic phage clone λhECH-2 containing exons II–VIII. Fluorescence *in situ* hybridization was performed as decribed previously (Rowley *et al.,* 1990). Biotin-labeled probes were prepared by nick-translation using Bio-11–dUTP (Enzo Diagnostics). Hybridization was detected with fluorescein-conjugated avidin (Vector Laboratories). Chromosomes were identified by staining with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI).

RESULTS AND DISCUSSION

Structure and Organization of the Human ECHS1 Gene

With a fragment of the previously cloned rat ECHS1 cDNA as hybridization probe, seven positive phage clones were isolated from a human placental genomic library. Restriction mapping followed by Southern blot hybridization analysis using appropriate cDNA fragments or synthetic oligonucleotides revealed that the two overlapping clones λhECH-2 and λhECH-6 (Fig. 1) span the entire ECHS1 gene. Gene fragments containing coding sequences and the 5′-flanking region were subcloned (Fig. 1) for sequence analysis. Exons, exon/intron boundaries, approximately 1500 bp of the 5′-upstream region, and 700 bp beyond the stop codon were sequenced. As shown in Fig. 1, the human ECHS1 gene locus extended over about 11 kb and consisted of eight exons interrupted by seven introns. Exon I contained the 5′-UTR and the putative N-terminal mitochondrial signal sequence. Exon VIII included the C-terminal portion of the coding sequence and the 382-bp 3′-UTR with a consensus polyadenylation signal (ATTAAA) located 359–364 bp downstream of the stop codon. Table 1 summarizes the sizes of the exons, the introns, and the exon/intron boundary sequences. All of the exon/intron junctions are consistent with the GT/AG rule (Breathnach and Chambon, 1981). The comparison of the exon sequences with the previously published human ECHS1 cDNA (Kanazawa *et al.,* 1993) revealed overall 11 exchanged nucleotides, and, as a result, five amino acid deviations. Nucleotide sequence changes were confirmed by sequencing both strands. The five deviating amino acids match the respective rat ECHS1 primary structure. A *Sfi*I restriction site that is present in the genomic sequence but not in the human cDNA results from a T to G (codon 82) and a G to C (codon 84) change.

Identification of Transcription Initiation Site

The transcription initiation site of the ECHS1 gene was mapped by primer extension analysis of human liver total RNA. The noncoding sequence was extended from the oligonucleotide HydG-5′as complementary to the 5′-end of the coding region of exon I. For analysis, the products of the primer extension reaction were sep-

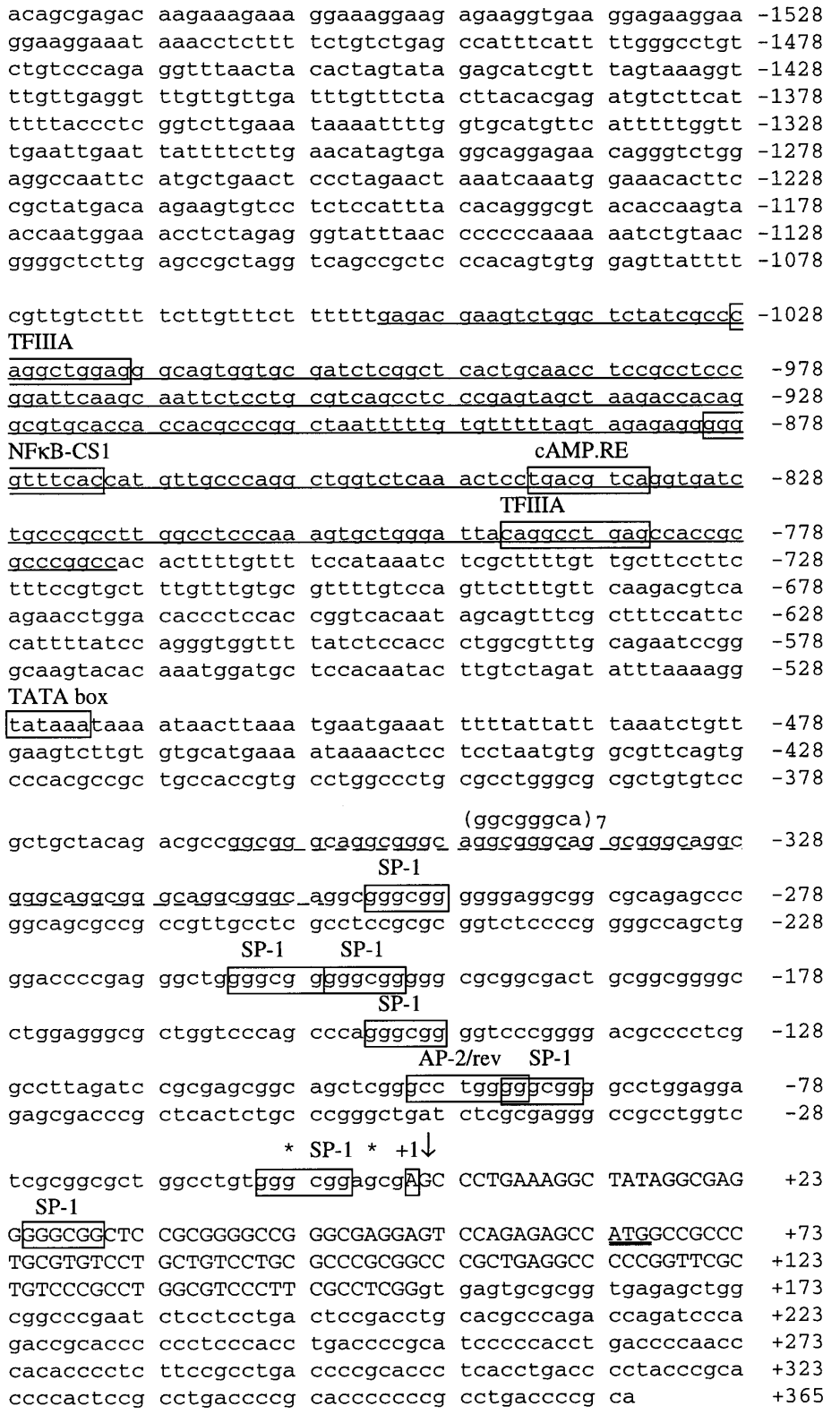

FIG. 3. Nucleotide sequence of the 5′-flanking region of the ECHS1 gene. The major transcription start site referring to nucleotide A is designated as +1 (boxed). The second major and two minor start sites are marked by an arrow and asterisks, respectively. The 5′-untranslated region and the subsequent coding region (ATG, double underlined) are in capital letters. Putative transcriptional regulatory elements are boxed. The *Alu* repeat element is marked by a solid underline, the $(ggcgggca)_7$ repeat by a dashed underline.

arated on a sequencing gel adjacent to a sequence ladder generated by the dideoxy chain termination reaction of subclone phECH6-3.3XbS (Fig. 1d) using oligonucleotide HydG-UTRas as primer. As a result, two major and two minor extension products were obtained (Fig. 2). The two major bands are located 62 and 63 bp upstream of the translation start codon. The most upstream of these two putative transcription start sites of similar intensity has been designated position +1 (nucleotide A) in the genomic sequence (Fig. 3).

Analysis of the 5′-Flanking Region

The nucleotide sequence of the 5′-flanking region of the human ECHS1 gene, contained in subclone phECH6-6.5BS (Fig. 1), was determined up to nucleotide 1577 upstream of the putative cap site (Fig. 3). Analysis of the upstream sequence was performed with a software program (Genetics Computer Group, 1991) that identified known regulatory sequences. The immediate 5′-flanking region of the ECHS1 gene is extremely GC-rich (≈75%). It contains six Sp-1 binding sites (GC boxes, GGGCGG) from −5 to −10, −89 to −94, −148 to −153, −201 to −206, −207 to −212, and −298 to −303 relative to the cap site. A further GC box is located in the 5′-UTR from +25 to +30. Although a TATA box-like sequence (TATAAA) is present between positions −522 and −527, it is located outside the putative promoter region of the ECHS1 gene. Thus, the ECHS1 gene promoter presents the typical features of TATA-less RNA polymerase II promoters (Blake *et al.*, 1990), which have been found in the two other characterized human genes encoding mitochondrial β-oxidation enzymes, the MCAD gene (Zhang *et al.*, 1992) and the DCI gene (Janßen *et al.*, 1994), and in many housekeeping genes (Dynan, 1986).

The 5′-flanking region contains several motifs known to be involved in transcription regulation. A reverse AP-2 binding site is present from −93 to −100, a NF-κB site from −871 to −880, and two TFIIIA binding sites from −785 to −794 and −1019 to −1028. A cAMP responsive element is located between positions −835 and −842. The role of these transcription factor binding sites in the regulation of ECHS1 gene expression remains to be established. Additional characteristics are an Alu-like sequence between nucleotides −770 and −1052 and a GC-rich repeat $(GGCGGGCA)_7$ from −307 to −362.

Chromosomal Localization of the Human ECHS1 Gene

In an initial analysis primer-specific PCR screening of a monochromosomal somatic cell hybrid panel (data not shown) assigned the ECHS1 gene locus to chromosome 10. The precise localization of the ECHS1 gene was resolved by fluorescense *in situ* hybridization of a biotin-labeled ECHS1 probe to normal human metaphase chromosomes. Hybridization of the ECHS1 probe resulted in specific labeling only of chromosome 10 (Fig. 4). Labeling of 10q25.3–q26 was observed on zero (1 cell), one (0 cells), two (3 cells), three (13 cells), or all four (8 cells) chromatids of the chromosome 10 homologues in 25 cells examined. Of 77 signals observed, 7 (9%) were located at the junction of 10q25.3–q26.1, 13 (17%) signals at 10q26.1, 38 (49%) signals at 10q26.2, 17 (22%) signals at 10q26.2–q26.3, and 2 (2%) signals at 10q26.3. One signal was located at 4q16 (1%). Similar results were obtained in an additional hybridization experiment using the same probe. These results suggest that the ECHS1 gene is localized to chromosome 10, bands q26.2–q26.3.

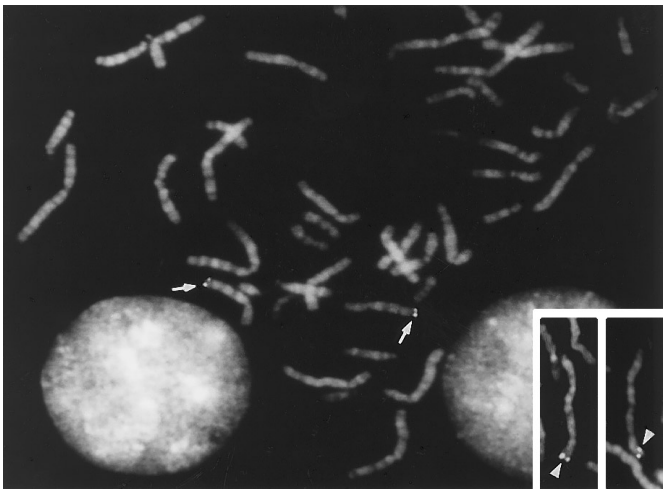

FIG. 4. Chromosomal assignment of the human ECHS1 gene. *In situ* hybridization of a biotin-labeled ECHS1 probe to human metaphase cells from phytohemagglutinin-stimulated peripheral blood lymphocytes. The chromosome 10 homologues are identified with arrows; labeling was observed at 10q26.2. The insets show partial karyotypes of two chromosome 10 homologues illustrating labeling at 10q26.2 (arrowheads). Images were obtained using a Zeiss Axiophot microscope coupled to a cooled, charge-coupled device (CCD) camera. Separate images of DAPI-stained chromosomes and the hybridization signal were merged using image analysis software (NIH Image 1.52).

Comparison with the Human DCI Gene

Mitochondrial ECHS1 and mitochondrial DCI are structurally, functionally, and evolutionary related enzymes. The amount of amino acid identity between the precursors of the respective human enzymes is 22.4%. Comparison of the structural organization of the genes encoding ECHS1 and DCI (Janßen *et al.*, 1994) showed no obvious similarities. The coding sequence of ECHS1 is spread over eight exons, whereas the DCI gene consists of seven exons. Additionally no exon/intron boundary position is conserved between the two genes.

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