Cloning of a cDNA coding for P-450 LM3c from rabbit liver microsomes and regulation of its expression

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ABSTRACT

Liver cytochromes P-450 LM3c in the rabbit and P-450p in the rat are two related forms, inducible by macrolide antibiotics such as triacetyloleandomycin (TAO) and glucocorticoids such as dexamethasone. We prepared a cDNA library from TAO induced rabbit liver mRNA and characterized a cDNA (pLM3c-4.1) that hybridized to pDex 3.22, a cDNA complementary to cytochrome P-450p mRNA. Northern blots of liver poly(A)RNA from untreated or TAO, erythromycin and rifampicin treated animals, revealed two mRNA species of approximately 1700 and 1850 nucleotides in length, that hybridized to LM3c cDNA and to pDEX 3.22. The level of both mRNAs was increased five fold over control by macrolide antibiotics but unaffected by both phenobarbital and B-naphthoflavone. After 5 days of TAO treatment LM3c mRNA had increased 5 fold while LM3c protein had increased 25 fold. However, the rate of P-450 LM3c gene transcription measured in isolated liver nuclei remained unchanged throughout five days of TAO treatment. We conclude that TAO may induce cytochrome P-450 LM3c by post-transcriptional effects.

INTRODUCTION

The several cytochromes P-450 (P-450) from liver microsomes constitute a family of hemoproteins involved in the detoxication and activation to mutagenic and carcinogenic metabolites of an impressive number of xenobiotics including most of drug and environmental pollutants, as well as in the oxidation of endogenous substrates such as steroids and prostaglandins (1), (2). At least ten different forms of microsomal P-450 have been isolated from animal liver (rat or rabbit) and characterized in terms of their molecular, spectral, enzymatic and immunologic properties (3-11). These various forms appear to be unique products of distinct genes. The level of some of them is increased following treatment of animals with such xenobiotic inducers (3), (12) as phenobarbital (PB) or polycyclic aromatic

compounds by a process involving increased gene transcription (13-17). We have found that macrolide antibiotics such as triacetyloleandomycin (TAO), erythromycin (ER) or rifampicin (RIF) represent a third class of inducers that increase the concentration of P-450 LM3c in rabbit liver (12), (18-20). Likewise, in the rat TAO and ER but not RIF, induce P-450p (21) a form already known to be inducible by pregnenolone 16 carbonitrile (PCN), dexamethasone (DEX) and other glucocorticoids (5). To investigate the molecular mechanism of induction of P-450 LM3c by macrolides, we took advantage of the homology between P-450 LM3c and P-450p(22) and used a cDNA (clone 3.22), previously shown to code for P-450p (22), as a probe to screen a rabbit cDNA library prepared from TAO treated rabbit poly(A)RNA. In this paper we report the isolation of a cDNA (pLM3C-4.1) coding for P-450 LM3c and the analysis of the induction of P-450 LM3c mRNA under macrolide antibiotics.

MATERIALS AND METHODS

Materials

Macrolide antibiotics, TAO, ER and RIF, were from Pfizer (Orsay, France), Roussel UCLAF (Paris, France) and Merrel Dow, (Neuilly, France), respectively. PB was from Specia (Paris, France) and -naphthoflavone (BNF) from Sigma (St Louis, Mo). Electrophoresis reagents and nitrocellulose were from BioRad (Richmond, Ca). Rabbit antigoat IgG and goat peroxidase anti-peroxidase complex were from Miles Scientific (Elkhart, In). Diaminobenzidine tetrahydrochloride was from Sigma (St Louis, Mo). [³⁵S] labelling reagent used to radiolabel P-450 LM3c was from Amersham International (Amersham, England). Oligo dT cellulose type III was from Collaborative Research (Lexington, MA). All enzymes used for cloning were from Pharmacia P.L. Biochemicals Inc. (Piscataway, NJ) and restriction nucleases were from Bethesda Research Laboratories (Gaithersburg, MD). [³²P] dCTP, [³²P] UTP, [³²P] ddATP, [³⁵S] Methionine and the rabbit reticulocyte cell free translation system were from Amersham International (Amersham, England). Plasmid pBR 322 was from New England Nuclear (Boston, Ma).

Animals and treatments

Male New Zealand white rabbits (2.0 - 2.5 kg) were used in this work. TAO and ER were administered orally (1 mmole per kg per day) for 5 days. RIF (50 mg/kg) and BNF (80mg/kg) were given by daily intraperitoneal injections for 4 and 3 days, respectively. PB was given in drinking water (0.1 % w/v, pH 7.4) for 5 days.

Microsomes, proteins preparation and monooxygenase activity

Liver microsomes and P-450 LM3c were prepared as described (20). P-450 LM3c was radiolabeled with t-butoxycarbonyl -L- [³⁵S] methionine N-hydroxysuccinimidyl ester. Briefly, 50 ul of 20 uM P-450 LM3c (in 0.1 M potassium phosphate pH 7.4, 0.1 mM EDTA, 20 % glycerol), 50 ul of 0.1 M sodium borate pH 9.0 and 0.625 nmol of the [³⁵S]-labelling reagent (specific activity 800 Ci/mmol) were incubated at 0 C for 30 minutes. The solution (100 ul) was then dialyzed first against 100 ml of 0.1 % trifluoroacetic acid, 0.5 mM DTT for 2 hours and finally against 100 ml of 0.1 M potassium phosphate pH 7.4, 20 % glycerol.

Erythromycin demethylase activity was determined according to the method of Nash (23). Microsomes were diluted to 1 mg/ml in 0.1 M potassium phosphate pH 7.4. Erythromycin (400 uM) was added to the suspension and the reaction was initiated by the addition of 0.5 mM NADPH. The assay was performed at 37 C for 10 minutes.

Preparation and purification of anti P-450 antibodies

Antibodies to highly purified P-450 LM3c were raised in a goat. The IgG fraction was isolated from the serum by repeated ammonium sulfate precipitations. Anti P-450 LM3c IgG were further purified by immuno-adsorption against liver microsomes from untreated rabbit coupled to a sepharose 4B gel, according to a procedure described elsewhere (5).

Western blot

Cytochrome P-450 LM3c was quantitated by immunoblot analysis of electrophoretically separated microsomal proteins as previously described (24) with the following modifications : lug of microsomal proteins were submitted to electrophoresis and anti P-450 LM3c IgG were used at 0.1mg/ml. Specific content of P-450 LM3c was estimated from densitometric analysis of the blot with a Shimadzu Dual-Wavelength Scanner.

RNA preparation

Total RNA was extracted from freshly excised rabbit liver by the procedure of Auffray and Rougeon (25).

Poly(A)RNA was isolated by two cycles of oligo dT-cellulose chromatography and fractionated by sedimentation through a 15-30 % (w/v) sucrose gradient in 10 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.1 % SDS for 15 hours at 32000 RPM (rotor SW41). Poly(A)RNA, collected from the various fractions was precipitated with ethanol and used to program a rabbit reticulocyte translation system.

Preparation and screening of the cDNA library

Double stranded cDNA was synthesized by the RNase H and DNA polymerase procedure as described by Gubler and Hoffman (26), using poly(A)RNA isolated from TAO induced rabbit liver as a template. The cDNA was then dC tailed and size fractionated through a Sepharose 4 B gel equilibrated with 10 mM Tris-HCl pH 7.5, 1 mM EDTA and 100 mM NaCl. cDNA fractions were annealed to oligo dG tailed pBR 322, at a ratio of vector to cDNA 60 : 1.2. Competent E. Coli RRI cells were transformed according to Kushner (27). Approximately 1500 colonies were obtained and screened on nitrocellulose filters as described by Hanahan and Meselson (28), using purified insert of pDEX 3.22 radiolabeled with [³²P] by nick translation (22). After baking in vacuo for 2 hours at 80 C and washing in 1 M NaCl 50 mM Tris-HCl pH 8.0 and 0.1 % SDS, 1 hour at 37 C, the filters were prehybridized overnight at 37 C in buffer A : 50 % formamide, 0.1 % ficoll, 0.1 % polyvinylpyrrolidone, 0.1 % BSA (fraction V), 0.5 % SDS, 100 ug/ml DNA from salmon sperm, 5 X SSPE (1 X SSPE : 0.15M NaCl, 10mM sodium phosphate pH 7.4, 1mM EDTA) and 10 % dextran sulfate. Hybridization was carried out in the same buffer for 24 hours at 37 C in the presence of 4 ng/ml of nick translated insert (210⁸ dpm/ug). Filters were washed twice for 15 minutes with 2 X SSC (1 X SSC : 0.15 M NaCl, 15mM sodium citrate pH 7.0) at room temperature and once for 10 minutes with 0.2 X SSC at 50 C. Autoradiography was carried out overnight at - 80 C.

Northern blots

Total liver RNA (10 ug) or poly(A)RNA (5 ug) from untreated and treated rabbits was electrophoresed on agarose gel (1.2 %) in

10 mM sodium phosphate buffer pH 7.0, 1.1 M formaldehyde, and transferred to nitrocellulose filter. Hybridization was carried out with 10 ng of pLM3C 4.1 or pDEX 3.22 radiolabeled by nick translation ($1-2 \cdot 10^8$ dpm/ug) in buffer A 18 hours at 37 C.

Hybrid selection

Plasmid DNA from 5 ml culture of clone 4.1 was prepared by the alkaline lysis procedure, dissolved in 50 ul of water and denatured by boiling 10 minutes in 0.3 M NaOH. The solution was then diluted to 1 ml with 5 M NaCl and immediately blotted on nitrocellulose filter pretreated with water (5 minutes) and 6 X SSC (15 minutes). After baking for 2 hours under vacuo, prehybridization and hybridization to poly(A)RNA prepared from TAO induced rabbit liver, were carried out according to the procedure of Fagan et al. (29). Hybrid selected RNA was translated in a rabbit reticulocyte lysate system and the proteins synthesized and immunoprecipitated with anti P-450 LM3C IgG analyzed by SDS polyacrylamide gel electrophoresis and autoradiography. Authentic [35 S] radiolabeled P-450 LM3C was also electrophoresed on the same gel for direct comparison.

Sequencing of Rsa I fragments of pLM3C 4.1 insert

pLM3C 4.1 insert was radiolabeled with terminal deoxynucleotidyl transferase and [32 P] dATP (3000 ci/mmol). After purification on low melting agarose gel, the insert was split with Rsa I : two fragments (75 and 80 bp) from both ends were obtained and sequenced by the procedure of Maxam and Gilbert (30).

In vitro transcription experiments

Approximately, 10^7 nuclei, purified from freshly excised liver of untreated and TAO treated rabbits for one and five days, were incubated for 5 minutes with 100-200 uCi of [32 P] UTP (400 Ci/mmol) as described (31). Nascent RNA chains were extracted in 6 M urea, 0.5 M NaCl, deproteinized by the hot phenol procedure and precipitated by 70 % ethanol, in the presence of 200 ug of yeast RNA. Five ug of DNA from pBR 322, pRGAPDH-13 and pLM3C-4.1, denatured by boiling for 10 minutes in 0.3 M NaOH, were blotted on nitrocellulose filter. Hybridization was carried out for 48 hours at 68 C with $5 \cdot 10^6$ cpm RNA in 50 mM sodium phosphate pH 7.0, 0.3 M NaCl, 1 mM EDTA, 0.1 % SDS

After a series of five washes (10 minutes each) in 2 X SSC at 68 C, once in 2 X SSC at 37 C (30 minutes) in the presence of 10 ug/ml RNase A and twice (5 minutes each) in 2 X SSC at room temperature, the filters were autoradiographed for 16 hours at - 80 C.

Southern blot analysis of pLM3C-4.1 related sequences in rabbit genomic DNA

Genomic DNA was prepared according to Gross-Bellard et al (32), digested with a 10 fold excess of EcoRI and electrophoresed in a 0.5 % agarose gel at 2V/cm for 20 hours in 90mM Tris-Borate buffer pH8.9, 2mM EDTA. Fragments of Adenovirus-2 DNA were used as molecular weight markers. Blotting on nitrocellulose was as described by Southern (33). Hybridization was carried out for 48 hours at 42 C with the nick-translated insert, washed and autoradiographed as described for northern blots.

RESULTS

Preparation and characterization of cytochrome P-450 LM3c cDNA

Poly(A)RNA isolated from untreated and TAO treated rabbit liver, was used to program a cell free translation system. Proteins synthesized were analyzed by immunoprecipitation with form specific anti P-450 LM3c IgG. The autoradiogram obtained after electrophoresis of both total and immunoprecipitated proteins (figure 1), revealed a polypeptide comigrating with authentic [³⁵S]-labeled P-450 LM3c, whose level was markedly increased (5 fold) in the translations of poly(A)RNA prepared from a TAO treated animal. From a portion of the liver used for poly(A)RNA preparation, microsomes were isolated and assayed for P-450 LM3c specific content and erythromycin demethylase, an activity known to be P-450 LM3c specific, (20-22). The results reported in Table I show that a high level of P-450 LM3c was present in the liver of the TAO treated rabbit.

Poly(A)RNA isolated from TAO induced rabbit liver was separated on a sucrose gradient and fractions enriched in P-450 LM3c mRNA, as determined by in vitro translation, were used as templates to synthesize a double stranded cDNA by the RNase H-DNA polymerase procedure (26). After insertion into the Pst I site of pBR322 by the dC-dG tailing method, a cDNA library of 1500

Figure 1 : Autoradiogram of electrophoretically separated total and immunoprecipitated proteins translated from poly(A)RNA from uninduced and TAO treated rabbit liver

Poly(A)RNA from uninduced and TAO treated rabbit liver was used to program a cell free translation system, containing 10 uCi [35 S] methionine. After translation for 90 minutes at 30 C, aliquots were immunoprecipitated with anti P-450 LM3c IgG. Both total translated proteins and immunoprecipitates were subjected to electrophoresis on SDS polyacrylamide (10 %) slab gel system, together with authentic [35 S]-labeled P-450 LM3c. Lane 1 : translation products without poly(A)RNA ; lanes 2 and 3 : total translated proteins from uninduced and TAO treated poly(A)RNA respectively ; lane 4 : immunoprecipitate of translation products without poly(A)RNA ; lanes 5 and 6 : immunoprecipitates from translations products of lanes 2 and 3, respectively. The arrow indicates the migration position of authentic P-450 LM3c.

colonies (E. Coli, RRI) was screened with [32 P] pDex 3.22 under low stringency.

Three putative clones containing 200, 300 and 1700bp insert respectively were obtained. These were also positive after a

Table I : Effect of TAO on total P-450 and P-450 LM3c specific content, microsomal erythromycin demethylase activity and level of translatable P-450 LM3c mRNA

Microsomes	Control	TAO induced
P-450 nmol/mg	0.9 $\pm$ 0.2	1.9 $\pm$ 0.2
Erythr.Demeth. nmol/min/mg	0.8 $\pm$ 0.2	4.2 $\pm$ 0.5
LM3c nmol/mg	0.07 $\pm$ 0.02	1.7 $\pm$ 0.2
m-RNA Translat.(cpm)		
Total	13600 $\pm$ 500	26000 $\pm$ 500
Immunop.	50 $\pm$ 20	520 $\pm$ 100

Microsomes were prepared from freshly excised liver of control and TAO treated animals. Total P-450 and P-450 LM3c specific contents were determined spectrally by the method of Omura and Sato and by immunoquantitative analysis, respectively. Erythromycin demethylase was determined as indicated under Methods. After cell free translation of poly(A)RNA, radioactivity associated to total and immunoprecipitated proteins was determined from aliquots of respective mixtures.

differential screening with [32 P] cDNA synthesized from poly(A)RNA prepared from TAO induced rabbits, (not shown). The plasmid containing the longest insert (pLM3c-4.1) was used to hybrid-select a poly(A)RNA coding for a polypeptide specifically immunoprecipitated with anti P-450 LM3c IgG and comigrating with authentic [35 S]-labeled P-450 LM3c, (not shown).

In order to characterize further pLM3c-4.1, the insert was digested with RSaI which generates two fragments of approximately 75-80 bp from each end. In the course of the extensive sequence analysis of pLM3c-4.1 insert these fragments were sequenced by the method of Maxam and Gilbert (30). The nucleotide sequence of the 5'end fragment (75bp) as well as the derived aminoacid sequence are reported in Table II, together with the available N-terminal sequence of cytochrome P-450 LM3c (Capony, J. P. and Derancourt, J. Centre de Biologie Macromoléculaire, CNRS, Montpellier, France, unpublished results). With the exception of the aminoacid at position 22 both sequences are homologous. These results allow us to conclude that pLM3c-4.1 insert is a cDNA encoding for P-450 LM3c or for a closely related protein. It can be seen from Table II that the 5'end RSA I fragment contains a

Table II : Nucleotide sequence of the 5' end Rsa I fragment of pLM3c-4.1 insert

N-term P-450 LM3c : Met Asp Leu Ile Phe Ser Leu Glu Thr Trp															
Val Leu Leu Ala Ala Ser Leu Val Leu Leu Tyr (?) Tyr															
1020															
cDNA (P-450 LM3c) : GCAGTGGCG ATG GAG CTG ATC TTT TCC															
Met Asp Leu Ile Phe Ser															
3040506070															
CTG GAA ACA TGG GTT CTC CTG GCT GCC AGC CTG GTG CTC CTG TAT															
Leu Glu Thr Trp Val Leu Leu Ala Ala Ser Leu Val Leu Leu Tyr															
CTA TAT															
Leu Tyr															

pLM3c-4.1 insert was split with Rsa I : two subfragments 78 and 80 bp were obtained from the 5' and 3' ends respectively. Nucleotide sequence of the 5' end Rsa I subfragment is reported here, as well as the derived aminoacid sequence. For comparison the available aminoacid sequence of P-450 LM3c is also reported. Aminoacid at position 22 could not be determined unequivocally.

small part (9 nucleotides) of the non coding 5' end of the mRNA. Thus pLM3c-4.1 insert (1700 bp long) is likely to code for the complete protein sequence of cytochrome P-450 LM3c.

Induction by macrolide antibiotics of mRNA coding for P-450 LM3c

In previous reports we showed that P-450 LM3c is induced by macrolide antibiotics such as TAO, ER and RIF (12), (18-20). Therefore, it appeared of interest to determine whether induction by these compounds was related to increased level of P-450 LM3c mRNA, as already shown with PB, PCN and polycyclic aromatic compounds, (13-16), (22).

Rabbits were treated with TAO, ER, RIF, PB and BNF. Both poly(A)RNA and microsomes were prepared simultaneously from portions of the same liver and analyzed by Northern and Western blots which were respectively probed with [³²P] pLM3c-4.1 and anti P-450 LM3c IgG. Under these conditions both levels of LM3c mRNA and protein (P-450 LM3c) could be directly compared.

From the Northern blots, (figure 2A and B) it appears that pLM3c-4.1 hybridizes with at least two mRNA species of approximately 1700 and 1850 bases in length in either untreated or treated rabbit liver. The amounts of these mRNAs is markedly increased (5 fold) in TAO, ER and RIF treated rabbit liver, but not after PB or BNF treatments. The relative intensity of the two bands is not drastically affected by the inducers. When pDEX 3.22

Figure 2 : Comparative levels of P-450 LM3c mRNA and P-450 LM3c isozyme under xenobiotics induction

Poly(A)RNA and microsomes were prepared from freshly excised liver of untreated and treated animals with TAO, ER, RIF, PB and BNF, as indicated in Methods. Poly(A)RNA (5 ug) were subjected to electrophoresis on agarose gel, transferred to nitrocellulose filter and hybridized to [32 P] pLM3c-4.1 radiolabeled by nick translation. After washing, filters were analyzed by autoradiography. Microsomal proteins (1 ug) were subjected to electrophoresis on SDS polyacrylamide slab gel and transferred to nitrocellulose filter. The western blot was developed with anti LM3c IgG.

A : Northern blot of poly(A)RNA from untreated (UT) and TAO

treated rabbit liver (T). B : Northern blot of poly(A)RNA from ER, PB, RIF and BNF treated rabbit. C : Filter presented in A, was dehybridized and rehybridized with [32 P] pRGAPDH-13 radiolabeled by nick translation. D : Western blot of authentic P-450 LM3c, 1 and 2 pmol, and microsomes from untreated (UT) and treated rabbits (1 ug).

(from rat liver), was used as a probe on the same nitrocellulose filter, the hybridization pattern was the same (results not shown). We confirmed that the observed differences were not due to variations in the amount of RNA loaded in the gel, by rehybridizing the filters with a probe complementary to glyceraldehyde-3P-dehydrogenase mRNA (pGAPDH-13) (34), which is not affected by TAO treatment (figure 2C).

Western blots of liver microsomes from untreated and treated rabbits are presented in Figure 2D. As shown in Table I, P-450 LM3c is markedly induced from 0.07 ± 0.02 nmol/mg in untreated animals to 1.7 ± 0.2 nmol/mg after macrolide antibiotic treatment ; on the other hand neither PB nor BNF stimulate this form significantly. Comparison with Northern blots shows that the level of mRNA hybridizing specifically to pLM3c-4.1 is in qualitative agreement with the level of immunoquantified P-450 LM3c in untreated and treated animals, whatever the inducer. However, the 5 fold increase in specific mRNA (Table III) does not adequately account for the 15 to 20 fold increase in protein level.

After one single dose of TAO (at 24 hours of treatment)

Table III : Kinetics of TAO effect on P-450 LM3c gene transcription rate and P-450 LM3c mRNA and P-450 LM3c levels

TAO dose (time of treatment)	Transcription rate	mRNA Relative to control	P-450 LM3c
0	1	1	1
1 (24 H)	1.1	2.3	1
5 (120 H)	1.1	4.8	25

Transcription rate and mRNA and P-450 LM3c levels were obtained from densitometric analysis of autoradiograms and western blots. Data from duplicate have been normalized to 1 for uninduced animals ; absolute value for P-450 LM3c level in these conditions is 0.07 ± 0.02 nmol/mg.

P-450 LM3c is not yet significantly induced (Table III) and P-450 LM3c mRNA level is increased only 2 to 3 fold.

Rate of P-450 LM3c gene transcription

The increased level of P-450 LM3c mRNA following TAO, ER or RIF treatment, could result either from an increased rate of synthesis or from a post transcriptional control (or from both). We have measured the rate of transcription of P-450 LM3c gene in isolated nuclei (31) prepared from untreated and TAO treated rabbit liver, after one and five days of treatment. Equivalent amounts of radioactive transcript were hybridized to nitrocellulose bound DNA dots carrying an excess of either pLM3c-4.1 or pRGAPDH 13. The later cDNA probe was used as an internal reference since this gene has been shown to be constitutively transcribed at similar rate in all tissues assayed (34). In parallel experiments (not shown), carried out in the presence of 2 ug/ml -amanitin, transcription was shown to be driven by RNA pol II. From the results reported in figure 3, it is clear that under these conditions (at one and five days of treatment) TAO does not modify the rate of transcription of P-450 LM3c gene. However, a very early and transient burst of TAO induced transcription cannot be ruled out.

Figure 3 : P-450 LM3c gene transcription after one and five days of TAO treatment

Nuclei were prepared from freshly excised liver of untreated and TAO treated rabbits for one and five days. Equivalent amounts of radioactive transcript were hybridized to nitrocellulose bound DNA from pRGAPDH-13, pLM3c-4.1 and pBR 322. After washing, filters were analyzed by autoradiography.

DISCUSSION

Based on our previous reports (21,22) showing that P-450 LM3c and P-450p were closely related homologous proteins, we isolated from a cDNA rabbit liver library, several clones that selectively hybridized to pDex 3.22, a plasmid harboring a cDNA to P-450p mRNA. Differential screening with (^{32}P) cDNA to total mRNA from untreated and TAO treated rabbits, hybrid-selection translation experiments and finally sequencing of the 5' end of the longest insert (pLM3c-4.1), allowed us to demonstrate that the cDNA were complementary to mRNA coding for P-450 LM3c or for closely related protein(s). In addition to molecular, enzymologic and immunologic data, cross-hybridization of P-450p cDNA (rat) with P-450 LM3c cDNA (rabbit) provides further support to the suggestion that P-450p and P-450 LM3c are homologous proteins.

Northern blots of rabbit liver RNA revealed two P-450 LM3c mRNA species of 1700 and 1850 nucleotides, regardless of whether pLM3c-4.1, or pDEX 3.22 served as probe. It is possible that there are two distinct mRNA_s sharing a repeated element although invariance of the hybridization pattern makes this unlikely. There may be two distinct forms of the same mRNA differing either in their extent of polyadenylation or in the length of their non coding regions, or again two distinct mRNA_s coding for two closely related proteins coinduced by macrolide antibiotics. In view of recent reports on the microheterogeneity of cytochrome P-450 forms at both protein (35-38) and nucleic

acid level (14,39), we decided to evaluate the extent of the P-450 LM3c gene family by Southern blot analysis of rabbit genomic DNA. As reported in figure 4, [^{32}P] pLM3c-4.1 hybridized to at least seven Eco RI restriction fragments, ranging from 0.6 to 8 kb (doublet at 6.2 kb), whose summation revealed a total of 30 kb of DNA. The number of LM3c related genes was also estimated from dot blot hybridization (not shown) of genomic DNA with [^{32}P] cDNA insert of pLM3c-4.1, by comparison of the intensity of the signal relative to a given amount of DNA, with that relative to a range of concentrations of authentic pLM3c-4.1. These experiments do not allow us to determine precisely the extent of the P-450 LM3c gene family but rather suggest two points : 1/ there could be more than one P-450 LM3c gene ; 2/ the LM3c family could be

Figure 4 : Southern blot analysis of pLM3c-4.1 related sequences in rabbit genomic DNA.

Genomic DNA was digested with ECORI, subjected to electrophoresis in a 0.5 % agarose gel and transferred to a nitrocellulose filter. Hybridization with [32 P] cDNA LM3c-4.1 and washing were carried out as described in Material and Methods A. Hind III fragments of Adenovirus-2 DNA were used as molecular weight markers ; B, ethidium bromide stained DNA ; C, autoradiography of the pLM3c-4.1 related sequences.

limited to less than five members. The two P-450 LM3c related mRNA species revealed on Northern blot (figure 3) could therefore derive from two P-450 LM3c related genes. In addition to these considerations concerning the polymorphism of P-450 LM3c, it is worth to mention here that in western blots of rabbit liver microsomes (under conditions of high protein concentration i. e. 25-50 ug) developed with anti P-450p IgG, two distinct proteins were visualized : in addition to the one migrating at 53 kd, most likely P-450 LM3c induced by TAO and RIF another protein migrating at 54 kd was revealed (22). This protein, which was

present in both untreated male and female animals, was induced by RIF but repressed by TAO and PB. However, Northern blots (figure 3) do not support this observation and rather indicate that both mRNAs are induced by TAO (as well as by RIF). Further experiments are therefore required before the nature of the two mRNAs species hybridizing to pLM3c-4.1 is fully established.

Induction of P-450 LM3c by macrolide antibiotics is at least partly accounted for by an increased level of its specific mRNA (figure 2). The finding that protein induction (15-20 fold) markedly exceeds mRNA induction (5 fold) suggests however that either the translatability of induced mRNA is increased or that other contributions are to be considered. In the rat, both in vivo and in primary culture of hepatocytes, TAO increases significantly (4 fold) the half life of P-450p (40). TAO may also stabilize LM3c and this could account for the disproportionate accumulation of this protein in the rabbit.

In contrast to previous reports (13-16), showing a strong and early increase in both specific mRNA level and transcription rate of several P-450 genes after administration of various inducers, the results reported in figure 3 and Table III suggest that mRNA induction by TAO is a slow process and show that the rate of transcription of the P-450 LM3c genes is not modified by TAO. Obviously, the possibility that the transcription rate is increased very early (a few hours) after the first injection of TAO and then returns to its control level at 24 hours, cannot be ruled out at the present time. Alternatively, a constitutive level of P-450 LM3c genes transcription would suggest that TAO could induce LM3c through a post-transcriptional process. Further data are needed in order to provide more experimental support, in favor or against this hypothesis.

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