Volume 236, number 2, 357-361 FEB 06238 August 1988

Differential splicing in the 3′ non-coding region of rat cytochrome P-452 (P450 IVA1) mRNA

David Earnshaw, Jeremy W. Dale, Peter S. Goldfarb and G. Gordon Gibson

Departments of Biochemistry and Microbiology, University of Surrey, Guildford GU2 5XH, Surrey, England

Received 8 July 1988

The gene coding for the clofibrate-induced cytochrome P-452 has been isolated from a cDNA library and a full-length cDNA clone sequenced. The sequence was identical with that reported by Hardwick et al. [(1987) J. Biol. Chem. 262, 801–810] apart from the presence of an additional 75 nucleotides inserted adjacent to the 3′-end of the coding region. Northern blot analysis indicates that this is probably a consequence of differential splicing, which may be relevant to the tissue-specific regulation of this gene.

Cytochrome P-450; Clofibrate; Gene regulation; Differential splicing

1. INTRODUCTION

The cytochrome P-450 proteins play an important role in the metabolism of endogenous substrates, detoxification of drugs and xenobiotics, and activation of environmental agents to toxic, mutagenic and carcinogenic forms. The broad specificity of these systems is due to the existence of a large family of related genes [2], the expression of each isoenzyme being modulated by endogenous factors or by exposure to various xenobiotics. This laboratory has already reported the purification and characterisation of a unique hepatic isoenzyme termed cytochrome P-452 (also known as P450 IVA1) induced by the hypolipidaemic drug clofibrate [3,4]. Unlike other cytochrome P-450 variants, this enzyme readily metabolises fatty acids but not drugs. Hardwick et al. [1] have shown that rat hepatic lauric acid ω-hydroxylase, or cytochrome P-450 LAω (thought to be the same as cytochrome P-452) shares less than 35% amino acid homology with cytochromes P-450 c,d,e and PCN. Another potential member of this new gene family, rabbit pulmonary prostaglandin ω-hydroxylase, cytochrome P-450 p-2, has recently been reported [5].

The process of induction of cytochrome P-452 is of substantial interest in relation to the hepatotoxicity of hypolipidaemics [6]. Clofibrate administration and transcriptional activation of the LAw gene in rat liver are closely linked [1] and a similar transcriptional activation of the first two peroxisomal β-oxidation enzymes has been observed in liver [7]. The results in this paper indicate a heterogeneity in the 3′-non-coding region of cytochrome P-452 mRNA which may be relevant to the regulation of this gene.

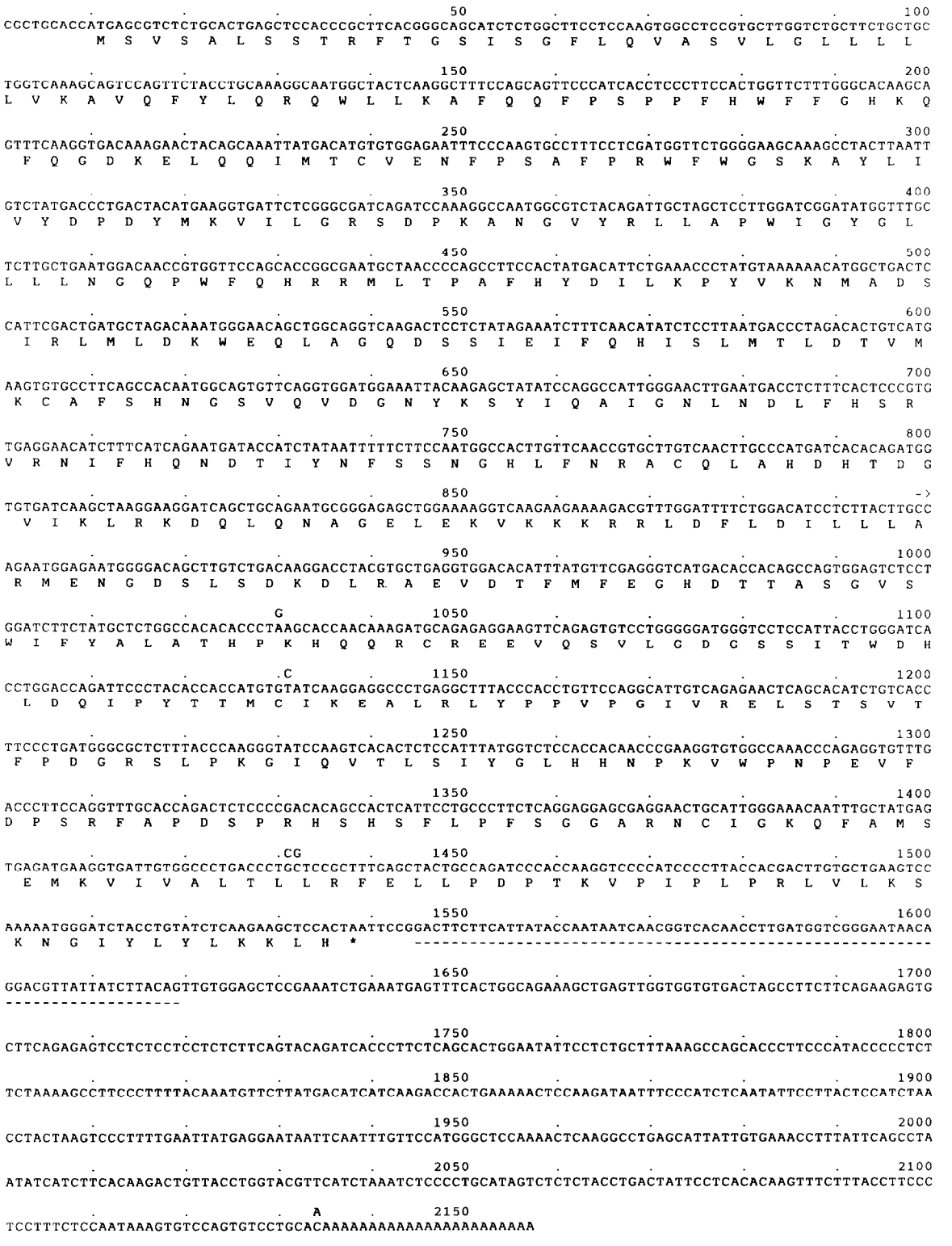
Correspondence (present) address: D. Earnshaw, Beecham Pharmaceuticals, Biosciences Research Centre, Great Burgh, Epsom KT15 8XQ, Surrey, England

2. MATERIALS AND METHODS

2.1. *Cloning and sequencing of cytochrome P-452 cDNA*

Clofibrate (400 mg/kg) was administered intraperitoneally to a 150 g male Wistar rat. After 16 h, total liver RNA was extracted using a guanidine thiocyanate/caesium chloride method [8]. The poly(A^+) mRNA was purified and cDNA synthesized by standard procedures [9]. *Eco*RI linkers were added for ligation with λ gt11 [10]. The cDNA library was screened with polyclonal antiserum to electrophoretically homogeneous cytochrome P-452 [3], using a biotin-streptavidin linked immunoassay system [11]. DNA sequence analysis was carried out by the dideoxy procedure after subcloning into M13 vectors. Smaller fragments were generated by digestion with specific restriction endonucleases (*Pst*I, *Sst*I, *Nco*I, *Bam*HI) and by progressive digestion with *Bal*31 nuclease.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50



2.2. *Oligonucleotide probes and hybridisations*

Oligonucleotides were kindly synthesized by C. Entwistle, and were 5′-labelled with ^{32}P using T_4 polynucleotide kinase (Amersham). Other probes were labelled with [^{32}P]dATP using an Amersham 'Multiprime' kit. After electrophoresis, RNA was transferred to Pall Biodyne membranes [12]. With the cDNA probe, the filters were pre-hybridised (50% formamide, 5 × SSC, 25 mM sodium phosphate, pH 6.5, 10 × Denhardt's solution, 200 μg/ml sonicated denatured herring testes DNA) at 42°C for 3 h; hybridisation was then carried out overnight at the same temperature. The filters were washed in 2 × SSC, 0.1% SDS and then in 0.5 × SSC, 0.1% SDS (all at 50°C) prior to autoradiography at −70°C. Hybridisation with oligonucleotide probes was at 55°C in a mixture of 6 × SSC, 10 × Denhardt's solution, 0.2% SDS and 100 μg/ml sonicated denatured herring testes DNA. Filters were washed at room temperature in 0.1 × SSC plus 0.1% SDS, and then in 2 × SSC containing 0.1% SDS at 50°C.

3. RESULTS AND DISCUSSION

Screening of the clofibrate-induced rat liver cDNA library with monospecific polyclonal antiserum yielded several positive clones. The largest insert (1.2 kb; clone gt11-A) was labelled and used to rescreen the library, yielding a clone (gt11-B) with an insert of 2.1 kb. This clone, from a previous Northern blot analysis (not shown), must contain a virtually full-length cDNA.

The cDNA inserts from gt11-A and gt11-B were sequenced. Fig.1 shows the sequence from the presumed full-length clone gt11-B. The sequence of the coding region is identical to that of Hardwick et al. [1], thus confirming that cytochrome P-452 is identical to cytochrome P-450 LAω. With the partial cDNA clone (A) four base differences were found which may arise from reverse transcriptase infidelity in the construction of this clone.

A more significant discrepancy was found in the 3′-non-coding region. Although clone A was identical to P-450LAω throughout this region, clone B possessed an additional 75 nucleotides shortly after the terminating codon (1545–1619, fig.1). The significance of this sequence was investigated by Northern blots with a P-452 cDNA probe and two synthetic 20-mer oligonucleotides (fig.2). Probe 1 is complementary to the 3′-end of the coding region, which is the same in both clones A and B,

```
          .      1520          .      1540          .      1560
AAAAATGGGATCTACCTGTATCTCAAGAAGCTCCACTAATTCCGGACTTCTTCATTATAC
 K  N  G  I  Y  L  Y  L  K  K  L  H  *
                                                  ----------

          .      1580          .      1600          .      1620
CAATAATCAACGGTCACAACCTTGATGGTCGGGAATAACAGGACGTTATTATCTTACAGT
-------------------------------------------------------------
```

Fig.2. Sequence of oligonucleotide probes. Oligonucleotide probes were synthesized, complementary to the indicated (underlined) regions of the DNA sequence of clone gt11-B. Probe 1 was complementary to the sequence from 1525 to 1544, while probe 2 was complementary to bases 1571–1590: The sequence from 1545 to 1619, indicated by a broken line, represents the additional 75 base sequence present only in gt11-B. (*) Stop codon.

and should therefore detect both mRNA species (and possibly others not yet identified). Probe 2 is complementary to part of the 75 nucleotide insert and is thus specific for the insert sequence. This was confirmed by dot hybridisations with the two clones.

The P-452 cDNA probe (fig.3A) showed the presence of low levels of mRNA in untreated kidney and liver and a marked increased after clofibrate administration. The two synthetic pro-

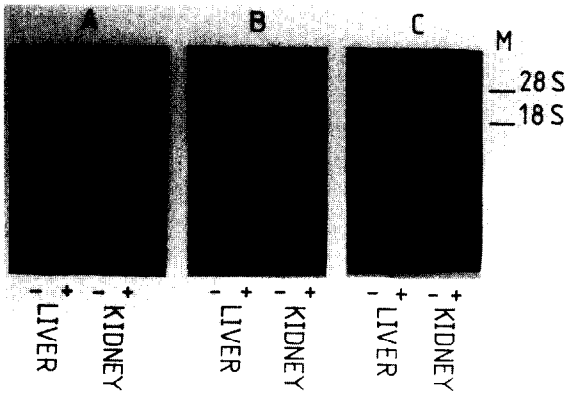


Fig.3. Northern blot analysis of cytochrome P-452 mRNA. 25 μg of total RNA isolated from liver or kidney from control (−) or clofibrate treated (+) rats was electrophoresed on 1.2% denaturing (formaldehyde) agarose gels and transferred to nylon filters. The probes were ^{32}P-labelled P-452 cDNA derived from clone gt11-B (A), or oligonucleotides 1 and 2 (B,C, respectively). Autoradiography was for 40 h. Ribosomal RNA size markers were used, as indicated.

Fig.1. Sequence of cytochrome P-452 as found in clone gt11-B. The arrow at position 899 indicates the 5′-end of the partial clone gt11-A. The isolated bases above the DNA sequence show the differences in the sequence determined for clone A. The sequence from 1545 to 1619, indicated by a broken line, is only present in gt11-B. (*) Stop codon.

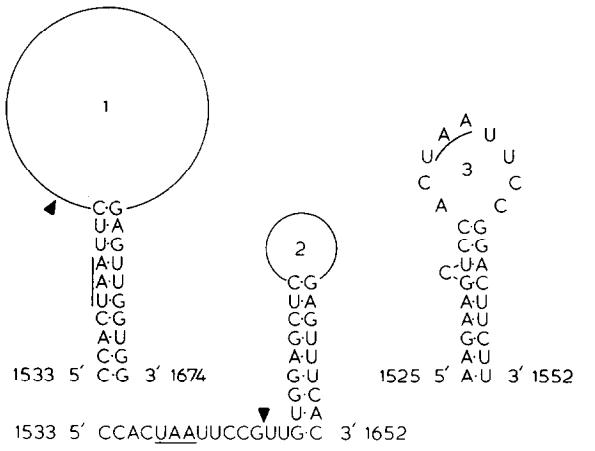


Fig.4. Potential stem-loop structures. Structures 1 and 2 are predicted in the mRNA corresponding to clone A. The arrowhead indicates the position of the 75 base insert in clone B. In the mRNA corresponding to clone B, structure 1 will be replaced by structure 3. The UAA stop codon is indicated by underlining; the numbering of all three structures is according to the sequence of clone B.

bes (fig.3B,C) detected a mRNA species of similar size that was present at a very low level in the control tissue and was dramatically induced by clofibrate. This establishes that mRNA containing the 75 nucleotide insert is present in the tissue and is induced by clofibrate. Therefore the presence of this sequence in clone B is not the result of a cloning artefact.

One possible explanation for this heterogeneity is that there are two almost identical copies of the gene, one of which has the extra 75 nucleotides adjacent to the termination codon. However, Southern blot analysis of rat genomic DNA digests using oligonucleotide 1 (which is common to both sequences) detects only a single band (not shown), which is consistent with the existence of a single gene.

A more likely explanation is that differential splicing of an intron can occur in the 3′-non-coding region. This intron would have a single 5′-splice donor site corresponding to position 1544 (fig.1) and two alternative 3′-splice acceptors. One of these, corresponding to position 1619 in fig.1, would give rise to the sequence seen in clone A, while the other acceptor site, at a position 75 nucleotides upstream, results in the gt11-B sequence which includes the 75 base insert. This is supported by the presence at 1619 of a canonical splice acceptor site TCTTACAG (fig.1).

Alternative RNA splicing has previously been shown for a human cytochrome P-450 [13]; this was within the coding region and the alternative mRNA did not appear to code for a functional protein. The results presented here are distinct in that the differential splicing occurs in the 3′-non-coding region, so both forms of the mRNA can code for a functional product. Differential splicing may however play a role in the regulation of gene expression. Analysis of the 3′-non-coding regions shows that different stem-loop structures are to be expected in the two forms of mRNA (fig.4). Such structures in the 3′-non-coding region have been reported to affect mRNA stability [14] or translational repression [15]. The biological significance of this phenomenon and its possible relevance to the control of expression of this gene is the subject of further investigations.

Acknowledgements: This work was supported by grants from the Wellcome Trust and the Medical Research Council. We are grateful to Claire Entwisle for the provision of oligonucleotide probes and to Catriona MacGeoch for supplying rat DNA and other assistance. The sequence data have been deposited with the EMBL Database under the accession number X07259.

REFERENCES

[1] Hardwick, J.P., Song, B.-J., Huberman, E. and Gonzalez, F.J. (1987) J. Biol. Chem. 262, 801–810.
[2] Nebert, D.W. and Gonzalez, F.J. (1987) Annu. Rev. Biochem. 56, 945–993.
[3] Tamburini, P.P., Mason, H.A., Bains, S.K., Makowski, R.J., Morris, B. and Gibson, G.G. (1984) Eur. J. Biochem. 139, 235–246.
[4] Bains, S.K., Gardiner, S.M., Mannweiler, K., Gillet, D. and Gibson, G.G. (1985) Biochem. Pharmacol. 34, 3221–3229.
[5] Matsubara, S., Yamamoto, S., Sogawa, K., Yokotani, N., Fujii-Kuriyama, Y., Haniu, M., Shively, J.E., Gotoh, O., Kusunose, E. and Kusunose, M. (1987) J. Biol. Chem. 262, 13366–13371.
[6] Reddy, J.K. and Lalwani, N.D. (1983) CRC Crit. Rev. Toxicol. 12, 1–58.
[7] Reddy, J.K., Goel, S.K., Nemali, M.R., Carrino, J.J., Laffler, T.G., Reddy, M.K., Sperbeck, S.J., Osumi, T., Hashimoto, T., Lalwani, N.D. and Rao, M.S. (1986) Proc. Natl. Acad. Sci. USA 83, 1747–1751.
[8] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[9] Perbal, B.V. (1984) A Practical Guide to Molecular Cloning, Wiley, New York.

[10] Huynh, T.V., Young, R.A. and Davis, R.W. (1985) in: DNA Cloning – A Practical Approach (Glover, D.M. ed.) vol.1, pp.49–78, IRL Press, Oxford.
[11] Buckland, R.M. (1986) Nature 320, 557–558.
[12] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[13] Okino, S.T., Quattrochi, L.C., Pendurthi, U.R., McBride, O.W. and Tukey, R.H. (1987) J. Biol. Chem. 262, 16072–16079.
[14] Shaw, G. and Kamen, R. (1986) Cell 46, 659–667.
[15] Dickey, L.F., Wang, Y.-H., Shull, G.E., Wortman, I.A. and Theil, E.C. (1988) J. Biol. Chem. 263, 3071–3074.