

Cloning of cDNA and genomic DNA for human cytochrome P-450_{11β}

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A full-length cDNA clone encoding steroid 11β-hydroxylase (P-450_{11β}) has been isolated from a cDNA library derived from human adrenal tumor. The insert of the clone contains an open reading frame encoding a protein of 503 amino acid residues together with a 4 bp 5'-untranslated region and a 576 bp 3'-untranslated region to which a poly(A) tract is attached. The promoter region of the P-450_{11β} gene has also been isolated from a genomic library derived from human pre-B cells. It contains a TATA box, a putative cAMP-responsive element, several repeated sequences and two sequence elements similar to the consensus sequence for binding of AP-1. A transient expression assay in Y-1 adrenal tumor cells demonstrates that the promoter activity is remarkably enhanced by treatment of the cells with cAMP. In addition, analysis using deletion mutants containing various lengths of the 5'-flanking region of the gene suggests that several *cis*-acting elements participate in transcriptional regulation of human P-450_{11β} gene.

Monooxygenase; P-450_{11β}; cDNA; Genomic DNA; Adrenal tumor; Steroidogenesis

1. INTRODUCTION

Steroid 11β-hydroxylase (P-450_{11β}), a monooxygenase [1], is an adrenal-specific cytochrome P-450 required for synthesis of both glucocorticoids and mineralocorticoids [2]. It has been shown that the enzyme purified from bovine adrenocortical mitochondria catalyzes not only 11β-, 18- and 19-hydroxylations of 11-deoxycorticosterone but also the conversion of corticosterone to aldosterone via 18-hydroxycorticosterone [3–5]. Clinical importance of human P-450_{11β} is evident because 5–8% of reported cases of congenital adrenal hyperplasia are caused by a defect of 11β-hydroxylation [6].

Recently, a cDNA clone containing a partial-length insert encoding human P-450_{11β} has been isolated from a fetal adrenal cDNA library and its nucleotide sequence determined [7]. More recently, a human genomic DNA for P-450_{11β} has been isolated and sequenced [8]. Nevertheless, a complete nucleotide sequence of human P-450_{11β} cDNA is not as yet definitely determined, although it is deduced by comparing the nucleotide sequence of human genomic DNA [8] with those of a partial-length human cDNA [7] and full-length bovine cDNAs [7,9,10]. In addition, how expression of human P-450_{11β} gene is regulated in living cells remains to be elucidated. Therefore, we attempted to isolate a full-length clone to define the entire nucleotide sequence of human P-450_{11β} cDNA. Simultaneously,

we made an effort to isolate a genomic DNA clone to elucidate the regulatory mechanisms of expression of the corresponding gene.

In this paper, we report the nucleotide sequence of a full-length human P-450_{11β} cDNA and that of the promoter region of the gene. Also, we present a line of evidence to show that the promoter region of human P-450_{11β} gene contains several *cis*-acting elements and that the gene expression is tissue-specifically regulable by cAMP at least in a cultured cell system.

2. MATERIALS AND METHODS

2.1. Molecular cloning and nucleotide sequencing

Total human RNA was prepared as described by Chirgwin et al. [11] from adrenal tumor of a patient suffering from primary aldosteronism. Poly(A)⁺ RNA was enriched therefrom by oligo(dT)-cellulose chromatography [12]. cDNA produced from the poly(A)⁺ RNA was fractionated by gel filtration and cDNA species longer than 1 kb were collected and used to construct a λgt10 cDNA library as described by Huynh et al. [13]. A full-length cDNA clone was isolated out of 1.3 × 10⁶ recombinants using as a probe the 45-mer synthetic oligonucleotide (5'-GGGCACGTGGTAGAAGTTCCTGCCGGAGCCCTTGATG-TCTAGCCA-3') of which 5' end was labeled with [γ-³²P]ATP and polynucleotide kinase. The probe was designed on the basis of the nucleotide sequence of a partial-length human P-450_{11β} cDNA [7]. The insert of the cDNA thus isolated was subcloned into pUC plasmid for further analysis. Nucleotide sequence was determined by the dideoxy chain termination method [14,15]. A genomic clone was isolated from a human genomic library which was derived from pre-B cells and packed in cosmid Lorist 2 vector. The total length of the insert was ≈ 40 kb, but the 1.4 kb *Hind*III-*Sma*I fragment containing the promoter region of the P-450_{11β} gene was subcloned into pUC119 plasmid and used for further studies.

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2.2. DNA transfection and chloramphenicol acetyltransferase (CAT) assay

To obtain various deletion mutants fused to the bacterial CAT gene, the clone containing the *Hind*III-*Sma*I fragment as described above was linearized with *Hind*III, treated with *Ba*131 exonuclease and then digested with *Eco*RI. The resulting fragments were subcloned into pUC118 plasmid and endpoints of various deletion mutants thus produced were determined by nucleotide sequencing. The insert of each deletion mutant was excised from pUC118 plasmid and inserted into the *Hind*III site of pSVOOCAT [16] after ligated with a *Hind*III linker.

Y-1 adrenal tumor cells [17] supplied from Japanese Cancer Research Resources Bank were cultured in Ham's F-12K medium supplemented with 15% fetal bovine serum (FBS). HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. The cells were plated at 3×10^6 for Y-1 cells or at 1×10^6 cells for HeLa cells per 90-mm dish, and cultured for 24 h. They were then transfected with CAT plasmids by the calcium phosphate-DNA coprecipitation method [18] followed by glycerol shock. CAT assays were performed according to the method of Gorman et al. [19]. In some experiments after DNA transfection, the cells were incubated in the culture medium supplemented with 1 mM 8-bromoadenosine 3':5'-cyclic monophosphate (8-Br cAMP).

3. RESULTS

Fig. 1 represents the restriction map of and the sequencing strategy for the full-length insert (pH11 β 1) of the cDNA clone isolated. Fig. 2 shows the nucleotide sequence of pH11 β 1 consisting of 2089 bp excluding a poly(A) tract. The translational initiation site is assigned to be the first ATG codon, because the transcriptional initiation site of the P-450_{11 β} gene is the nucleotide C, 7 bp upstream from the ATG codon (Fig. 3). Also, the sequence around the first ATG triplet agrees well with the favoured sequence which flanks the functional initiation codon $\hat{A}XXATG$, where X is any nucleotide [20]. The open reading frame starting from this initiation codon consists of 1509 nucleotides encoding a polypeptide composed of 503 amino acid residues, followed by the translational termination codon TAA. A poly(A) tract is attached to the 3'-terminus of 576 bp 3'-untranslated region. The amino acid sequence

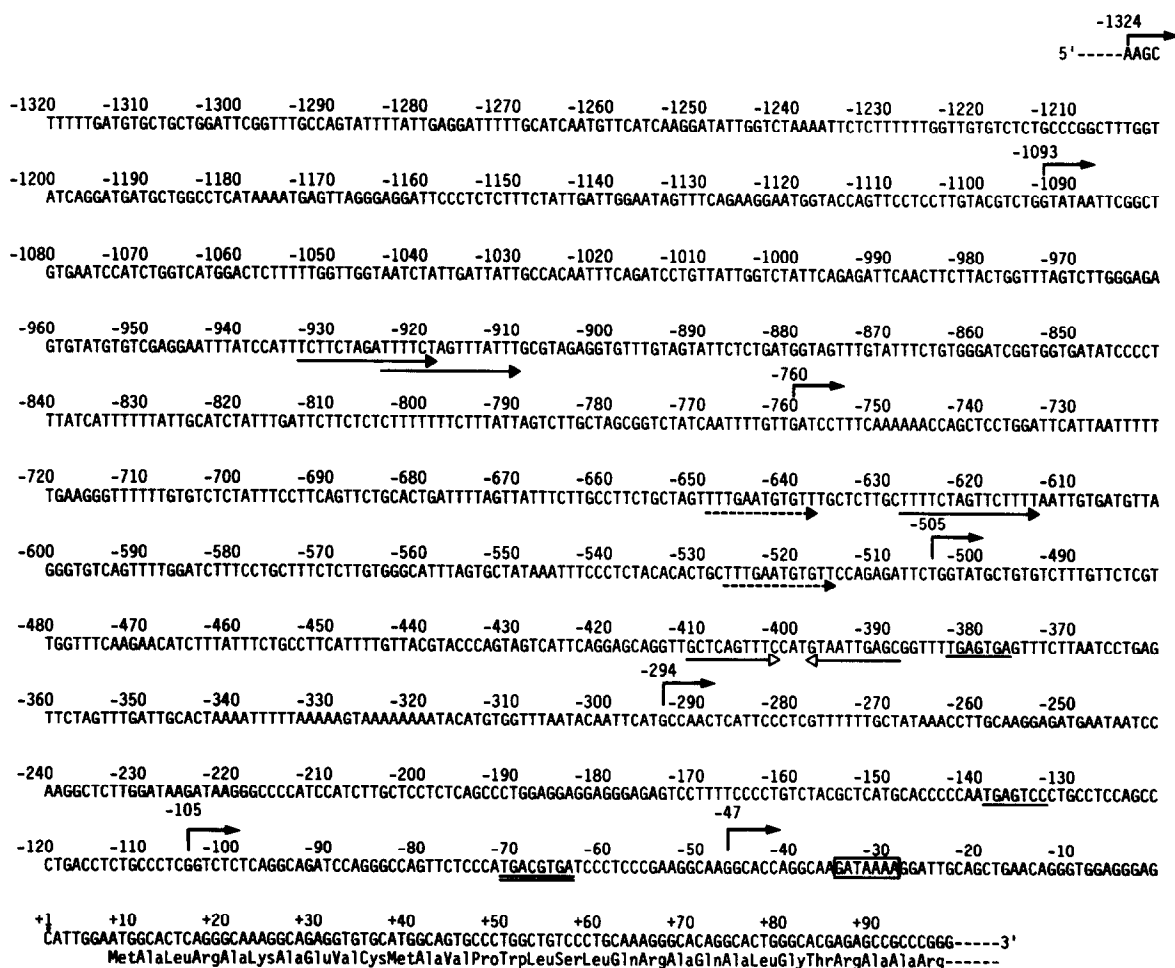


Fig. 3. Nucleotide sequence of the 5'-flanking region and exon 1 of the P-450_{11 β} gene. Numbers above the nucleotide sequence refer to nucleotide positions relative to the transcriptional initiation site taken as +1 [8]. The deduced amino acid sequence is shown below the nucleotide sequence. A TATA element is boxed, and putative cAMP-responsive and AP-1 elements are shown by double and single underlines, respectively. Palindromic and two types of repeated sequences are indicated by solid and dashed arrows with open and closed arrowheads, respectively. The endpoints of 5'-deletion mutants used for transient expression assays in Fig. 4 are also shown by solid broken arrows.

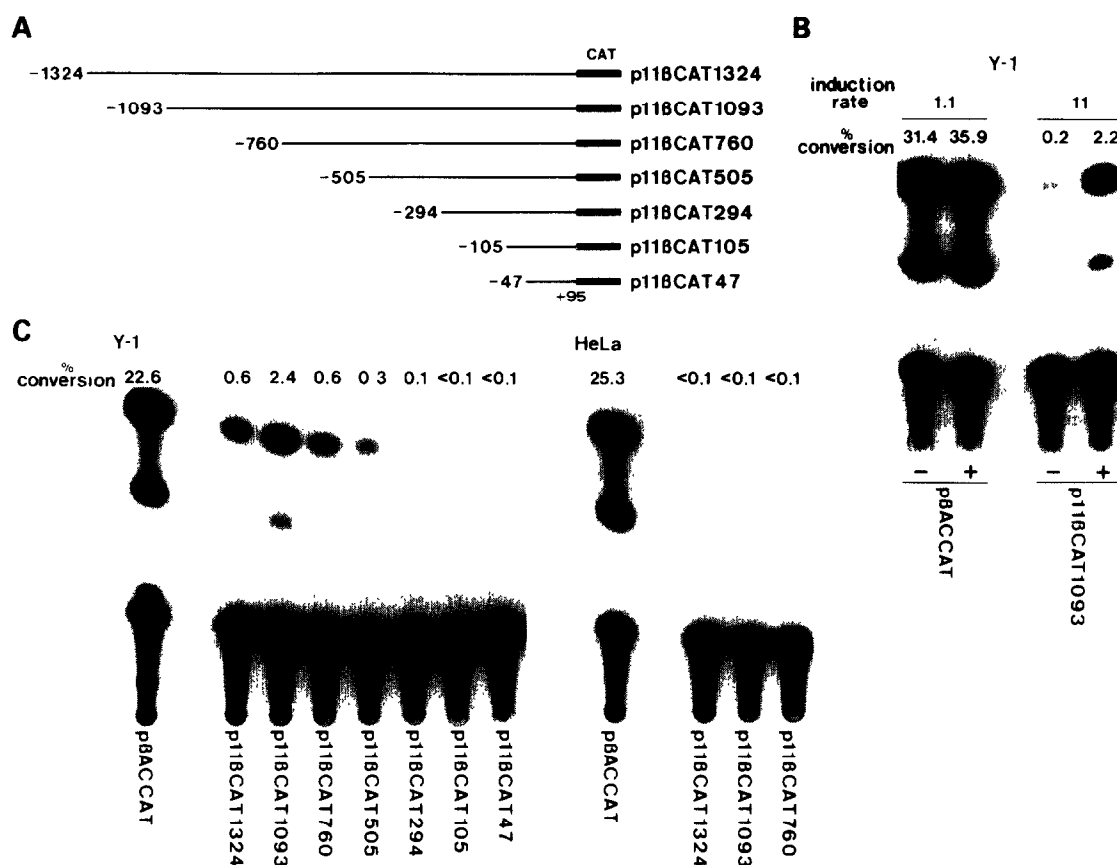


Fig. 4. Transient expression assay of the P-450_{11β} gene. (A) Structures of 5'-deletion mutants used in these experiments are depicted. Numbers on the left of diagrams indicate the 5'-endpoints of deletion mutants. (B) Cyclic AMP-inducible expression of the P-450_{11β} gene. Y-1 cells were transfected with the chimeric construct, p11βCAT1093, or the β-actin-CAT construct, pβACCAT. They were incubated in the absence (–) or presence (+) of 1 mM 8-Br cAMP for 40 h. Percentages of chloramphenicol conversion to its acetylated derivatives and induction rates are shown by numerals in the figure. (C) Analysis of promoter activity of 5'-deletion mutants. Y-1 or HeLa cells were transfected with various deletion mutants and incubated in the medium supplemented with 1 mM 8-Br cAMP for 40 h. Then, CAT activities were measured as described [19].

deduced from the nucleotide sequence of pH11β1 (Fig. 2) is strikingly homologous to those of bovine [7,9,10] and rat [21] cDNAs for P-450_{11β} (73% homologous with the bovine amino acid sequence and 64% homologous with the rat amino acid sequence; unpublished results).

Using the same oligonucleotide probe as that used for screening the cDNA library, we simultaneously isolated a genomic clone containing the 5'-flanking region of the gene. As shown in Fig. 3, nucleotide sequence analysis reveals that the region contains a TATA box (–35 to –29), a putative cAMP-responsive element, CRE, (–71 to –64) [22] and two putative AP-1 elements (–383 to –377 and –139 to –133) [23,24]. It is noticed that two types of repeated sequences and a palindromic sequence are present in the region shown in Fig. 3.

In the next experiments, we examined whether the promoter is responsive to chemical stimuli such as cAMP by using CAT assay. As shown in Fig. 4, treatment of Y-1 cells with 8-Br cAMP results in a marked

increase in promoter activity up to 11-fold when a chimeric construct containing the 5'-flanking region up to –1093 relative to the transcriptional initiation site is used. In contrast, the same treatment has substantially no effect on the transient expression of a human β-actin-CAT construct, pβACCAT [25].

In order to localize *cis*-acting element(s) in the 5'-flanking region, promoter activities of various 5'-deletion mutants were measured by transient expression assays (Fig. 4). When the region between –1324 and –1094 is deleted, promoter activity increases 4-fold, suggesting that the region between –1324 and –1094 contains negative *cis*-acting element(s). On the other hand, further deletion extending to position –761 causes a marked reduction of promoter activity, indicating that the region between –1093 and –761 is required for efficient transcriptional activity. A further decrease in the activity occurs when the 5'-flanking region is deleted to position –506. Finally, deletion to position –295 results in a considerable loss of promoter activity. When HeLa cells are used in place of

Y-1 adrenal cells, no promoter activity is detectable (Fig. 4C), suggesting that expression of human P-450_{11 β} gene is highly tissue-specific.

The above results, taken together, indicate that the 5'-flanking region of the gene we isolated has promoter activity and that the promoter region has various *cis*-acting elements which might participate in regulating expression of human P-450_{11 β} .

4. DISCUSSION

In the present study, we have isolated a full-length cDNA for human P-450_{11 β} and simultaneously a genomic DNA having the promoter region of the P-450_{11 β} gene. As a whole, our nucleotide sequence data of the cDNA agree well with those deduced from human genomic DNA for P-450_{11 β} [8], although minor differences are detectable. Differences of the nucleotide sequences are more marked when our data of a full-length cDNA are compared with those of a partial-length cDNA [7]. The minor differences between the former data [8] and ours are probably due to allelic variants, but the reason for considerable differences between the latter data [7] and ours is unclear.

Promoter activity of human P-450_{11 β} gene is markedly enhanced by treatment of Y-1 cells with cAMP (Fig. 4B) as is observed with mouse P-450_{11 β} gene [26]. It is noted that the chimeric construct (p11 β CAT105, in Fig. 4C) contains a CRE-like element and yet it does not exhibit any detectable promoter activity even after treatment of the cells with cAMP. Nevertheless, this CRE-like element may be involved at least in part in transcription of human P-450_{11 β} gene, because mutation of the CRE present in mouse P-450_{11 β} gene abolishes the gene expression [27]. Whether various elements present in the 5'-flanking region of human P-450_{11 β} gene exhibit any regulatory significance in living cells must wait for further study.

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REFERENCES

- [1] Hayaishi, O. (1974) *Molecular Mechanisms of Oxygen Activation*, Academic Press, New York.
- [2] Sato, R. and Omura, T., eds. (1978) *Cytochrome P-450*, Kodansha, Tokyo, Academic Press, New York.
- [3] Momoi, K., Okamoto, M., Fujii, S., Kim, C.Y., Miyake, Y. and Yamano, T. (1983) *J. Biol. Chem.* 258, 8855-8860.
- [4] Wada, A., Okamoto, M., Nonaka, Y. and Yamano, T. (1984) *Biochem. Biophys. Res. Commun.* 119, 365-371.
- [5] Katagiri, M., Takemori, S., Itagaki, E. and Suhara, K. (1978) *Methods Enzymol.* 52, 124-132.
- [6] White, P.C., New, M.I. and Dupont, B. (1987) *N. Engl. J. Med.* 316, 1519-1524, 1580-1586.
- [7] Chua, S.C., Szabo, P., Vitek, A., Grzeschik, K.-H., John, M. and White, P.C. (1987) *Proc. Natl. Acad. Sci. USA* 84, 7193-7197.
- [8] Mornet, E., Dupont, J., Vitek, A. and White, P.C. (1989) *J. Biol. Chem.* 264, 20961-20967.
- [9] Morohashi, K., Yoshioka, H., Gotoh, O., Okada, Y., Yamamoto, K., Miyata, T., Sogawa, K., Fujii-Kuriyama, Y. and Omura, T. (1987) *J. Biochem.* 102, 559-568.
- [10] Kirita, S., Morohashi, K., Hashimoto, T., Yoshioka, H., Fujii-Kuriyama, Y. and Omura, T. (1988) *J. Biochem.* 104, 683-686.
- [11] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) *Biochemistry* 18, 5294-5299.
- [12] Okayama, H., Kawaichi, M., Brownstein, M., Lee, F., Yokota, T. and Arai, K. (1987) *Methods Enzymol.* 154, 3-28.
- [13] Huynh, T.V., Young, R.A. and Davis, R.W. (1985) *DNA Cloning: A Practical Approach* (Volume 1) (Glover, D.M., eds.), IRL Press Limited, Oxford, England, pp. 49-78.
- [14] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5463-5467.
- [15] Vieira, J. and Messing, J. (1987) *Methods Enzymol.* 153, 3-11.
- [16] Araki, E., Shimada, F., Shichiri, M., Mori, M. and Ebina, Y. (1988) *Nucleic Acids Res.* 16, 1627.
- [17] Yasumura, Y., Buonassisi, V. and Sato, G. (1966) *Cancer Res.* 26, 529-535.
- [18] Graham, F.L. and van der Eb, A.J. (1973) *Virology* 52, 456-467.
- [19] Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) *Mol. Cell. Biol.* 2, 1044-1051.
- [20] Kozak, M. (1981) *Nucleic Acids Res.* 9, 5233-5252.
- [21] Nonaka, Y., Matsukawa, N., Morohashi, K., Omura, T., Ogihara, T., Teraoka, H. and Okamoto, M. (1989) *FEBS Lett.* 255, 21-26.
- [22] Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G. and Goodman, R.H. (1986) *Proc. Natl. Acad. Sci. USA* 83, 6682-6686.
- [23] Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P. and Karin, M. (1987) *Cell* 49, 729-739.
- [24] Lee, W., Mitchell, P. and Tjian, R. (1987) *Cell* 49, 741-752.
- [25] Kawamoto, T., Makino, K., Orita, S., Nakata, A. and Kakunaga, T. (1989) *Nucleic Acids Res.* 17, 523-537.
- [26] Rice, D.A., Aitken, L.D., Vandenberg, G.R., Mouw, A.R., Franklin, A., Schimmer, B.P. and Parker, K.L. (1989) *J. Biol. Chem.* 264, 14011-14015.
- [27] Mouw, A.R., Rice, D.A., Meade, J.C., Chua, S.C., White, P.C., Schimmer, B.P. and Parker, K.L. (1989) *J. Biol. Chem.* 264, 1305-1309.