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Molecular cloning and sequence analysis of cDNA encoding Δ^4-3-ketosteroid 5β-reductase of rat liver

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A cDNA clone encoding Δ^4-3-ketosteroid 5β-reductase was isolated from rat liver cDNA libraries using antibodies specific for the enzyme and oligonucleotides as probes. The cDNA contained 981-base pair open reading frame encoding 327 amino acid residues (M, 37 376) and an unusually long 3'-untranslated region rich in AT sequence in the total length of 3189 base pairs. The predicted amino acid sequence contains the sequences similar to the putative NADPH- and steroid-binding regions.

Δ^4-3-Ketosteroid 5β-reductase; cDNA cloning

1. INTRODUCTION

In bile acids synthesis and steroid hormone metabolism, Δ^4-3-ketosteroid 5β-reductase plays an important role to catalyze reduction of the Δ^4-double bond to give A/B-*cis* conformation [1]. The enzyme was recently purified to homogeneity in this laboratory [2,3]. Subsequent studies have shown that the N-terminal amino acid of this enzyme is blocked, prompting us to determine the amino acid sequences of peptides obtained by peptidase-treatment and prepare the specific monoclonal and polyclonal antibodies against the enzyme (Onishi et al., to be published). In this paper, we describe the isolation of a cDNA clone encoding Δ^4-3-ketosteroid 5β-reductase from rat liver cDNA libraries using the specific antibodies and synthetic oligonucleotides corresponding to the partial amino acid sequences as probes.

2. MATERIALS AND METHODS

Δ^4-3-Ketosteroid 5β-reductase was purified from male rat liver cytosol 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 previously [4]. Oligonucleotides were synthesized based on the amino acid sequence of a peptide fragment of the enzyme ((Lys)-Thr- Phe-Ile-Ala-Val-Lys) as follows 5'-TT IAC IGC G(A/T)AT A(G)AA IGT T(C)TT-3'.

The cDNA libraries were prepared from liver poly(A)$^+$RNA of male rats [5], using λgt 11 and λZAP vectors. A λgt 11 oligo(dT)-primed cDNA library was screened with the specific polyclonal antibodies and a ^{32}P-labeled family of twelve 20-mer oligonucleotides.

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The cDNA obtained was used as a probe to isolate a complete cDNA clone from these libraries [6]. Positively reacted clones isolated through several rounds of screening were subcloned into pBluescript SK(−) plasmid. DNA sequencing was performed by using Exo III/Mung bean nuclease deletion system (Takara Co.) [7] and sequenase kit (United States Biochemical Corp.). Southern hybridization and Northern hybridization were performed by the method of Maniatis et al. [6].

3. RESULTS AND DISCUSSION

Specific polyclonal antibodies were prepared against rat liver Δ^4-3-ketosteroid 5β-reductase. The antibodies specifically reacted to the enzyme as examined by Western blotting and therefore were used for screening. Out of 1×10^5 clones of λgt 11 oligo(dT)-primed cDNA library, 8 immunoreactive clones were isolated and subjected to Southern hybridization using a mixture of ^{32}P-labeled oligonucleotides corresponding to the amino acid sequence of the internal peptide as a probe. Seven clones (λ2, 5, 7, 9, 11, 12 and 13) which hybridized positively with the oligonucleotides were subjected to restriction mapping. As shown in fig. 1, λ5, 7, 9, 12 and 13 clones showed a common size (1.8 kbp) and the same restriction map, and λ2 and 11 clones which had 3.0 kbp long insert contained the longer 3'-end. The mRNA size of Δ^4-3-ketosteroid 5β-reductase was estimated to be about 3.2 kb long by Northern hybridization using λ2 cDNA clone insert as a probe (Fig. 2). The insert of λ2 cDNA clone was then subcloned into pBluescript (p5β-2) and subjected to nucleotide sequencing. The nucleotide sequence of p5β-2 contained 903 bp long open reading frame consisting of 301 amino acid residues (M_r 34311). The deduced amino acid sequence contained the sequences of all the peptides obtained by

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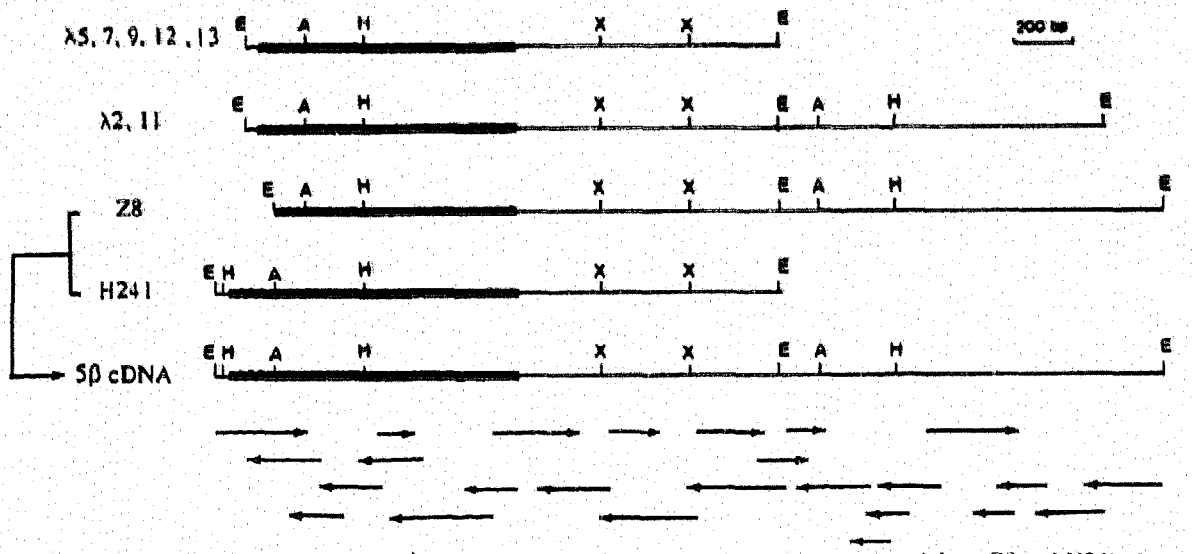

Fig. 1. Restriction map and sequencing strategy of Δ^4-3-ketosteroid 5β-reductase cDNA which constructed from Z8 and H241. Arrows indicate the directions and extents of sequencing. In the diagram, the restriction sites are abbreviated: A is *Acc*I, E is *Eco*RI, H is *Hin*dIII, and X is *XBa*I.

peptidase-treatment of the purified enzyme (Onishi et al., to be published) except one (SNCATWEYLEACK) (Fig. 3). Furthermore, the molecular weight calculated from the deduced sequence was much smaller than that of the purified protein (M_r 37000). These results indicated that the isolated clones might be incomplete. In order to isolate a full size clone therefore, oligo(dT)-primed λZAP cDNA library and random-primed λgt 11 cDNA library were rescreened using ^{32}P-labeled p5β-2 cDNA insert as a probe. A number of positive clones were isolated and two of them had the region missing in the clones isolated previously. The restriction map (Fig. 1) and nucleotide sequence (Fig. 3) revealed that Z8 clone isolated from the oligo(dT)-primed λZAP cDNA library contained the polyadenylation signal and

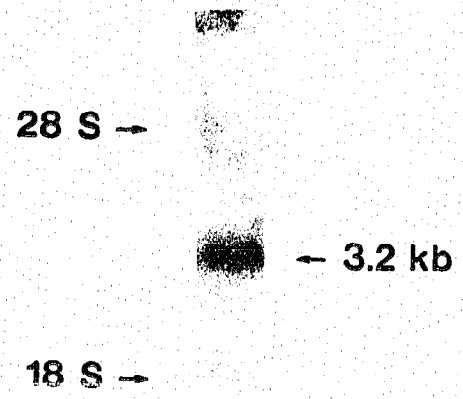

Fig. 2. Northern hybridization of rat liver poly(A)$^+$RNA. Five μg of poly(A)$^+$ RNA was electrophoresed on agarose gel containing formaldehyde [6]. A ^{32}P-labeled insert of p5β-2 was used as a probe.

H241 clone isolated from the random-primed λgt 11 cDNA library had a larger coding region which codes the amino acid sequence missing in p5β-2. The overlapping portion of the two sequences was identical. Consequently, the entire sequence for Δ^4-3-ketosteroid 5β-reductase was constructed from Z8 and H241. The nucleotide sequence thus constructed was 3189 bp long and contained 978 bp long open reading frame which codes 326 amino acid residues. The calculated molecular weight (M_r 37376) was in agreement with that of the purified enzyme (M_r 37000). It is not known at this moment why many clones missing the amino acid sequence such as p5β-2 are abundant in the liver cDNA libraries.

As shown in Fig. 3, 5β cDNA clone contained a long 3′-noncoding region which was rich in AT nucleotides and often contained ATTTA motifs, 5′-AAT-3′ or 5′-TAA-3′ trinucleotides in the single strand region of the secondary structure. Such unique structures in 3′-noncoding region are known to exist in rapidly degrading mRNA [8,9]. Recently, a similar unique structure was observed in 3′-noncoding region of P-450$_{ch7\alpha}$ which plays an important role in the conversion of cholesterol to bile acids whose mRNA showed rapid degradation and circadian rhythm [10,11]. Although these unique structures are suggestive of the rapid turnover of the mRNA of Δ^4-3-ketosteroid 5β-reductase, circadian rhythm was not observed in the mRNA level (data not shown).

Comparison of the amino acid sequence with the NADPH-binding enzyme and sex hormone-binding globulin revealed that the two unique amino acid regions exist in Δ^4-3-ketosteroid 5β-reductase. One region from residue 86–101 in Fig. 3 seems to be involved in steroid-binding. Although this sequence is not homologous to the common sequence of steroid-

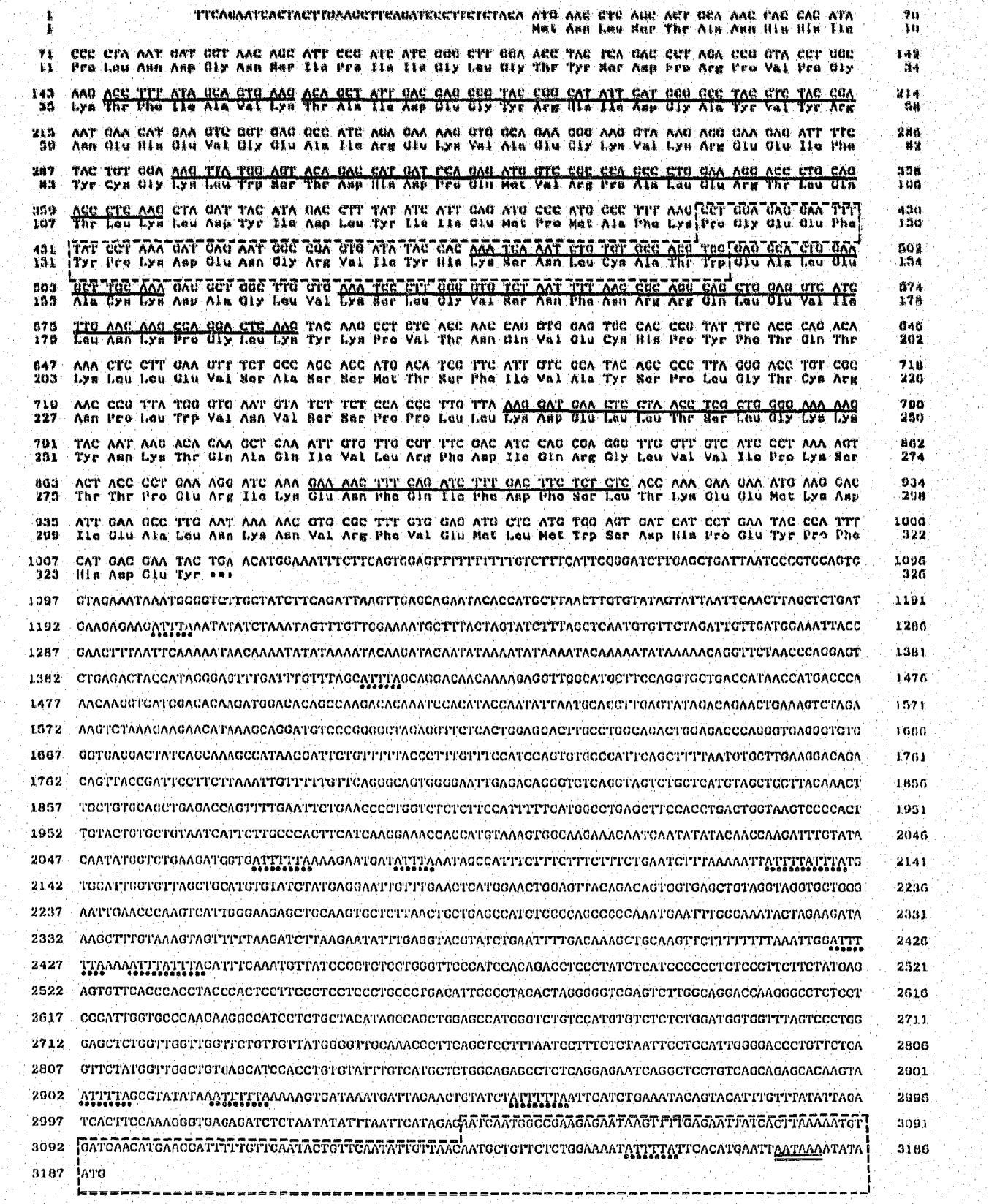

Fig. 3. Nucleotide sequence of Δ^4-3-ketosteroid 5β-reductase cDNA and the predicted amino acid sequence of the protein. Amino acids determined by peptide sequence analysis (Onishi et al., to be published) are overlined. The nucleotide sequences missing in p5β-2 are boxed with broken lines. ATTTA motif and similar sequences to it within 3'-noncoding region are marked by dot lines. A sequence of AATAAA indicates a polyadenylation signal.

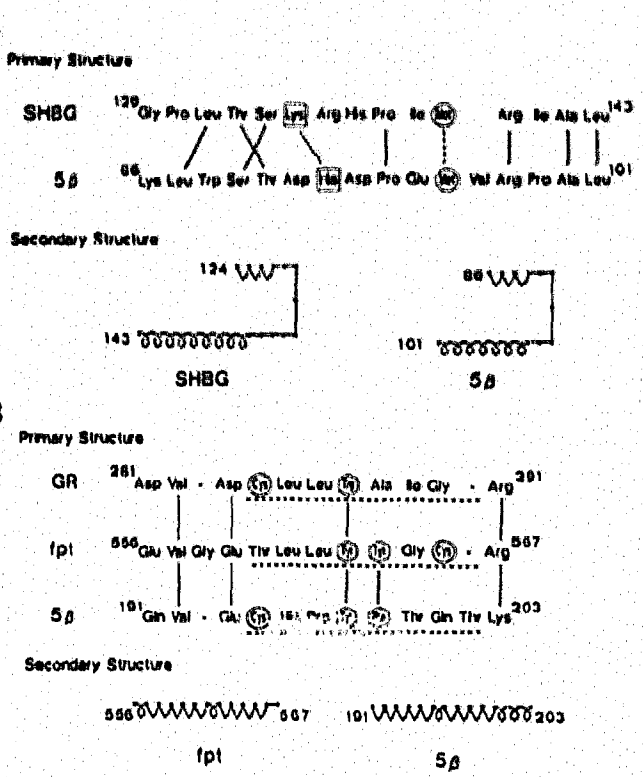

Fig. 4. Two unique regions of Δ^4-3-ketosteroid 5β-reductase, steroid-binding (A) and NADPH-binding (B) regions. (A) Residues 86–101 of Δ^4-3-ketosteroid 5β-reductase (5β) and the steroid-binding region of sex hormone-binding globulin (SHBG) are compared. Methionine residues are circled. Positively charged residues which are postulated to bind to the D-ring are boxed. Homologous residues are denoted with vertical lines. In the figures of secondary structure, α-helical regions are denoted by coils, β-sheets by zig-zag lines, and random coils by straight lines. (B) Residues 191–203 of Δ^4-3-ketosteroid 5β-reductase (5β) and NADPH-binding regions of glutathione reductase (GR) and NADPH cytochrome P-450 reductase (fpt) are compared. Cysteine residues are circled and aromatic residues are encircled hexagonally. Homologous residues are denoted with vertical lines, and hydrophobic or nonpolar regions are shown by broken lines. Secondary structures were drawn as indicated above.

binding in the sex hormone-binding globulin [12], the predicted secondary structure of this region calculated by Chou – Fasman methods [13] was very similar to that of the sex hormone-binding globulin (Fig. 4A) and moreover, there exists a methionine residue which is postulated to be required for binding of protein to the A-ring of the steroid nucleus. However, lysine residue which is reported to bind to the D-ring was not found. Instead, positively charged histidine residue was found near the position corresponding to it. These observations suggest that this region may function for substrate binding.

As Δ^4-3-ketosteroid 5β-reductase requires NADPH as a cofactor, we have surveyed if there is any similar structure to the NADPH-binding site reported in glutathione reductase [14] or NADPH-cytochrome P-450 reductase [15–18]. As a result, it was found that the region from residue 191–203 in Fig. 3 retains the similar charged groups and hydrophobic sequences. The location of the aromatic amino acid which is present close to the adenine molety of NADPH (Fig. 4B) was also acknowledged. The secondary structure around this region calculated by Chou – Fasman method [13] also showed the similarity to the structure of NADPH-binding site of rat hepatic NADPH-cytochrome P-450 reductase [15]. From these results we postulate that this region may be NADPH-binding site of this enzyme.

Curiously, however, the entire amino acid sequence of Δ^4-3-ketosteroid 5β-reductase did not show any significant homology to that of Δ^4-3-ketosteroid 5α-reductase [19] which works toward the same or similar substrates and requires NADPH as a cofactor.

In conclusion, we have isolated a cDNA clone and determined the primary structure of Δ^4-3-ketosteroid 5β-reductase, which catalyzes an important reaction of 5β-reduction of Δ^4-3-ketosteroid in the catabolism of cholesterol and metabolism of steroid hormones. The availability of the cDNA probe should lead to insights into the nature and the detailed regulatory mechanism of this important enzyme.

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