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Molecular cloning of cDNA for cholesterol 7α-hydroxylase from rat liver microsomes

Nucleotide sequence and expression

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A complete cDNA clone encoding cholesterol 7α-hydroxylase was isolated from a rat liver cDNA library by the use of specific antibodies to the enzyme. The isolated cDNA clone was 3.6 kbp long and contained a 1509-bp open reading frame encoding 503 amino acid residues ($M_r = 56\,880$). The identity of the cDNA was confirmed by expression of cholesterol 7α-hydroxylase activity and the immunoreactive protein in COS cells transfected with pSVL expression vector carrying the cDNA insert. The primary structure of cholesterol 7α-hydroxylase deduced from the nucleotide sequence of the cDNA indicated that the enzyme constitutes a novel P-450 family.

Cholesterol 7α-hydroxylase; Cytochrome P-450; cDNA cloning; (COS cell)

1. INTRODUCTION

Cholesterol 7α-hydroxylase (EC 1.14.13.17) is a major regulatory enzyme for bile acid biosynthesis in vertebrates [1]. This enzyme is a microsomal monooxygenase consisting of a cytochrome P-450 and NADPH-cytochrome P-450 reductase. In spite of its importance as a regulatory enzyme functioning in disposal of cholesterol from the body in the form of bile acids, little is known about the regulatory mechanism of this enzyme at the molecular level.

Recently, cytochrome P-450 ch7α catalyzing cholesterol 7α-hydroxylation has been purified and characterized in this laboratory [2]. The successful purification of the enzyme has facilitated exploration of the mechanism of the enzyme regulation using immunochemical analysis and/or molecular biology technique. The present paper describes the isolation of a complete cDNA encoding rat cholesterol 7α-hydroxylase from a rat liver cDNA library using specific antibodies. The results obtained indicate that cholesterol 7α-hydroxylase is a unique form of the cytochrome P-450 superfamily. The identity of the isolated cDNA clone was confirmed by expression of the enzyme activity in COS 7 cells transfected by pSVL vector carrying the cDNA insert.

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2. MATERIALS AND METHODS

Cytochrome P-450 ch7α was purified from liver microsomes of cholestyramine-treated male rats as described previously [2]. Specific polyclonal antibodies were prepared by immunizing BALB/c female mice with the purified protein mixed with Ribi adjuvant as described before [3].

A λ gt11 cDNA library and a λ ZAP cDNA library were prepared from liver poly(A^+) RNA of cholestyramine-treated male rats [4]. The liver λ gt11 cDNA library was screened with specific antibodies to cholesterol 7α-hydroxylase as described by Young and Davis [5]. Positive plaques with immunoreactive signals were isolated, and the insert DNA of a positive clone was excised by *Eco*RI digestion and used as a probe to screen the λ ZAP cDNA library for the isolation of full-length cDNA clones.

DNA sequence analysis of cDNA clone was carried out by the dideoxy chain termination method of Sanger et al. [6] with a modification which used 7-deaza-dGTP [7] and Sequenase [8]. Expression of cDNA clone was performed by inserting the 2.2-kbp *Xho*I-*Xho*I fragment (see fig.2) into pSVL vector and transfecting COS 7 cells with the constructed plasmid DNA [9] by an electropolation method. Northern hybridization [4], Western blotting [10], and determination of 7α-hydroxycholesterol [2] were carried out as described previously.

3. RESULTS AND DISCUSSION

In the course of several trials to produce specific antibodies to cholesterol 7α-hydroxylase, strong and specific polyclonal antibodies were obtained from an immunized mouse. This preparation of antibodies inhibited strongly and specifically the hydroxylase activity in liver microsomes (data not shown). We therefore used the antibodies to screen liver cDNA libraries to

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isolate cDNA clones encoding cholesterol 7α-hydroxylase. The rat liver cDNA library constructed in λ gt11 was screened with the antibodies. Out of 2×10^5 clones, 3 immunoreactive clones were isolated and analyzed. The 2.5-kbp insert was excised by *Eco*RI digestion from one clone and used to check the size of cholesterol 7α-hydroxylase mRNA by Northern hybridization. As shown in fig.1, the size of the hydroxylase mRNA was approximately 3.6 kbp. The 2.5-kbp insert was used as a probe to screen the λ ZAP cDNA library for isolation of full-length cDNA clones. Four out of 18 isolated clones from the λ ZAP cDNA library had approximately 3.6 kbp inserts. These clones were converted to pBluescript phagemid clones by the in vivo excision method [11] and were subjected to restriction mapping. Since all of the 4 clones were found to have the same restriction map, a plasmid clone, called p7α-11, was selected to determine the nucleotide sequence of cholesterol 7α-hydroxylase cDNA.

Fig.2 shows the restriction map of p7α-11 and the sequencing strategy. A 1.6-kbp DNA fragment from the 5′ end was sequenced and found to contain a 1509-bp open reading frame (fig.3) encoding 503 amino acids (M_r = 56880). This agrees with the molecular weight of the purified protein as estimated by SDS-polyacrylamide gel electrophoresis [2]. The amino terminal 6 residues deduced from the nucleotide sequence of p7α-11 were somewhat different from our previous result (Met-Phe-Glu-Val-(Ile)-Ser-Leu) [2]. We repeated the amino terminal sequence analysis of the purified protein and found that the sequence is identical with that deduced from the cDNA. The previous report should therefore be read as Met-Met-Thr-Ile-Ser-Leu-.

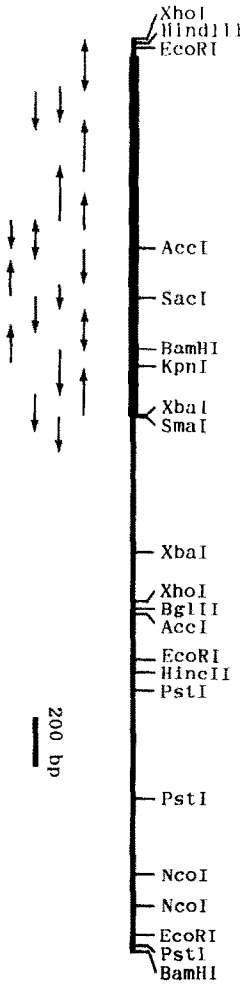

Fig.2. Restriction map and sequencing strategy of p7α-11. Arrows indicate the directions and extents of sequencing. Restriction sites outside of *Eco*RI site at both ends are some of multicloning sites of pBluescript vector.

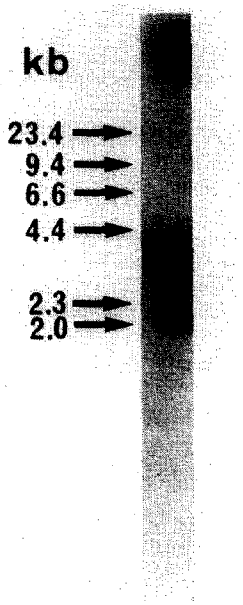

Fig.1. Northern hybridization of liver poly(A^+) RNA of cholestyramine-treated rats. Five μg of poly(A^+) RNA was electrophoresed on agarose gel containing formaldehyde [4]. A ^{32}P-labeled insert (2.5-kbp) was used as a probe.

The primary structure deduced from the nucleotide sequence of p7α-11 was compared with those of other forms of cytochrome P-450 by a computer homology search (NBRF data base). As a result, we could not find out any P-450s exhibiting more than 30% sequence similarity to cholesterol 7α-hydroxylase. Although the overall sequence similarity of the hydroxylase to other P-450s is less than 30%, its heme binding region, located between residues 437 and 457, is somewhat conserved, though the sequence similarity of this region to other P-450s is at most in the range 40–47%. The low similarity of the amino acid sequence of cholesterol 7α-hydroxylase to other P-450s demonstrates that this enzyme cannot be classified as a member of any P-450 gene families listed by Nebert et al. [12]. The enzyme is thus concluded to constitute a novel P-450 family.

To confirm that p7α-11 DNA actually encodes cholesterol 7α-hydroxylase, the *Xho*I-*Xho*I fragment (2.2-kbp) of the cDNA was introduced to the expression vector pSVL and transfected into COS 7 cells. Since COS 7 cells contain NADPH-cytochrome P-450 reductase and the substrate cholesterol, we expected that the product, 7α-hydroxycholesterol, should accumulate in the transfected cells during the culture. We harvested cells at 0, 6, 12, 24, 36 and 48 h after transfection, and analyzed the product and immunoreactive protein in the microsomal fraction. Fig.4A shows that the product was detected after cultivation for 12 h and then increased sharply to 0.5 nmol/mg protein after 48 h cultivation. Neither the cells at time zero nor control cells transfected with only pSVL vector showed any product at all. The product showed the same retention time on HPLC and the same R_f value on thin-layer chromatography as 7α-hydroxycholesterol (data not shown). Western blotting analysis (fig.4B) showed that the amount of a protein reactive with anti-7α-hydroxylase antibodies increased also in parallel with the accumulation of the product. These results confirmed that the isolated clones undoubtedly encoded cholesterol 7α-hydroxylase.

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   1                                                Met Met Thr Ile Ser Leu Ile Trp Gly Ile Ala
   1  GGTCTCCCCTTTGGAAATTTTCCTGCTTTTGCAAA ATG ATG ACT ATT TCT TTG ATT TGG GGA ATT GCC

  12  Val Leu Val Ser Cys Cys Ile Trp Phe Ile Val Gly Ile Arg Arg Arg Lys Ala Gly Glu
  69  GTG TTG GTG AGC TGT TGC ATA TGG TTT ATT GTT GGA ATA AGG AGA AGG AAA GCT GGT GAA

  32  Pro Pro Leu Glu Asn Gly Leu Ile Pro Tyr Leu Gly Cys Ala Leu Lys Phe Gly Ser Asn
 129  CCT CCT TTG GAG AAC GGG TTG ATT CCG TAC CTG GGC TGT GCT CTG AAA TTT GGA TCT AAT

  52  Pro Leu Glu Phe Leu Arg Ala Asn Gln Arg Lys His Gly His Val Phe Thr Cys Lys Leu
 189  CCT CTT GAG TTC CTA AGA GCT AAT CAA AGG AAG CAT GGT CAC GTT TTT ACC TGC AAA CTG

  72  Met Gly Lys Tyr Val His Phe Ile Thr Asn Ser Leu Ser Tyr His Lys Val Leu Cys His
 249  ATG GGG AAA TAT GTC CAT TTC ATC ACA AAC TCC CTG TCA TAC CAC AAA GTC TTA TGT CAT

  92  Gly Lys Tyr Phe Asp Trp Lys Lys Phe His Tyr Thr Thr Ser Ala Lys Ala Phe Gly His
 309  GGA AAA TAT TTT GAC TGG AAA AAA TTT CAT TAC ACT ACT TCT GCG AAG GCA TTT GGA CAC

 112  Arg Ser Ile Asp Pro Asn Asp Gly Asn Thr Thr Glu Asn Ile Asn Asn Thr Phe Thr Lys
 369  AGA AGC ATT GAC CCA AAT GAT GGA AAT ACC ACG GAA AAT ATA AAC AAC ACT TTT ACC AAA

 132  Thr Leu Gln Gly Asp Ala Leu Cys Ser Leu Ser Glu Ala Met Met Gln Asn Leu Gln Ser
 429  ACC CTC CAG GGA GAT GCT CTG TGT TCA CTT TCT GAA GCC ATG ATG CAA AAC CTC CAA TCT

 152  Val Met Arg Pro Pro Gly Leu Pro Lys Ser Lys Ser Asn Ala Trp Val Thr Glu Gly Met
 489  GTC ATG AGA CCT CCT GGC CTT CCT AAA TCA AAG AGC AAT GCC TGG GTC ACG GAA GGG ATG

 172  Tyr Ala Phe Cys Tyr Arg Val Met Phe Glu Ala Gly Tyr Leu Thr Leu Phe Gly Arg Asp
 549  TAT GCC TTC TGT TAC CGA GTG ATG TTT GAA GCT GGC TAT CTA ACA CTG TTT GGC AGA GAT

 192  Ile Ser Lys Thr Asp Thr Gln Lys Ala Leu Ile Leu Asn Asn Leu Asp Asn Phe Lys Gln
 609  ATT TCA AAG ACA GAC ACA CAA AAA GCA CTT ATT CTA AAC AAC CTT GAC AAC TTC AAA CAA

 212  Phe Asp Gln Val Phe Pro Ala Leu Val Ala Gly Leu Pro Ile His Leu Phe Lys Thr Ala
 669  TTT GAC CAA GTC TTT CCG GCA CTG GTG GCA GGC CTT CCT ATT CAC TTG TTC AAG ACC GCA

 232  His Lys Ala Arg Glu Lys Leu Ala Glu Gly Leu Lys His Lys Asn Leu Cys Val Arg Asp
 729  CAT AAA GCT CGG GAA AAG CTG GCT GAG GGA TTG AAG CAC AAG AAC CTG TGT GTG AGG GAC

 252  Gln Val Ser Glu Leu Ile Arg Leu Arg Met Phe Leu Asn Asp Thr Leu Ser Thr Phe Asp
 789  CAG GTC TCT GAA CTG ATC CGT CTA CGT ATG TTT CTC AAT GAC ACG CTC TCC ACC TTT GAC

 272  Asp Met Glu Lys Ala Lys Thr His Leu Ala Ile Leu Trp Ala Ser Gln Ala Asn Thr Ile
 849  GAC ATG GAG AAG GCC AAG ACG CAC CTC GCT ATT CTC TGG GCA TCT CAA GCA AAC ACC ATT

 292  Pro Ala Thr Phe Trp Ser Leu Phe Gln Met Ile Arg Ser Pro Glu Ala Met Lys Ala Ala
 909  CCT GCA ACC TTT TGG AGC TTA TTT CAA ATG ATC AGG AGT CCT GAA GCA ATG AAA GCA GCC

 312  Ser Glu Glu Val Ser Gly Ala Leu Gln Ser Ala Gly Gln Glu Leu Ser Ser Gly Gly Ser
 969  TCT GAA GAA GTG AGT GGA GCT TTA CAG AGT GCT GGC CAA GAG CTC AGC TCT GGA GGG AGT

 332  Ala Ile Tyr Leu Asp Gln Val Gln Leu Asn Asp Leu Pro Val Leu Asp Ser Ile Ile Lys
1029  GCC ATT TAC TTG GAT CAA GTG CAA CTG AAT GAC CTG CCG GTA CTA GAC AGC ATC ATC AAG

 352  Glu Ala Leu Arg Leu Ser Ser Ala Ser Leu Asn Ile Arg Thr Ala Lys Glu Asp Phe Thr
1089  GAG GCT CTG AGG CTT TCC AGT GCA TCC TTG AAT ATC CGC ACA GCT AAG GAG GAC TTC ACT

 372  Leu His Leu Glu Asp Gly Ser Tyr Asn Ile Arg Lys Asp Asp Met Ile Ala Leu Tyr Pro
1149  CTC CAT CTT GAG GAC GGT TCC TAT AAC ATC CGA AAA GAT GAC ATG ATA GCT CTT TAT CCA

 392  Gln Leu Met His Leu Asp Pro Glu Ile Tyr Pro Asp Pro Leu Thr Phe Lys Tyr Asp Arg
1209  CAG TTA ATG CAC TTG GAT CCT GAA ATC TAC CCA GAC CCT TTG ACT TTC AAA TAT GAC CGG

 412  Tyr Leu Asp Glu Ser Gly Lys Ala Lys Thr Thr Phe Tyr Ser Asn Gly Asn Lys Leu Lys
1269  TAC CTT GAT GAA AGC GGG AAA GCA AAG ACC ACC TTC TAC AGT AAT GGA AAC AAG CTG AAG

 432  Cys Phe Tyr Met Pro Phe Gly Ser Gly Ala Thr Ile Cys Pro Gly Arg Leu Phe Ala Val
1329  TGT TTC TAC ATG CCC TTC GGA TCA GGC GCG ACA ATA TGT CCT GGA AGA CTC TTT GCC GTC

 452  Gln Glu Ile Lys Gln Phe Leu Ile Leu Met Leu Ser Cys Phe Glu Leu Glu Phe Val Glu
1389  CAA GAA ATC AAG CAG TTT TTG ATC CTG ATG CTC TCC TGC TTT GAA CTG GAG TTT GTG GAG

 472  Ser Gln Val Lys Cys Pro Pro Leu Asp Gln Ser Arg Ala Gly Leu Gly Ile Leu Pro Pro
1449  AGC CAA GTC AAG TGT CCC CCT CTA GAC CAG TCC CGG GCA GGC TTG GGA ATT TTG CCA CCA

 492  Leu His Asp Ile Glu Phe Lys Tyr Lys Leu Lys His ***
1509  CTA CAT GAT ATT GAG TTT AAA TAT AAA CTG AAA CAC TGA TACGTGGTTGGAAGAAGCGAACACTGGA

1576  TGATGTCACTTGGCGGCTGAGAGTCATCACTAAACAGG
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Fig.3. Nucleotide sequence of a part of p7α-11 and predicted amino acid sequence. A DNA fragment of cDNA (1.6 kbp) including total coding region was sequenced. The consensus sequence for the heme binding domain of cytochrome P-450 is underlined.

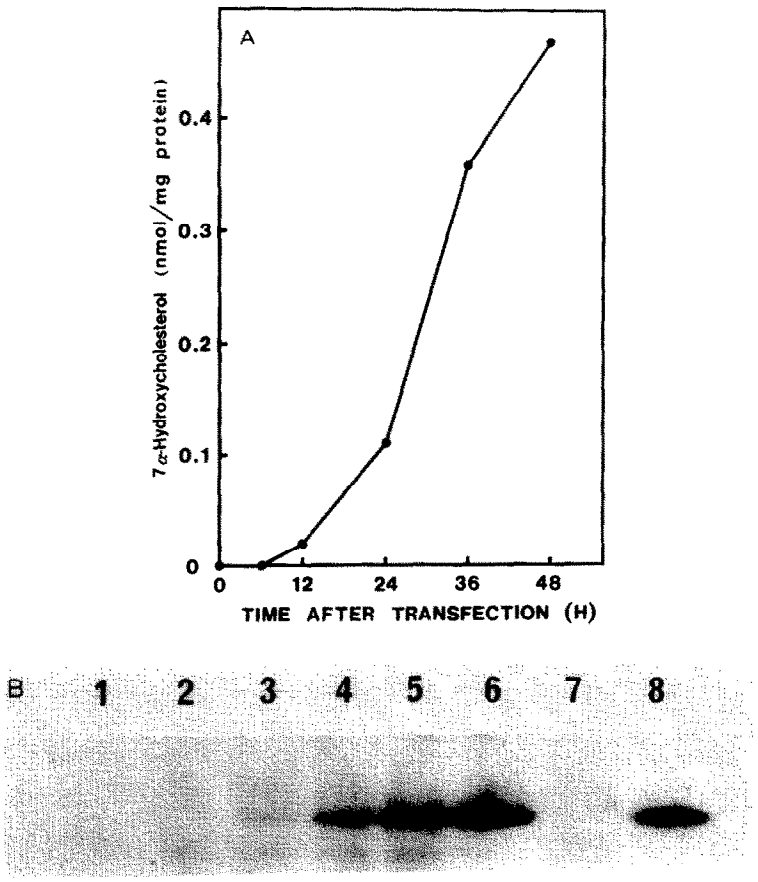

Fig.4. Expression of cholesterol 7α-hydroxylase cDNA in COS cells. Microsomal fractions of COS cells harvested at various culture times were subjected to analysis. (A) The amount of 7α-hydroxycholesterol, and (B) the immunoreactive protein with antibody to cholesterol 7α-hydroxylase in microsomes of COS cells transfected with the recombinant expression vector. Cells were harvested at 0, 6, 12, 24, 36 and 48 h after the transfection (lanes 1–6, respectively). Lane 7, control COS cells transfected with the non-recombinant vector at 48 h culture; lane 8, cholesterol 7α-hydroxylase purified from rat liver as the standard.

In conclusion, we have isolated cDNA clones for cholesterol 7α-hydroxylase, which is coded by a gene belonging to a unique family of cytochrome P-450. The availability of the cDNA probe should lead to insights into the detailed regulatory mechanism of this important enzyme. Expression of this enzyme under various physiological conditions as well as the isolation and sequence analysis of the genomic DNA for the enzyme are now in progress.

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