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Regulated expression of cytochrome *P*-450scc (cholesterol-side-chain cleavage enzyme) in cultured cell lines detected by antibody against bacterially expressed human protein

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The first step in the synthesis of steroids is catalysed by cytochrome *P*-450ssc (cholesterol-side-chain cleavage enzyme). We have investigated the synthesis of this enzyme in three cultured cell lines at the protein and hormone secretion levels. Hormone levels were measured by an enzyme immunoassay using a monoclonal antibody against progesterone. The protein level was detected using polyclonal antibodies directed against a *P*-450scc fusion protein overproduced in *Escherichia coli*. Utilizing a bacteriophage T7 promoter expression system, a large amount of human *P*-450scc fusion protein was produced and easily purified. *P*-450scc was synthesized in the mouse adrenal tumour cell line Y1 and human choriocarcinoma cell line JEG-3, but not in monkey kidney cell line COS-1. The production of *P*-450scc in Y1 and JEG-3 cells was stimulated by 8-bromo cyclic AMP, the effect of which was not observed until 6 h after induction and was more pronounced at 24 h. Y1 and JEG-3 cells exhibited a difference in progesterone secretion after induction.

INTRODUCTION

The first step in the synthesis of steroid hormones is the conversion of cholesterol to pregnenolone [1]. This step is catalysed by a mitochondrial electron transport system which includes adrenodoxin reductase, adrenodoxin and a terminal cytochrome *P*-450 termed CYPXIA1 (*P*-450scc; cholesterol-side-chain cleavage enzyme) [2]. *P*-450scc is a haem-containing protein which is synthesized as a precursor and subsequently cleaved to yield a mature peptide of 49 kDa upon insertion into the mitochondrial inner membrane [3].

Bovine *P*-450scc has been purified from the adrenal gland by a number of methods and characterized [4–6]. Its cDNA codes for a protein of 520 amino acids, of which 39 are a signal peptide [7]. The human *P*-450scc cDNA sequence is 82% identical to the bovine sequence and is present as a single-copy gene (*CYP11A1*) on chromosome 15 [8]. Expression of *P*-450scc mRNA is stimulated by adrenocorticotropin in the adrenal gland [9] and by gonadotropin in the placenta and gonads [8,10]. These peptide hormones exert their effects through cyclic AMP as an intracellular messenger [8,10,11].

Mouse adrenocortical Y1 cells and human choriocarcinoma JEG-3 cells accumulate *P*-450scc RNA after stimulation by 8-bromo cyclic Amp (8-Br-cAMP) [12,13]. In this report we have analysed this stimulated expression further by determining the time course of the induction. We have also utilized a non-expressing cell line COS-1 for comparison.

Despite considerable knowledge about *P*-450scc gene expression and regulation, very little is known about the characteristics of the human *P*-450scc protein because of difficulty in obtaining enough human tissue for its purification. To circumvent this problem, we have utilized a bacterial expression system [14] to produce a recombinant fusion protein of human *P*-450scc in *Escherichia coli*. Milligram quantities of the protein have been produced and used to raise antibody reactive towards both recombinant and authentic *P*-450scc.

MATERIALS AND METHODS

Plasmids, reagents and cell lines

Bacterial strain BL21(DE3)pLysS and cloning vector pET-3c were obtained from Dr. William Studier (Brookhaven National Laboratory, Long Island, NY, U.S.A.). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) plasmid was provided by Dr. Selina Chen-Kiang (Mount Sinai Medical School, New York, NY, U.S.A.). The cloning of plasmid phtscc2 was described previously [8]. Alkaline-phosphatase-conjugated goat anti-rabbit IgG, NitroBlue Tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were purchased from Sigma. JEG-3 and Y1 cell lines were generously provided by Dr. Jerome Strauss (University of Pennsylvania, Philadelphia, PA, U.S.A.) and Dr. Bernard Schimmer (University of Toronto, Toronto, Canada) respectively.

Plasmid construction

The expression vector pET-3c has a single *Bam*HI site at the 12th codon of phage T7 gene 10, which allows the insertion of a foreign gene [14]. The plasmid phtscc2 was digested with *Xho*II to release a 2.44 kb fragment which was then ligated into pET-3c at the compatible *Bam*HI site. The resulting recombinant plasmid pET-scc (Fig. 1) has the *P*-450scc coding sequence starting from codon 51 connected to the first 12 amino acids of phage gene 10. It would have the proper transcriptional and translational start sites contributed by the phage sequence. A T7 transcriptional termination site is also located 3′ to the insert in the vector.

Protein purification and blotting

The bacterial strain BL21(DE3)pLysSpET-scc was grown in ZY medium supplemented with 0.4% glucose to an absorbance of 0.2–0.4, induced with 0.4 mM-isopropyl β-D-thiogalactoside (IPTG) and harvested 2 h afterwards [15]. The fusion protein was purified by a procedure modified from that of Kaplan &

Abbreviations used: *P*-450scc, cytochrome *P*-450scc (cholesterol-side-chain cleavage enzyme; 8-Br-cAMP, 8-bromo cyclic AMP; IPTG, isopropyl β-D-thiogalactoside; NBT, NitroBlue Tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GdnHCl, guanidine hydrochloride; PBS, phosphate-buffered saline.

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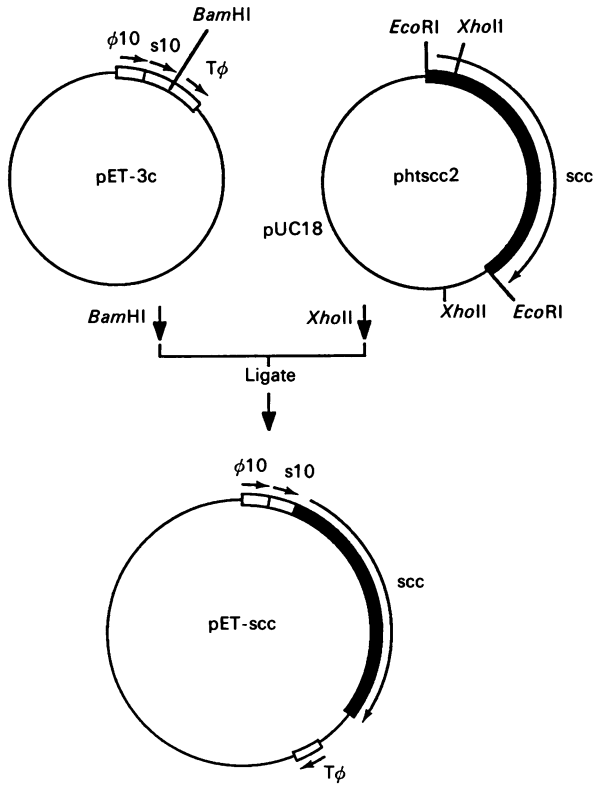

Fig. 1. Construction of pET-scc

The T7 promoter (ϕ10) and the gene 10 translation start site (s10) are 5′ to the scc cDNA, whereas the transcription terminator (Tϕ) is at the 3′-end of the scc cDNA in the resulting plasmid pET-scc.

Greenberg [16]. The cell pellet was resuspended in R buffer [50 mM-Tris/HCl, pH 8.0, 200 mM-NaCl, 0.1 mM-EDTA, 0.1 mM-dithiothreitol and 5% glycerol] and lysed with 0.1 vol. of 50 mM-EDTA/10% (v/v) Triton X-100. This suspension was incubated for 30 min at room temperature, sonicated and centrifuged at 150000 *g* in an SW41 rotor for 30 min. The pellet was dissolved in 6 M-guanidine hydrochloride (GdnHCl) in R buffer and centrifuged again. The protein in the supernatant was again precipitated in 1 M-GdnHCl by a dropwise addition of R buffer. The pellet was redissolved in 6 M-GdnHCl/10 mM-potassium phosphate, pH 7.2, and loaded on to a hydroxyapatite column. The recombinant protein was eluted with the flow-through fraction. For immunoblotting, proteins were electroblotted on to nitrocellulose paper in 20% (v/v) methanol/25 mM-Tris/0.19 M-glycine/0.1% SDS at 25 V for 3 h. For amino acid sequencing, the proteins were transferred to polyvinyldifluoride membrane in 250 mM-sodium borate (pH 9.6)/20% methanol/0.5% SDS using a semi-dry electroblotting procedure [17]. The blot was then stained with 0.5% Coomassie Blue R-250 in 30% propan-2-ol/10% acetic acid for 2 min, destained in 50% ethanol/10% acetic acid, and then rinsed in water. After drying, the desired band was excised and subjected to *N*-terminal sequence analysis.

Antibody production

About 1 mg of the partially purified *P*-450scc in 6 M-GdnHCl was injected intradermally into a rabbit. The same amount was given in each of two booster shots about 4 weeks and 8 weeks later. Blood was collected 10 days after the boost. Serum was separated from clotted blood cells and stored at −20 °C.

Protein sequencing

Automated cycles of Edman degradation were performed with a gas/liquid phase sequencer with an on-line phenylthiohydantoin-amino acid analyser (Applied Biosystems, Foster City, CA, U.S.A.) according to the procedure of Hsieh *et al.* [18].

Antibody reactions

Two procedures were used, with modifications, for antibody detection [19,20]. When using colour reactions, the protein blots were first blocked with 3% gelatin in TBST buffer (20 mM-Tris/HCl, pH 7.4, 50 mM-NaCl and 0.05% Tween 20) for 1 h at room temperature. They were then treated with antiserum at 1:1000 dilution for 1 h. After three washes with TBST, goat anti-rabbit IgG conjugated with alkaline phosphatase was applied and incubated for 1 h. Having been washed with TBST, the blots were incubated with substrate solution (330 μg of NBT/ml and 165 μg of BCIP/ml in 100 mM-Tris/HCl, pH 9.5, 100 mM-NaCl and 5 mM-$MgCl_2$) until colour developed.

Alternatively, the blot was stained with 0.1% Amido Black in 45% methanol/10% acetic acid and then destained in 75% methanol/2% acetic acid to reveal the protein bands. After rinsing in phosphate-buffered saline (PBS; 0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4 and 0.024% KH_2PO_4, pH 7.2), the blot was blocked with 5% non-fat milk in PBST buffer (0.2% Tween-20 in PBS, pH 7.2) for at least 1 h at room temperature. It was then treated with antiserum at 1:1000 dilution, washed with PBST three times and incubated with ^{125}I-Protein A for 1 h. The blot was washed with PBST, dried on Whatman 3MM paper and autoradiographed at −70 °C with two intensifying screens.

Production of anti-progesterone antibody

A monoclonal antibody against progesterone was obtained using a procedure described by Fantl *et al.* [21]. This antibody, code no. G7, is highly specific and has a binding affinity of approx. 1.1×10^{10} M^{-1} [22].

Progesterone measurement

The progesterone assay was modified from a direct enzyme immunoassay [23] using monoclonal antibody G7. A 5 μl portion of culture medium together with 200 μl of progesterone–horseradish peroxidase were added to star tubes (Nunc 470319) coated with anti-progesterone antibody. After incubation at room temperature for 15 min and two washings with 0.01 M-PBS, the colour was developed in 1 ml of 2 M-*o*-phenylenediamine in 0.003% H_2O_2 for 30 min. The reaction was stopped by 200 μl of 3 M-sulphuric acid and the absorbance at 492 nm was compared with that of a progesterone standard curve. The variation coefficients within and between assays were approx. 7% and 10% respectively. The sensitivity was 3 pg/tube.

RESULTS

Construction and overexpression of pET-scc plasmid

The expression vectors we used (pET vectors), developed by Rosenberg *et al.* [14], contain a strong gene 10 promoter from T7 bacteriophage which can direct high levels of expression. Human *P*-450scc cDNA was inserted into pET-3c as described in the Materials and methods section to form pET-scc (Fig. 1).

The *E. coli* strain BL21(DE3)pLysS carries an integrated T7 RNA polymerase gene under the control of the *lac* UV5 promoter [15]. After induction with IPTG, T7 RNA polymerase which recognizes its specific promoter accumulates and transcribes its target gene at a high rate. This transcription-linked translation process produces a large amount of *P*-450scc fusion protein,

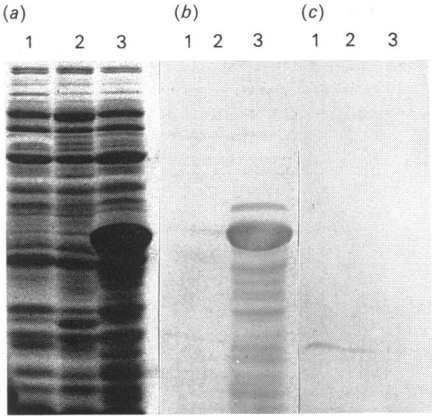

Fig. 2. Immunoblot analysis of bacterial lysate

Portions of 40 μl (lane 1) or 30 μl (lanes 2 and 3) of lysate from 1 ml of cells were loaded on to a 10% polyacrylamide gel in the presence of SDS and electrophoresed until the Bromophenol Blue dye ran to the bottom of the gel. Three identical gels were prepared. One gel was stained with Coomassie Blue (*a*); the others were transferred to nitrocellulose membrane and treated either with anti-*P*-450scc antibody at 1:1000 ratio (*b*) or with non-immune serum (*c*). Positive bands were detected by colour reaction. Lane 1, BL21(DE3)pLysS pET-3c; lane 2, BL21(DE3)pLysS/pET-scc without IPTG induction; lane 3, BL21(DE3)pLysS/pET-scc after IPTG induction.

Table 1. *N*-Terminal amino acid sequence of bacterially expressed *P*-450scc

The sequence was determined as described in the Materials and methods section. The amino acid released as the phenylthiohydantoin derivative at each cycle is shown.

Cycle	Amino acid	Predicted sequence	Origin of amino acid
1	–	Met	Gene 10, residue 1
2	Ala	Ala	Gene 10, residue 2
3	Ser	Ser	Gene 10, residue 3
4	Met	Met	Gene 10, residue 4
5	Thr	Thr	Gene 10, residue 5
6	Gly	Gly	Gene 10, residue 6
7	Gly	Gly	Gene 10, residue 7
8	Gln	Gln	Gene 10, residue 8
9	Gln	Gln	Gene 10, residue 9
10	Met	Met	Gene 10, residue 10
11	Gly	Gly	Gene 10, residue 11
12	Arg	Arg	*Bam*HI linker
13	Ile	Ile	*P*-450scc, residue 12
14	Pro	Pro	*P*-450scc, residue 13
15	Ser	Ser	*P*-450scc, residue 14
16	Pro	Pro	*P*-450scc, residue 15

termed B-scc. A prominent band of B-scc was produced (Fig. 2*a*, lane 3) compared with that in cells without induction (lane 2) or in vector-only-containing cells (lane 1). From the intensity of the gel, it is estimated that 10–20 mg of B-scc is produced per litre in this culture.

The overproduced B-scc protein forms an insoluble inclusion body in the cell and can be easily purified by repeated centrifugation/washing steps. *N*-Terminal sequencing of the purified protein revealed that the first 11 amino acids belong to phage gene 10, and that the 12th amino acid (Arg) is encoded by the *Bam*HI linker, as predicted (Table 1). Starting from amino acid 13, the sequence is identical with that of mature *P*-450scc

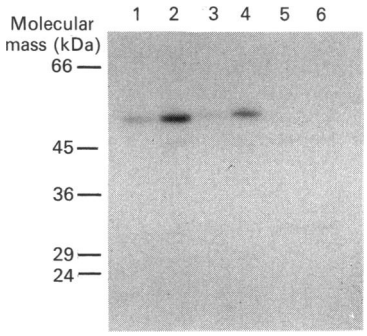

Fig. 3. Immunoblot analysis of cultured cells treated with cyclic AMP

Cells were treated with 8-Br-cAMP for 24 h in serum-free medium (lanes 2, 4 and 6) or were not treated (lanes 1, 3 and 5). Then 50 μg of cell lysate was electrophoresed in a 10% polyacrylamide gel in the presence of SDS, transferred on to nitrocellulose paper and treated with a 1:1000 dilution of anti-*P*-450scc antiserum. ^{125}I-Protein A was used to detect antibody binding. Lanes 1 and 2, JEG-3 cells; lanes 3 and 4, Y1 cells, lanes 5 and 6, COS-1 cells.

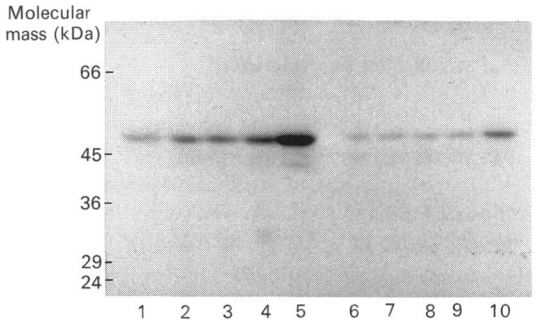

Fig. 4. Time course of 8-Br-cAMP stimulation of *P*-450scc

Portions of lysates (50 μg) from Y1 cells (lanes 1–5) and JEG-3 cells (lanes 6–10) treated with 1 mM-8-Br-cAMP for 0 h (lanes 1 and 6), 1 h (lanes 2 and 7), 2 h (lanes 3 and 8), 6 h (lanes 4 and 9) or 24 h (lanes 5 and 10), were separated by SDS/PAGE, transferred to nitrocellulose, treated with anti-*P*-450scc antiserum and detected with ^{125}I-Protein A.

from amino acid 12, thereby demonstrating the identity of the overexpressed protein.

Property of antibody against B-scc

A polyclonal antibody raised against the purified protein was evaluated in immunoblot analyses. A strong band showed up for the overexpressed B-scc (Fig. 2*b*, lane 3) and a weak band could be detected at the uninduced state (lane 2). This antiserum has a high titre and is specific for B-scc, since it did not react with other proteins in control bacteria (lane 1), and non-immune serum did not react with the proteins either (Fig. 2*c*). The other protein bands seen in Fig. 2(*b*) (lane 3) are probably degradation products of B-scc, since degradation of overexpressed foreign protein is a common phenomenon, and minor degraded protein bands can be detected when the gel is overloaded with B-scc.

P-450scc production in cultured cell lines and its stimulation by cyclic AMP

Three mammalian cell lines were characterized for *P*-450scc production. Among them, COS-1 does not produce steroids [24], whereas JEG-3 and Y1 do [12,25]. In Y1 and JEG-3 cells a 50 kDa protein band was detected which increased in intensity upon stimulation by 8-Br-cAMP (Fig. 3, lanes 2 and 4). COS-1 cells, on the other hand, did not produce *P*-450scc even after

Table 2. Regulation of the levels of *P*-450scc in Y1 and JEG-3 cells by 8-Br-cAMP at different time points

Cells were cultured in 3 cm dishes and 1 mM-8-Br-cAMP was added to culture media at different times as indicated in the first column. The dish labelled time zero received no 8-Br-cAMP. The level of *P*-450scc protein was detected by immunoblot analysis, and *P*-450scc activity by measuring the amount of progesterone (P4) secreted into culture media. Each P4 measurement was repeated five times in triplicate and the data are expressed as means ± S.E.M.

	Y1			JEG-3		
		Fold increase			Fold increase	
Time (h)	P4 (ng/ml)	Protein	P4	P4 (ng/ml)	Protein	P4
0	6.4±2.0	1.0	1.0	6.5±1.3	1.0	1.0
1	8.8±2.7	1.4	1.4	7.8±1.1	1.4	1.2
2	12.6±0.9	1.8	2.0	7.1±1.8	1.9	1.1
6	24.5±7.4	2.1	3.8	8.2±1.9	2.2	1.3
24	100.2±24.0	4.2	15.7	14.0±1.8	5.5	2.2

8-Br-cAMP treatment. The presence or absence of *P*-450scc in these three cell lines correlates well with their ability to synthesize steroids.

Time course of stimulation by cyclic AMP

To analyse cyclic AMP-stimulated *P*-450scc expression in more detail, we incubated cells with 8-Br-cAMP for different time periods to monitor the course of induction. Protein accumulation after different periods of incubation was measured in an immunoblot experiment (Fig. 4). The amount of *P*-450scc present in both Y1 and JEG-3 cells increased slowly and was clearly evident after 24 h of incubation.

The enzymic activity of *P*-450scc was determined by measuring the concentration of progesterone that had accumulated in the culture media (Table 2). Both Y1 and JEG-3 cells had secreted about the same amount of progesterone (about 6 ng/ml per plate) at time zero. After the addition of 8-Br-cAMP the amount of progesterone increased steadily in both cell culture systems throughout the entire 24 h incubation period; however, Y1 cells showed a bigger increase than JEG-3 cells.

The levels of *P*-450scc protein were semi-quantified by densitometric scanning and compared with control values where cells had not been treated with 8-Br-cAMP. As shown in Table 2, we observed a 4–5-fold induction in the amount of protein. The induction of progesterone accumulation in JEG-3 cells was only 2-fold, although there was a 5-fold increase in *P*-450scc protein. The increase in progesterone accumulation in Y1 cells was more pronounced, reaching up to 15-fold.

DISCUSSION

P-450scc catalyses the first step in the synthesis of all steroids [1] and is regulated by various peptide hormones in different steroidogenic tissues [8–10] at different developmental stages [26,27]. The effect of adrenocorticotropin on steroidogenesis involves both short-term and long-term action [28]. The acute action increases *P*-450scc activity due to increased binding of cholesterol to the enzyme [29–31]. The long-term action is due to increased *P*-450scc synthesis [9,11]. This increased synthesis is observed in both Y1 and JEG-3 cell lines. However, the amount of progesterone secretion was different between the two cell lines. In Y1 cells the progesterone level increased steadily, reaching 15-fold at 24 h. In JEG-3 cells, however, there was only a 2-fold increase. One possibility is that in JEG-3 cells the progesterone-metabolizing enzyme may also be stimulated, leading to quick conversion of progesterone into other steroids, and thus the accumulation of progesterone would be less pronounced.

We describe in this paper the first purification of human *P*-450scc fusion protein (B-scc) using recombinant DNA techniques. The bacterial strain BL21(DE3) used for protein expression produces a low level of T7 RNA polymerase that can be inhibited by T7 lysozyme [15]. Thus the introduction of T7 lysozyme can increase tolerance towards possibly deleterious effects that are generated by the recombinant plasmid [32]. The T7 lysozyme can be provided by either pLysE, a strong producer, or pLysS, a weak producer. pET-c21 in pLysE-containing cells did not overproduce the protein (results not shown), probably because of the growth-inhibitory effect generated by high levels of lysozyme. Cells carrying pLysS appear to be the best system for B-scc expression.

B-scc is predicted to be identical in sequence with the authentic protein except for substitution of the first 12 amino acids (Table 1). This substitution apparently did not affect the antigenicity of the protein, as its antibody still reacted with *P*-450scc produced by mammalian cells (Figs. 3 and 4). The essentially unlimited supply of B-scc and availability of its antibody make its characterization much easier.

JEG-3 cells are of human origin, whereas Y1 cells are derived from murine cells. The *P*-450scc proteins produced from both cell lines are from different species, yet they react with the same antiserum, demonstrating that they share common antigenic sites. Anti-(bovine *P*-450scc) antibody also cross-reacts with porcine *P*-450scc [33,34]. The higher intensity of protein bands in JEG-3 than in Y1 cells is probably due to a better reaction with human than murine *P*-450scc (Fig. 3). This shows that *P*-450scc is moderately conserved in mammals at both the nucleic acid and amino acid levels.

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