FEBS Letters 397 (1996) 250–252 FEBS 17793

Fish testicular 11β-hydroxylase: cDNA cloning and mRNA expression during spermatogenesis

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Received 3 September 1996; revised version received 7 October 1996

Abstract We isolated and characterized a cDNA encoding testicular 11β-hydroxylase, cytochrome P450(11β) from the Japanese eel (*Anguilla japonica*) testis. The cDNA contains an open reading frame that encodes a protein of 511 amino acids. The predicted amino acid sequence shares 38–48% homology with those of adrenal P450(11β) from mammals and frog. Transient expression in COS 1 cells confirmed that the protein encoded by this cDNA had P450(11β) activity. Northern blotting revealed a single 1.8 kb long transcript of P450(11β). This transcript was not found in immature eel testes prior to an injection with human chorionic gonadotropin (hCG), but it was present in eel testes after hCG injection.

Key words: Cytochrome P450(11β); cDNA cloning; Spermatogenesis; Japanese eel

1. Introduction

11β-Hydroxylase, cytochrome P450(11β), is an important steroidogenic enzyme responsible for the biosynthesis of both glucocorticoids and mineralocorticoids in the vertebrate adrenal cortex. In addition, in fish testes P450(11β) is also involved in the biosynthesis of 11-ketotestosterone, a potent androgen in fishes. 11-Ketotestosterone is biosynthesized from testosterone by the actions of two enzymes, P450(11β) and 11β-hydroxysteroid dehydrogenase (11β-HSD).

Recently, 11-ketotestosterone has been identified as the spermatogenesis-inducing hormone in the Japanese eel (*Anguilla japonica*) [1–3]. Under cultivation conditions, male Japanese eels have immature testes containing spermatogonia together with inactive testicular somatic cells such as Leydig and Sertoli cells. Using a recently developed organ culture system for eel testes, we found that the hormonal induction of spermatogenesis in eel testes involves gonadotropin stimulation of Leydig cells to produce 11-ketotestosterone. In turn, 11-ketotestosterone activates Sertoli cells to stimulate premitotic spermatogonia to complete spermatogenesis [4].

It is of considerable interest, both from the standpoint of investigation of gonadotropin regulation of P450(11β) gene expression and with respect to evolutionary and structural aspects of this enzyme, to obtain and characterize a full-length cDNA clone encoding eel testicular P450(11β). Although complete sequences of adrenal cortex P450(11β) have been determined in several mammalian species and frog [5–12], no attempts have been made to clone gonadal P450(11β) cDNAs from any species. We report here the isolation, characterization and expression of a full-length cDNA encoding P450(11β) from a eel testicular cDNA library.

2. Materials and methods

2.1. Cloning and sequencing

Based on a search of Genebank sequences, two conserved amino acid regions in P450(11β) (LFELAR and NYHIPA) (Fig. 1) were selected and their degenerated oligonucleotides were synthesized and used as primers for PCR reaction. PCR reaction was carried out using cDNA template from testes of eels killed 9 days after a single injection of human chorionic gonadotropin (hCG). The predicted PCR product was subcloned and sequenced. A testis cDNA library made from Japanese eels killed 3 days after hCG was constructed in λZAP II. A total of 500 000 phages from this library were screened using PCR product as probe. Subclones were prepared by in vivo excision. The insert cDNA was nest-deleted at both ends using *Exo*III/Mung bean nuclease. The cDNA was sequenced using an ABI 370A DNA Sequencer.

2.2. Expression of the cDNA clone

Eel P450(11β) cDNA containing the entire coding region was cut by *Eco*RI and *Ssp*I, yielding a 1.6 kb fragment without poly(A). This fragment was inserted into the *Sma*I site of expression vector pSVL (Pharmacia LKB) to construct the recombinant expression vector pSVL-E11h. Plasmids pSVL-E11h or pSVL (40 μg) without an insert as a control were transfected into COS 1 cells according to Tanaka et al. [13]. Thin layer chromatography (TLC) was performed using ^{14}C-labeled testosterone as a substrate according to Sakai et al. [14] except that the TLC plates were exposed to an imaging plate and visualized by a BAS 2000 image analyzer (Fuji Photo Film, Tokyo).

Testis total RNA was extracted from Japanese eels killed on day 0 (before hCG injection) and on days 1–9 (after hCG injection). Total RNA (10 μg) from each day was resolved by formaldehyde denaturing agarose gel electrophoresis, then transferred to a nylon membrane (Amersham). The full-length cDNA of eel testis P450(11β) was excised from vector, labeled with [^{32}P]dCTP using a Random Extension Plus Kit (Dupont), and hybridized for at least 18 h at 60°C. The membrane was washed at 65°C, then exposed to an imaging plate. The Northern blots were examined using a BAS 2000 image analyzer. After stripping the signals, the same membrane was rehybridized with a Japanese eel β-actin cDNA probe.

3. Results and discussion

Using a PCR amplified product as a probe, a testis cDNA library from Japanese eels killed 3 days after hCG injection was screened. Based on partial sequence analysis of positively hybridized clones, one clone named pE11h with an insert of 1774 bp was fully sequenced. The results indicated that this 1774 bp insert contains a 30 bp 5′-noncoding region, a 1533 bp open reading frame, a 173 bp 3′-noncoding region and a 38 residue poly(A) tail. The open reading frame begins at the first ATG codon of the 5′ terminus and encodes a predicted

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PII S0014-5793(96)01187-8

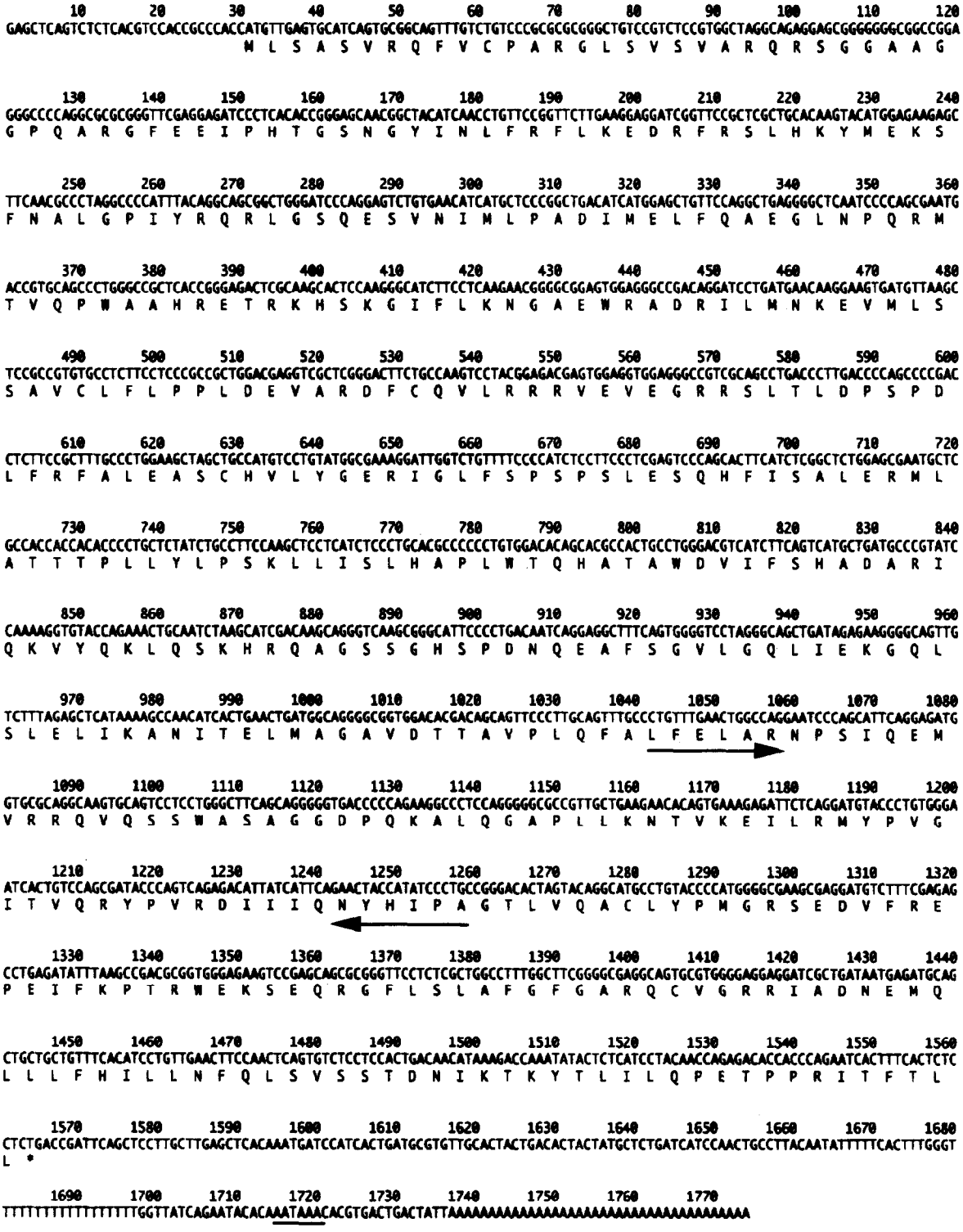

Fig. 1. Nucleotide sequence of Japanese eel P450(11β) and the deduced amino acid sequence. The AATAAA polyadenylation signal is underlined by a solid line. The primer regions used in PCR is underlined by arrows.

protein consisting of 511 amino acids (Fig. 1). The predicted amino acid sequence of eel testicular P450(11β) shares 38–48% homology with those of adrenal P450(11β) from mammals and frog (Table 1).

To confirm that the protein encoded by pE11h has P450(11β) activity we assayed enzymatic activity by expressing the recombinant expression vector pSVL-E11h in COS 1 cells. In this study, testosterone was used as a substrate to obtain detectable signals of 11β-hydroxytestosterone. TLC results indicated that most testosterone was converted to androstenedione with only a little amount of testosterone remaining unconverted (data not shown). This may be caused by endogenous 17β-hydroxysteroid dehydrogenase activity in COS 1 cells [15]. No detectable band corresponded to 11β-

Table 1
Identities of amino acids between Japanese eel P450(11β) and those of other species

Species	Identity
Frog (11β0)	48%
Bovine (11β0)	43%
Porcine (11β0)	42%
Ovine (11β)	43%
Human (11β1)	42%
Mouse (11β1)	38%
Rat (11β1)	38%
Human (11β2)	42%
Mouse (11β2)	43%
Rat (11β2)	42%

hydroxytestosterone. On the other hand, a band corresponding to 11β-hydroxyandrostenedione was obvious in the experimental group, but virtually absent in the controls. These findings were also confirmed by two-dimensional TLC analysis (data not shown). The conversion percentage of 11β-hydroxyandrostenedione from androstenedione was deduced by their relative density on TLC plates. Conversion was obvious by pSVL-E11h (20%), but not by the control vector pSVL (0.4%). These results, together with amino acid similarity to mammalian and frog P450(11β), indicate that the cDNA insert of pE11h encodes an eel testicular P450(11β).

Northern blotting of testis total RNA from Japanese eel with or without hCG treatment detected a band of about 1.8 kb and the hCG-induced expression of the P450(11β) gene. The size of the 1.8 kb mRNA species is in good agreement with that of the eel P450(11β) cDNA clone. As shown in Fig. 2, there was no apparent hybridized signal of P450(11β) in testis total RNA from eels without hCG treatment. The results were the same even when 50 μg total RNA was applied (data not shown). The P450(11β) message was detected in the testes of eels on days 1–9, but at various expression levels. The signal was intense on days 1 and 3. On days 6 and 9, the signal decreased greatly. The expression level of P450(11β) on days 1 and 3 demonstrated that hCG injection induces P450(11β) expression in eel testis. The time course of this expression was consistent with that of Leydig cell activation

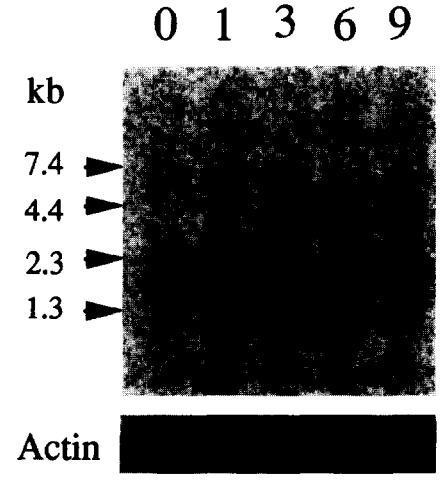

Fig. 2. Northern blots of P450(11β) expression in Japanese eel testis. 0: before hCG injection. 1–9: 1–9 days after a single injection of hCG.

(as early as day 1 after the hCG injection) and the increased production of 11-ketotestosterone (1–3 days after injection) in hCG-treated eels [1]. The decrease in the P450(11β) transcripts on days 6 and 9 may be due to feedback from increased serum levels of 11-ketotestosterone. The repression effect of androgen on expression of steroidogenic enzymes has been reported in mammals [16].

In summary, this paper reports the first isolation and characterization of a cDNA encoding a testicular P450(11β). The eel testicular P450(11β) amino acid sequence shares 38–48% homology with that from mammalian and frog adrenal glands. Northern blotting suggested that hCG induces the synthesis of P450(11β) by increasing the content of translatable mRNA. The eel testis should continue to be a valuable model with which to gain a better understanding of not only the hormonal regulation of spermatogenesis but also the hormonal regulation of P450(11β) gene expression in vertebrates.

Acknowledgements: This research was aided in part by Grants-in-Aid for Primary Research (07283104) and by the International Scientific Research Program grant (06044235) from the Ministry of Education, Science, Culture, and Sports, Japan, and by a Grant-in-Aid (Bio Media Program) from the Ministry of Agriculture, Forestry and Fisheries (BMP 95-II-2-6). Research funds from the Mitsubishi Foundation and the Uehara Memorial Foundation are also acknowledged.

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