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Re-expression of C/EBPα induces CYP2B6, CYP2C9 and CYP2D6 genes in HepG2 cells

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Abstract Cytochrome P450 (CYP) activity is very low or even absent in human hepatomas, a phenomenon that is accompanied by low levels of some liver transcription factors, notably C/EBPα. To investigate a possible link between this transcription factor and hepatic CYP expression, we have stably transfected HepG2 cells with a C/EBPα vector containing a Zn-inducible metallothionein promoter. Expression of functional C/EBPα up to liver levels concomitantly increased the mRNAs of several members of the CYP2 family (2B6, 2C9 and 2D6), suggesting that this transcription factor may play a relevant role in controlling the hepatic expression of CYP enzymes.

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Key words: Cytochrome P450 gene regulation; C/EBPα; Human hepatocyte; HepG2 cell

1. Introduction

The cytochromes P450 (CYP) constitute a superfamily of monooxygenases that participate in the metabolism of endogenous substrates and play a key role in the detoxification as well as in the metabolic activation of xenobiotics [1,2]. Constitutive liver CYP expression (and induction by exogenous/endogenous compounds) appears to be regulated mainly at the transcriptional level [3]. However, research into the precise modulation mechanisms has been hampered by the fact that CYP expression is almost absent in hepatoma cells and rapidly declines in primary cultured hepatocytes [4–6].

Studies on gene promoter and enhancer sequences have led to the identification of several liver-specific transcription factors that control the activation of many hepatic genes [7] and may also possibly regulate CYP expression [8]. Human hepatoma HepG2 is a cell line that retains many hepatic biochemical functions [9,10] with the notable exception of cytochrome P450 [4]. Analysis of several hepatocyte-specific transcription factors (HNF-1, HNF-3, HNF-4, C/EBPα) in HepG2 revealed that their levels were lower than in human hepatocytes, especially in the case of C/EBPα. In order to investigate whether this factor could play a role in the control of human CYP transcription, we stably transfected HepG2 cells with a C/EBPα expression vector containing a Zn-inducible metallothionein promoter. Our results demonstrate that the expression of functional C/EBPα in HepG2 cells at levels like those found in hepatocytes results in increased levels of CYP2B6, CYP2C9 and CYP2D6 mRNAs, suggesting a probable role for this transcription factor in the regulation of the constitutive expression of genes of the CYP2 family.

2. Materials and methods

2.1. Cell culture and transfection

HepG2 cells were plated in Ham's F-12/Leibovitz L-15 (1:1, v/v) supplemented with 7% newborn calf serum, 50 U/ml penicillin, 50 mg/ml streptomycin, and cultured to 70% confluence. The plasmid pPC22-C/EBPα (a kind gift of J. Patrick Condreay, Wellcome Research Laboratories, NC) [11] is a 9.0-kb expression vector containing a zinc-inducible, human metallothionein IIA promoter, driving expression of the rat C/EBPα gene (r-C/EBPα). This expression vector was transfected into the human hepatoma HepG2 (ECACC 85011430) using lipofectin (Gibco-BRL) according to the recommendations of the manufacturer. Following transfection, cells were grown in medium supplemented with 900 μg/ml Geneticin (Gibco-BRL), and stable antibiotic-resistant clones were isolated. In parallel, control HepG2 were transfected with the insertless vector. To induce maximal expression of C/EBPα, 100 μM zinc-sulfate was added to the culture medium for 18–20 h.

2.2. Isolation and culture of human hepatocytes

Surgical liver biopsies (1–5 g) were obtained from patients undergoing cholecystectomy after informed consent was obtained. Patients had no known liver pathology nor did they receive medication during the weeks prior to surgery. None of the patients were habitual consumers of alcohol or other drugs. A total of six liver biopsies (2 males and 4 females) were used. Patients' ages ranged from 26 to 71 years. Human hepatocytes were isolated using a two-step perfusion technique [5] and seeded on plates coated with fibronectin (3.6 μg/cm^2) at a density of 8×10^4 cells/cm^2. Culture medium was Ham's F-12/Leibovitz L-15 (1:1, v/v) supplemented with 2% newborn calf serum, 5 mM glucose, 50 U/ml penicillin, 50 mg/ml streptomycin, 0.2% bovine serum albumin and 10^{-8} M insulin. The medium was changed one hour later to remove unattached hepatocytes.

2.3. Extraction of nuclear proteins and Western blot analysis

HepG2 cells were lysed with Nonidet P-40 lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% Nonidet P-40). The cell lysates were then incubated on ice for 15 min, and nuclei were pelleted at $1000\times g$ for 5 min at 4°C. The pellets were washed again with lysis buffer, resuspended in 50 mM Tris-HCl (pH 7.4), 10 mM magnesium acetate, 40% glycerol, and 1 mM dithiothreitol, and stored at −70°C until ready for use. Nuclear extracts were prepared as described [12] and electrophoresed in an SDS-polyacrylamide gel (20 μg proteins/lane). Proteins were transferred to Immobilon membranes (Millipore) and sheets were incubated with rabbit polyclonal antibody raised against a peptide corresponding to amino acids 253–265 of rat C/EBPα (Santa Cruz Biotechnology, Inc.). After washing, blots were developed with horseradish peroxidase-labelled goat anti-rabbit IgG, using an Enhanced Chemiluminescence Kit (Amersham Life Sciences).

2.4. Albumin synthesis in HepG2 cells

The synthesis and secretion of albumin were monitored by means of an ELISA in samples of culture media taken at regular time intervals [13].

2.5. Analysis of mRNA by semiquantitative RT-PCR

Total cellular RNA was extracted with RNeasy Total RNA Kits

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Abbreviations: CYP, cytochrome P450; C/EBP, CCAAT/enhancer-binding protein; r-C/EBPα, rat CCAAT/enhancer-binding protein; HNF, hepatocyte nuclear factor

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(Qiagen) and contaminating genomic DNA was removed by incubation with DNase I Amplification Grade (Gibco-BRL). RNA (1 μg) was reverse transcribed as described [14]. Diluted cDNA (3 μl) was amplified in 40 μl of 20 mM Tris-HCl (pH 8.4) containing 50 mM KCl, 1.5 mM $MgCl_2$, 50 μM of each deoxynucleotide triphosphate, 1 U Taq DNA polymerase (Gibco-BRL) and 0.2 μM of each primer. After denaturing for 4 min at 94°C, amplification was performed by 27–35 cycles of 35 s at 94°C, 45 s at 60°C and 1 min at 72°C, and a final extension of 5 min at 72°C. Appropriate cDNA dilutions and cycles were empirically determined for each mRNA to ensure that the PCR-products would not reach the plateau of the amplification. Under these conditions, the yield of the PCR is proportional to the input cDNA [15]. For quantitative analysis, aliquots of the PCR reaction (15 μl) were mixed with diluted picogreen reagent (1:200 in TE buffer) (Molecular Probes), stained samples were excited at 485 nm, and the emission at 538 nm was read in a micro-plate spectrofluorimeter [16]. Appropriate blank samples containing non-amplified cDNA, primers, and PCR-mix were also included. Aliquots (25 μl) of the PCR reaction were subjected to electrophoresis on 1.5% agarose gel, for size and purity confirmation. Sample-to-sample variations were normalized by analysis of β-actin and/or GPDH from the same cDNA dilution series.

2.6. Oligonucleotides

Oligonucleotides are mentioned in Table 1.

3. Results and discussion

3.1. CYP mRNA levels and expression of liver transcription factors in the human hepatoma HepG2 and in cultured human hepatocytes

The expression of CYP genes in HepG2 cells is very low when compared with human hepatocytes (see as an example CYP2C9, Fig. 1A). This phenomenon could be due to a transcriptional block of CYP genes or to lack of expression of appropriate transactivating factors in the tumor cell line. Analysis of four major hepatocyte-specific transcription factors (HNF-1, HNF-3, HNF-4, C/EBPα) in HepG2 cells showed low levels of C/EBPα mRNA (ca. 15% of human hepatocytes), followed by HNF-3 (25%) and HNF-1 (40%). This contrasts with the measured HNF-4 mRNA which was close to that found in 20-h cultured hepatocytes (Fig. 1A).

A decrease in the mRNA of the above mentioned transcription factors is also observed during the isolation and culture of hepatocytes, a phenomenon that is concomitant with the decrease in CYP mRNAs. Of the various transcription factors investigated, C/EBPα showed the earliest and fastest decay during the initial stages of culture (Fig. 1B).

Recent results in our laboratory showed that culturing hepatocytes in collagen gels, a technique that greatly improves the expression and inducibility of CYPs, resulted in the maintenance of key transcription factors, notably C/EBPα, at the levels found in freshly isolated hepatocytes [17]. This finding together with the data reported here led us to consider C/EBPα a potential transcription factor regulating CYP expression in hepatocytes.

3.2. Stable transfection of HepG2 cells with C/EBPα

To test the previous hypothesis we stably transfected the plasmid pPC22-C/EBPα into HepG2. It has been reported that C/EBPα has a strong antiproliferative role [18,19]. Consequently, high expression levels of this factor in transfected cells could be incompatible with cell growth and make the cloning stage more difficult. To overcome this potential drawback a plasmid containing a zinc-inducible human metallothionein IIA promoter was used [11]. This vector ensures low basal expression of C/EBPα and cell growth in the absence of the cation, but promotes high expression of the transgene when the culture medium is supplemented with Zn^{2+}. Upon lipofectin transfection the antibiotic-resistant clones were screened by Western blot analysis of nuclear extracted proteins.

As shown in Fig. 2A, a strong antibody-stained p42-C/EBPα protein was observed in transfected HepG2 cells upon stimulation with 100 μM $ZnSO_4$, but not in the nuclear extracts of cells transfected with an insertless vector. The level of the expressed protein was similar to that found in freshly isolated hepatocytes. A second protein band of ca. 30 kDa was also recognized in nuclear extracts of hepatocytes, which evidences the presence of the two translation products derived from the endogenous C/EBPα mRNA (p42 and p30 proteins) [20].

To verify that the expressed factor was functional, its effects on albumin, a well characterized target gene [21], were examined. Addition of Zn^{2+} to culture media of C/EBPα-expressing cells resulted in a significant increase in albumin synthesis (Fig. 2B), which reached values similar to those found in 24-h cultured human hepatocytes (18.5 ± 2.56 ng albumin/min/mg). These results proved that in transfected HepG2 clones re-expression of functional C/EBPα up to a level sufficient to transactivate an endogenous hepatic-specific gene was possible.

3.3. Human CYP mRNA levels in C/EBPα-expressing cells

To determine whether C/EBPα plays a role in CYP expression in hepatic cells, we measured CYP mRNA levels in HepG2 clones stably transfected with C/EBPα. The human cytochrome P450 gene superfamily comprises more than 35 different isoenzymes grouped in 14 families [22]. In the present

Table 1

Gene	Accession	5′ nt	Forward primer	5′ nt	Reverse primer
CYP2B6	M29874	1256	atg ggg cac tga aaa aga ctg a	1538	aga ggc ggg gac act gaa tga c
CYP2C9	M61855	1285	ttc atg cct ttc tca gca gg	1668	ttg cac agt gaa aca tag ga
CYP2D6	X08006	1169	cta agg gaa cga cac tca tca c	1457	ctc acc agg aaa gca aag aca c
CYP2E1	J02625	913	aca gag acc acc agc aca act	1492	atg agc ggg gaa tga cac aga
CYP3A4	M14096	1353	cct tac aca tac aca ccc ttt gga agt	1734	agc tca atg cat gta cag aat ccc cgg tta
CYP3A5	J04813	855	gaa gaa aag tcg cct caa c	1533	aag aag tcc ttg cgt gtc ta
C/EBPα	U34070	1513	gtg gag acg cag cag aag	1962	ttc caa ggc aca agg tta tc
HNF-1(α)	M57732	1223	cca gaa cct cat cat ggc ctc act	1550	cac ctc ggg ctt gtg gct gta gag
HNF-3α	U39840	1571	cag caa aca aaa cca cac aaa	1859	taa ata acc ctc cac aaa cta
HNF-4	X87871	916	gcc tac ctc aaa gcc atc at	1190	gac cct ccc agc agc atc tc
G6PDH	X03674	1293	aag ccc gcc tcc acc aac tca	1528	ggc acc cca tcc cac ctc tca t
β-Actin	X00351	480	cgt acc act ggc atc gtg at	931	gtg ttg gcg tac agg tct ttg
r-C/EBPα	X12752	679	ccc gtg ccc agc cct cat	942	cac ctt ctg ctg cgt ctc cac

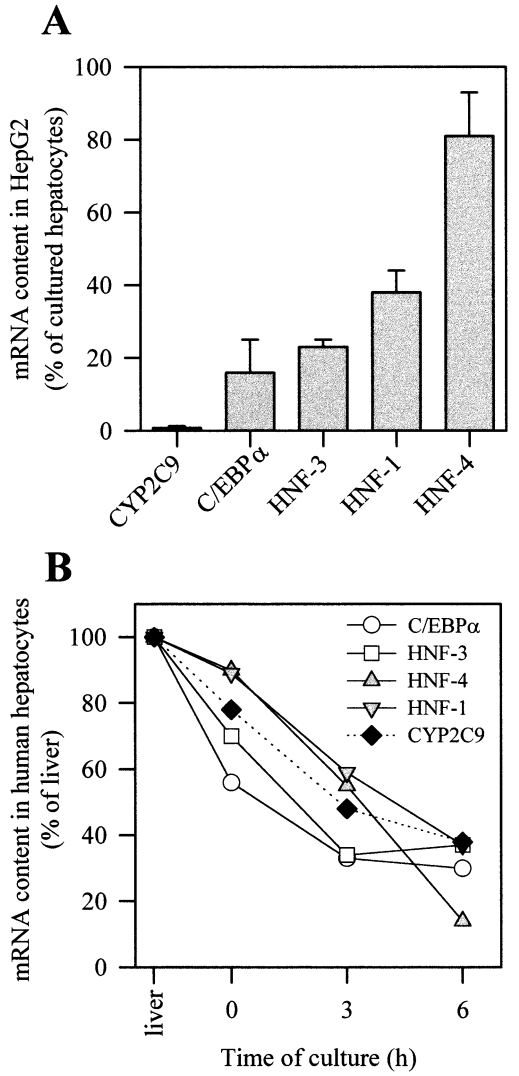

Fig. 1. Liver transcription factors and CYP2C9 mRNA in the human hepatoma HepG2 (A) and in human hepatocytes (B). Total RNA was purified from HepG2 cells, liver biopsy, isolated (0 h) and cultured hepatocytes (3–6 h), and specific mRNA levels were measured by semi-quantitative RT-PCR. Sample-to-sample variations were normalized by comparative analysis of β-actin. Bars represent mean ± S.D. ($n = 3$) (A), and symbols represent averaged results from 3–4 independent experiments (B).

study we focused on six P450 isoenzymes belonging to the CYP2 and CYP3 families, which encompass the most relevant xenobiotic-metabolizing P450s in man [23]. Upon activating the expression of C/EBPα with Zn^{2+}, the mRNA levels of CYP2B6, 2C9 and 2D6 clearly increased (ca. 5.5-, 12.8- and 2.2-fold, respectively, relative to the 'housekeeping' GPDH mRNA; averaged results from 3–4 independent experiments). We did not observe significant changes in the levels of CYP2E1 and 3A4/5 mRNA (Fig. 3).

The increased expression of these CYP genes upon expression of ectopic C/EBPα mRNA indicates that this transcription factor must play a relevant role. The experimental evidence reported in our paper certainly does not prove the direct effect of C/EBPα on the promoter and enhancer sequences of these CYP genes, but some indirect evidence points to the feasibility of a direct transactivation mechanism. Computer sequence analysis of the 5′-flanking regions of the human CYP2C9 and CYP2D6 genes (the CYP2B6 gene has not been cloned yet) has revealed several consensus binding sites for C/EBP proteins [24,25]. In addition, transient transfection experiments have shown that C/EBPα could have a direct activating effect on rat CYP2B [26] and CYP2C [27] promoters, but not on the rat CYP2E1 promoter [28]. Finally, the mRNA levels of human HNF-1, HNF-3 and HNF-4 were not altered in the C/EBPα expressing clones (data not shown). This rules out the possibility of cross-activation among C/EBPα and the above mentioned liver-enriched transcription factors, which could indirectly affect CYP gene transcription. Rather, it suggests that C/EBPα is probably the regulator that transactivates CYP2B6, CYP2C9 and CYP2D6 genes. This study also shows for the first time that a simultaneous reactivation of several CYP genes can be elicited by re-expressing a missing transcription factor in human hepatoma cells.

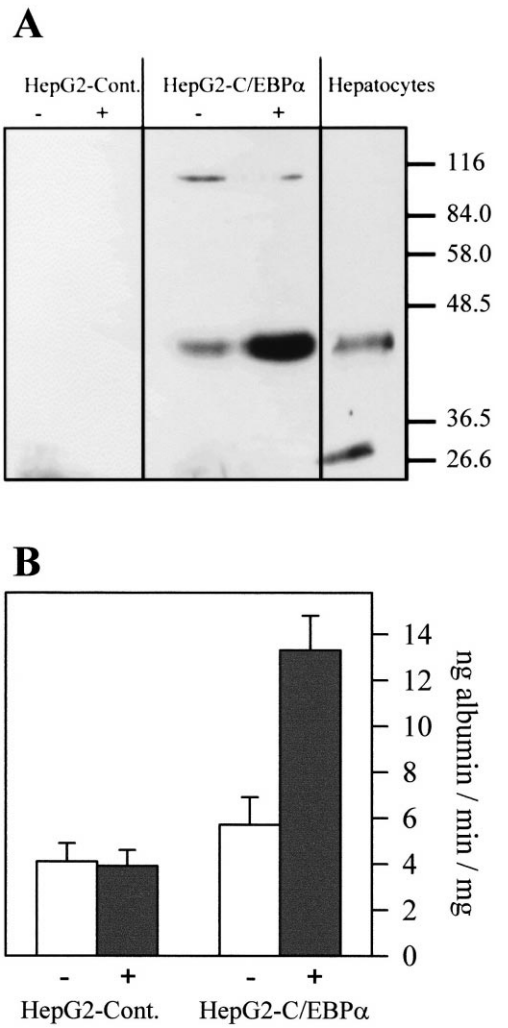

Fig. 2. Stable transfection of functional C/EBPα in HepG2. Hepatoma cells were transfected with the plasmid pPC22-C/EBPα (HepG2-C/EBPα) or the insertless vector (HepG2-Control). G418-resistant clones were exposed (+) or not (−) to 100 μM $ZnSO_4$ for 18 h. A: Western blot analysis of nuclear extracts (20 μg) from representative clones (lanes 1–4) and freshly isolated hepatocytes (lane 5) stained with rabbit anti-C/EBPα antibody. B: ELISA quantification of albumin synthesis in control and C/EBPα expressing cells. Bars represent mean ± S.D. ($n = 4$).

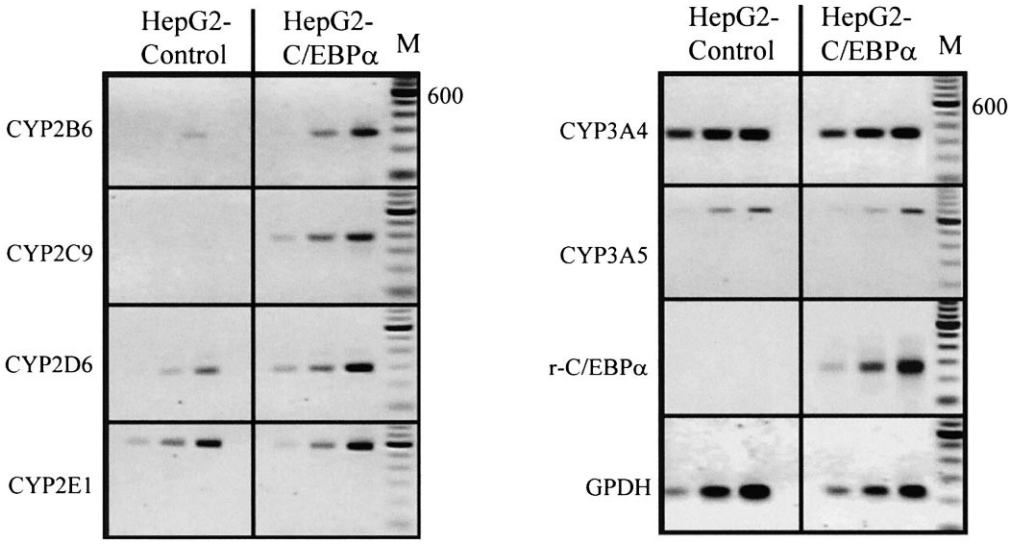

Fig. 3. RT-PCR analysis of CYP mRNAs in C/EBPα expressing cells. cDNAs from transfected HepG2 cells incubated with 100 μM $ZnSO_4$ for 18 h were serially diluted and amplified with specific primers. After gel electrophoresis of the PCR products and staining with ethidium bromide, the fluorescent bands were recorded with a video camera. M: 100-bp DNA ladder.

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References

[1] Gonzalez, F.J. and Gelboin, H.V. (1994) Drug Metab. Rev. 26, 165–183.
[2] Wrighton, S.A. and Stevens, J.C. (1992) Crit. Rev. Toxicol. 22, 1–21.
[3] Shaw, P.M. (1994) in: Liver Gene Expression (Tronche, F. and Yaniv, M.R.G., Eds.) pp. 87–111, Landes, Austin, TX.
[4] Sassa, S., Sugita, O., Galbraith, R.A. and Kappas, A. (1987) Biochem. Biophys. Res. Commun. 143, 52–57.
[5] Gómez-Lechón, M.J., López, P., Donato, T., Montoya, A., Larrauri, A., Giménez, P., Trullenque, R., Fabra, R. and Castell, J.V. (1990) In Vitro Cell. Dev. Biol. 26, 67–74.
[6] Kocarek, T.A., Schuetz, E.G. and Guzelian, P.S. (1993) Mol. Pharmacol. 43, 328–334.
[7] Cerghini, S. (1996) FASEB J. 10, 267–282.
[8] Gonzalez, F.J. and Lee, Y.H. (1996) FASEB J. 10, 1112–1117.
[9] Knowles, B.B., Howe, C.C. and Aden, D.P. (1980) Science 209, 497–499.
[10] Javitt, N.B. (1990) FASEB J. 4, 161–168.
[11] Watkins, P.J., Condreay, J.P., Huber, B.E., Jacobs, S.J. and Adams, D.J. (1996) Cancer Res. 56, 1063–1067.
[12] Rana, B., Mischoulon, D., Xie, Y., Bucher, N.L. and Farmer, S.R. (1994) Mol. Cell. Biol. 14, 5858–5869.
[13] Castell, J.V., Montoya, A., Larrauri, A., López, P. and Gómez-Lechón, M.J. (1985) Xenobiotica 15, 743–749.
[14] Jover, R., Lindberg, R.L. and Meyer, U.A. (1996) Mol. Pharmacol. 50, 474–481.
[15] Murphy, L.D., Herzog, C.E., Rudick, J.B., Fojo, A.T. and Bates, S.E. (1990) Biochemistry 29, 10351–10356.
[16] Ahn, S.J., Costa, J. and Emanuel, J.R. (1996) Nucleic Acids Res. 24, 2623–2625.
[17] Gómez-Lechón, M.J., Jover, R., Donato, T., Ponsoda, X., Rodriguez, C., Stenzel, K., Klocke, R., Paul, D., Guillén, I., Bort, R. and Castell, J.V. (1998) J. Cell. Physiol., in press.
[18] Mischoulon, D., Rana, B., Bucher, N.L. and Farmer, S.R. (1992) Mol. Cell. Biol. 12, 2553–2560.
[19] Timchenko, N.A., Wilde, M., Nakanishi, M., Smith, J.R. and Darlington, G.J. (1996) Genes Dev. 10, 804–815.
[20] Ossipow, V., Descombes, P. and Schibler, U. (1993) Proc. Natl. Acad. Sci. USA 90, 8219–8223.
[21] Friedman, A.D., Landschulz, W.H. and McKnight, S.L. (1989) Genes Dev. 3, 1314–1322.
[22] Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C. and Nebert, D.W. (1996) Pharmacogenetics 6, 1–42.
[23] Shimada, T., Yamazaki, H., Mimura, M., Inui, Y. and Guengerich, F.P. (1994) J. Pharmacol. Exp. Ther. 270, 414–423.
[24] de Morais, S.M., Schweikl, H., Blaisdell, J. and Goldstein, J.A. (1993) Biochem. Biophys. Res. Commun. 194, 194–201.
[25] Quandt, K., Frech, K., Karas, H., Wingender, E. and Werner, T. (1995) Nucleic Acids Res. 23, 4878–4884.
[26] Luc, P.V., Adesnik, M., Ganguly, S. and Shaw, P.M. (1996) Biochem. Pharmacol. 51, 345–356.
[27] Tollet, P., Lahuna, O., Ahlgren, R., Mode, A. and Gustafsson, J.A. (1995) Mol. Endocrinol. 9, 1771–1781.
[28] Liu, S.Y. and Gonzalez, F.J. (1995) DNA Cell. Biol. 14, 285–293.