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Genetics

Molecular cloning of cDNAs encoding rat and human medium-chain acyl-CoA dehydrogenase and assignment of the gene to human chromosome 1

(cDNA library screening with immunopurified mRNA/fatty acid oxidation/mitochondrial biogenesis)

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ABSTRACT **Rat liver mRNA encoding the precursor of medium-chain acyl-CoA dehydrogenase was purified to near homogeneity by polysome immunoadsorption using a polyclonal, monospecific antibody. A single-stranded, ^{32}P-labeled cDNA probe was synthesized using the enriched mRNA as template and was used to screen directly 15,000 colonies from a total rat liver cDNA library constructed in pBR322. One clone [600 base pairs (bp)] was positively identified by hybrid-selected translation combined with mitochondrial processing of translated products. Using the isolated rat cDNA as probe, 43,000 colonies from a human liver cDNA library were screened. Three overlapping clones (1100 bp, 500 bp, and 400 bp) were isolated and positively identified by hybrid-selected translation. The largest human cDNA clone was subcloned into the transcription vector pGEM-2, which contains a bacteriophage T7 RNA polymerase promoter. *In vitro* transcription of this recombinant, followed by *in vitro* translation, showed that the cDNA clone coded for ≈80% of the medium-chain acyl-CoA dehydrogenase protein. The sizes of rat and human mRNAs encoding the precursor of medium-chain acyl-CoA dehydrogenase were 2.2 and 2.4 kilobases long, respectively, as determined by blot hybridization analysis of electrophoretically fractionated poly(A)$^+$ RNA. Southern blot analysis of DNAs from human–rodent somatic cell hybrids with an isolated human cDNA assigned the gene coding for this enzyme to the short arm of chromosome 1, band p31. The chromosomal assignment was confirmed by *in situ* hybridization of the probe to human metaphase cells. Direct screening of cDNA libraries using a highly enriched mRNA to generate a probe, as demonstrated in this study, may provide the most rapid and convenient approach to cDNA cloning of low-abundance mRNAs.**

Five acyl-CoA dehydrogenases are known to date (1). They are short-chain, medium-chain, and long-chain acyl-CoA dehydrogenases, isovaleryl-CoA dehydrogenase, and 2-methyl branched-chain acyl-CoA dehydrogenase. Each of these dehydrogenases is a mitochondrial flavoprotein, catalyzing α,β-dehydrogenation of acyl-CoA esters. The former three enzymes catalyze the first reaction step of the β-oxidation cycle in the catabolism of fatty acids, while the latter two enzymes catalyze the dehydrogenation of branched short-chain acyl-CoAs in the metabolism of the branched-chain amino acids. All these acyl-CoA dehydrogenases share similar structural and functional features, but they differ distinctly in the chain length and configuration of the acyl chain of their substrates (2–4). Their similarities suggest that these five enzymes may share a common ancestral gene and have acquired their structural diversity and distinctive substrate specificities in the course of evolution. Among the five acyl-CoA dehydrogenases, medium chain acyl-CoA dehydrogenase (EC 1.3.99.3, MCADase) has the broadest substrate specificity, covering C_4 to C_{12} straight-chain acyl-CoAs. MCADase isolated from rat liver is a homotetramer with a subunit molecular weight of 45,000 (4). Like other nuclear-coded mitochondrial proteins, rat MCADase is synthesized on cytoplasmic ribosomes as a precursor (preMCADase; M_r 49,000) distinctly larger than its mature subunit. The precursor is then imported by an energy-dependent mechanism into mitochondria where its leader sequence is proteolytically cleaved, producing the mature form (5). Human preMCADase has an apparent molecular weight of 50,000; its leader peptide has been estimated at 4000 (6).

Inherited deficiency of MCADase in man was characterized in 1982 (7); at least 24 cases with this disorder are now known (8–11). The clinical features of the disorder are intolerance to prolonged fasting, recurrent episodes of hypoglycemic coma accompanied by medium-chain dicarboxylic aciduria, impaired ketogenesis, and low plasma and tissue carnitine levels. This disorder may be severe, even fatal, in some patients. Among 13 human MCADase-deficient fibroblast cell lines studied, residual enzymatic activity ranged from 6 to 13% of the mean control value (6). The MCADase in these cell lines was indistinguishable in size from normal human MCADase (6).

cDNA cloning of MCADase would provide a valuable tool for the molecular study of MCADase and its genetic deficiency in man. In this report, we describe the isolation of partial cDNAs encoding rat and human MCADase. Using the cloned human cDNA as probe, we assigned the gene for MCADase to the short arm of human chromosome 1, band p31.

EXPERIMENTAL PROCEDURES

Antibody. Preparation of antiserum raised against purified rat liver MCADase has been described (4). The antiserum was monospecific to rat and human MCADase and did not crossreact with other acyl-CoA dehydrogenases (4, 6). The IgG fraction of the antiserum was purified using a protein A-Sepharose (Pharmacia) column and used for polysome immunopurification (12, 13).

Enrichment of preMCADase mRNA from Rat Liver. mRNA encoding preMCADase was enriched by polysome immunopurification (12, 13). Polysomes were isolated from six livers of male Sprague–Dawley rats in the presence of trichodermin and vanadyl ribonucleoside. The isolated polysomes were incubated with the purified IgG and then applied to a protein A-Sepharose column. After the column

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Abbreviations: MCADase, medium-chain acyl-CoA dehydrogenase; bp, base pair(s); kb, kilobase(s).

was washed extensively to remove unbound polysomes, the ribosomal subunits and the specific mRNA were eluted from the column. The eluate was then applied to an oligo(dT)-cellulose (type 2, Collaborative Research, Waltham, MA) column, and the isolated preMCADase mRNA was further purified by centrifugation on a linear sucrose gradient (5–20%, wt/wt).

***In Vitro* Translation of the Enriched mRNA.** A rabbit reticulocyte lysate translation kit (Bethesda Research Laboratories) employing 0.05 mCi (1 Ci = 37 GBq) of [^{35}S]methionine (Amersham) was used to translate the enriched mRNA. The translated polypeptides were analyzed, either directly (3 μl) or after immunoprecipitation (27 μl), by NaDodSO$_4$/10% PAGE as described by Laemmli (14). The gels were subjected to fluorography.

Preparation of a Rat Liver cDNA Library and a Human Liver cDNA Library. Total rat liver mRNA was used to prepare a cDNA library in *Escherichia coli* of the DH1 strain transformed with recombinant pBR322 as previously reported (15). This rat liver cDNA library is the same library from which a full-length ornithine transcarbamoylase cDNA clone was isolated (15). Likewise, a total human liver cDNA library was prepared from a postmortem liver, except that cDNA synthesis was carried out by another method (16).

Screening of the Libraries. A single-stranded, ^{32}P-labeled cDNA probe was synthesized from the highly enriched mRNA by use of reverse transcriptase from avian myeloblastosis virus (Life Sciences, St. Petersburg, FL) and [α-^{32}P]dCTP (Amersham) (16). This cDNA was used as probe to screen directly the total rat liver cDNA library by *in situ* colony hybridization (17). Plasmid DNA from positive clones was isolated on a small scale (18), digested with *Pst* I (New England Biolabs), and analyzed by 1.5% agarose gel electrophoresis. The rat preMCADase cDNA plasmid was purified by CsCl centrifugation, and the cDNA insert was nick-translated and used to identify human preMCADase clones in the human liver cDNA library.

Hybrid-Selected Translation of mRNA Using the Isolated cDNA Clones. The positive cDNA clones were isolated and subjected to hybrid-selected translation (19). An aliquot of translation products was further processed with intact rat liver mitochondria (20).

Subcloning of cDNA into a Transcription Vector and *in Vitro* Transcription/Translation of the cDNA. The cDNA insert of an isolated clone was subcloned into pGEM-2 transcription vector (Promega Biotec, Madison, WI) containing T7 and SP6 RNA polymerase promoters. The newly constructed plasmid was used to transform *E. coli* strain DH5 (Bethesda Research Laboratories), and recombinant plasmids were recovered and purified as before. *In vitro* transcription of the cDNA was carried out according to the manufacturer's protocol, and the synthesized mRNA was translated *in vitro* as described above.

RNA Blot Analysis. Rat and human liver mRNAs were each fractionated by electrophoresis in 2.2 M formaldehyde/1% agarose gels, transferred to nitrocellulose filters (Schleicher & Schuell), and hybridized with the ^{32}P-labeled cDNA clone encoding rat or human preMCADase.

Hybrid Cell Lines. Two sets of somatic cell hybrids were used to localize the gene coding for MCADase. For the primary assignment to a human chromosome, Chinese hamster–human hybrids of series XII, XIII, XV, XVII, XVIII, and XXI were used. Their derivation has been summarized (21, 36). The chromosomal content of the hybrids was determined at the same passage as DNA extraction.

Regional assignment of MCADase was carried out by analyzing six hybrid cell lines with distinct rearrangements of chromosome 1. The hybrid clones XV-16A-F4 and X-5F-q were formed between Chinese hamster cells and human fibroblasts with balanced translocations of chromosome 1 (22, 23). Two clones (XVI-10C and XVI-18A) of hybrid series XVI had acquired different *de novo* translocations involving the short arm of chromosome 1 (24, 25). In hybrid series XIX, two independent clones contained either the short arm (XIX-23A) or the long arm (XIX-48A) of chromosome 1 (26).

Southern Blot Hybridization. Genomic DNA was extracted from hybrids and parental control cell lines as described (27). DNA samples were digested with *Eco*RI, electrophoresed, transferred to nitrocellulose filters, and hybridized to the human preMCADase cDNA as previously reported (21).

***In Situ* Hybridization.** The human preMCADase cDNA probe was nick-translated with [^{3}H]dATP, [^{3}H]dCTP, and [^{3}H]dTTP to a specific activity of 2×10^7 cpm/μg. *In situ* hybridization to human chromosome preparations was performed according to published procedures (21, 28).

RESULTS

Purified Rat Liver preMCADase mRNA. mRNA encoding rat preMCADase was purified by polysome immunopurification. The enriched mRNA and total liver mRNA were translated in a cell-free system, and the polypeptides produced were analyzed by NaDodSO$_4$/PAGE (Fig. 1). Translation of the immunopurified preMCADase mRNA yielded a major unique band (lane 2) immunoprecipitable with monospecific anti-MCADase antiserum (lane 5). This major unique band was indistinguishable in size from rat preMCADase (lane 4). In addition, a few shorter polypeptides were observed in lane 2. All of these polypeptides were immunoprecipitable (lane 5) and probably derived from early termination of translation or from initiation at internal methionines. Therefore, this result indicates that preMCADase mRNA was purified to near homogeneity. A faint band of preproalbumin was, however, observed in some *in vitro* translations of the enriched mRNA, although it is not clearly seen in lane 2.

Synthesis of a Single-Stranded cDNA and Its Use as a Probe in Screening a Rat Liver cDNA Library. A single-stranded, ^{32}P-labeled cDNA probe was synthesized from the enriched preMCADase mRNA and used to screen directly 15,000 independent colonies from a total rat liver cDNA library. Approximately 500 positive colonies were identified in the primary screening. Thirty-six colonies were tentatively taken through further screening (total of three rounds) until single well-isolated colonies were obtained. Among them, plasmid cDNA from four clones exhibited the same restriction fragment patterns as those of a full-length preproalbumin cDNA when digested with *Pst* I, *Hha* I, or *Hin*dIII (29). One of the four clones was subjected to hybrid-selected translation and positively identified as a full-length preproalbumin cDNA clone (Fig. 2, lanes 1 and 5). The cDNA insert of this cDNA clone was isolated, radiolabeled by nick-translation, and used

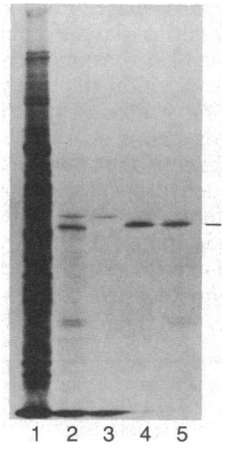

FIG. 1. NaDodSO$_4$/10% PAGE of polypeptides translated from total rat liver mRNA and from immunopurified preMCADase mRNA. Lane 1: translation of total mRNA. Lane 2: translation of immunopurified preMCADase mRNA. Lane 3: translation with no added mRNA, showing the position of [^{35}S]methionine-reticulocyte protein adduct (12). Lane 4: translation products shown in lane 1, after immunoprecipitation with anti-MCADase antiserum. Lane 5: translation products shown in lane 2, after immunoprecipitation with anti-MCADase antiserum. Marker at right indicates position of preMCADase.

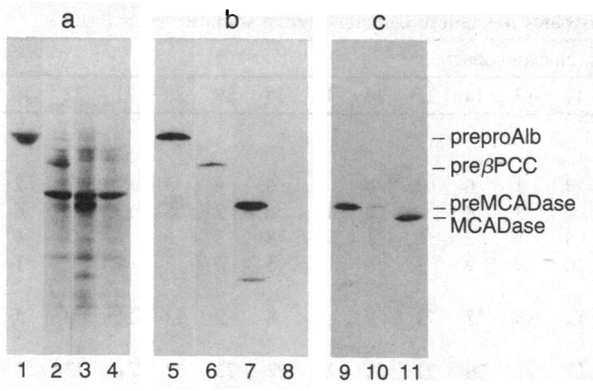

FIG. 2. Identification of rat preMCADase cDNA by hybrid-selected translation. Fluorography was for 25 min (lane 5), 2 hr (lane 1), 14 hr (lanes 9–11), 24 hr (lanes 3, 4, 7, and 8), or 39 hr (lanes 2 and 6). (*a*) Direct analysis of translation products of hybrid-selected mRNAs. cDNAs used for hybrid-selection were preproalbumin (preproAlb) cDNA (lane 1); cDNA coding for the precursor of the β subunit of propionyl-CoA carboxylase (preβPCC) (lane 2); cDNA coding for rat preMCADase (R-1) (lane 3); pBR322 DNA (lane 4). (*b*) Immunoprecipitation of the translation products shown in lanes 1–4 with corresponding antisera. Lane 5: immunoprecipitation of products shown in lane 1 with antialbumin antiserum. Lane 6: immunoprecipitation of products shown in lane 2 with anti-βPCC antiserum. Lane 7: immunoprecipitation of products shown in lane 3 with anti-MCADase antiserum. Lane 8: immunoprecipitation of products shown in lane 4 with anti-MCADase antiserum. (*c*) Mitochondrial processing of preMCADase. Lane 9: immunoprecipitated translation products of mRNA selected by R-1. Translation products shown in lane 9 were incubated with intact rat liver mitochondria at 27°C for 1 hr and the mitochondria were sedimented by centrifugation for 5 min at 13,000 × *g*. The mitochondrial pellet (lane 11) and the supernatant (lane 10) were immunoprecipitated with anti-MCADase antiserum, electrophoresed, and fluorographed.

for Southern blot analysis of other isolated cDNA clones. Only one clone [R-1, 600-base-pair (bp) insert] did not hybridize to the preproalbumin cDNA probe.

Hybrid-selected translation with clone R-1 using rat liver mRNA yielded a 49-kDa polypeptide indistinguishable in size from rat preMCADase (Fig. 2, lane 3). This polypeptide was immunoprecipitable with anti-MCADase antiserum (lane 7). In this experiment, cDNA encoding the precursor of the β subunit of propionyl-CoA carboxylase (16) was used as a positive control (lanes 2 and 6), and unmodified pBR322 served as a negative control (lanes 4 and 8). In order to demonstrate the posttranslational processing of preMCADase translated from hybrid-selected mRNA, the translation product (lane 9) was incubated with intact rat liver mitochondria. Most of the polypeptide was taken up by mitochondria and cleaved to yield a polypeptide (lane 11) identical in size to mature rat MCADase (localized in the same lane by Coomassie blue stain), while only a small fraction of the polypeptide remained as precursor (49 kDa) in the supernatant (lane 10).

Screening of a Human Liver cDNA Library. The cDNA insert of R-1 was radiolabeled by nick-translation and used to screen 43,000 independent colonies from a human cDNA library. One positive clone (H-1, 400-bp insert) was isolated. With this human cDNA clone as probe, additional colonies were screened under more stringent washing conditions. Two additional positive clones (H-2, 1100-bp insert; H-3, 500-bp insert) were identified. Hybrid-selected translation with each of the three clones using human liver mRNA yielded a 50-kDa protein band indistinguishable in size from human preMCADase and specifically immunoreactive with anti-rat MCADase antibody (data not shown).

***In Vitro* Expression of Human MCADase.** The cDNA insert of the largest human preMCADase cDNA (H-2) was isolated along with minimal flanking pBR322 sequences by digestion with *Msp* I. This fragment was ligated with *Acc* I-cut, dephosphorylated pGEM-2 transcription vector (Fig. 3*a*). The transcription of the plasmid (linearized with *Bam*HI), using T7 RNA polymerase followed by *in vitro* translation, yielded nine polypeptides (Fig. 3*b*, lane 3), all immunoprecipitable with anti-MCADase antiserum (lane 4) but not with normal rabbit serum (lane 5). These polypeptides were probably derived from initiation of translation at different internal methionine codons. The largest synthesized peptide was 40 kDa in size. On the other hand, transcription of the other strand with SP6 RNA polymerase following *Hin*dIII digestion did not produce translatable mRNA (lane 2).

RNA Blot Analysis. The lengths of rat and human preMCADase mRNAs were 2.2 and 2.4 kilobases (kb), respectively, as determined by blot hybridization analysis of electrophoretically fractionated rat liver and human liver mRNAs using either rat or human preMCADase cDNAs as probes (data not shown).

Human Chromosomal Mapping. In the first set of hybrids studied, the human specific 4.2-kb and 2.7-kb *Eco*RI fragments hybridized with the human preMCADase cDNA probe H-1 only in hybrids carrying human chromosome 1 (data not shown). In mouse DNA and in DNA from mouse-derived hybrids, a 9.4-kb *Eco*RI band cross-hybridized with this probe. In rat control DNA and hybrids from rat origin, a 5.6-kb *Eco*RI fragment was detected. No cross-hybridizing fragment was detected in Chinese hamster DNA. Correlation between the human-specific fragments and various human chromosomes in somatic cell hybrids is summarized in Table 1. Human chromosome 1 is the only chromosome that segregated perfectly with the human MCADase fragments.

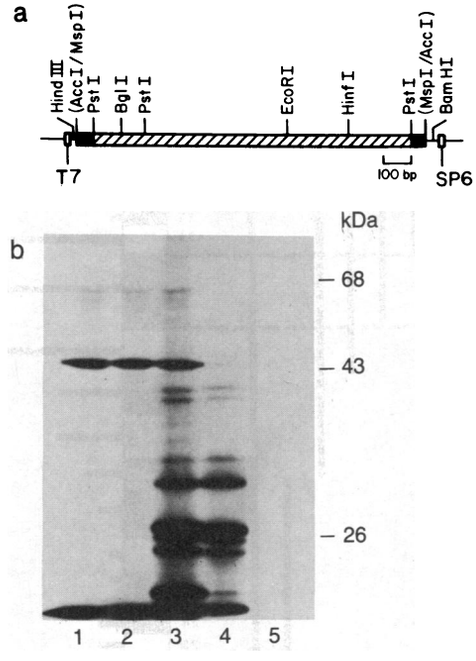

FIG. 3. (*a*) Partial restriction map of H-2 inserted into pGEM-2. Hatched segment shows the cDNA flanked by pBR322 sequences (black boxes). Straight line indicates the pGEM-2 sequence, which contains T7 RNA polymerase promoter and SP6 polymerase promoter (shown as open boxes). (*b*) *In vitro* transcription and translation of H-2. Lane 1: translation with no added mRNA. Lane 2: translation of the mRNA transcribed with SP6 RNA polymerase. Lane 3: translation of the mRNA transcribed with T7 RNA polymerase. Lane 4: translation products shown in lane 3 after immunoprecipitation with anti-MCADase antiserum. Lane 5: translation products shown in lane 3 after immunoprecipitation with normal rabbit serum.

Table 1. Correlation of human MCADase sequences with human chromosomes in Chinese hamster–human somatic cell hybrids

	Human chromosomes																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
Hybridization/ chromosome																								
+/+	5	5	6	3	2	5	2	6	3	1	3	4	2	6	3	6	3	6	6	3	7	7	2	1
−/−	11	11	3	9	8	5	9	8	9	10	7	7	3	3	6	5	11	8	7	6	3	6	6	11
+/−	0	2	3	5	5	3	5	3	6	8	3	5	7	2	6	3	6	3	3	6	2	2	4	8
−/+	0	2	8	3	5	8	4	4	2	3	6	6	9	9	7	6	2	5	6	7	9	7	1	2
Discordant hybrids	0	4	11	8	10	11	9	7	8	11	9	11	16	11	13	9	8	8	9	13	11	9	5	10
Informative hybrids	16	20	20	20	20	21	20	21	20	22	19	22	21	20	22	20	22	22	22	22	21	22	13	22
% discordant	0	20	55	40	50	52	45	33	40	50	47	50	76	55	59	45	36	36	41	59	52	41	38	47

Data on rearranged chromosomes or chromosomes present in fewer than 10% of the cells were excluded.

All other chromosomes were excluded by four or more discordant hybrids as possibly carrying the MCADase gene.

The second set of hybrids, containing partially overlapping regions of chromosome 1 in the absence of an intact chromosome 1, allowed us to precisely map MCADase to a single chromosome band. Hybrids carrying region B (XVI-10C), C (XVI-18A), D (XIX-23A), or F (X-5F-q) of chromosome 1 were positive for the two human DNA fragments, whereas the hybrids that contained region A (XV-16A-F4) or E (XIX-48A) were negative (Fig. 4 *Left*). The shortest region of overlap of all positive hybrids was band 1p31, localizing the MCADase gene to this band.

The regional assignment determined by somatic cell hybrid analysis was further confirmed by *in situ* hybridization of ^{3}H-labeled preMCADase cDNA H-1 to human metaphase chromosomes. Twenty-two percent of cells analyzed had silver grains at bands p22–p32 of one or both copies of chromosome 1. Of 107 grains observed on chromosome 1, 61 (57%) were found over this specific region, with a peak at band p31 (Fig. 4, *Right*). The peak of the silver grain distribution coincides precisely with the single band localization of MCADase derived from the completely independent study of somatic cell hybrids (Fig. 4).

DISCUSSION

In this report, we describe the isolation of partial cDNA clones coding for rat and human MCADase. Immunochemical purification of mRNAs has been successfully used for cDNA cloning of various low-abundance mRNAs (13, 16, 19, 31, 32). In most of the previous studies, purified mRNAs were used for preparation of cDNA probes to screen already enriched cDNA libraries prepared from immunopurified mRNAs. An efficient method for preparation of a cDNA library from minute amounts (usually nanograms) of purified

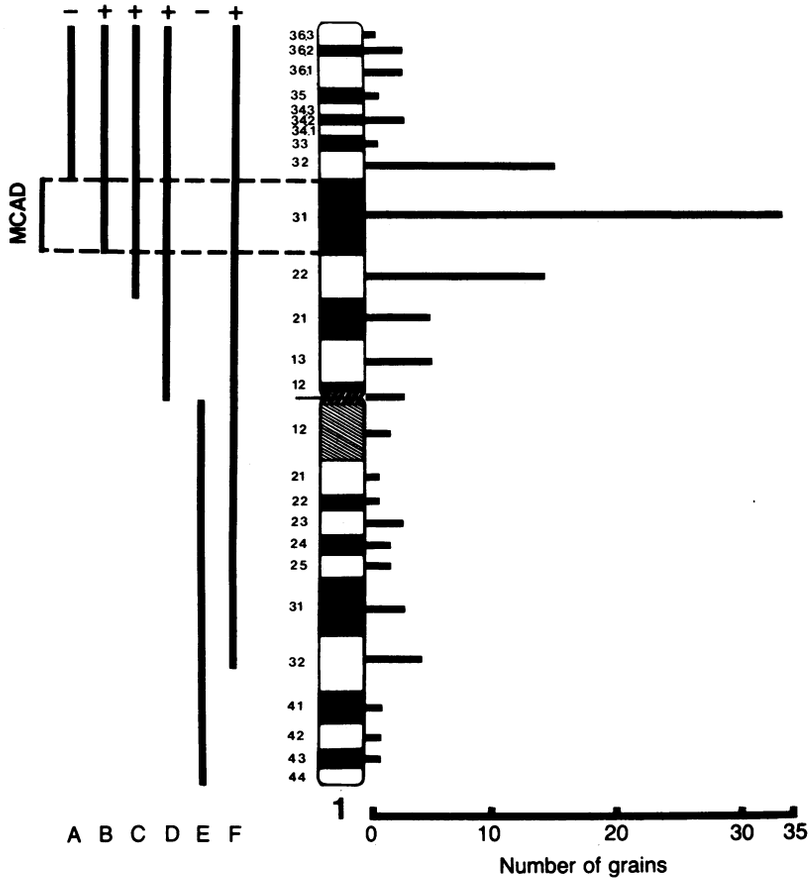

FIG. 4. Ideogram of G-banding patterns of human chromosome 1 (30). (*Left*) Regional mapping data with vertical bars illustrating different portions of chromosome 1 present in somatic cell hybrids. + = hybrid positive for human MCADase sequences, and − = hybrid negative. (*Right*) Silver grain distribution along chromosome 1 after *in situ* hybridization; horizontal bars indicate number of grains over each band.

mRNA has been devised and proven to be successful (16), but the procedure still involves several difficult steps, and significant losses of material may occur. In the present study, we screened directly a total rat liver cDNA library, using the single-stranded radiolabeled cDNA synthesized from the highly enriched preMCADase mRNA. Numerous false positive colonies were observed in the primary screening and, subsequently, were identified as preproalbumin cDNA clones. This was probably due to contamination of the mRNA preparation with a minute amount of preproalbumin mRNA, as was detected in some of the *in vitro* translation experiments. This observation is consistent with the fact that preproalbumin mRNA is the most abundant message among hepatic mRNAs (33), 100- to 200-fold more abundant than the preMCADase mRNA. However, the preproalbumin cDNA clones were subsequently eliminated by Southern blot analysis using a full-length preproalbumin cDNA as a probe, and a preMCADase cDNA clone was obtained. Thus, when monospecific antibody is available, a highly purified mRNA can be prepared by immunopurification of specific polysomes. Our findings indicate that such enriched mRNA can be used for direct screening of an existing cDNA library derived from total mRNA. This may provide a more rapid and convenient approach to cDNA cloning of low-abundance mRNAs than methods in current use.

The identification of the isolated rat preMCADase cDNA (R-1) was verified by hybrid-selected translation, yielding a 49-kDa polypeptide. The polypeptide was indistinguishable in size from the precursor of the subunit of MCADase and was immunoprecipitable with anti-MCADase antibody. Mitochondrial processing of the translation product indicated that the newly synthesized peptide had a 4-kDa leader sequence that directed the peptide into mitochondria and was cleaved off to produce a 45-kDa polypeptide during or after the translocation. This observation is consistent with the result obtained in a study of MCADase biosynthesis (5). This experiment provided additional evidence that the isolated clone indeed encoded preMCADase mRNA.

A human liver cDNA library was screened with the radiolabeled rat preMCADase cDNA clone to obtain human preMCADase cDNAs. The identities of the human preMCADase cDNA clones (H-1, H-2, and H-3) were similarly confirmed by hybrid-selected translation. Further, one of the clones (H-2) was subcloned into a transcription vector and shown to encode an open reading frame that generated polypeptides recognizable by anti-MCADase antiserum. The size of the largest synthesized polypeptide was 40 kDa, suggesting that H-2 contained ≈80% of the coding region of human preMCADase mRNA (Fig. 3).

The apparent sizes of rat and human preMCADase mRNAs were 2.2 kb and 2.4 kb, respectively, as determined by blot analysis. These data explain why preproalbumin mRNA, which is ≈2026 bases long (29), was not separated from rat preMCADase mRNA on linear sucrose gradient centrifugation. Because the estimated number of nucleotides in the coding region of rat preMCADase is ≈1340 (4, 5), these data suggest the presence of a long 3′ untranslated sequence in both rat and human preMCADase mRNAs similar to that observed in porcine mitochondrial aspartate aminotransferase precursor (34).

By using the isolated human cDNA clone as a probe, the gene for MCADase was assigned to chromosome 1, band p31. This result places the MCADase locus just distal to the loci *NRAS, PGM1, NGFB, TSHB,* and *MSK1* and immediately proximal to *FUCA1, UROD, AK2, UMPK, BLYM,* and *MYCL* (35). Thus, the assigned position of the human MCADase gene makes it a reference point for a detailed linkage map of chromosome 1. If restriction fragment length polymorphisms are detected with the MCADase probe, they will be useful for completing the map of the short arm of human chromosome 1. In addition, it will be of interest to compare the location of genes for other acyl-CoA dehydrogenases when cDNA probes for these enzymes become available.

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