Vol. 160, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1989 Pages 276-281

THE 5'-REGION OF THE P450XIA1 (P450scc) GENE CONTAINS A BASAL PROMOTER AND AN ADRENAL-SPECIFIC ACTIVATING DOMAIN

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Received February 2, 1989

The first step to the synthesis of all steroids is catalyzed by P450scc. We constructed nine deletion mutants of the 5'-region of the P450scc gene and connected them to a CAT reporter gene to assay transcriptional activity of the P450scc promoter. A short 145 bp fragment stimulated transcription by two fold. This DNA was active in all cells tested irrespective of their tissue origin and steroidogenic activity. DNA at -145/-573 of the upstream region did not increase transcription any further. DNA including 2500 bp of the upstream region stimulated transcription by 10 fold only in adrenal Y-1 cells. Hence in the -145 region contains a low level P450scc promoter and the 2500 bp DNA possesses an adrenal specific enhancing element.

Cytochrome P450scc (cholesterol side-chain cleavage enzyme) is a mitochondrial monooxygenase converting cholesterol to pregnenolone and isocaproic acid. This first and rate-limiting step in steroid synthesis is the site of regulation by peptide hormones in steroidogenic tissues (1-5). In the adrenal, ACTH stimulates P450scc synthesis through cAMP intermediate (2, 6). The precise mechanism of the activation, however, is unknown.

There is a single human P450scc gene (7), termed P450XIA1(8). This gene, which contains 10 exons and 9 introns (9), is located on chromosome 15 (10). A 5.4 kb fragment at the 5'-flanking region can confer cAMP responsiveness (11). The precise location of the cAMP responsive element, however, has not been delineated. To analyze the promoter activity in P450scc transcription, we have constructed a series of nine deletion mutants and transfected them into both steroidogenic and nonsteroidogenic cells. We report the identification of a basal level general promoter and an adrenal specific enhancing element.

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0006-291X/89 $1.50

MATERIAL AND METHODS

Isolation and Characterization of the P450scc Genomic Clone

The library screening procedure with an oligonucleotide probe was described previously (12) and a single positive clone was purified. A 6 kb *Eco*RI fragment and a 1 kb *Pst*I /*Eco*RI fragment in this clone which hybridized to the oligonucleotide probe were subcloned into pUC18 for more detailed analysis. The appropriate subfragments were again subcloned into M13 for dideoxynucleotide sequence determination (12).

Primer Extension and S1 Nuclease Mapping

For S1 mapping, a 27mer (base 53 to 79) primed the synthesis of a DNA probe from an M13 template containing 1000 bp of the *Pst*I/*Eco*RI fragment of the P450scc 5'-flanking region. The reaction mixture was then digested with *Pst*I and DNA probe corresponding to -90/+79 region of the gene was purified from the gel. The resulting 169 nucleotide probe was hybridized with 10 μg of human testis RNA and treated with S1 nuclease (Pharmacia) as described earlier (13).

Plasmid Construction for Transfection

Plasmid vector pUC13CAT was constructed by ligating the *Bam*HI/*Hind*III fragment of the CAT gene into pUC13 after the addition of *Sal*I linkers. An *Eco*RI/*Sph*I fragment spanning -5400/+60 of the P450scc gene was cloned into pUC18 at the *Eco*RI/*Sma*I site to become pSCCdel. pSCC2500 and 573 were cloned by inserting the 2500 bp *Hind*III/*Xba*I or the 573 bp *Pvu*II/*Xba*I fragment of pSCCdel into pUC13CAT respectively. pSCC600 was obtained by digesting pSCCdel with *Pvu*II and cloned into pUC13CAT. Deletion mutants were prepared by linearizing parent plasmids, digesting with Bal 31 for 1 to 3 mins, cut again with *Xba*I, and the shortened fragments were subcloned into the *Sma*I/*Xba*I sites of pUC13CAT. pSCC459 and 531 were derived from pSCC600, pSCC346 and 236 from pSCC573, while pSCC76 and 145 were from pSCC236. The deletion end points were all confirmed by restriction mapping and DNA sequencing.

Cell Culture and Chloramphenicol Acetyltransferase Assay

JEG-3 and Y-1 cells were obtained from Drs. J. F. Strauss III and B. P. Schimmer, respectively, and cultured accordingly (14). Gene transfer experiments were performed by the calcium phosphate precipitation procedure (15) using 6-well dishes. CAT activity was determined either using [^{14}C]-chloramphenicol (15) or [^{3}H]-acetyl CoA (16).

RESULTS AND DISCUSSION

Characterization of the P450scc gene and the mRNA initiation site

We isolated a clone containing the first exon of the P450scc gene and about 5 kb of its 5'-flanking area (Fig. 1). The sequence in this region will be published elsewhere (in preparation). To determine the mRNA initiation site, a synthetic oligonucleotide complementary to bases 53-79 was used to prime the synthesis of cDNA with human testis tumor RNA as a template. The resulting extension product was 79 nucleotides (lane 1 in Fig.2A) when it was compared with a sequence ladder (lanes 5, 6, 7, 8). This result was confirmed by S1 analysis where undigested S1 probe is 169 nucleotides (lane 4 in Fig. 2A and lane 1 in Fig. 2B). The S1 probe protected a DNA fragment of the same size as the extension

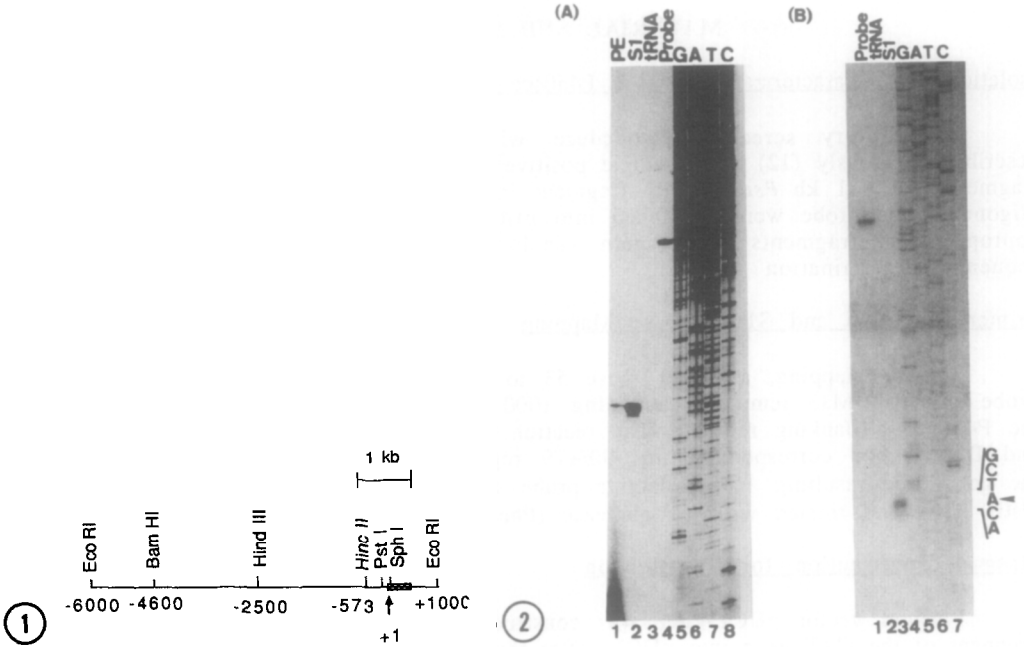

Fig.1 Structure of the 5'-region of the human P450scc gene. Untranscribed and intron sequences are represented by a line. The hatched box represents the first exon. The transcriptional start site (indicated by an arrow) is numbered as nucleotide +1.

Fig. 2(A) Determination of the mRNA start site by primer extension and S1 nuclease mapping. Lane 1 represents primer extension product from human testicular tumor RNA. Lane 2 is the S1 nuclease protected fragment from testis RNA. Lane 3 is S1 digestion of probe hybridized with tRNA only. Lane 4 is the size of the probe. Lanes 5, 6, 7, 8, consist of a ladder G, A, T, C. of a random sequence. (B) S1 nuclease mapping. Lane 1 is undigested probe. Lane 2 is S1 digestion of probe which is hybridized with tRNA only. Lane 3 is protection seen in testis RNA. Lanes 4, 5, 6, 7 are a sequence ladder initiated at the the same 5'-end as the S1 probe. The sequence where mRNA starts is indicated by an arrow.

product (lane 2 of Fig. 2A and lane 3 of Fig. 2B), whereas tRNA showed no protection (lane 3 of Fig. 2A and land 2 of Fig. 2B). Hence the cap site was located at 62 nucleotides upstream from the ATG initiation codon.

Promoter Analysis

A deletion mapping procedure was used to locate and to measure the strength of the promoter. The 5' deletion end points, spanning from -2500 to -76, are diagrammed in Fig. 3A. These DNA fragments were linked to the CAT gene and the resulting fusion plasmids were transfected into Y-1 and JEG-3 cells, both of which were steroidogenic (17, 18).

As observed in many other systems (19), the promoterless pUC13CAT produced some background expression presumably due to nonspecific promoters in the pUC13 region of the vector (Fig. 3B). Transfection of pSCC76 results in expression higher than background, indicative of some promoter activity. This activity plateaued when DNA was 145 bp in length. pSCC236, 346, or 459 produced

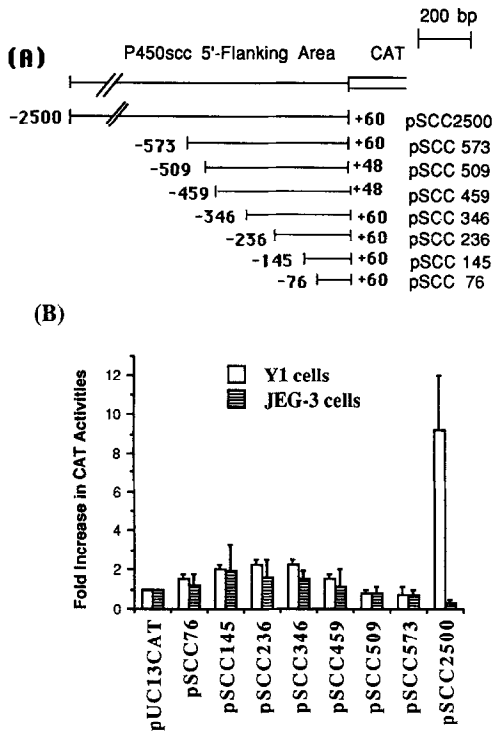

Fig. 3(A).Deletion plasmids containing P450scc 5'-flanking DNA linked to the CAT gene. The plasmids are named after the end points of the 5'-flanking deletions. (B) Expression of CAT activity in cells transfected with deletion plasmids. The values shown in the figure are the average of seven independent experiments with their standard deviation shown.

similar CAT levels as pSCC145, approximately two fold over background. Hence the P450scc promoter resides in a 145 bp fragment that includes the TATA (-25/ -29) and the CATT (-64/ -67) boxes. This basal promoter has a low activity which is only two fold over background.

CAT activity started to decrease when DNA longer than 346 bp were used. pSCC573 retained only 60% of the activity compared with vector pUC13CAT (Fig. 3B). The lower level of expression could be due to the presence of an inhibitory element situated in this region. Yet linking DNA at -314 / -573 to the thymidine kinase promoter (20) did not inhibit the expression of CAT in transfected JEG-3 cells (data not shown). Hence, if sequence between -314 and -573 contained a negative element, it did not affect a heterologous promoter. Alternatively the lower CAT activity associated with this region could be due to decreased translation of readthrough transcripts into the CAT protein. The insertion of 400 bp or more of the genomic sequence between the spurious promoter and the CAT initiation codon would render the RNA less likely to be translated and thus lower the background. This phenomenon has been reported elsewhere (19).

Plasmids containing 2500 bp of the P450scc 5'-flanking sequence gives a high level of transcription in adrenal Y-1 cells showing it contains an activating

Table 1

CAT Activities of Transfectants expressed as fold increase in activity over pUC13CAT

DNA	HeLa	JEG-3	Y1	CHO	COS-1
pUC13CAT	1	1	1	1	1
pSCC236	3.68	1.6	2.3	1.64	5.12
pSCC573	0	0.7	0.7	0	0.056
pSCC2500	0.10	0.3	9.19	0.077	0.33
pSV2CAT	200	256	378	250	574

Cells in 6 cm dishes were transfected with 5 μg of plsmids using the calcium phosphate precipitation procedure and harvested after 48 hrs. Fifty μg of proteins in the extract were used in each assay in a scintillation vial containing Econofluor plus 0.5 μCi of [^{3}H]-acetyl CoA, 1 mM chloramphenicol in 100 mM Tris-HCl, pH7.8. The CAT activities were scored as the cpm transfered into the product which partitioned into the organic layer. Two to seven experiments were performed for each cell line and the average value of the fold increase over vector is presented here.

domain which can direct high levels of transcription (Fig. 3B). Yet this is not seen in the same construct transfected into JEG-3 cells (Fig. 3B). Therefore this activating element in the 2500 bp fragment appears to be adrenal specific.

Tissue Specificity of the Regulatory elements

To investigate the cell-type specific transcription further, we transfected plasmids into five cell lines of different tissue origins, including HeLa (cervical carcinoma), CHO (ovary), COS-1 (kidney), JEG-3 (placental trophoblast), and Y-1 (adrenal) to check if there is any tissue specificity. pSV2CAT which contains the SV40 promoter and enhancer (15) is used as a positive control. As shown in table 1, although the background exhibited by pUC13CAT is high, pSCC236 yielded a higher amount of CAT in each cell line. This confirmed that TATA and CATT boxes acted as a general promoter and should be functional in every cell line. pSCC573 produced lower CAT activities in all cell lines consistent with previous results. Transfection with pSCC2500 resulted in low CAT activities in all cell lines except Y-1, which again had elevated activity. Thus we concluded that the activating domain present at -573/ -2500 was cell type specific. Whether this cell type specific activator functions as an enhancer should await further characterization.

ACKNOWLEDGMENTS

The authors would like to thank Drs. Ru-Chih Huang and Walter Miller for discussions and encouragement and Dr. Ming Tam for the synthesis of oligonucleotides. This work was supported by National Science Council and Academia Sinica, Republic of China.

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