Volume 259, number 1, 175-180 FEB 07949 December 1989

Establishment of mouse and rat hepatoma cell clones showing stable expression of rabbit cytochrome P450 IA2

L. Corcos, C. Rechenmann, M.C. Weiss and D. Pompon*

Unité de Génétique de la Différenciation, URA 152 du CNRS, Département de Biologie Moléculaire, Institut Pasteur, 25 rue du Dr Roux, Paris and *Centre de Génétique Moléculaire du CNRS, Laboratoire Propre Associé à l'Université Pierre et Marie Curie, Gif-sur-Yvette, France*

Received 10 October 1989

Cytochrome P450 IA2, a liver-specific member of the 3-methylcholanthrene-inducible family, is never detected in established cell lines. With the aim of isolating cells stably producing this protein, we have used rat and mouse hepatoma cells as recipients in transfection experiments involving rabbit cytochrome P450 IA2 cDNA. We report here the isolation of five hepatoma cell clones expressing functional P450 IA2. The level of expression is comparable to that found in COS cells transiently transformed by other P450 cDNAs. It ranges between 0.4 and 1.6 pmol P450 IA2/mg total cell protein.

Cytochrome P450; cDNA expression vector; Transfection; (Hepatoma cell)

1. INTRODUCTION

Cytochromes P450 constitute a large family of hemoproteins expressed mainly in hepatocytes. They are involved in the metabolism of endogenous substrates and in the first step (Phase I) of the detoxication pathway of xenobiotics [1]. Toxicological studies involving cytochrome P450 would profit from the availability of mammalian cell lines of hepatic origin showing stable expression of one or more P450 enzymes. Such lines should retain activity of the Phase II conjugating enzymes necessary for the final steps of drug metabolism [2]. Rat and mouse hepatoma cells have been shown to express the P450 IA1 isoenzyme of the MC-inducible family [3,4]. While hepatoma cell lines show promise as in vitro models for toxicology, their usefulness would be greatly enhanced by increasing the spectrum of the cytochromes P450 that are expressed.

The second member of the MC-inducible family, cytochrome P450 IA2, is first expressed in rat and mouse liver near birth [5,6]. This enzyme is always absent in hepatomas, and in all liver derived permanent lines, although it may be maintained in short-term non-proliferating hepatocytes [7]. Several cDNAs encoding this isoenzyme have been cloned recently [8,9] and successfully expressed in heterologous yeast expression systems [9,10].

Our goal is to transfer a P450 IA2 encoding cDNA into rat and mouse hepatoma cells that already express P450 IA1 in order to generate cell lines showing expression of both members of the MC-inducible P450 family of enzymes. Therefore, we have transfected these cells with rabbit cytochrome P450 IA2 cDNA [9] in vectors where expression is directed by either the strong SV40 early promoter, or the weaker but inducible MMTV promoter.

Correspondence address: L. Corcos, Unité de Génétique de la Différenciation, URA 152 du CNRS, Département de Biologie Moléculaire, Institut Pasteur, 25 rue du Dr Roux, Paris, France

Abbreviations: SV40, Simian virus 40; MMTV, mouse mammary tumor virus; MC, 3-methylcholanthrene; DEX, dexamethasone; HAT, hypoxanthine aminopterin thymidin; HPRT, hypoxanthine-guanine-phosphoribosyl-transferase; gpt, guanine-phosphoribosyl-transferase; neo, neomycin; 7-EOC, 7-ethoxyresorufin; 7-EOR, 7-ethoxycoumarin; CHP, cumene hydroperoxide

2. MATERIALS AND METHODS

2.1. *Cell lines and cell culture conditions*

H4II rat and BTG9 mouse hepatoma cells have been previously described [11,12]. BTG9 cells carry a thioguanine resistance marker and are thus unable to grow in HAT medium unless a functional HPRT or Eco-gpt enzyme is introduced. Both cell lines were cultivated in modified Ham's-F12 medium as described [13]. Transfection experiments were carried out according to [12]. For neo selections, G418 (Gibco) was added to the culture medium at a final concentration of 600 μg per ml. For DEX inductions, the cells were treated with 2×10^{-6} M DEX for 48 h from the day after transfection.

2.2. *Plasmid vectors*

The P450 expression plasmids were constructed by inserting the (5')*Bam*HI-*Eco*RI(3') restriction fragment of pΔ703/VGAL [9], coding for rabbit P450 IA2, downstream of either the SV40 or the MMTV promoter (fig.1). The P450 IA2 cDNA fragment does not contain any 5' or 3' non-coding sequences. The remainder of the

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

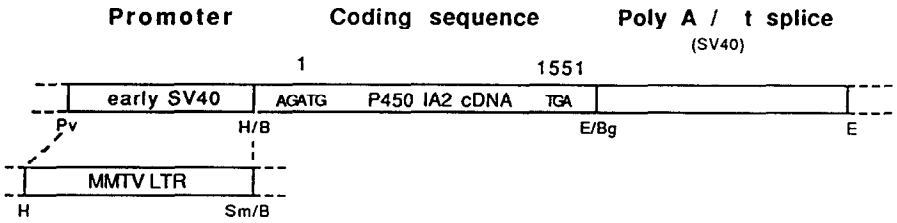

Fig.1. Structure of rabbit cytochrome P450 IA2 expression cassettes. Correspondence for the unique restriction sites indicated is as follows: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; E, *Eco*RI; Pv, *Pvu*II; Sm, *Sma*I. Numbers indicate the positions of the first (1) and last (1551) nucleotide of the P450 IA2 coding sequence.

structure of the P450 expression plasmids is that of pSV2 [14]. pSVIA2 and pMMTVIA2 are 5770 and 6935 base pairs long, respectively.

The pSVgpt plasmid has been described previously [15]. The pSVneo plasmid was a gift from P. Herbomel.

2.3. *Enzyme assays and P450 contents*

The names used for the P450s studied here respect the new recommended nomenclature (see [16]). The P450 IA1 content was deduced from its NADPH-dependent 7-EOR *O*-deethylase activity using published procedures [8] and a turnover number of 3 pmol resorufin formed per pmol of mouse P450 IA1 and per min. The substrate concentration was 2.5 μM and activities were corrected as appropriate for the contribution of P450 IA2 (EOR *O*-deethylase, turnover number: 0.03 min^{-1}). The P450 IA2 content was deduced from the CHP-dependent 7-EOC *O*-deethylase activity [9]. Using 660 μM CHP and 100 μM EOC, a turnover number of 0.1 min^{-1} was observed for purified or microsome bound P450 IA2 [9].

2.4. *Nucleic acid analysis*

DNA and RNA preparations, Northern and Southern blots have been carried out following standard procedures [17]. Northern and Southern blot hybridization and washes were performed as described [18]. Autoradiography was performed using Dupont Quanta III intensifying screens.

3. RESULTS AND DISCUSSION

3.1. *P450 contents*

Measurements of P450 activities making it possible to distinguish unambiguously between P450 IA1 (endogenous) and IA2 (introduced) activities were carried out as described [9,10]. The rationale for the calculation is the following. Although substrate specificities of rat or mouse P450 IA1 and rabbit P450 IA2 show partial overlap, the choice of a particular set of tests allowed calculation of the respective P450 IA1 and IA2 amounts. P450 IA1 content was deduced from NADPH-dependent 7-EOR *O*-deethylase activity for which the IA2 contribution is only 1% at an identical enzyme concentration. The P450 IA2 determination resulted from measurement of a CHP-dependent 7-EOC *O*-deethylase activity for which the IA1 interference is low and decreases rapidly with time due to selective inactivation of P450 IA1 by hydroperoxides. The P450 IA1 and IA2 contents were deduced from the values of these two assays assuming specific activities given in section 2. Thus, the calculated P450 amount corresponds only to the amound of catalytically competent enzyme. All the results presented here will thus refer to P450 contents instead of activities.

3.2. *Transfection and selection to obtain cell clones showing stable expression of P450 IA2*

Two hepatoma cell lines, H4II (rat) and BTG9 (mouse) that differ in their expression of cytochromes P450 and of fetal and neonatal hepatic functions (table 1) were chosen for this work. The P450 expression plasmids were constructed as specified in section 2, and their main features are shown in fig.1. That both plasmids encode functional P450 IA2 was demonstrated by transitory expression experiments with the two cell lines (data not shown). With the MMTV promoter no enzyme at all is present in uninduced cells, indicating a basal expression more than 15 times lower than that of the SV promoter. Using the induction conditions that we have determined to be optimal (2×10^{-6} M DEX for 48 h from the day after transfection), expression corresponding to at least one third of that from the SV promoter is obtained.

3.2.1. Isolation of pools of transfected colonies

Two plasmids, pSVgpt and pSVneo carrying respectively the Eco-gpt and neoselective markers were employed in cotransfection experiments with P450 IA2 plasmids.

H4II and BTG9 cells cotransfected with pSVneo and pSVIA2 gave rise to colonies growing in the presence of G418 after 2–4 weeks. These colonies were pooled and analyzed for P450 IA2 expression (fig.2). The mRNA level was weak for H4II cells, for which the signal inten-

Table 1

Characteristics of recipient cells

	H4II	BTG9
Cytochrom P450	IA1, IIB1/2, IIC6	IA1
Phase II enzymes	+	+
Neonatal enzymes	+	–
Albumin	+	+
Alphafoetoprotein	–	+

Phenotypic properties of H4II rat and BTG9 mouse hepatoma cells. Cytochrome P450 names are according to the new nomenclature [16]. + and – represent semi-quantitative estimates based on Northern blots and/or protein contents [11,21] and unpublished results

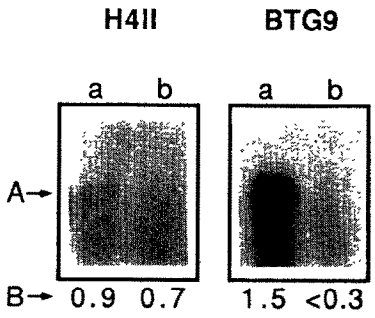

Fig.2. Expression of P450 IA2 mRNA and enzyme in pools of pSVIA2 transfected hepatoma cells during selection. (A) Northern blot analysis of total cellular RNA in pools of transfected H4II or BTG9 clones, probed with the rabbit cytochrome P450 IA2 cDNA. 15 μg total RNA from pools after two weeks (a) or five weeks (b) of selection. The longer time corresponds to the isolation of individual clones (30 cell generations). (B) Cytochrome P450 IA2 content was calculated from 7-EOC *O*-deethylase activity measurements (see section 2). Values are expressed in pmol cytochrome P450 IA2 per mg of total cell protein.

sity was clearly not proportional to the enzyme content measured. When examined at 5 weeks, the mRNA level was below the limit of detection but the enzyme level remained almost unchanged. For BTG9 cells, both the mRNA signal and enzyme content became undetectable after 5 weeks, indicating for these cells a clear tendency toward loss of P450 IA2 expression. To investigate the nature of this apparent instability, two experiments were carried out: determination of the frequency of colony formation with different combinations of plasmids, and study of the evolution of individual clones of transfected cells.

3.2.2. Isolation of individual clones

Table 2 shows the frequency of colony formation under selective conditions. H4II cells transfected with pSVneo alone or in combination with either one of the P450 expression vectors gave the same frequency of G418 resistant colonies, of generally healthy appearance. In all cases, the transfection efficiency of H4II cells is lower than that observed for BTG9 cells.

The mouse BTG9 hepatoma cells, when transfected with pSVneo or pSVgpt alone, or in combination with pMMTVIA2 give rise to colonies at high frequency. However, when the pSVIA2 plasmid is introduced, the number of colonies is dramatically decreased, by a factor of 10–100. In addition, examination of the dishes revealed that BTG9 colonies developed normally in all sets of dishes that did not involve pSVIA2. When the P450 expression vector included the SV promoter, the BTG9 colonies failed to grow vigorously, and were composed of vacuolated unhealthy cells; many colonies died altogether. This phase of 'crisis' lasted for 10–20 generations, at which time the cells recovered, or were lost. These observations suggest that high level expression of the P450 IA2 protein impairs the growth of most of the BTG9 cells. In view of the striking behaviour of BTG9 cells transfected with pSVIA2, a large number of clones were isolated and characterized. From the other transfection series of the two hepatoma lines, at least 5 colonies were picked and expanded.

3.3. *Analysis of isolated hepatoma cell clones*

The DNA extracted from BTG9 clones selected after transfection in the presence of pSVgpt was digested by *Eco*RI, blotted onto filters and hybridized with the P450 IA2 insert (fig.3). This enzyme cuts once in the two P450 expression vectors outside of the P450 IA2 coding sequence. A very different pattern of plasmid incorporation was observed depending upon the promoter driving P450 IA2 expression. The MMTVIA2 clones all carry a large number of tandem-integrated intact plasmids (more than 50 copies). Among clones transfected with pSVIA2, only 5 out of 9 contain at least one intact plasmid copy per cell; the others carry either no hybridizing material or smaller fragments. We have obtained similar results in the case of the cotransfection experiment involving both pSVneo and pSVIA2 plasmids (data not shown). No change in the patterns of plasmid integration was observed after prolonged culture (data not shown). The fact that only about half of the pSVIA2 transfected clones retain intact copies of the plasmid may be due to a negative selection over P450 IA2 expressing cells during clone isolation. In addition, it is striking that massive incorporation of plasmid copies occurs only when IA2 expression is driven by a weak promoter.

H4II cells demonstrated a pattern of plasmid integration similar to that for BTG9: large numbers of tandem-

Table 2

Frequencies of neo^R or HAT^R colonies after co-transfection

Selection plasmid		pSVneo			pSVgpt		
P450 expression plasmid		None	pSVIA2	pMMTVIA2	None	pSVIA2	PMMTVIA2
Cells	H4II	1	2 (5)	1.7 (5)	–	–	–
	BTG9	100	10 (9)	–	30	0.5 (9)	80 (6)

Frequencies are expressed as transfectants per 10^4 cells; –, not determined; in parentheses, number of clones isolated

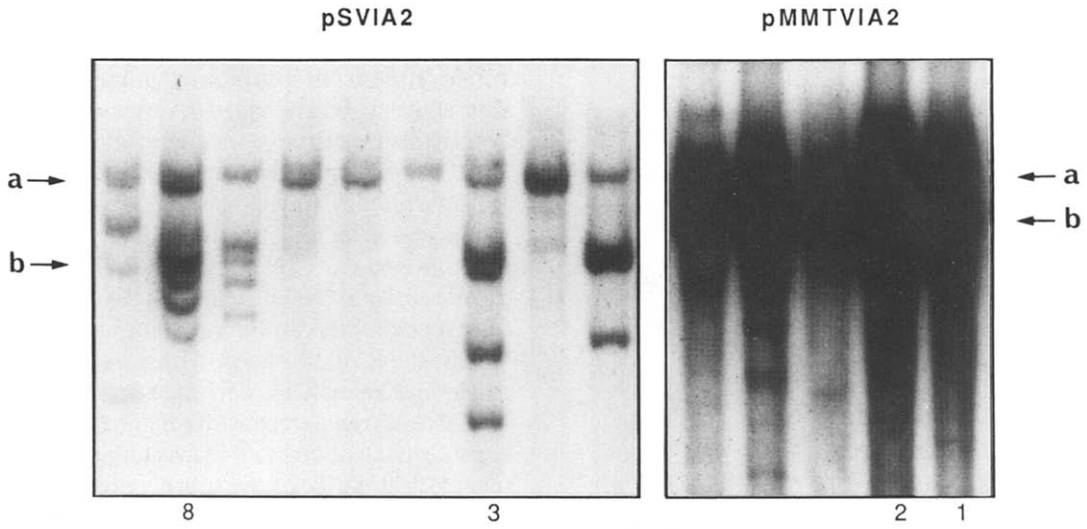

Fig.3. Cytochrome P450 IA2 cDNA sequences in individual clones of BTG9 transfected cells. Southern blot analysis of BTG9 transfected clones. Genomic DNA (11 μg) from each cell clone were digested with *Eco*RI, electrophoresed through a 0.8% agarose gel, transferred to nylon N-Hybond membranes and probed with P450 IA2 cDNA. (a) Endogenous P450 IA2 mouse gene; (b) Integrated copies of P450 IA2 containing plasmids. Numbers at the bottom refer to cell clones showing expression of the rabbit P450 IA2 enzyme.

integrated intact copies for MMTVIA2, and fewer than five copies, showing variable integration sites, for SVIA2 (see below).

Cytochrome P450 IA2 expression was assayed in each of the isolated H4II or BTG9 clones. Table 3 summarizes the results obtained soon after clone isolation (early) or after more than 50 cell generations (late). In H4II cells, the SVIA2 vector was expressed in most clones, and good P450 IA2 expression retained for two of them. Detectable expression of the MMTV construct was rare, and the single clone that maintained P450 IA2 expression showed an enzyme level close to the limit of detection.

Among the BTG9 clones transfected with the SVIA2 vector, only 3 out of 8 (HAT plus G418 selections) which contain at least one intact copy of the pSVIA2 plasmid actually produce the enzyme. Two of these clones proved to be stable upon long term culture. In addition, the BTG9 clones transfected with MMTVIA2 fail to express P450 IA2 upon long term cultivation. For comparison, the P450 IA1 contents of pools of transfectants from both H4II and BTG9 cells are shown.

Fig.4 presents the profile of plasmid incorporation, of P450 IA2 transcripts, and of P450 IA2 enzyme content for each of the five transfected clones that show stable expression of the introduced P450 IA2. It will be noted for one of the H4II clones that a weak mRNA signal may be associated with significant enzyme content, as noted above (fig.2) for pooled clones. The absence of correlation between the P450 IA2 mRNA and enzyme contents in these cells suggests that there is a limitation in activity of the rabbit enzyme, due for example to a reduced coupling efficiency between the rat

Table 3

Characterization of P450 IA2 expressing clones

		P450 IA2				
		pSVIA2		pMMTVIA2***		P450 IA1††
		early*	late**	early	late	
H4II	number of positive	4 (5)	2 (5)	1 (5)	1 (5)	
	P450 content†	0.6–2.2	0.6–0.8	0.4	0.4	0.45
BTG9	number of positive	3 (18)	2 (18)	2 (6)	0 (6)	
	P450 content	1.6–2.0	1.6–1.8	1.5–1.6	<0.3	10

The number of positive clones and their corresponding ranges of P450 IA2 content are indicated; †, pmol P450 IA2 per mg of total cell protein, range among expressing clones; *, 30 cell generations after transfection; **, >50 cell generations after transfection; ***, dexamethasone induction, 2×10^{-6} M for 48 h from the day after transfection; ††, P450 IA1 expression (pmol per mg of total cell protein) from the endogenous gene in pools of transfectants; in parentheses, total number of isolated clones tested for P450 IA2 content

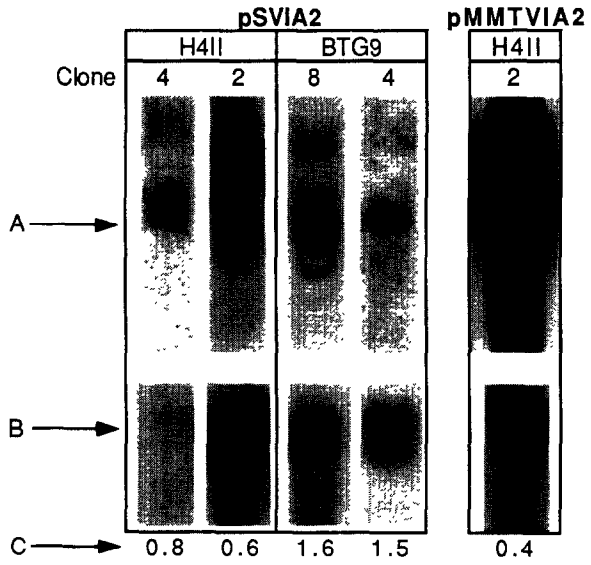

Fig.4. Characteristics of P450 IA2 expressing clones. For each clone, integrated plasmid copies, mRNA level and protein content are shown. (A) Southern blot analysis of genomic DNA from H4II and BTG9 cell clones digested with *Bam*H1 or *Eco*R1, respectively, probed with P450 IA2 cDNA. The arrows show the position of intact integrated plasmids. (B) Northern blot of RNA from the same clones. For the H4II clones transfected by pMMTVIA2, the mRNA level corresponds to DEX-treated cells (2×10^{-6} M for 48 h from the day after transfection). (C) P450 IA2 enzyme level (see legend of fig.2).

(endogenous) NADPH-P450 reductase and the rabbit (introduced) P450 form. For individual as well as pooled clones of BTG9, a strong RNA signal is correlated with generally higher enzyme content. The fact that two mRNA bands are found for both vectors and in both H4II and BTG9 cells may be due to an alternative splicing of the SV40 intron present downstream of the TGA termination codon of the P450 IA2 cDNA. The stably expressing clones produce significant P450 IA2 enzyme, giving values ranging from 0.4 to 1.6 pmol P450 IA2/mg total cell protein.

4. CONCLUSIONS

Two lines of evidence presented here indicate that production of large amounts of the P450 IA2 enzyme is not compatible with cell growth for the majority of BTG9 cells. A phenomenon of negative selection for such cells is indicated by the drastic reduction in the frequency and the vigor of clones surviving selection when the transfection involved pSVIA2. The second argument comes from the observation of loss of P450 IA2 protein upon prolonged culture both from pooled colonies and from the majority of isolated clones. In some cases however, the apparent toxicity of the enzyme can be overcome, since clones that show stable expression of the introduced plasmid were obtained. H4II cells did not seem to be affected by the presence of the enzyme, and clones showing stable expression of the plasmid vector were isolated at a reasonable frequency.

A difference in behaviour of the two hepatoma lines may not be surprising: H4II cells express a large number of hepatic functions restricted to postnatal hepatocytes, the stage when the P450 IA2 gene is first expressed, while BTG9 cells do not. It can be suggested that P450 IA2 enzyme interferes with the metabolism of endogenous substrates, possibly steroid compounds, when produced in a metabolic context devoid of other postnatal hepatic functions.

We report here the first example of the establishment of liver cell lines stably expressing rabbit P450 IA2. The enzyme level is about 100 times lower than that obtained in mammalian cells infected by vaccinia viruses that permit transient expression of other P450 activities, or in stably transformed yeast cells [9,19]. However, the enzyme levels we have observed are comparable to those found in COS cells transfected with other P450 cDNAs in expression vectors [20], and they range between 2.5×10^4 and 10×10^4 P450 IA2 molecules per cell.

The methodology we used can be applied to other xenobiotic-metabolizing enzymes. Hepatoma cells showing stable expression of a large spectrum of both Phase I and Phase II detoxication enzymes should be of great value in toxicology.

Acknowledgements: We are grateful to Peter Shaw for critical reading of the manuscript, to Anne-Marie Catherin who prepared the plasmids for transfection experiments, and to Solange Papelard for help in preparing the manuscript. This work was supported by grants from the CEE Biotechnology Action Program and the Association française de Recherche sur le Cancer (ARC). L.C. was a fellow from the ARC.

REFERENCES

[1] Wolf, C.R. (1986) Trends Genet. 8, 209-214.
[2] Okey, A.B., Roberts, E.A., Harper, P.A. and Denison, M.S. (1986) Clinical Biochem. 19, 132-141.
[3] Corcos, L. and Weiss, M.C. (1988) FEBS Lett. 233, 37-40.
[4] Israël, D.I. and Whitlock, J.P. (1983) J. Biol. Chem. 258, 10390-10394.
[5] Atchison, M. and Adesnik, M. (1986) CRC Critical Rev. Biochem. 19, 247-305.
[6] Tuteja, N., Gonzalez, F.J. and Nebert, D.W. (1985) Dev. Biol. 112, 177-184.
[7] Pasco, D.S., Boyum, K.W., Merchant, S., Chalberg, S.C. and Fagan, J.B. (1988) J. Biol. Chem. 263, 8671-8676.
[8] Gonzalez, F.J., Mackenzie, P.I., Kimura, S. and Nebert, D.W. (1984) Gene 29, 281-292.
[9] Pompon, D. (1988) Eur. J. Biochem. 177, 285-293.
[10] Cullin, C. and Pompon, D. (1988) Gene 65, 203-217.
[11] Deschatrette, J. and Weiss, M.C. (1974) Biochimie 56, 1603-1611.
[12] Lutfalla, G., Blanc, H. and Bertolotti, R. (1985) Somat. Cell Mol. Genet. 11, 223-238.
[13] Coon, H.G. and Weiss, M.C. (1964) Proc. Natl. Acad. Sci. USA 62, 852-859.
[14] Mulligan, R.C. and Berg, P. (1981) Mol. Cell. Biol. 1, 449-459.
[15] Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Mol. Cell. Biol. 2, 1044-1051.

[16] Nebert, D.W., Adesnik, M., Coon, M.J., Estabrook, R.W., Gonzalez, F.J., Guengerich, F.P., Gunsalus, I.C., Johnson, E.F., Kemper, B., Levin, W., Phillips, I.R., Sato, R. and Waterman, M.R. (1987) DNA 6, 1-11.
[17] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Cold Spring Harbor, New York.
[18] Church, G. and Gilbert, W. (1984) Proc. Natl. Acad. Sci. USA 81, 1991-1995.
[19] Aoyama, T., Korzekwa, K., Nagata, K., Gillette, J., Gelboin, H.V. and Gonzalez, F.J. (1989) Eur. J. Biochem. 181, 331-336.
[20] Gonzalez, F.J., Skoda, R.C., Kimura, S., Umeno, M., Zanger, U.M., Nebert, D.W., Gelboin, H.V., Hardwick, J.P. and Meyer, U.A. (1988) Nature 331, 442-446.
[21] Szpirer, C. and Szpirer, J. (1975) Differentiation 4, 85-91.