Vol. 186, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1992 Pages 426-431

AMPLIFICATION OF P450c21 EXPRESSION IN CULTURED MAMMALIAN CELLS

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Received May 21, 1992

We describe in this paper an investigation of mammalian expression systems for P450c21 (21-hydroxylase). Four different promoters, the SV40 early and late promoters, MMTV-LTR, and CMV immediate early promoter were tested for their ability to drive the expression of P450c21 in cultured COS-1 cells. With the exception of MMTV-LTR, all drove the expression of similar levels of functional 21-hydroxylase. In addition, the Rat-1 cell line was tested and shown to be suitable for the stable expression of functional P450c21. We have established cell lines derived from Rat-1 either normal or mutant P450c21 stably expressed together with amplifiable markers. The expression of P450c21 was further increased by selection in methotrexate.

P450c21 (21-hydroxylase) is a membrane-bound mixed-function oxidase of the P450 superfamily. The enzyme catalyses the 21-hydroxylation of progesterone and 17α-hydroxyprogesterone during the biosynthesis of mineralocorticoid and glucocorticoid hormones in the adrenal cortex (1-3). Gene mutations affecting the activity of P450c21 results in defective production of cortisol and an overproduction of androgenic steroids (due to accumulation of 17-hydroxyprogesterone), resulting in congenital adrenal hyperplasia (CAH). CAH affects about 1 in 15000 Caucasians (2,3), with symptoms ranging from virilization of females to acute salt-wasting (2,4). P450c21 is encoded by the human CYP21B gene, and mutations of this gene have been found in more than 90% of CAH patients analyzed (2). Many of these mutations arise from gene conversion events involving the adjacent CYP21A pseudogene (5-10).

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0006-291X/92 $4.00

P450c21 is expressed only in the adrenal cortex in low abundance, making its purification and characterization difficult. Human P450c21 has never been purified from the adrenal. Our investgations are directed at maximizing the expression of P450c21 for functional studies. In this paper we compare the efficiency of different promoters to direct P450c21 expression and describe the establishment of cell lines supporting high-level, stable expression of the same enzyme.

MATERIALS AND METHODS

Source of Plasmids: pMAMneo and pSVL were purchased from Clontech (California, USA) and Pharmacia (Uppsala, Sweden) separately. pCMV-3 was modified by adding a 135 bp bidirectional polyadenylation signal from the SV40 (the blunt-ended *Bam*HI/*Hpa*I fragment) to its *Sma*I site (11). pCMV-3A contains the polyadenylation signal in the same orientation (BamHI to HpaI) as the CMV promoter. pCMV-3BB contains two polyadenylation signals in the reverse orientation.

Construction of the plasmids: The plasmid pc21 which contains full-length P450c21 cDNA was constructed by mutagenizing phc21 DNA at the initiation codon ATG to form a *Cla*I site. P450c21 cDNA, relieved from pc21 by *Cla*I/*Bam*HI digestion and blunt-ended with Klenow enzyme, was cloned into pSVL at the SmaI site, pCMV-BB or pCMV-A at blunt-ended XbaI site, to form pSVL-c21, pCMV-c21BB, or pCMV-c21A. Plasmids were named c21A or c21B depending on the orientation of the transcription terminator. Two terminators were also placed at the end of the cDNA to test whether more efficient termination would increase the efficiency of gene expression.

DNA transfection and Enzyme Assay : For transient assays cells were transfected with 5 μg of each of pRSV-β-gal (12) and phc21 (8) by calcium phosphate precipitation as described (13). Cells were assayed for 21-hydroxylase activity after 48 hours. For stable transfectants, cells were similarly transfected with normal or mutated phc21, pSV2Neo (14) and pFD11 (15) (5, 0.5 and 0.5 μg respectively, without carrier DNA) per 6-cm culture dish. After 24 hours the cells were passed to 10-cm dishes containing 0.4 mg/ml G418 (Sigma Chemical Co., USA). 21-Hydroxylase activity assay and selection in G418 and methotrexate were essentially as described (10,16).

RESULTS AND DISCUSSION

Four different promoters were compared for their ability to support P450c21 expression in COS-1 cells: SV40 early promoter and enhancer, SV40 late promoter, the long terminal repeat sequences from MMTV (MMTV-LTR), and the immediate early promoter of cytomegalovirus (CME-IE). In addition, the effect of transcriptional terminators on gene expression was also tested by placing a bidirectional SV40 transcriptional terminator at the end of the cDNA (11). 21-Hydroxylase activities expressed in cells transfected with these plasmids are presented in Table 1. With the exception of MMTV-LTR, which was weakest (13%

Table 1. Comparison of Mammalian Promoters for Expression of P450c21 by Transient Transfection. [^{14}C]-17-hydroxyprogesterone was incubated with COS-1 cells in 6-cm dishes 48 h after transfection with 5 μg each of pRSV-β-gal and the indicated P450c21-expression plasmids. The amount of [^{14}C]-deoxycortisol formed was quantitated by counting the radioactive spots on the thin layer chromatography plate which separates the substrate from the product.

Plasmids	Promoter	% Product Formation	Promoter Strength
—	—	0.3	-
phc21	SV40 Early	77	++
phcNeo	MMTV-LTR	13	+
pSVL-c21	SV40 Late	82	++
pCMV-c21A	CMV-IE	54	++
pCMV-c21BB	CMV-IE	60	++

conversion of substrate to product), the other promoters were equally efficient, giving between 54% to 82% conversion of substrate to product. Neither the presence of the transcriptional terminator, nor its orientation and copy number affected the production of P450c21.

To achieve very high levels of protein expression, a scheme for co-transfection of three plasmids (encoding P450c21, neomycin resistance, and dihydrofolate reductase) followed by G418 selection and amplification by selection with methotrexate of the transfected sequences was devised. An altered mouse dihydrofolate reductase cDNA (pFD11) was cotransfected with neomycin resistance gene as a dominant selectable marker (15). The whole cell population was subjected to selection at a higher methotrexate concentration,individual colonies were then isolated for analysis at different levels of selection.

Rat-1 and CHO cells have been used as recipients for stable amplification of c-H-*ras*1 and dihydrofolate reductase genes (15,16). We tested these cell lines to see if they had necessary co-factors to support 21-hydroxylase activity. After transfection, both COS-1 and Rat-1 but not CHO cells showed 21-hydroxylase activity towards both progesterone and 17-hydroxyprogesterone. Rat-1 was therefore selected as the recipient for stable transfection.

Both normal or mutated phc21 were transfected into Rat-1 cells. After selection in G418, clones were isolated, expanded and assayed for 21-hydroxylase activity. Fig. 1 shows the expression in individual clones, isolated after transfection with phc21 (lanes 1-6) or with the I172N mutant plasmid (lanes 8-13). Although there are different amounts of expression in the randomly selected clones, expression of active enzyme is clear in all but one (lane 5) of the wild-type transfectants. Likewise only one of the I172N mutants (lane 10) did not show partial enzymatic activity. The I172N mutation of P450c21 is causally associated with steroid 21-hydroxylase deficiency (6,10) and its activity is about 4% of the wild-type enzyme

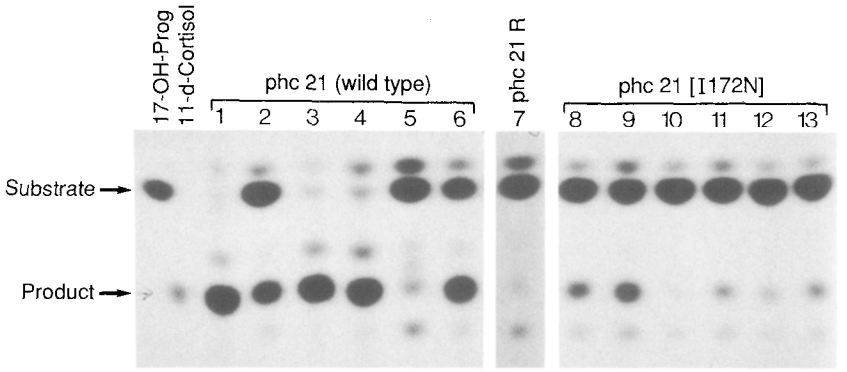

Fig. 1. Assay of 21-hydroxylase activity in G418-selected Rat-1 cells. Cells were transfected with normal phc21 (lanes 1-6), phc21R (a control plasmid in which the P450c21 cDNA is present in reverse orientation, lane 7), or mutated phc21 (I172N, lanes 8-13). Near-confluent clones in a 24-well dish were incubated with [^{14}C]-17-hydroxyprogesterone in 1 ml medium for 20 hours, after which the medium was extracted and analyzed.

in COS-1 cells (8,10). No enzymatic activity was detected from the clone transfected by the plasmid phc21R where P450c21 cDNA was cloned in the reverse orientation from the promoter (lane 7). The V281L and R356T mutant clones were also selected and found to exhibit reduced activity in Rat-1 cells (data not shown), in agreement with studies using transient expression in COS-1 cells (10,17). More than 80% of the clones assayed at the primary selection level exhibited clearly detectable 21-hydroxylase activity.

Cells from the total G418-selected population were passaged to 100 and 250 nM methotrexate. Smaller increments in methotrexate concentration appears to be preferable to obtain higher overall levels of amplification (data not shown). Fig. 2A shows immunoblot analysis of P450c21 obtained from a clonal cell line isolated after G418 selection (Lane 4) and the other from the total G418-resistant population directly selected in 250 nM methotrexate (lane 5). There is higher production of P450c21 (lane 5) when cells were selected after methotrexate amplification. When 21-hydroxylase activity from these two clones were compared (Fig. 2B), the conversion of substrate to product was 15%, 66% and 92% for the "non-amplified" clone and 54%, 94% and 97% for the 250 nM methotrexate-selected clone at 15, 60 and 120 minute time points, respectively. This level of expression is much higher than that achieved using the total population of G418-resistant cells, demonstrating that a high level of active P450c21 expression has been achieved after methotrexate selection and cloning.

In summary, we have produced P450c21 in cell lines cotransfected with P450c21 cDNA and amplifiable markers. Selection in 250 nM methotrexate enables

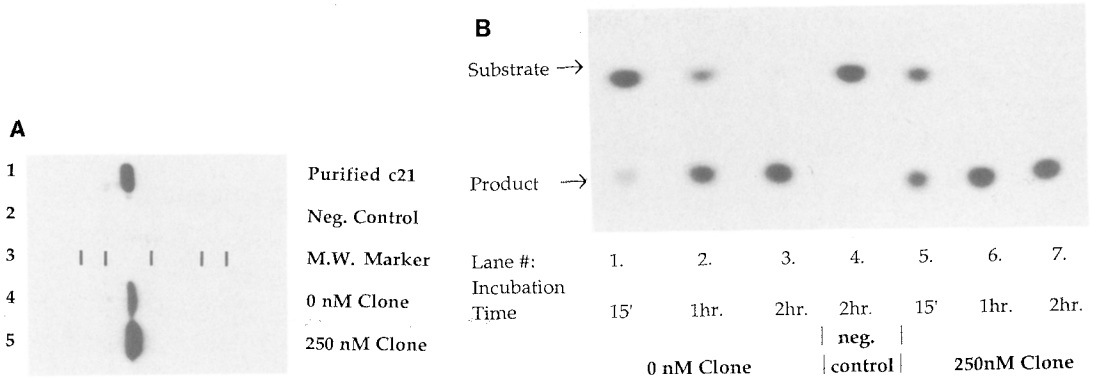

Fig. 2. A: Immunoblot analysis of P450c21 protein from clonal cell lines isolated at the primary selection level (0 nM clone, lane 4) and at 250 nM methotrexate (250 nM clone, lane 5). In lane 1, purified P450c21 expressed bacterially was loaded on the gel. Negative control (lane 2 in Panel A and lane 4 in Panel B) refers to cells transfected with only neomycin resistance gene and pFD11 without P450c21 plasmid and selected with 250 nM methotrexate. Lane 3 is protein size markers with the following sizes: 97.4, 69, 46, 30, 14.3 kDa. B: Autoradiograph showing conversion of 17-hydroxy-progesterone by the two clones used in (A).

the amplification of P450c21 production. This scheme should be useful in the production of not only P450c21 but also other proteins of mammalian origin.

ACKNOWLEDGMENTS

We thank Dr. Lih-Hwa Hwang (Development Center for Biotechnology, Taiwan) and Genentech Inc. (California, U.S.A.) for the gifts of pCMV-3, Rat-1 cells and pFD11. This work was supported by National Science Council (NSC #79-0412-B001-16 and #NSC-79-0412-B001-19) and Academia Sinica, Republic of China.

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