Differential regulation of 3β-hydroxysteroid dehydrogenase type II and 17α-hydroxylase/lyase P450 in human adrenocortical carcinoma cells by epidermal growth factor and basic fibroblast growth factor

J Doi, H Takemori, M Ohta1, Y Nonaka2 and M Okamoto

Department of Molecular Physiological Chemistry, Osaka University Medical School, Suita, Osaka, Japan

1Laboratory of Nutrition, Koshien College, Nishinomiya, Hyogo, Japan

2College of Nutrition, Koshien University, Takarazuka, Hyogo, Japan

(Requests for offprints should be addressed to M Okamoto, Department of Molecular Physiological Chemistry, Osaka University Medical School, 2–2 Yamadaoka, Suita, Osaka, 565–0871, Japan; Email: mokamoto@mr-mbio.med.osaka-u.ac.jp)

Abstract

Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) are pluripotent growth factors that stimulate both the proliferation and steroidogenesis of adrenocortical cells. Here we demonstrate that EGF and bFGF specifically induce mRNA of 3β-hydroxysteroid dehydrogenase type II (3βHSD II) and suppress that of 17α-hydroxylase/lyase P450 (CYP17) in human adrenocortical H295R cells. The induction of 3βHSD II mRNA did not occur until 6 h after the growth factor treatment and was completely abolished in the presence of a protein synthesis inhibitor, cycloheximide (CHX), suggesting that the induction required de novo protein synthesis. The CYP17 mRNA suppression began at almost the same time as the induction of the 3βHSD II mRNA. Interestingly, the CYP17 mRNA level was increased by the CHX treatment. Both the 3βHSD II and CYP17 mRNAs were repressed by treatment with a calmodulin kinase II (CaMK II) inhibitor, KN-93, and were enhanced by a mitogen-activated protein kinase (MAPK) inhibitor, PD98059. The PD98059-mediated induction of the 3βHSD II mRNA was completely blocked by the CHX treatment. Interestingly, treatment with EGF in the presence of both PD98059 and CHX produced a greater increase in the CYP17 mRNA than did treatment in the presence of PD98059 alone. These results suggest that CHX-sensitive factor(s) and CaMK II- and MAPK-signaling pathways may have important roles in both induction of 3βHSD II and suppression of CYP17 by EGF or bFGF in H295R cells.


Introduction

Numerous investigators have reported that epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) influence steroidogenesis in adrenocortical cells (Gospodarowicz & Handley 1975, 1986, McAllister & Hornsby 1987a, Fisher & Lakshmanan 1990, Mesiano et al. 1993, Mesiano & Jaffe 1997). Singh and coworkers reported that EGF stimulated cortisol secretion from cultured bovine (Singh & Waters 1983) and sheep (Singh et al. 1985) adrenal cortical cells. The stimulation was abolished when inhibitors of cholesterol biosynthesis, compactin and AY9944, were added. Further studies showed that the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and the rate of cholesterol synthesis were increased in the EGF-treated cells. In contrast, the rates of conversion of radioactive pregnenolone to steroid intermediates were little influenced by the growth factor. On the basis of these results, they concluded that EGF activated the biosynthesis of the steroid hormone precursor, rather than the steroidogenic enzymes themselves.

bFGF has been suggested as a physiological regulator of adrenocortical functions both in vivo (Mesiano et al. 1991, Basile & Holzwarth 1994) and in vitro (Crickard et al. 1981, Hornsby et al. 1983, Hotta & Baird 1986, Ho & Vinson 1995). It was purified from extracts of bovine adrenals, and a large amount was detected in the zona fasciculata and the medulla (Basile & Holzwarth 1993). It was also detected immunochemically and by northern blot analysis in bovine (Gospodarowicz et al. 1986), rat (Basile & Holzwarth 1993) and human (Mesiano et al. 1991) adrenal cortex. Basile & Holzwarth (1993) observed that, in a unilaterally adrenalectomized rat, the remaining adrenal gland underwent compensatory hypertrophy, and they suggested that bFGF might be involved in this phenomenon. Recently, Thomas et al. (1997) showed that transplantation of bovine adrenocortical cells with
bFGF-overproducing mouse fibroblasts into immuno-
deficient mice resulted in both cell proliferation and
activation of steroidogenesis of the transplants. Receptors
for bFGF were also shown to exist in the rat zona
glomerulosa (Basile & Holzwarth 1994). These previous
reports strongly suggest that EGF and bFGF act as para-
crine effectors in the adrenal cortex and may modulate
steroidogenesis in adrenocortical cells.

H295R cells were originally isolated from human
adrenal carcinoma cells (Gazdar et al. 1990, Rainey
et al. 1994). The cells were reported to possess five
steroidogenic P450s – namely, side-chain cleavage P450
(CYP11A), 17α-hydroxylase/lyase P450 (CYP17), 21-
hydroxylase P450 (CYP21), 11β-hydroxylase P450
(CYP11B1) and aldosterone synthase P450 (CYP11B2) –
and 3β-hydroxysteroid dehydrogenase type II (3βHSD II)
(Bird et al. 1996). Because of this, these cells have often
been used for studying the molecular mechanism under-
lying human adrenocortical steroidogenesis. In the present
study, we investigated the effects of EGF and bFGF on
gene expression of steroidogenic enzymes in H295R
cells.

Materials and Methods

Cell culture and treatment with EGF and bFGF

H295R cells were generous gifts from J Ian Mason at
University of Edinburgh, UK. The cells were maintained
in Dulbecco’s Modified Eagle’s/Ham’s F-12 medium
(DMEM/F-12; Gibco BRL, Grand Island, NY, USA)
containing 1% ITS plus (insulin/transferrin/selenium/linoleic
acid; Becton Dickinson Labware, Lincoln Park, NJ, USA), 2% Ulitroser G (BioSepra Inc., France) and
antibiotics, at 37 °C under an atmosphere of 5% CO2–95%
air, as described before (Bird et al. 1996). The cells
(5 × 105) were cultured in 100 mm dishes for 48 h and
then incubated for further 24 h in serum-free medium
(DMEM/F-12 containing antibiotics and 0.01% BSA). To
initiate the experiments, the medium was exchanged for
one containing EGF (Austral Biochemicals, San Ramon,
CA, USA) or bFGF (Austral Biochemicals) at the final
concentration of 10 ng/ml. After the 24 h incubation, the
cells were washed with PBS, and the total RNA was
extracted. Protein kinase inhibitors (bisindolylmaleimide I,
H-8, H-89, PD98059 and KN-93) and a protein synthesis
inhibitor (cycloheximide; CHX) were purchased from
Calbiochem Ltd (La Jolla, CA, USA) and Sigma, respect-
ively, dissolved in dimethylsulfoxide, and stored at
−20 °C until required for use. Cells that had been
maintained in the serum-free medium for 24 h, were first
incubated with or without bisindolylmaleimide I, H-8,
H-89, PD98059 or KN-93 for 1 h, and then stimulated
with EGF or bFGF dissolved in the freshly prepared
inhibitor-containing medium.

RNA extraction

Isolation of the total RNA was performed according to the
method of Chomczynski & Sacchi (1987) with some
modifications (Takemori et al. 1997). Briefly, the cells
were washed with 1 ml PBS, and then dissolved in 1·6 ml
guanidine isothiocyanate solution (Trizol reagent; Gibco
BRL). The cell lysates were transferred into micro-
centrifuge tubes and mixed with 0·4 ml chloroform. After
the centrifugation, an aqueous phase was taken, to which
ethanol was added to precipitate total RNAs. The RNAs
were washed with 70% ethanol, dried and dissolved in
200 µl distilled water. DNA contaminant in the RNA
preparation was digested by 1 U DNase I (Gibco BRL)
using the buffer supplied by the manufacturer. The RNAs
were again extracted by phenol–chloroform (1:1) and
reprecipitated by ethanol.

Northern blot analysis

To prepare a cDNA probe for 3βHSD II, a DNA
fragment of the exon 3 was amplified by PCR using
primers shown in Table 1 and ligated into the Smal site of
pUC18. cDNA fragments of CYP11A and CYP21 were
also prepared, using RT-PCR with sets of primers listed
in Table 1, and introduced into pH7R (Novagen,
Madison, WI, USA). A NdeI–HindIII cDNA fragment of
CYP17 was excised from pCW (Katagiri et al. 1995) that
had been kindly provided by M Katagiri at Osaka Kyokai
University, Japan. We utilized a rat glycolaldehyde–3-
phosphate dehydrogenase (G3PDH) cDNA fragment
(Halder et al. 1998) as the control probe. Total RNAs
(5 µg) were separated by electrophoresis in 1% agarose gel
in the presence of 6% formaldehyde and then transferred
onto nylon membrane (Hybond-N+; Amersham,
Arlington Heights, IL, USA) by capillary action in
20 × SSPE (0·2 M phosphate buffer, pH 7·7, containing
3·6 M NaCl and 0·02 M EDTA). The RNA on the
membrane was hybridized with [32P]-labeled DNA frag-
ments in 5 × SSPE containing 50% formamide, 0·5% SDS,
0·1% polyvinylpyrrolidone, 0·1% Ficoll and 0·1% BSA at
42 °C for 16 h. After the hybridization, the membrane was
washed sequentially in 2 × SSPE/0·1% SDS, 1 × SSPE/
0·1% SDS, and 0·2 × SSPE/0·1% SDS at 60 °C, and then
exposed to X-ray film at −80 °C with an intensifying
screen. The radioactivity in hybridized signals was
quantified with a phosphoimager (BAS2000; Fuji-film,
Tokyo, Japan).

Determination of mRNAs for CYP11B1 and CYP11B2 by
RT-PCR followed by Southern blot analysis

Total RNAs (2 µg) were reverse-transcribed by 200 U
reverse transcriptase (Super Script II; Gibco BRL) in 20 µl
reaction mixture containing 150 ng random hexamer. An
aliquot (5 µl) of the reaction mixture was used for PCR

amplification with 20 pmol primer sets (common primer) shown in Table 1 in 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. The amplified cDNA fragments were separated in 1-5% agarose gel and transferred onto a nylon membrane. To distinguish the cDNA of CYP11B1 from that of CYP11B2, the membrane was subjected to Southern blot analyses using \(^{[32P]}\)-labeled oligonucleotide probes specific to either the type I or the type II isoform of \(3\beta\)HSD revealed that only the type II cDNA was clearly amplified from the total RNAs extracted from H295R cells (Fig. 1D).

**Time courses of \(3\beta\)HSD and CYP17 mRNA expression**

Time courses of the EGF- and bFGF-mediated induction of \(3\beta\)HSD mRNA and suppression of CYP17 mRNA were examined (Fig. 2). A distinct increase in \(3\beta\)HSD mRNA was seen after the 6 h incubation with EGF, its level becoming maximal after 12 h and gradually diminishing thereafter. When bFGF instead of EGF was used as the stimulator, the mRNA level continued to increase after 12 h. In contrast, EGF or bFGF decreased the level of CYP17 mRNA, which reached the lowest point after 24 h. There was no significant change in the cell number under these growth factor treatments.

**Effect of various protein kinase inhibitors on EGF- and bFGF-mediated gene expression**

Numerous investigators have reported that protein kinases are involved in the signal transduction system of \(3\beta\)HSD and CYP17 gene expression (McAllister & Hornsby 1990, Chris et al. 1990, Bird et al. 1996). Therefore, several protein inhibitors were tested to explore the signaling pathway occurring in the EGF- and bFGF-treated H295R cells. As shown in Fig. 3, protein kinase A inhibitors, H–8 (15 µM) and H–89 (20 µM), or a protein kinase C (PKC) inhibitor, bisindolylmaleimide I (10 µM), seemed to have no effect on the levels of \(3\beta\)HSD and CYP17 mRNAs in both EGF- and bFGF-treated cells. A mitogen-activated protein kinase (MAPK) inhibitor, PD98059 (20 µM), in contrast, further increased the growth factor–dependently increased \(3\beta\)HSD mRNA level. PD98059 appeared to restore the growth factor–mediated suppression of CYP17 mRNA, but had little effect on the basal levels of expression of both genes (data not shown).

### Table 1 Sequence of oligonucleotides used in the experiments

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Forward primers ((5'\rightarrow3'))</th>
<th>Reverse primers ((5'\rightarrow3'))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(3\beta)HSD type II</td>
<td>GCA CAT GGA TCT GTG GTT GCA G‡</td>
<td>GAC CTG GCC TTG TGC CCC TGT TGC C‡</td>
</tr>
<tr>
<td>(3\beta)HSD type I-specific</td>
<td>TGG TCC GCC TGT TGG TGG AA‡</td>
<td>CTA CCT CTA TCC TAC TGG TGT AG‡</td>
</tr>
<tr>
<td>(3\beta)HSD type II-specific</td>
<td>TCA TCC GCC TCT TGG TGA AG‡</td>
<td>CAG CAG TAC TGG GAG CAT GTA GT†</td>
</tr>
<tr>
<td>CYP11A</td>
<td>TGC CTT TIG AGT CCA TCA CT†</td>
<td>ATG TTC AAG GCC GTT CTT GT‡</td>
</tr>
<tr>
<td>CYP21</td>
<td>CTC AGC TGC TTC CAT CAG TCC†</td>
<td>CCA ATG TCG CTT GTG CTT GT‡</td>
</tr>
<tr>
<td>Common to CYP11B and CYP11B2</td>
<td>CAC AA CC GCC GTT CTT GT‡</td>
<td>CCC AAC GCT GTG CA§</td>
</tr>
<tr>
<td>CYP11B1-specific</td>
<td>CCC AAC GCT GTG CA§</td>
<td>CCC AAG GCC GTG CA§</td>
</tr>
<tr>
<td>CYP11B2-specific</td>
<td>CCC AAC GCT GTG CA§</td>
<td>CCC AAG GCC GTG CA§</td>
</tr>
</tbody>
</table>

\(^{†}\)Primers to generate cDNA probes for northern blot analysis.

\(^{‡}\)Primers for RT-PCR analysis.

\(^{§}\)Oligonucleotide probes for Southern blot analysis after RT-PCR.
Next, the inhibitor of Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMK II), KN-93, was tested. KN-93 (5 µM) markedly decreased the level of 3βHSD mRNA in the EGF- and bFGF-treated cells. It exerted little effect on the basal mRNA expression in the non-treated cells (data not shown).

**Figure 1** Effect of EGF and bFGF on mRNA expression of steroidogenic enzymes. (A) Total RNAs (10 µg) were prepared from H295R cells that had been incubated without or with EGF (10 ng/ml) and bFGF (10 ng/ml) for 24 h. mRNAs of 3βHSD, CYP17, CYP11A and CYP21 were subjected to northern blot analyses. mRNA of G3PDH was visualized as the internal standard. (B) The RNAs were reverse-transcribed and the cDNA fragments amplified by PCR using primers hybridizable with both CYP11B1 and CYP11B2 cDNAs. The PCR products were then subjected to Southern blot analyses using oligonucleotide probe specific to the respective enzymes. Plasmid DNAs inserted with CYP11B1 or CYP11B2 cDNA fragments (two lanes on the left) were used for the positive controls of the specific hybridization. Each blot (A and B) is representative of three independent experiments. (C) The levels of mRNAs of 3βHSD, CYP17, CYP11A and CYP21 (in A) and CYP11B1 and CYP11B2 (in B) were determined by a phosphoimager (BAS 2000) after northern blot analysis and Southern blot analysis coupled with RT-PCR, respectively. Each mRNA level was first normalized to the level of G3PDH mRNA and then expressed as the fold change (means ± SEM; n = 3). (D) The cDNA fragments were amplified with 3βHSD type I- or type II-specific primers. The DNA fragments were separated on an agarose gel, and visualized by ethidium bromide staining. Total RNAs of placenta were used as the positive control for 3βHSD type I and type II mRNAs. cont, Control; M, molecular markers.

**Figure 2** Time course of 3βHSD and CYP17 mRNA expression stimulated by growth factors. Total RNAs (10 µg) were isolated from H295R cells at the indicated times after the addition of EGF (10 ng/ml: A, C) and bFGF (10 ng/ml: B, D). The levels of mRNA of 3βHSD (A, B) and CYP17 (C, D) were determined by a phosphoimager (BAS 2000) after northern blot analysis, and then normalized by those of G3PDH. Each point represents a mean value of duplicate experiments.
shown). Another CaMK II inhibitor, KN-62 (10 µM), or a calmodulin antagonist, W-7 (30 µM), also inhibited the 3βHSD expression data (not shown). It should be noted that KN-93 further decreased the growth-factor-dependent repressed mRNA level of CYP17.

**Effect of CHX on EGF- and bFGF-mediated gene expression**

Because the induction of 3βHSD and suppression of CYP17 appeared to be late events of growth factor treatment, we examined whether de novo protein biosynthesis was required for this phenomenon. An inhibitor of protein synthesis, CHX (10 µg/ml), was added to the culture medium together with EGF and bFGF. As shown in Fig. 4A, CHX completely suppressed the EGF- and bFGF-mediated induction of 3βHSD mRNA, suggesting that de novo protein synthesis is required for the induction of 3βHSD mRNA. The CHX treatment also completely abolished the PD98059-mediated enhancement of 3βHSD mRNA in EGF-stimulated H295R cells (Fig. 4B). To our surprise, CHX seemed to prevent the EGF- and bFGF-mediated repression of CYP17 mRNA. The treatment with EGF in the presence of both PD98059 and CHX produced a more increased level of CYP17 mRNA than did treatment in the presence of PD98059 alone (Fig. 4B). Similar results were obtained by treatment with the two inhibitors in the bFGF-stimulated cells (data not shown).

**Discussion**

Previous studies of bovine (Singh & Waters 1983) and sheep (Singh et al. 1985) adrenal cortical cells demonstrated that the addition of EGF to culture media resulted in stimulated cortisol secretion from and cholesterol synthesis in the cells, without significant effect on the production rate of steroid intermediates. Abolition of the EGF-mediated stimulation of cortisol secretion by inhibitors of cholesterol synthesis led to the idea that HMG-CoA...
EGF and bFGF induce mRNA of 3βHSD II and suppress that of CYP17


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reductase, the rate-limiting enzyme of cholesterol synthesis, may be the major target of EGF action in the cells. The presence of EGF receptor has been reported in human normal, tumor (Sasano et al. 1994), and fetal (Smikle et al. 1996) adrenocortical cells. Coulter et al. (1996b) reported that the administration of EGF increased the volume of the definitive zone of fetal adrenals of rhesus monkeys in late gestation. This zone-specific increase seemed to be the result of cellular hypertrophy, not of cell proliferation. It was also found that EGF treatment induced 3βHSD II protein in the definitive and transitional zones without significant effect on CYP17. Similar induction of 3βHSD II occurred in fetal rhesus monkeys in which endogenous adrenocorticotropic hormone (ACTH) secretion was stimulated by administration of an inhibitor of CYP11B1, metyrapone (Coulter et al. 1996a). Coulter and colleagues were unable to determine whether the effect on adrenocortical function that was produced by the EGF administered to the animals had occurred as the result of the activation of the hypothalamic–pituitary–adrenal axis, as described by Lugr et al. (1988) and Polk et al. (1987), or as the result of the direct action of EGF on adrenocortical cells. Our present study with H295R cells, however, demonstrated that treatment of the cells with EGF or bFGF stimulated transcription of the 3βHSD II gene in the absence of ACTH or cAMP, the second messenger of ACTH.

Interestingly, the EGF or bFGF treatments resulted in a decrease in CYP17 mRNA levels in H295R cells. Welsh & Hsueh (1982) also reported that gonadotropin-stimulated production of testosterone, androstenedione, and 17α-hydroxypregesterone in cultured testicular cells was inhibited by EGF. Because EGF also inhibited the conversion of oxenous 17α-hydroxypregesterone to androstenedione, they proposed that the EGF effect occurred via inhibition of 17α-hydroxylase and 17,20-lyase. These results suggested that EGF and bFGF could directly modulate the gene expression of steroidogenic enzymes in steroidogenic cells.

Hornsby et al. (1983) reported that bFGF acted as a mitogen in human fetal adrenal cortex, and that the mitogenic effect of bFGF was greater in the definitive zone than in the fetal zone. The mRNA of bFGF in cultured human adrenocortical cells was increased by about threefold by the addition of ACTH. These results suggest that bFGF may be one of the autocrine factors of adrenocortical cells, in which secretion is regulated by ACTH. Li et al. (1998) reported that bFGF treatment increased the level of CYP17 mRNA in cultured bovine fasciculata cells. Our results, in contrast, showed that both bFGF and EGF decreased the level of CYP17 mRNA in H295R cells. This discrepancy may have resulted from the species difference or from the difference in the zonal origin of the cells.

Membrane-associated receptor tyrosine kinases such as EGF/TGFα receptor and bFGF receptor are believed to stimulate several protein-kinase-mediated signaling pathways, such as those involving protein kinase C (PKC) and MAPK (Kim & Muller 1999, Rowan et al. 2000). Several investigators (Leers et al. 1997, Bird et al. 1998) have demonstrated that a PKC agonist, phorbol-12-myristate-13-acetate (PMA), increased 3βHSD II mRNA expression. MAPK was immunochemically detected in the rat zona glomerulosa, and MAPK seemed to be involved in gene expression of 3βHSD II, and thus activate the production of aldosterone (McNeil et al. 1998). These findings indicate that PKC- and MAPK-mediated signaling pathways may be involved in regulating 3βHSD II gene expression, suggesting the possibility that the effect of EGF and bFGF on 3βHSD II gene expression described here could be mediated via these signaling systems. The PKC inhibitor, bisindolylmaleimide I, however, did not block the EGF- or bFGF-mediated induction of 3βHSD II mRNA in a concentration of 10 µM – a concentration sufficient to block the PMA-mediated induction of 3βHSD II mRNA (data not shown). Moreover, the level of 3βHSD II mRNA was further increased, not inhibited, by the addition of the MAPK inhibitor PD98059 in the presence of EGF or bFGF. These results suggest that a signaling system mediated by a member(s) other than PKC or MAPK may be important for the EGF- and bFGF-stimulated induction of 3βHSD II mRNA. A possible candidate signal would be CaMK II, because KN-93, an inhibitor of CaMK II, markedly inhibited the EGF- and bFGF-stimulated induction of 3βHSD II mRNA. However, this inhibitor also inhibited the expression of CYP17 mRNA. This indicates that the CaMK II-mediated pathway may be essential for the gene expression of 3βHSD II, but this signaling system could not account for the repressive effects of EGF and bFGF on the expression of the CYP17 gene.

It took several hours for EGF or bFGF to increase the 3βHSD II mRNA level in H295R cells (Fig. 2). As in the PKC-mediated induction of 3βHSD II mRNA (Leers et al. 1997), CHX blocked the effect of EGF and bFGF on 3βHSD II mRNA expression (Fig. 4), indicating that de novo protein synthesis is required for the EGF- and bFGF-mediated induction of 3βHSD II gene expression. In contrast, EGF or bFGF decreased the level of CYP17 mRNA in H295R cells (Fig. 1). Interestingly also, the EGF- or bFGF-mediated suppression of CYP17 mRNA was abolished by CHX treatment (Fig. 4). These results may suggest the presence of a CHX-sensitive protein factor(s) that is induced in H295R cells by the growth factor treatment, and which stimulates the expression of the 3βHSD II gene but inhibits that of the CYP17 gene.

Treatment of the cells with EGF in the presence of both CHX and PD98059, however, revealed the complicated involvement of the MAPK signaling pathway in the expression of these genes. The incubation with both CHX and PD98059 resulted in further increase in the level of CYP17 mRNA in EGF-stimulated H295R cells, whereas
the presence of PD98059 seemed not to influence the blocking effect of CHX on 3βHSD II gene expression (Fig. 4B). These results indicate that more than one factor – one sensitive to the CHX-treatment and the other related to the MAPK signaling pathway – is involved in the regulation of 3βHSD II and CYP17 gene expression. In the EGF-mediated activation of the 3βHSD II gene, the CHX-sensitive factor may act more strongly than that involved in the MAPK pathway, because treatment with both CHX and PD98059 completely abolished expression of the gene. In contrast, in the EGF-mediated suppression of the CYP17 gene, the CHX-sensitive factor and the factor involved in the MAPK signaling pathway seemed to repress the gene independently. To date, we have failed in our attempts to search the database of the promoter regions of the 3βHSD II and CYP17 genes for sequences typical of cis elements of transcription factors that may act downstream of either the MAPK or CaMK II-mediated signaling pathway.

Further investigation is in progress in our laboratory to elucidate the precise mechanism underlying the enhancement of 3βHSD II mRNA and suppression of CYP17 mRNA by EGF and bFGF.

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