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ANALYSIS OF THE HUMAN ADRENODOXIN PROMOTER: EVIDENCE FOR ITS ACTIVITY

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Adrenodoxin is an iron-sulfur protein serving as an electron transfer intermediate in the mitochondrial cytochrome P450 system. To study its transcriptional regulation we construct a human adrenodoxin genomic clone which includes 333 bp of DNA upstream of the mRNA start site. This DNA contains a TATA box and two GC boxes. When we place it in front of the CAT reporter gene and transfect it into the recipient cell lines, it directs transcription of the CAT gene. This DNA in reverse orientation does not show any transcriptional activity. This promoter element functions in three mammalian cell lines: JEG-3, COS-1, and Y-1.

The steroid hydroxylase systems in the mitochondria contain the terminal cytochrome P450, adrenodoxin, and adrenodoxin reductase. Adrenodoxin is an iron-sulfur protein that transfers electrons in the hydroxylase system. It is expressed abundantly in steroidogenic tissues and in lower amount in liver and kidney (1). The expression of adrenodoxin is stimulated by cAMP (1). This regulated expression is controlled at the transcriptional level (2). The human adrenodoxin gene is located on chromosome 11 and its pseudogenes on chromosome 20 (3). In order to understand the mechanism of transcriptional regulation, we cloned the human adrenodoxin gene and sequenced all its four exons and the 5'-flanking region (4). We now report the promoter activity of this gene.

MATERIALS AND METHODS

Cell Lines and Transfection Assays

Y-1 cells and human choriocarcinoma cell line JEG-3 were obtained from B. P. Schimmer and J. F. Strauss III. Cells were plated onto 6 cm

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dishes 24 h before transfection and cultured in F-10 medium supplemented with 15 % horse serum and 2.5 % bovine fetal serum (5). Six μg of plasmids per plate were transfected using the standard calcium phosphate precipitation procedure (6). Cells were harvested 48 hrs later and 10 μg of proteins were used to assay for CAT activity either using thin layer chromatography (6) or by direct liquid scintillation counting (7).

Plasmid Construction

The ends of the *Eco*RI/*Pvu*II fragment (-333/+81) of the cloned adrenodoxin genomic DNA were made double stranded using the Klenow fragment, then blunt-end ligated in both orientations into the *Sma*I site of pUC13CAT which has the CAT gene inserted into pUC13 at the *Sal*I site. The resulting plasmids are named pAdx330-CAT and pAdx330R-CAT depending on their orientations.

RESULTS

The 5'-Flanking Region of the Human Adrenodoxin Gene

We isolated several clones by screening the human genomic DNA libraries (8, 9) with the adrenodoxin cDNA probe (1). Among them clone h-5 contains the 5'-region of the gene including the first exon (4). The structure of the 3.2 kb *Eco*RI fragment harboring the first exon is shown in Fig. 1A. This DNA contains 333 bp of the 5'-flanking area, the first exon, and 2.5 kb of the first intron. The *Eco*RI/*Puv*II fragment of the DNA was again subcloned into the vector pUC13CAT. As shown in Fig. 1B, pAdx330-CAT contains DNA at -333/+81 in the correct 5' to 3' orientation. pAdx330R-CAT includes the same DNA in reverse orientation. The sequences of both plasmids at the junctions were verified by the dideoxynucleotide sequencing procedure using double-stranded plasmid DNA as a template. This region includes a TATA box at position -27 /-30 (shown in black boxes in Fig. 1B) and two GC rich regions at positions -54 /-63 and -94 / -103. The GC boxes which have been shown to bind transcription factor Sp1 (10) are in opposite orientations at the adrenodoxin 5'-region.

Adrenodoxin Gene Promoter Activity

Since both TATA and GC boxes have been shown to function as promoter elements in other genes (11, 12), we would like to determine whether they have any promoter function for the adrenodoxin gene. We transfected both pAdx330-CAT and pAdx330R-CAT into the steroid secreting JEG-3 cells and measured CAT activity to reflect its transcriptional activity. Fig. 2 shows the results of a typical transfection experiment performed in duplicate. There is a high level of CAT

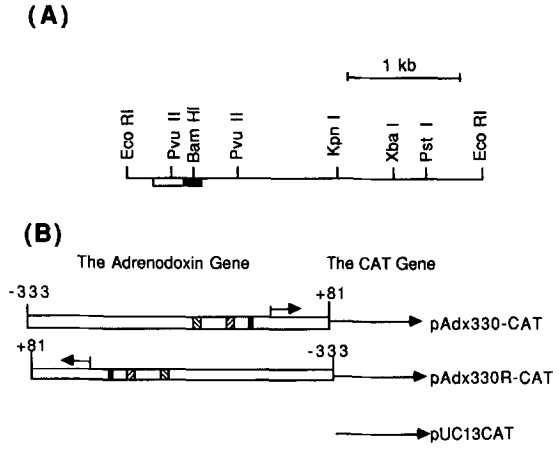

Fig. 1(A). Restriction map of the clone containing the 5'-region of the human adrenodoxin gene. The open box represents the 5'-untranslated region of the first exon, while the black box represents coding region of the first exon.

(B). Construction of plasmids used in CAT assays. Open box: 5'-region of the adrenodoxin gene; slashed boxes: GC boxes; black box: TATA box. The numbers above the open box correspond to the start and end of the gene fragments. The arrows above the open box represent the orientation and mRNA initiation sites. The line with arrows denote CAT genes and the orientation of transcription.

expression driven by the adrenodoxin promoter (pAdx330-CAT) when it is in the right orientation. The SV40 promoter and enhancer containing plasmid pSV2CAT is used as a positive control. The vector pUC13CAT gives a low level of background (Figs. 2 and 3) which may have come from spurious promoters located in the plasmid, as observed in many systems (13, 14). Hence pAdx330R-CAT serves as a better negative control which gives very little background. Comparing the amount of expression in pAdx330-CAT and pAdx330R-CAT, we conclude that this 333 bp DNA fragment contains a promoter element.

The Adrenodoxin Promoter Is Active in Many Cell Types

Adrenodoxin is expressed in mouse adrenal tumor Y-1 cells (Chung et al., unpublished result), choriocarcinoma cells JEG-3 (1), and in lower amount in monkey kidney COS-1 cells (15). The level of expression in cultured cells reflects that in the corresponding tissues. To investigate whether the adrenodoxin promoter is active in these cells, we transfected plasmids which contain the adrenodoxin promoter into all of them. The results (Fig. 3) showed similar expression patterns in all three cell lines. CAT activity increased only when the promoter was inserted in the right orientation. Hence we conclude that the adrenodoxin promoter functions

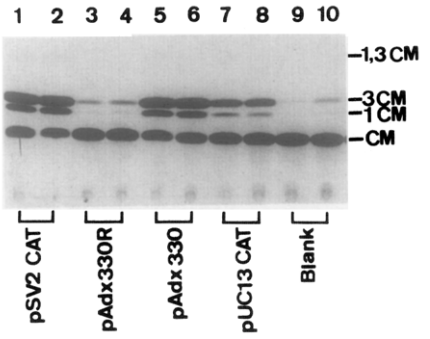

Fig. 2. CAT activity in transfected JEG-3 cells. Six μg of plasmids were used to transfect per plate of cells and 10 μg of proteins were used to measure CAT activity on a TLC plate. Duplicate experiments were performed in each case. CM denotes chloramphenicol, 1 CM denotes 1-acetylated chloramphenicol, 3 CM denotes 3-acetylated chloramphenicol, and 1,3 CM denotes 1, 3-diacetylated chloramphenicol.

as a general promoter which acts in all three cell lines tested. Since the TATA and GC boxes bind common transcription factors (14), it is not surprising that all three cell lines contain these transcription factors.

DISCUSSION

We have identified an adrenodoxin promoter which is active in Y-1, JEG-3 and COS-1 cells. This promoter is composed of a TATA box and two GC boxes. It confers a basal activity to the adrenodoxin transcription. We

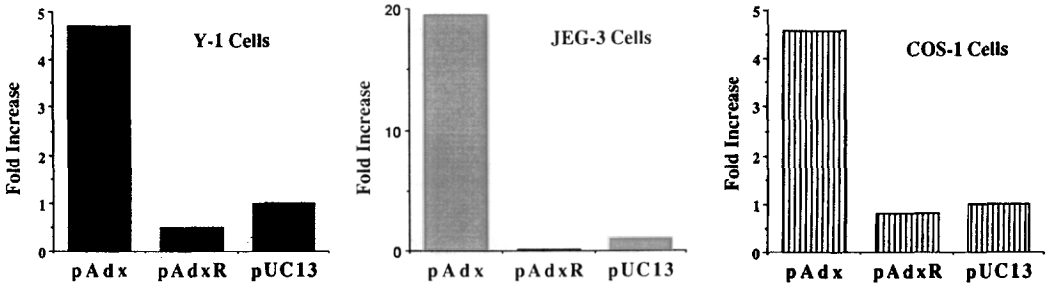

Fig. 3. CAT assays of cells transfected with CAT plasmids linked to the adrenodoxin promoter. pAdx plasmid has the promoter in the forward orientation (pAdx330-CAT). pAdxR has the promoter in the reverse orientation (pAdx330R-CAT). pUC represents the vector plasmid (pUC13CAT). Plasmid DNAs were transfected into cells. After 48 hrs, CAT activity in the cell lysate was measured and expressed as fold increase over vector pUC13CAT. Duplicate transfection was performed in each case. The JEG-3 plot is the average of eight experiments. The COS-1 graph is the average of three experiments, while the Y-1 data was performed once.

have not detected other regulatory elements that might contribute to the adrenodoxin transcription yet. This basal promoter is a general one, active in all three cell types tested. And it appears to be a strong one.compared to many other promoters (16, 17). This strong promoter may explain why adrenodoxin mRNA is an abundant message.

One of the problems encountered in the transfection experiments is that the supposedly promoterless vector often gives rise to some CAT transcription due to the spurious promoter coming from the vector portion of the plasmid (13, 14). The use of pAdx330R-CAT that inserts a DNA fragment of the same length but in reverse orientation as the test plasmid pAdx330-CAT serves as a better negative control than the vector pUC13CAT. It alleviates the background problem to ascertain the validity of data interpretation. The inactivity of the adrenodoxin promoter in reverse orientation also demonstrates its orientation dependence.

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