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COMPLETE cDNA SEQUENCE OF A MAJOR 3-METHYLCHOLANTHRENE-INDUCIBLE CYTOCHROME P-450 ISOZYME (P-450AFB) OF SYRIAN HAMSTERS WITH HIGH ACTIVITY TOWARD AFLATOXIN B_1

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SUMMARY: Cytochrome P-450AFB is a major isozyme inducible by 3-methylcholanthrene in Syrian golden hamsters and shows high potency toward aflatoxin B_1 activation. We have isolated and sequenced cDNA clones to P-450AFB by immunoscreening a hamster liver cDNA library in λ gt11. The longest clone contains an open reading frame of 1482 nucleotides and encodes a protein of 494 amino acids with a molecular weight of 57,420. The sequence of P-450AFB shares a 73 % and 65% homology with that of mouse P-450 15α (IIA3) and rat P-450a (IIA1), respectively, indicating that P-450AFB is a unique gene of the P-450 IIA subfamily. The apparent concentration of a mRNA species hybridizable to the clone as well as the concentration of a protein immunoreactive to P-450AFB was increased significantly by the treatment with 3-methylcholanthrene, which indicates that the increase in P-450AFB protein is due mainly to an elevation of the mRNA.

Cytochrome P-450 (P-450) is the terminal enzyme of the pathway involved in the metabolism of a wide variety of xenobiotics including carcinogens. The extremely broad substrate specificity of P-450 is partly a result of various forms of P-450. Extensive studies have revealed the existence of different forms of P-450 in various animal species, and cDNA specific for each of the P-450s has been isolated and classified into

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several subfamilies (1). However, the determination of cDNA for hamster P-450s has not yet been reported.

We demonstrated previously that liver fractions of PCB-treated Syrian golden hamsters possessed high potency toward aflatoxin B_1(AFB_1) activation and isolated a unique P-450 isozyme (P-450AFB or P-450-I) which exhibited a high specific activity to AFB_1 compared to the isozymes from other animals (2). More recently, we have provided immunochemical evidences using monoclonal and polyclonal antibodies that this isozyme is inducible by 3-methylcholanthrene-type inducers in hamsters (3) but not in other animal species (4,5).

To more characterize this P-450 isozyme of hamsters and establish its structural relationship to those of other animal species, we have isolated cDNA clones of this isozyme and sequenced the entire nucleotide sequence of the cDNA, which would be the first report on cDNA sequence of hamster P-450s. Sequence comparison indicates that this isozyme, most similar to mouse P-450$15\alpha$(IIA3) (6) and rat P-450a (IIA1) (7), is a hamster homologue of the P-450IIA gene subfamily.

MATERIALS AND METHODS

Protein Sequence Analysis of P-450AFB. P-450AFB protein was purified from livers of 3-methylcholanthrene-treated Syrian golden hamsters (Nippon SLC Co., Hamamatsu, Japan) as described in our previous paper (2) and analyzed for the N-terminal amino acid sequence by automatic Edman degradation using a gas phase protein sequencer (Applied Biosystems 4704) (8). Phenylthiohydantoin-amino acid derivatives were identified by a HPLC using a Senshupak Aquasil SE-4 column.

Screening of cDNA Library and Sequencing. cDNA library was constructed in the λgt11 expression vector (9) essentially as described (10, 11) with poly(A)$^+$ RNA isolated from livers of male hamsters treated with 3-methylcholanthrene (12,13). Recombinants from the cDNA library were screened with the use of the polyclonal antibodies against P-450AFB (14). Positive clones were isolated and the cloned cDNA inserts were purified after EcoRI digestion and were subcloned into pUC19 for the production of large amounts of DNA. A library of M13 clones was produced (15) by random sonication of the self-ligated insert cDNA, followed by ligation into SmaI-digested M13mp8 vector DNA. M13 DNA was sequenced by the dideoxynucleotide sequencing method

(16). Sequence data were analysed by MicroGenie software (Beckman Instruments Inc., Fullerton, CA).

Hybridization of RNA. Poly(A)$^+$ RNA was isolated from livers of hamsters that were untreated or treated i.p. either with phenobarbital (60 mg/kg) or with 3-methylcholanthrene (25 mg/kg) 20 hr prior to sacrifice (12,13). Poly(A)$^+$RNA was subjected to electrophoresis (17) and transferred to Nytran membranes (Schleicher & Schuell, West Germany). The membranes were hybridized with the probe prepared by nick translation of the fragment of the cloned DNA. The hybridized bands were visualized by autoradiography (18).

Immunoblot Analysis of Microsomal Protein. Hepatic microsomes were prepared from hamsters that were untreated or treated either with phenobarbital or 3-methylcholanthrene, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19). The proteins on the gels were transferred to nitrocellulose sheets (Schleicher & Schuell, West Germany) and immunostained for P-450AFB (20).

RESULTS AND DISCUSSION

A hamster liver cDNA library was screened with polyclonal antibodies raised against P-450AFB. From $6x10^5$ recombinants, 80 positive clones were obtained and cDNA inserts of 24 clones were isolated. Two clones with relatively longer cDNA inserts were characterized. The clones contained an internal EcoRI site and were digested into two fragments with approximate length of 0.6 kilobases (kb) and 1.2 kb. Subsequent sequence analysis has shown that the clones were identical in their overlapping regions and that the fragments of 0.6 kb and 1.2 kb encode the 5' end and 3' end regions of P-450AFB mRNA, respectively. As presented in Fig. 1, the entire cDNA clone for P-450AFB contains 12 nucleotides of 5' noncoding region and a reading frame nucleotide of 1482 which encodes a protein of 494 amino acids for a molecular weight of 57,420, which is approximately equivalent to the value of 56,000 estimated from SDS-PAGE (2). The 3' noncoding region contains 300 nucleotides including a poly(A)$^+$ tail and a putative poly(A)$^+$ addition site signal AATAAT. As for N-terminal amino acid sequence, the first 20 amino acids sequence determined by Edman degradation of the purified protein was identical to the residues 1-20 predicted

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                                                                    TCCACTGCCACC    12

ATGCTGGTGTCCGGGATGCTCCTCGTGGTTGTGCTAACCTGCCTCAGCGTCATGATCATAATGTCTGTGTGGAGGCAGAGGAGACTGTTG   102
MetLeuValSerGlyMetLeuLeuValValValLeuThrCysLeuSerValMetIleIleMetSerValTrpArgGlnArgArgLeuLeu
                                                                                          30
AGGAAGATGCCTCCAGGACCCACCCCACTGCCCTTTATTGGGAACTTTCTGGAGCTGGACACAGAGAAGTTTTATGACTGCCTTTCAAAG   192
ArgLysMetProProGlyProThrProLeuProPheIleGlyAsnPheLeuGluLeuAspThrGluLysPheTyrAspCysLeuSerLys
                                                                                          60
ATGAGGGAGCGGTATGGCCCTGTGTTCACCATCCACCTGGGGCCTCGACCTGCTGTGATGCTGTGGGGTTACGATGCTGTGAAGGAGGCT   282
MetArgGluArgTyrGlyProValPheThrIleHisLeuGlyProArgProAlaValMetLeuTrpGlyTyrAspAlaValLysGluAla
                                                                                          90
CTCATTGACCAGGCTGAGGAGCTCAGTGACCGAGGAGAGCAAGCTTTCTTCGACTGGTTCTTCAAAGGCTATGGTGTGGTGTTCAGCTCC   372
LeuIleAspGlnAlaGluGluLeuSerAspArgGlyGluGlnAlaPhePheAspTrpPhePheLysGlyTyrGlyValValPheSerSer
                                                                                         120
GGGGAGCGCGCCAAGCAACTCAGGCGCTTCTCCATCGCCACGCTGAGGGACTTCGGCTTTGGAAAACGTGGCATTGAGGAGCGCACCATA   462
GlyGluArgAlaLysGlnLeuArgArgPheSerIleAlaThrLeuArgAspPheGlyPheGlyLysArgGlyIleGluGluArgThrIle
                                                                                         150
GAGGAGACCAGCTTTCTCATACAGGCCCTGCGGGACACAAACGGTGCCACAATAGACCCCACCTTCTACATGAGCCGGACAGTCTCCAAC   552
GluGluThrSerPheLeuIleGlnAlaLeuArgAspThrAsnGlyAlaThrIleAspProThrPheTyrMetSerArgThrValSerAsn
                                                                                         180
GTCATCAGTTCCATTGTGTTTGGGAACCGCTTTGAATATGACGACAAGGAATTCTTGTCACTGTTGGGCATGATAATGCGAAGTTTCCAG   642
ValIleSerSerIleValPheGlyAsnArgPheGluTyrAspAspLysGluPheLeuSerLeuLeuGlyMetIleMetArgSerPheGln
                                                                                         210
TTCATGTCTACTTCAACAGGACAGCTCTTTGAGATGTTCTATTCAGTGATGAAGCACCTGCCAGGATGCCAGCACCAGGCCTATAAGGAA   732
PheMetSerThrSerThrGlyGlnLeuPheGluMetPheTyrSerValMetLysHisLeuProGlyCysGlnHisGlnAlaTyrLysGlu
                                                                                         240
ATGCAGGGACTGGAGGACTTCATAGCCAGGAAGGTGGAAGAGAACCAACGCACCCTGGACCCCAACTCCCCCCGGGACTTCATCGACTCC   822
MetGlnGlyLeuGluAspPheIleAlaArgLysValGluGluAsnGlnArgThrLeuAspProAsnSerProArgAspPheIleAspSer
                                                                                         270
TTCCTCATCCGCATGCAGGAGGAGAAGAAGAACCCTCGAACTCAGTTTCACATGAGGAACCTGCTCATGACCACACTGAACCTTTTCTTC   912
PheLeuIleArgMetGlnGluGluLysLysAsnProArgThrGlnPheHisMetArgAsnLeuLeuMetThrThrLeuAsnLeuPhePhe
                                                                                         300
GCGGGTACAGAGACCGTCAGCACAACCACGCGTTACGGCTTCCTGCTGCTCATGAAGTACCCTCACATTGCAGCCAAGATGCATGAGGAA  1002
AlaGlyThrGluThrValSerThrThrThrArgTyrGlyPheLeuLeuLeuMetLysTyrProHisIleAlaAlaLysMetHisGluGlu
                                                                                         330
ATTGACCAGGTGATTGGCAGGAACAGGCAGCCCAAGTATGAGGACCATTTGAAGATGCCCTACACTGAGGCTGTCATCTACGAGATCCAG  1092
IleAspGlnValIleGlyArgAsnArgGlnProLysTyrGluAspHisLeuLysMetProTyrThrGluAlaValIleTyrGluIleGln
                                                                                         360
AGATTTGTAGATGTGGTTCCTTTGGGTCTGCCCCGTAGCACCACCAAGGACATCAAGTTTCGGGACTTCCTCATTCCCAAGGGCACTGAC  1182
ArgPheValAspValValProLeuGlyLeuProArgSerThrThrLysAspIleLysPheArgAspPheLeuIleProLysGlyThrAsp
                                                                                         390
GTTTTCCCTGTACTGAGCTCTGTGCTGAAGGACCCCAAGTTCTTCTCCAACCCCAACGACTTTAACCCCCAGCACTTCCTGGATGACAAG  1272
ValPheProValLeuSerSerValLeuLysAspProLysPhePheSerAsnProAsnAspPheAsnProGlnHisPheLeuAspAspLys
                                                                                         420
GGACAGTTTAAGAAGAGCAATGCTTTTATGCCCTTCTCCGTTGGAAAGCGATACTGTTTTGGAGAAAGCCTGGCTAAGATGGAGCTCTTC  1362
GlyGlnPheLysLysSerAsnAlaPheMetProPheSerValGlyLysArgTyrCysPheGlyGluSerLeuAlaLysMetGluLeuPhe
                                                                                         450
ATCTTCTTCACAACCATCATGCAGAATTTCTGCTTCAAGTCCCCACAGGCACCCCAAGACATAGATGTGACCCCACAATATTTCAGCTTT  1452
IlePhePheThrThrIleMetGlnAsnPheCysPheLysSerProGlnAlaProGlnAspIleAspValThrProGlnTyrPheSerPhe
                                                                                         480
GCCGCAATCCCTCCAAAATTCACCATGAGCTTCCTGCCTCGCTGAGCGGGAACTCTGATGGGTGGAGACAATGAGCATGTCCAGAAACAG  1542
AlaAlaIleProProLysPheThrMetSerPheLeuProArgEnd
                                                494
GGCGGGGCTAATGGGGTGGGGCCAATCCGGGTAGGGCTAAAGGAGAGCATGAAAATTAGAGGGAAGTCTGGGGCCTGAAGTATTACACAG  1632

AGAGAGAGAGAGAGCTGAGCAGAGTGATCACCTTCCTGAAGACGGGTTCTTCAAAGTTGGGAAGAGAGGCTGGGATGCCTTCCCGTCGTA  1722

TCTGAACACCGATCGTAATAATTAAAGCTATTGTTGATTGTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                  1794
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Fig. 1. Nucleotide and deduced amino acid sequence of P-450AFB cDNA. Internal EcoRI site and the homologous C-terminal cysteine region are underlined. Boxed sequence denotes poly(A)$^+$ addition site signal. Nucleotides are numbered to the right of each line and amino acids are numbered below the corresponding residue.

Table 1. Comparison of deduced amino acid sequences between P-450AFB and other cytochorme P-450s

Subfamily	Animal	Protein	Amino acid homology (%)		
			Whole region	N-terminal region	C-terminal cysteine region
IIA3	Mouse	P-45015α	73.4	55	80.1
IIA1	Rat	P-450a	65.3	65	66.6
IIA2	Rat	P-450a2	61.6	65	71.4
IIB1	Rat	P-450b	54.5	30	61.9
IIC7	Rat	P-450f	48.3	10	66.6
IIE1	Rat	P-450j	42.5	10	66.6
IID1	Rat	P-450db1	38.1	20	66.6
IA2	Rat	P-450d	32.4	20	47.6
IIIA4	Human	P-450NF	25.0	20	33.3
IVA1	Rat	P-450LAω	24.0	25	38.0

The results are given as the percent homology with P-450AFB in the whole sequence, in the N-terminal sequence of 20 amino acids and in the C-terminal sequence of 21 amino acids surrounding cysteine residue. The subfamily names are taken from the nomenclature proposed by Nebert et al. (1).

from cDNA sequence, which confirmed that the clones were derived from a P-450AFB mRNA.

As presented in Table 1, the comparison with P450s of various gene subfamilies has shown that P-450AFB protein sequence has high similarity with those of P-450IIA subfamily, sharing a 73 %, 65 % and 62 % homology with mouse P-45015α (IIA3) (6), rat P-450a (IIA1) (7) and P-450a2 (IIA1) (21), respectively. This and the characteristics of P-450AFB, especially its extremely high inducibility by 3-methylcholanthrene (more than 100-fold) and high potency toward AFB_1 activation, suggests that P-450AFB belongs to IIA subfamily but is a unique gene in this subfamily. In addition, P-450AFB showed higher similarity with phenobarbital-inducible P-450s of IIB family (P-450b, P-450e) (22) than with 3-methylcholanthrene-inducible P-450s of IA subfamily (450c,

P-450d) (23,24). It is to be noted that P-450AFB has very low similarity with P-450NF (IIIA4) that was reported responsible for AFB_1 activation in human livers (25).

Comparison of the N-terminal region shows that P-450AFB shares 55 - 65 % identical residues in the first 20 amino acids with P-450s of IIA subfamily, while it shares less than 30 % identical residues with other subfamilies. On the contrary, the C-terminal region containing cysteine residue is more conserved, and similarity in this region can reach 80 % between P-450AFB and P-45015α and over 60 % with P-450s of II gene family. This confirms further that P-450AFB belongs to P-450IIA subfamily.

The size of the mRNA encoding P-450AFB was determined by hybridization of poly(A)$^+$ RNA from hamster livers with the probe prepared from 5' end half of the cDNA consisting of 600 base pairs. A strongly hybridizing band of mRNA species was detected

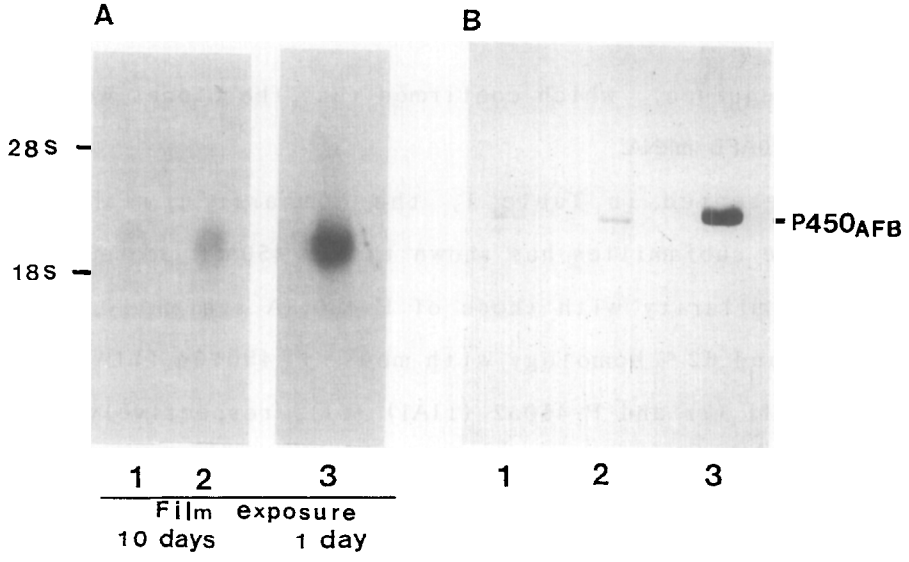

Fig. 2. Hybridization analysis of mRNA and immunoblot analysis of microsomal proteins prepared from the livers of hamsters. Panel A; autoradiograph of mRNA (2 µg) hybridized to the nick translated probe of the fragment of the isolated clone. Autoradiographic exposure time is 10 days for lane 1 and 2, and 1 day for lane 3. Panel B; immunoblot of microsomal proteins (4 µg) immunostained using the antibodies against P-450AFB. In both A and B, lane 1, untreated hamsters; lane 2, phenobarbital-treated hamsters; lane 3, 3-methylcholanthrene-treated hamsters.

with an approximate size of 2.3 kb. An additional faint band was detected in the ranges of 4.0-4.5 kb. The apparent concentration of the major mRNA species was much higher in the livers of hamsters treated with 3-methylcholanthrene than in those of untreated or phenobarbital-treated hamsters (Fig. 2A), which correlated well with the levels of the protein determined by immunoblot analysis (Fig. 2B). These results suggest that the induction of P-450AFB protein is due mainly to an elevation of its mRNA.

The present study has shown for the first time the isolation of cDNA clones for a P-450 isozyme of hamsters, which might be a unique gene of P-450IIA subfamily. Further study would contribute to elucidate the role of the isozyme in AFB_1-toxicity and the mechanism of induction in hamsters by 3-methylcholanthrene.

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REFERENCES

1. Nebert, D. W., Nelson, D. R., 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, R. (1989) DNA 8, 1-13.
2. Mizokami, K., Nohmi, T., Fukuhara, M., Takanaka, A., and Omori, Y.(1986) Biochem.Biophys. Res. Commun. 139, 466-472.
3. Sunouchi, M., Fukuhara, M., Ohno, Y., and Takanaka, A. (1988) J.Toxicol. Sci.(Tokyo) 13, 193-204.
4. Sunouchi, M., Ishimi, Y., Mizokami, K., Fukuhara, M., Takanaka, A., Hanaoka, F., and Yamada, M. (1988) Biochem. Biophys. Res. Commun. 153, 441-447.
5. Fukuhara, M. Nohmi, T., Mizokami, K., Sunouchi, M., Ishidate, M. Jr., and Takanaka, A. (1989) J.Biochem.(Tokyo) in press.
6. Squires, E. J., and Negishi, M. (1988) J. Biol. Chem. 263, 4166-4171.
7. Nagata, K., Matsunaga, T., Gillette, J., Gelboin, H. V.,and Gonzalez, F. J. (1987) J.Biol.Chem. 262, 2787-2793.
8. Hunkapiller, M. W., Hewick, R. M., Dreyer, W. J., and Hood, L. E. (1983) In Methods in Enzymol. (C.H.W. Hirs, and S.N. Timasheff,Ed.) Vol.91, pp.399-413. Acad.Press, New York.
9. Young, R.A. and Davis, R.J. (1983) Proc. Natl. Acad. Sci. USA 80, 1194-1198.

10. Watson, C. J. and Jackson, J. F.(1985) In DNA Cloning (D.M. Glover, Ed.) Vol.1, pp.79-88. IRL Press, Oxford.
11. Gubler, U. and Hoffman, B. J. (1983) Gene 25, 263-269.
12. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutler, W. J. (1979) Biochemistry 18, 5294-5299.
13. Aviv, H., and Leder, K. (1972) Proc. Natl. Acad. Sci. USA 69, 1408-1412.
14. Young, R. A. and Davis, R.W. (1983) Science 222, 778-782.
15. Deininger, P.L. (1983) Anal. Biochem. 129, 216-223.
16. Sanger, F., Niklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
17. Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, p.202. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
18. Southern, E. M. (1975) J. Mol. Biol. 98, 503-517.
19. Laemmli, U. K. (1970) Nature 227, 680-685.
20. Guengerich, F.P., Dannan, G. A., Wright, S. T., Martin, M. V. and Kaminsky, L. S. (1982) Biochemistry 21, 6019-6030.
21. Matsunaga, T., Nagata, K., Holsztynska, E. J., Lapenson, D. P., Smith, A., Kato, R., Gelboin, H. V., Waxman, D.J. and Gonzalez, F. J. (1988) J. Biol. Chem. 263, 17995-18002.
22. Fujii-Kuriyama, Y., Mizukami, Y., Kawajiri, K., Sogawa, K. and Muramatsu, M. (1982) Proc. Natl. Acad. Sci. USA 79,2793-2797.
23. Sogawa, K., Gotoh, O., Kawajiri, K. and Fujii-Kuriyama, Y. (1984) Proc. Natl. Acad. Sci. USA 81, 5066-5070.
24. Kawajiri, K., Gotoh, O., Sogawa, K., Tagashiro, Y., Muramatsu, M. and Fujii-Kuriyama, Y. (1984) Proc. Natl.Acad. Sci. USA 81, 1649-1653.
25. Shimada, T. and Guengerich, F.P. (1989) Proc. Natl. Acad. Sci. USA 86, 462-465.