Identification of a second human acetyl-CoA carboxylase gene

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INTRODUCTION

Acetyl-CoA carboxylase (ACC), an important enzyme in fatty acid biosynthesis and a regulator of fatty acid oxidation, is present in at least two isoenzymic forms in rat and human tissues. Previous work has established the existence of a 265 000 Da enzyme in both the rat and human (RACC265; HACC265) and a higher-molecular-mass species (275 000–280 000 Da) in the same species (RACC280; HACC275). An HACC265 gene has previously been localized to chromosome 17. In the present study, we report cloning of a partial-length human cDNA sequence which appears to correspond to HACC275 and its rat homologue, RACC280, as judged by mRNA tissue distribution and cell-specific regulation of mRNA/protein expression. The gene encoding this isoenzymic form of ACC has been localized to the long arm of human chromosome 12. Thus, ACC is represented in a multigene family in both rodents and humans. The newly discovered human gene and its rat homologue appear to be under different regulatory control to the HACC265 gene, as judged by tissue-specific expression in vitro and by independent modulation in cultured cells in vitro.

EXPERIMENTAL

cDNA library screening

A ZAP II human white adipose tissue cDNA library (gift of Dr. David Bernlohr, University of Minnesota) was screened with rat ACC probes, as previously detailed [12]. This screening yielded human ACC sequences, HACC-1 and HACC-2. HACC-2 was then used to rescreen the library to isolate overlapping sequences. Probe labelling and library screening of 2 x 10^6 plaques was carried out, as in [15]. Positive plaques were purified through three rounds of screening and phagemid from positive clones rescued with helper phage (Stratagene).

Plasmid preparation and DNA sequencing

Plasmid DNA was purified from bacterial culture using a standard alkaline lysis protocol [16]. DNA to be used for sequencing was further purified using Qiagen Mini or Midi-columns, according to the manufacturer’s instructions. DNA was sequenced using an Applied Biosystems Prism® ready reaction Dye Deoxy Terminator Cycle Sequencing kit, and cycled in a Perkin-Elmer PCR Thermocycler, according to the manufacturers’ instructions. For initial sequence analysis, T7

Abbreviations used: ACC, acetyl-CoA carboxylase; HACC, human acetyl-CoA carboxylase, as either the 275 kDa (HACC275) or 265 kDa (HACC265) isozyme; RACC, rat acetyl-CoA carboxylase, as either the 280 kDa (HACC280) or 265 kDa (RACC265) isozyme; DMEM, Dulbecco’s modified Eagle medium; PVDF, poly(vinylidene difluoride).

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The nucleotide sequence of HACC-2 and overlapping clones (contiguous sequence termed HACC-β) has been deposited in the GenBank under accession number U34591.
Two principal cell lines were chosen for the current investigations, the H4IIE rat hepatoma cell and the rat H9c2 embryonic cardiomyocyte, the latter of which differentiates from myoblasts to cardiomyocyte, the latter of which differentiates from myoblasts to cardiomyocyte.

Promoter and M13 reverse primers were used to prime sequence reactions. Subsequently gene-specific primers were employed to completely sequence both DNA strands of each plasmid. ExTRANeous dye terminators were then removed from the resulting sequence reactions using a CentrI-Sep column (Princeton Separations, Inc.). The purified sequencing reactions were then dried in a Speed-Vac and analysed on an automated DNA sequencer (Applied Biosystems Model 373). DNA sequences were analysed and aligned using MacVector® software and the GCG software package. Sequences were additionally formatted using an Excel® macro, as in [15].

Southern blot analysis and analysis of somatic cell hybrid DNA

Southern blotting was carried out by a standard method [16]. Genomic DNA from human, mouse and hamster (CHO cells) and from two somatic cell hybrid panels (Coriell; Bios Laboratories) was digested with various restriction enzymes previously detailed [15]. Filters were washed at high stringency in 0.1 SSC for 20 min (2 changes) at room temperature followed by 0.1 SDS at 55 °C for 1.5 h (3 changes); SSPE: 0.15 M NaCl/0.1 M sodium phosphate (pH 7.4)/1 mM EDTA.

Electroblotting of ACC

After separation of proteins on a 5%, SDS/acylamide gel and transfer to poly(vinylidene difluoride) (PVDF) membrane, lysate proteins were probed with either a monoclonal antibody specific for RACC280 (ACC 7AD3) [2], an anti-(N-terminal peptide) antibody (N) specific for RACC265 [2], or streptavidin–peroxidase, which detects both ACC isoenzymes [2,7]. Antibody binding was detected with peroxidase-labelled secondary antibodies and chemiluminescence (ECL; Amersham).

Northern blot analysis and RNA preparation

Total RNA was isolated from tissues and cell lines using a guanidinium isothiocyanate–lithium chloride method, as described in [19]. Northern blot analysis was carried out, as previously detailed [15]. Filters were washed at high stringency in all experiments [2]× SSPE for 20 min (2 changes) at room temperature followed by 0.1× SSPE/0.1% SDS at 55 °C for 1.5 h (3 changes); SSPE: 0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA.

Materials

H9c2 cells were obtained from the American Type Culture Collection. H4IIE cells were a kind gift from Dr. Daryl Granner (Vanderbilt). Cell media and sera were obtained from Gibco/BRL. PVDF membranes were purchased from Millipore. Secondary antibodies and most chemical reagents were purchased from Sigma. α-[32P]CTP for cDNA probe labelling was purchased from DuPont/New England Nuclear. Nitrocellulose for North-
Figure 2  Comparison of HACC-α and HACC-β deduced amino acid sequences

Shown is the alignment of the deduced HACC-β amino acid sequence with residues 1181–2000 of the HACC-α (HACC265) [14]. Alignments with gap assignments were performed using the Pileup program of the GCG software package. Non-identical amino acids are indicated by shading.

RESULTS

Isolation of unique HACC cDNAs

During screening of a human adipose tissue cDNA library with a partial-length RACC265 cDNA, two partially overlapping but non-identical cDNAs, termed HACC-2 (785 bp) and HACC-1...
(752 bp) were isolated (Figure 1). The DNA sequence of HACC-1 corresponds to nt 3672 to 4423 of the reported HACC265 cDNA [14]. The HACC-2, as a putative new ACC sequence, was used to rescreen the library to isolate overlapping clones; two such clones were identified that extended this sequence in both the 5’ and 3’ directions, yielding a contiguous cDNA sequence of about 2.5 kb. An open reading frame was identified within this contiguous sequence, which encodes for a protein sequence highly homologous to, yet significantly different from, the reported HACC265 deduced amino acid sequence (Figure 2) [14]. In this alignment, we have assigned the name HACC-alpha (α) to the HACC265 sequence and HACC-beta (β) to the new sequence; the reasons for this designation are detailed in the Discussion. We were unable with further screening to extend the HACC-β sequence in either the 3’ or 5’ direction. While thus lacking a full-length coding sequence, sufficient sequence has been obtained to indicate that this unique sequence encodes an ACC. Comparison with the database of the HACC-β contiguous sequence yields alignment with ACC species from several organisms (results not shown).

**Identification of unique mRNAs recognized by HACC-2**

In order to characterize further this new ACC sequence, Northern blot analysis was performed. The hybridization profile of HACC-2 was compared with that of HACC-1 against RNA isolated from several human cell lines, from representative rat tissues and from rat cell lines after manipulation of ACC polypeptide content.

We have reported heterogeneity of HACC isoenzyme expression in several human breast cancer cell lines [7]. As previously determined by immunoblotting and streptavidin blotting, the MCF7 and ZR-75 lines express both HACC265 and HACC275 polypeptides, while the T47D line expresses only HACC265 [7]. In parallel to these observations of expressed protein, HACC-1, corresponding to HACC265 sequence, recognizes a 9 kb mRNA in all three of these cell lines with the highest apparent expression in the T47D line (Figure 3). However, HACC-2 hybridizes with a similar sized mRNA predominantly in the MCF7 and ZR-75 cells, corresponding to the distribution of HACC275.

Since HACC275 appears to be the human homologue of RACC280 and HACC265 the human homologue of RACC265 [7], the hybridization patterns of HACC-2 and HACC-1 to rat tissue RNAs were compared in an effort to further distinguish these cDNAs. As shown in Figure 3, HACC-1 hybridizes to a 9 kb mRNA in rat white adipose, but no hybridization to any mRNA in either rat skeletal muscle or heart is seen. This parallels the expected distribution of RACC265 polypeptide [2,3]. In contrast, HACC-2 hybridizes to a 9 kb mRNA in both heart and skeletal muscle, but not in white adipose tissue, corresponding to the polypeptide distribution of RACC280 [2,3]. Weak hybridization was seen in this experiment to either probe with total brain RNA; in other experiments (results not shown) the HACC-1 reactive species appeared to predominate. A pattern of hybridization identical to that of HACC-2 was seen with both the 3'- and 5'-overlapping clones in the HACC-β contiguous sequence that were obtained by further library screening (results not shown). Taken together, these data suggest that HACC-1 is highly homologous to the RACC265 sequence, while HACC-2 is highly homologous to the RACC280 sequence.

RACC280 isoenzyme content can be selectively altered in both H4IIE hepatoma cells and in the differentiating H9c2 cell, providing other sources of mRNA for cDNA characterization. As shown in Figure 4, during differentiation of the H9c2 cell...
from myoblast to myotube, RACC280 is selectively induced. As determined by streptavidin blotting of cell lysates, ACC induction is first detectable on day 4 of differentiation and plateaus between days 6 and 7 (Figure 4, upper panel). With the use of ACC isoenzyme-specific antibodies, this ACC species is identified as the RACC280 isoenzyme (Figure 4, middle panel); no reactivity with an ACC265 species can be observed (Figure 4, bottom panel).

In H4IIE hepatoma cells, glucose and a fibric acid derivative, ciprofibrate, both lead to the selective induction of RACC280 with no change in RACC265 content (Figure 5). As judged by streptavidin–horseradish peroxidase blotting, RACC265 is the predominant form of ACC present in these cells in basal medium (Figure 5A). No change in ACC content is observed over the 48 h of substrate deprivation. However, after 24 h of glucose repletion, there is a marked induction of RACC280 with no change in RACC265. The identification of this band as RACC280 is indicated by its reactivity with the RACC280-specific antibody (Figure 5B). It should be noted that the biotin-containing protein at 120 kDa is unchanged over this time period (Figure 5A); this protein is mitochondrial pyruvate carboxylase, which is solubilized in the presence of SDS [4]. In the H4IIE hepatoma cells, ciprofibrate also leads to a selective induction of RACC280 with no change in RACC265 after 72 h of drug stimulation (Figure 5C). This increase is observed in the digitonin lysate, although other biotin-containing proteins of mitochondrial origin (e.g. pyruvate carboxylase) are seen in this fraction.

To reinforce the conclusion that HACC-1 is highly homologous to RACC265 sequence, while HACC-2 is highly homologous to RACC280 sequence, we examined the hybridization patterns of HACC-2 and HACC-1 cDNAs with the rat cell RNAs after manipulation of ACC polypeptide expression. As shown in Figure 6, a 9 kb mRNA, recognized by HACC-2, is increased in all of these cell lines after RACC280 induction, but...
Genomic DNA from hamster (Chinese hamster ovary cell line; CHO), mouse and human were digested with EcoRI, HindIII, PstI and Taq and then analysed by Southern blotting. The blot on the left, after hybridization with 32P-HACC-2, was stripped and then reprobed with 32P-HACC-1 to allow overlay alignment.

the 9 kb mRNA recognized by HACC-1 is unchanged under all three conditions of induction. Both mRNAs are, however, induced in liver by fasting/refeeding of the intact rat in vivo, a nutritional manipulation known to induce both ACC species [2,9].

Given the unique hybridization patterns of these cDNAs in the human cell lines, rat tissues and in rat cell lines, we conclude that the HACC-2 sequence (and overlapping extension sequences in the HACC-β contiguous sequence) represents the homologue of RACC280, namely HACC275, while HACC-1 sequence, corresponding to partial HACC265 sequence [14], represents the homologue of RACC265. These conclusions, based on available sequence information and mRNA distribution, must be regarded as tentative until such time as the full-length cDNA sequence, in vitro expression of both isoforms and human enzyme isolation are completed. However, irrespective of the completion of this work in progress, as discussed below, a second and novel human ACC gene has clearly been identified in the present study.

Identification of a second human ACC gene

Inspection of the sequences of HACC265 and the partial sequence of HACC275 suggests strongly that they are derived from different genes and are not related by alternative splicing of a single gene (Figure 2). Southern blot analysis of human genomic DNA digested with nine different restriction enzymes and probed with either HACC-2 or HACC-1 reveals non-overlapping patterns of hybridization, also strongly suggesting a multigene family (Figure 7). Similar analysis of rat genomic DNA by this same technique was also consistent with more than one rat ACC gene (results not shown).

The gene encoding HACC275 was localized by hybridization of DNA fragments derived from two separate somatic cell hybrid panels (Bios and Coriell). Before these studies, Southern blot hybridization of human, mouse and hamster genomic DNA was carried out in order to assure that human DNA sequence could be recognized against a background of either mouse or hamster DNA. As shown in Figure 8, both the HACC265 and HACC275 gene sequences can be recognized against this rodent background after digestion with four different restriction enzymes. These results further indicate the unique hybridization patterns of these two cDNAs, not only in the human, but in the mouse and hamster, consistent with a multigene family in these species as well. We previously documented the existence of more than one ACC isoenzyme in cell lines derived from both mouse and hamster [3,4].

Hybridization of the EcoRI-digested Bios cell panel DNA with HACC-1 showed recognizable human sequence only in cell lines 811 and 937 (Figure 9). Concordance analysis showed that this corresponds to hybridization with human chromosome 17 sequences, confirming the previous localization of HACC265 to chromosome 17 by in situ hybridization [13,14]. An identical chromosomal localization was assigned after analysis of the DNA from the Coriell cell panel (results not shown).

Hybridization of the EcoRI-digested Coriell cell panel DNA...
Southern blot analysis of Coriell cell line DNA (results not cDNAs of HACC-2 gave the identical localization pattern on the latter is known to contain a fragment of chromosome 12 (see text). Human genomic DNA is recognized only in the cell line chromosome 12 and chromosome 6; to the predominant or exclusive chromosome represented in each of the cell lines. Hybridizing and hamster, shown in the left-hand three lanes of each panel. The lane designations correspond to the predominant or exclusive chromosome represented in each of the cell lines. Hybridizing human genomic DNA is recognized only in the cell line chromosome 12 and chromosome 6; the latter is known to contain a fragment of chromosome 12 (see text).

with HACC-2 showed recognizable human sequence only in cell lines corresponding to chromosomes 12 and 6 (Figure 10). The latter cell line is known to contain a piece of chromosome 12, represented by the mitochondrial aldehyde dehydrogenase gene at the 12q24.2 locus [20,21]. In contrast, the chromosome 12 cell line is not known to contain any chromosome 6 fragments. Analysis of the Bios cell panel DNA with this same probe also showed recognizable human sequence only in cell lines (Coriell) after digestion with EcoRI. These membranes were probed with 32P-HACC-2. The hybridization pattern of these DNAs is compared with total genomic DNA from human, mouse and hamster, shown in the left-hand three lanes of each panel. The lane designations correspond to the predominant or exclusive chromosome represented in each of the cell lines. Hybridizing human genomic DNA is recognized only in the cell line chromosome 12 and chromosome 6; the latter is known to contain a fragment of chromosome 12 (see text).

**DISCUSSION**

These studies indicate that ACC is represented in the human genome by a multigene family of related sequences. In addition, we have presented evidence that a multigene family is also present in the rat, mouse and hamster.

The 265000 Da form of ACC, present in both the rat and human (RACC265 and HACC265, respectively), has previously been extensively characterized [10–14]. The deduced amino acid sequence of HACC265 has been in some dispute, based on results obtained from different laboratories [12,14]. Recently, Abu-Elheiga and co-workers have reported a cDNA sequence for human ACC265 that is different at the 3'-end from that reported earlier by Ha and co-workers in one of our laboratories (K.H.K.) [12,14]. The former suggested the possibility of the earlier sequence being a hybrid sequence between the two different forms of ACC. Subsequent work to clarify this discrepancy, as reported herein by independent cloning of HACC sequences in one of our laboratories (L.A.W.), indicates that this is indeed the case. The sequence from nt 4404 to 7020, reported by Ha et al. [12], is not that of HACC-β (HACC265) as originally reported, but that of HACC-β. Therefore, in comparing the HACC-β sequence to the HACC-α sequence, we have compared our sequences in the studies reported herein to that to Abu-Elheiga, which we now believe to be the correct full-length HACC265 sequence.

Given that full-length ACC sequences, which have regions of absolute nucleotide identity, have been assembled by cDNA library walking and/or PCR extension techniques, it becomes important to ascertain that each piece of overlapping cDNA is aligned in its correct full-length clone. As we have demonstrated in this study, the profiling of select tissue and cell mRNAs may be useful in making the correct contiguous alignment of large cDNAs assembled in this manner. Southern blot hybridization of total genomic DNA and/or DNA derived from somatic cell hybrid panels can also be used to maintain the proper assignments.

The partial-length cDNA contiguous sequence that has been assembled in the present study from HACC-2 appears to correspond to the 275000 Da form of ACC (HACC275) and its rat homologue, RACC280, based on patterns of RNA hybridization. Its gene has been localized to chromosome 12, and, based on hybridization to genomic DNA in one somatic cell hybrid line (Coriell Chromosome 6; HHW484), probably to its distal long arm [20,21]. The mRNA derived from this human gene (and from its rat homologue) appears to be highly expressed both in tissues that are classically either lipogenic (liver) or non-lipogenic (heart, skeletal muscle) [1–3]. All of these tissues are mitochondria-rich and can have high rates of fatty acid oxidation. It is therefore tempting to speculate that this gene product is perhaps most important in the provision of malonyl-CoA, not for fatty acid synthesis, but for the allosteric regulation of fatty acid oxidation through modulation of carnitine palmitoyltransferase I [4,5].

The discovery of isoforms increases the complexity of the understanding of the role and regulation of ACC. One factor contributing to this complexity is the tissue-specific expression of the isoenzymes [1–4]. In rat, both isoforms (RACC265 and RACC280) are expressed in the liver, lactating mammary gland, pancreatic islets and brown adipose tissue. The heart and skeletal muscle predominantly express RACC280 and the brain and white adipose tissue solely express RACC265. In the human, two isoforms (HACC265 and HACC275) are expressed in adipose tissue, while only the higher-molecular-mass form is expressed in skeletal muscle. Human breast cancer cell lines have varying tissue-specific expression of one or both isoenzymes [7]. The ACC isoforms also differ in their enzyme kinetics. RACC280 has increased citrate dependence (higher $K_c$) and a 2-fold higher $K_m$ for acetyl-CoA [2,22]. In tissues where both isoenzymes are expressed, they are also associated in a complex composed of both types of polypeptide chains [2,3]. It seems possible that...
numerous forms of total ACC actually exist, depending on the relative mix of isoenzyme content in each tissue.

In addition to tissue-specific expression, the two isoforms can be co-ordinately or independently regulated in vivo [2,23] and in vitro in cultured cell lines. In vivo in the liver, starvation (where lipogenesis is low) represses the content of both isoenzymes, and high carbohydrate feeding (in which lipogenesis is increased) induces both. However, in heart and skeletal muscle, nutritional state does not alter RACC280 content [2]. Similarly, the induction of diabetes mellitus in the rat by streptozotocin markedly diminishes expression of both isoenzymes in the liver and RACC265 in adipose tissue, but the cellular content of RACC280 in heart and skeletal muscle is unaltered (A. Bianchi and L. A. Witters, unpublished work). In cell lines cultured in vitro, shown in the present study, RACC280 expression can be independently regulated by myodifferentiation, glucose and ciprofibrate. These in vitro observations provide some model systems where the dissection of the individual roles of ACC isoforms and the mechanisms underlying their unique regulation can be addressed in future studies.

There is adequate precedent for multigene families of metabolic enzymes with selective expression in liver, heart and skeletal muscle. Several of the enzymes of glycogen metabolism are prime examples of such multigene families. Given this precedent, it is possible that the HACC275/RACC280 expressed in liver may not necessarily be the product of the same gene as the enzyme expressed in heart or skeletal muscle. In order to avoid confusion with the designation of ACC isoenzymes based solely on molecular mass, we propose a nomenclature change. We propose that the 265000 Da form, as extensively characterized in the rat and human and represented by an HACC265 sequence reported [2], be referred to as ACC-alpha (α) and that the larger-molecular-mass form, which is the subject of the present report (275000 Da in the human; 280000 in the rat), be referred to as ACC-beta (β). Human species are thus designated as HACC-α and HACC-β. Such a designation leaves open the possibility that there are other as yet unidentified ACC genes encoding enzymes of similar/identical molecular masses. Indeed, by in situ chromosomal hybridization, there are conflicting reports as to the localization of the human gene encoding for the 265000 Da enzyme. One group has reported localization to 17q12, using an HACC265 PCR-derived genomic probe [14], while another has reported localization to 17q21 using an RACC265 cDNA [13]. This suggests the possibility of three HACC genes, two on chromosome 12 and one on chromosome 17.

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