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Human NADPH-P450 Oxidoreductase: Complementary DNA Cloning, Sequence and Vaccinia Virus-Mediated Expression and Localization of the *CYPOR* Gene to Chromosome 7

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SUMMARY

The cDNA containing the full coding sequence of human NADPH-P450 oxidoreductase was isolated and completely sequenced. The cDNA contained 2398 base pairs, including 9 and 358 base pairs of 5′ and 3′ noncoding sequences, respectively. The human NADPH-P450 oxidoreductase protein deduced from the cDNA has 677 amino acids, with a calculated molecular weight of 76,656. The cDNA nucleotide and deduced amino acid sequences displayed 83 and 92% similarities, respectively, with those of the rat NADPH-P450 oxidoreductase. By use of somatic cell hybrids, the NADPH-P450 oxidoreductase gene was regionally localized to human chromosome 7 (7p15–q35). The levels of NADPH-P450 oxidoreductase protein and mRNA were analyzed in 13 human liver specimens and less than 3-fold variation was found among the different livers. The NADPH-P450 oxidoreductase cDNA was inserted into vaccinia virus and expressed in cell culture. The cDNA-expressed enzyme was active in reducing the electron acceptor cytochrome *c*. In addition, the NADPH-P450 oxidoreductase stimulated the enzymatic activity of vaccinia virus-expressed human P_3450 when both recombinant viruses were used to coinfect human cells in culture. An approximate equal mole level of NADPH-P450 oxidoreductase and P_3450 was required to achieve maximal activity for both ethoxycoumarin *O*-deethylase and aryl hydrocarbon hydroxylase.

OR is a flavoprotein that contains 1 mol each of FMN and FAD (1). The enzyme is anchored to the lipid bilayer of the endoplasmic reticulum (2, 3) via a hydrophobic amino terminal peptide (4, 5) and it is presumed that the bulk of the enzyme is exposed to the cytoplasmic surface of this intracellular membrane system. It is here that OR functions to transfer electrons from NADPH to the different forms of cytochrome P450. The amino acid sequences of rat (6, 7), rabbit (8), and yeast (9) OR were determined through cDNA sequencing, whereas the structures of the pig (10, 11) and trout (12) enzymes were determined by direct protein sequencing.

In the present report, we have isolated and sequenced the human OR cDNA. We have used this cDNA as a probe to map the chromosomal location of the OR gene in humans. The levels of OR protein and mRNA were also analyzed in several human liver specimens. Finally, the OR cDNA was inserted into vaccinia virus and the resultant recombinant virus was used to produce active enzyme in cell culture. The expressed OR was able to supply electrons to vaccinia virus-expressed P_3450[1] when both recombinant viruses were used for coinfecting human cells.

Experimental Procedures

Antibody production and immunoblotting. Rat OR was prepared from animals that had received phenobarbital, using the procedure of Yasukochi and Masters (13). P450d was prepared from 3-methylcholanthrene-treated rats by the protocol of Goldstein *et al.* (14). These preparations of antigens yielded single bands when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15). Young female New Zealand white rabbits were used to prepare polyclonal antibodies against OR and P450. These antibodies were used to quantitate levels of human OR and P_3450 by Western immunoblotting analysis (16). The immunoblots were developed using alkaline phosphatase-conjugated goat anti-rabbit IgG (KPL Laboratories, Gaithersburg, MD).

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[1] P_3450 has been designated IA2 and is coded by the *CYP1A2* gene (51). P450d is the rat IA2 and P450MC is rat IA1.

ABBREVIATIONS: OR, NADPH-P450 oxidoreductase; vWT, vOR and vP_3450, wild type and recombinant vaccinia viruses containing oxidoreductase and P_3450 cDNAs, respectively; TK, thymidine kinase; bp, base pairs; kb, kilobases.

RNA purification and analysis and cDNA cloning and sequencing. The liver samples were from kidney donors and were provided by Drs. T. Inaba and W. Kalow at the University of Toronto (K series), Dr. U. A. Meyer at the Biocenter, University of Basel (KDL series), and the University of Miami kidney donor program (M series). RNA was prepared from 5-g portions of frozen liver using the technique of Chirgwin *et al.* (17), except that cesium trifluoroacetate (Pharmacia Fine Chemicals, Piscataway, NJ) was substituted for CsCl. Total RNA was analyzed by electrophoresis on 2.2 M formaldehyde-containing 1% agarose gels (18), blotting to Nytran membranes (Schleicher and Schuell, Keene, NH) and hybridization to nick-translated cDNA probes. Filter hybridizations and washings were carried out as described by Church and Gilbert (19).

The OR and P_3450 cDNA clones were isolated from a λgt11 (20) library that was prepared from human liver poly(A) RNA using the procedure described by Watson and Jackson (21). This library was used for earlier studies (22, 23). Nick-translated rat OR (24) and mouse P_3450 (25) cDNA inserts were used to screen the library. The longest cDNAs obtained were cloned into the M13 mp11 *Eco*RI site and their 3′- and 5′-most ends were sequenced by the dideoxy chain-termination method (26). The human OR cDNA sequence was completely determined using the shotgun cloning method of Deininger (27). The human P_3450 cDNA sequence has already been established (28, 29).

Expression of OR and P_3450 cDNAs. OR and P_3450 were expressed using the vaccinia virus expression system (30). Vaccinia virus strain WR, plasmid pSC11, and TK⁻ 143 cells were kindly provided by Dr. Bernard Moss at the National Institute of Allergy and Infectious Diseases. CV-1 cells, used in the production of recombinant Vaccinia, were obtained from the American Type Culture Collection (ATCC CCL70). The cDNAs were inserted into the plasmid pSC11 (31). The recombinant plasmids were then allowed to integrate into wild type Vaccinia virus strain WR using the TK gene inactivation selection protocol (32). vOR and vP_3450 were used to infect TK⁻ cells. After 48 hr of infection, the cells were harvested and lysed by sonication for 5 sec, and protein levels were assayed by immunoblotting with antibody prepared against the rat enzymes. OR activities were monitored by reduction of cytochrome *c*. P_3450-mediated ethoxycoumarin *O*-deethylase (33) and aryl hydrocarbon hydroxylase (34) activities were assayed by standard procedures. For mixing experiments, vOR and vP_3450 were used to coinfect TK⁻ cells and immunoblotting was performed using a mixture of antibodies.

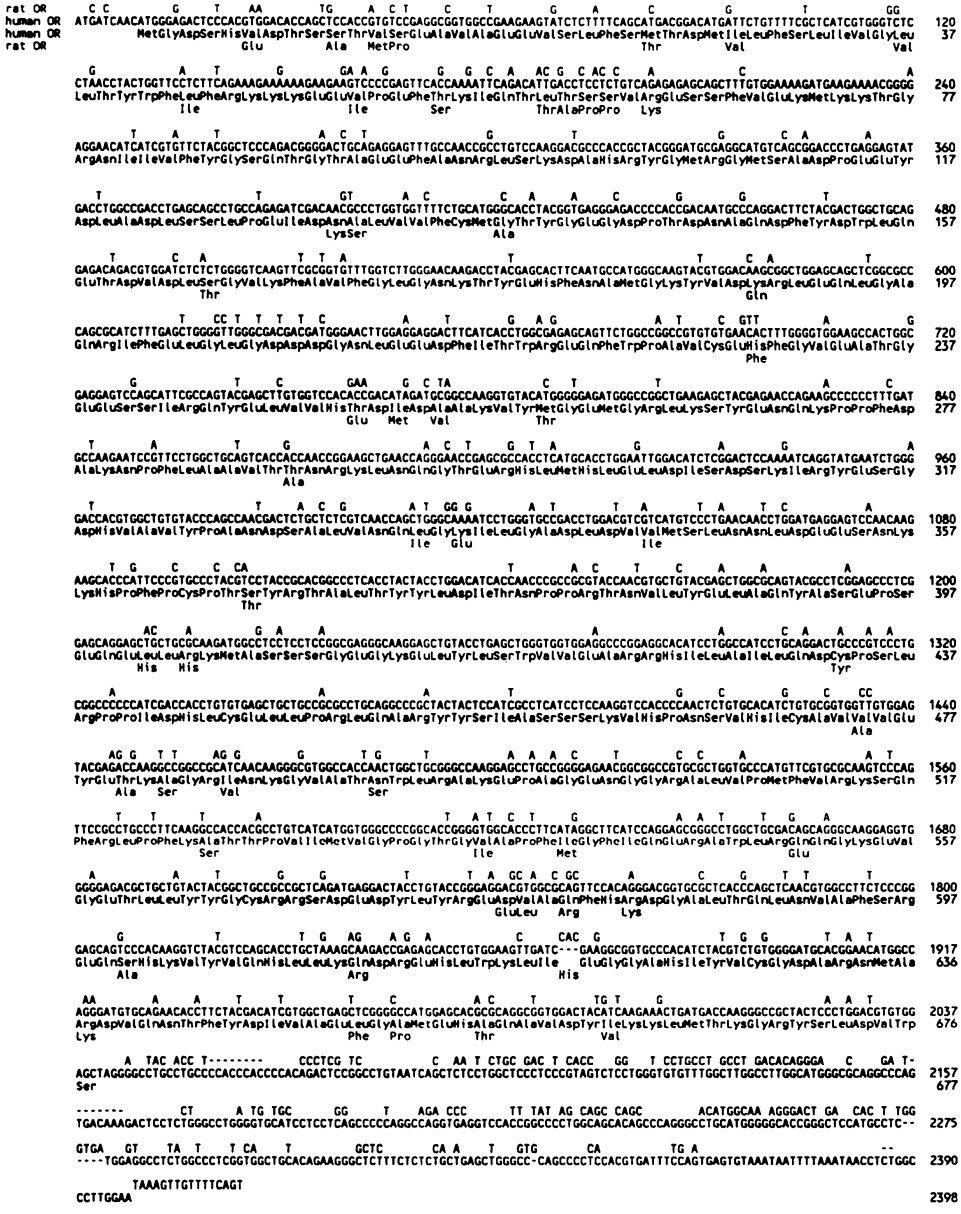

Fig. 1. Sequence of the human OR cDNA. The complete cDNA and deduced amino acid sequences of OR are presented. The nucleotide and amino acid residues of rat OR that differ from the human enzyme are displayed *above* and *below* the human sequences, respectively.

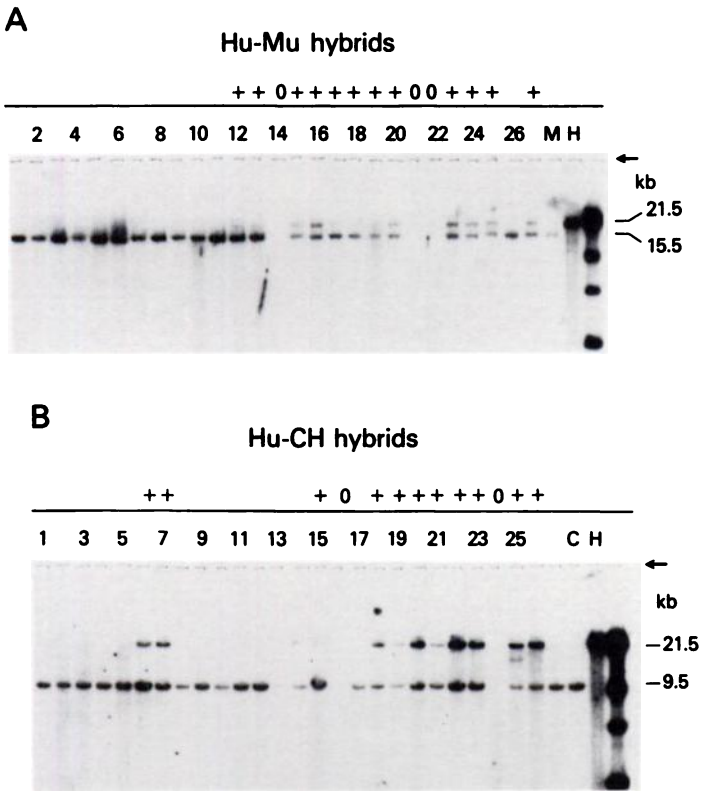

Fig. 2. Southern analysis of representative *Eco*RI-digested human-mouse (*Hu-Mu*) (A, *lanes 1–27*) and human-Chinese hamster (*Hu-CH*) (B, *lanes 1–27*) somatic cell hybrid DNAs. DNAs (10 μg/lane) from individual cell lines were digested to completion with *Eco*RI, electrophoresed on agarose gels, transferred to nylon membranes, and annealed with the full-length OR cDNA. The filters were washed under high stringency (55° in 0.015 M NaCl, 0.2% sodium dodecyl sulfate) and subjected to autoradiography. Chinese hamster (*C*), mouse (*M*), and human (*H*) parental cell DNAs were also analyzed. The standards at the *right* of each panel are ^{32}P-labeled *Hind*III-digested λ DNA markers of 23, 9, 6.6, and 4.4 kb. The *horizontal arrow* denotes the origin of migration of the DNAs and the + represents the presence of a human OR-specific band. The *lanes* labeled *0* were not scored.

Chromosome localization of the OR gene. The somatic cell hybrid procedure was used to map the OR gene. Panels of human-mouse and human-Chinese hamster cell lines that contain subsets of human chromosomes in a rodent chromosome background were prepared. The cell lines used in this report have been described (35–37) and they were characterized by karyotypic analyses of banded mitotic chromosomes and electrophoretic assays of human biochemical markers (35–37). The presence of the OR gene in the hybrid cell lines was assayed by Southern blotting (38) using a nick-translated cDNA insert. The hybridization and washings were performed using high stringency conditions, in which only sequences of greater than 90% similarity are detected (39).

Results and Discussion

Cloning and sequencing of human OR. The human OR cDNA was isolated from a λgt11 library, using a portion of the rat OR cDNA as a probe for screening (24). The cDNA contained 2398 bp, including a complete open reading frame coding for 677 amino acids and 9 bp and 358 bp of 5′ and 3′ noncoding sequences, respectively (Fig. 1). Two in-frame ATG codons were found at the 5′ end of the human OR cDNA. However, the first ATG was not found in the rat OR sequence. We, therefore, assigned the second ATG as the initiation Met residue for the human OR. The OR cDNA-deduced protein had a calculated M_r of 76,656 and displayed 92% amino acid sequence similarity with the rat enzyme. The putative functional domains for interactions of the protein with FAD, FMN, and NADPH, as previously described (11, 40), were well conserved with the domains in the rat, rabbit, and pig enzymes (data not shown). Interestingly, the histidine residue at position 621 in the rat enzyme (Fig. 1), also found in rabbits, pigs, and trout, is deleted in human OR, due to the absence of the CAC codon.

TABLE 1

Segregation of OR gene with human chromosome 7

The human OR gene was detected as a 21.5-kb hybridizing band in *Eco*RI digests of human-rodent somatic cell hybrid DNAs after Southern hybridization with a 2.4-kb full-length human OR cDNA probe. This band was well resolved from cross-hybridizing 9.5-kb and 15.5-kb hamster and mouse sequences, respectively. Detection of the gene is correlated with the presence or absence of each human chromosome in the group of somatic cell hybrids. Discordancy represents presence of the gene in the absence of the chromosome (+/−) or absence of the gene despite the presence of the chromosome (−/+), and the sum of these numbers divided by total hybrids examined (×100) represents per cent discordancy. The human-hamster hybrids consisted of 28 primary hybrids and 14 subclones (7 positive of 42 total) and human-mouse hybrids represented 14 primary clones and 37 subclones (29 positive of 51 total). The hybrid cell DNAs were also analyzed with human chromosome 7-specific probes including P450 PCN (7q21–q22), T cell receptor β (7q32–q35), T cell receptor γ (7p15), and the *Met* protooncogene (7q21–q31). One human-hamster hybrid and subclone retained T cell receptor γ but none of the other chromosome 7 markers, whereas another hybrid retained T cell receptor β alone. The results with these hybrids containing spontaneous breaks involving chromosome 7 permit regional localization of the OR to 7p15–q35.

Human Chromosome	Gene/Chromosome +/+	+/−	−/+	−/−	Discordancy
					%
1	20	16	15	42	33
2	14	22	9	48	33
3	12	24	24	33	52
4	20	16	39	28	59
5	13	23	18	39	44
6	26	10	23	34	35
7	36	0	0	57	0
8	18	18	19	38	40
9	16	20	16	41	39
10	15	21	5	52	28
11	13	23	20	37	46
12	13	23	22	35	48
13	24	12	9	48	23
14	21	15	20	37	38
15	28	8	16	41	26
16	12	24	23	34	51
17	29	7	27	30	37
18	23	13	25	32	41
19	17	19	11	46	32
20	20	16	21	36	40
21	27	9	33	24	45
22	13	23	20	37	46
X	19	17	28	29	48

Chromosome localization of the OR. The OR gene was mapped using the somatic cell hybrid strategy. Panels of human-mouse and human-Chinese hamster hybrids were analyzed (Fig. 2). The human gene was detected as a 21.5-kb *Eco*RI fragment, which was clearly resolved from the mouse 15.5-kb and hamster 9.5-kb hybridizing fragments. The results, summarized in Table 1, demonstrated that the presence or absence of the OR gene was correlated with the presence or absence of human chromosome 7. The OR gene segregated discordantly (≥23%) with all other human chromosomes. Two hybrids were analyzed that contained spontaneous breaks involving chromosome 7. These cell lines were also examined with probes for genes previously localized to specific regions of this chromo-

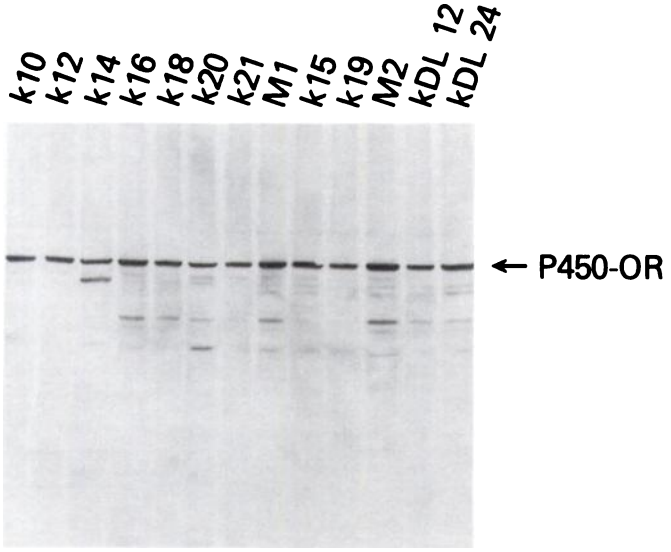

Fig. 3. Immunoblotting analysis of OR in different human liver specimens. Liver microsomal protein (50 μg) was electrophoresed on a 10% sodium dodecyl sulfate-containing polyacrylamide gel, transferred to nitrocellulose filter, and reacted with antibody to rat OR. The labeled proteins were developed using alkaline phosphatase-conjugated goat anti-rabbit IgG.

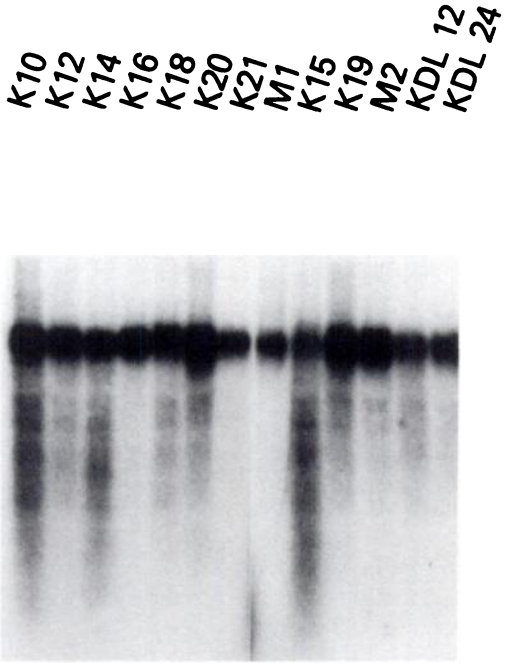

Fig. 4. Expression of OR mRNA in human liver. Total RNA (10 μg/lane) was electrophoresed on 2.2 M formaldehyde-containing agarose gels, transferred to nylon membranes, and hybridized to ^{32}P-labeled OR cDNA. The filter was washed and subjected to autoradiography. In a separate experiment, the OR mRNA size was estimated at 2.6 ± 0.2 kb.

some (Table 1, legend). One cell retained the T cell receptor γ gene located at 7p15 but lacked other genes on chromosome 7, whereas another cell line retained only T cell receptor β located at 7q21–35. These results permit the assignment of the OR gene *CYPOR* to 7p15–q35. It is noteworthy that the human *CYP3A* P450 gene family locus is located on 7q21.3–q22.1 (41). In addition, both the OR locus and the *CYP3A* locus reside on mouse chromosome 6 (42).

Expression of the OR gene in human liver. The level of OR in 13 human liver specimens was examined by Western immunoblotting analysis (Fig. 3). The enzyme was found in every liver sample examined. Surprisingly, there was less than 2-fold variation between samples in the amount of expressed protein. The amount of OR mRNA was also similar between liver specimens, except for slightly lower levels in K21, M1, K15, and KDL 12 (Fig. 4). The K15 mRNA appears to be slightly degraded, however. The rather constant level of OR expression between liver samples is in marked contrast to the 5- to 10-fold variability in the expression of some forms of P450 in human liver specimens, including P450d (43), P450PCN1 (22, 44), P450j (45, 46), and P450 IID1 (23).

Expression of the OR and $P_3$450 in vaccinia virus. The OR and $P_3$450 cDNAs were inserted into vaccinia virus and the recombinant viruses were used to infect TK$^-$ cells. The vOR produced a protein of M_r 78,000 and the v$P_3$450 produced a protein of M_r approximately 52,500 (Fig. 5). Infection of cells with wild type virus vWT yielded no immunodetectable bands. The ratios of infecting virus expressing OR and $P_3$450 influenced the levels of immunodetectable proteins; the amount of expressed OR increased with increasing amounts of vOR while the level of expressed $P_3$450 slightly decreased. The decrease in $P_3$450 is probably due to competition of the two infecting viruses in the cell.

The activities of OR and $P_3$450 also changed when the ratios of vOR and v$P_3$450 were altered (Table 2). The activity of OR in cells infected with 15×10^7 plaque-forming units of vOR was 50 nmol of cytochrome *c* reduced/min/mg of cell lysate protein. Only trace levels of OR activity were found in cells infected with vWR or v$P_3$450 alone. Analysis of the $P_3$450-associated activities, ethoxycoumarin *O*-deethylase and aryl hydrocarbon hydroxylase, revealed that cells infected with v$P_3$450 had both

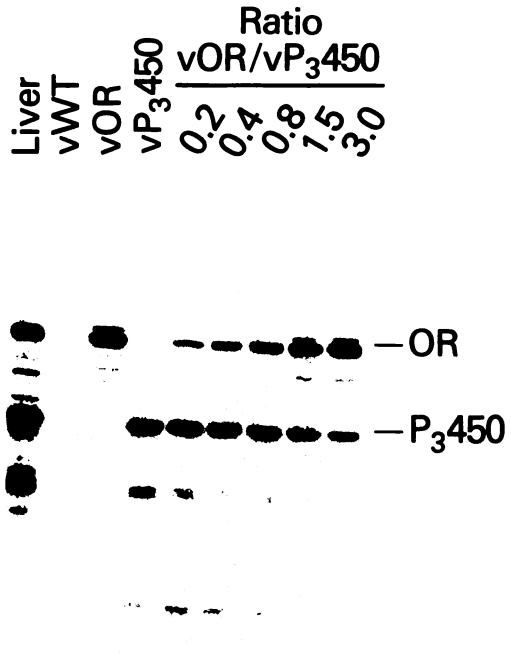

Fig. 5. cDNA-directed expression of OR and $P_3$450 using vaccinia virus. Human microsomal protein (50 μg) and total protein lysate (50 μg) from vaccinia-infected cells were subjected to immunoblotting analysis, using a mixture of antibodies to rat OR and P450d proteins. The *ratio* refers to the infecting OR and $P_3$450 vaccinia virus particles (see details of Table 2).

TABLE 2

Activities of OR ethoxycoumarin-*O*-deethylase, and aryl hydrocarbon hydroxylase in recombinant vaccinia-infected cells

TK⁻ cells were infected with VOR and/or vP$_3$450 at the pfu (plaque-forming units) indicated. Cell extracts were then prepared and assayed for the three activities.

Cell infection			P$_3$450[a]	OR[b]	Ethoxycoumarin *O*-Deethylase	Aryl hydrocarbon hydroxylase
vOR	vP$_3$450	vOR/vP$_3$450				
pfu × 10^7/175-cm^2 flask			*pmol/mg*	*nmol/min/mg*	*pmol/min/nmol of P$_3$450*	
0	0		ND[c]	0.59 —[d]	ND	ND
	0		ND	49.8 —	ND	ND
0	10		5	0.97 —	60	19
1.5	10	0.15	13	7.32 (6)	360	61
4	10	0.37	12	13.4 (12)	500	69
7	10	0.75	11	21.3 (18)	700	98
15	10	1.50	8	30.8 (26)	750	94
30	10	3.00	7	42.7 (34)	780	96

[a] The level of P$_3$450 was spectrally quantitated using the reduced carbon monoxide-binding extinction coefficient of Omura and Sato (50).

[b] The numbers in parenthesis represent the pmol of OR/mg of protein calculated from a Western blot using a standard curve of purified OR, as described by McManus *et al.* (48). The filters were scanned using a Beckman DU-8 spectrophotometer. The standard curve was linear from 0.2 to 2 pmol of enzyme. The purified preparation had a specific activity of about 45 units/mg. One unit of activity corresponds to 1 μmol of cytochrome *c* reduced/min.

[c] ND, Not detectable.

[d] —, Not determined.

activities and these activities were absent in cells infected with only vWR and vOR (Table 2). When vOR was used with vP$_3$450 to coinfect cells, P$_3$450 activities increased with increasing levels of expressed OR and then plateaued at OR/P$_3$450 ratios of greater than 1.5. Ethoxycoumarin *O*-deethylase activities increased from 60 pmol/min/nmol of P$_3$450, when no recombinant OR was expressed, to a level of 700 pmol/min/nmol of P$_3$450, at a vOR/vP$_3$450 ratio of 0.75 (Table 2). Aryl hydrocarbon hydroxylase activity increased from 19 pmol/min/nmol of P$_3$450 to a maximal level of 98 pmol/min/nmol of P$_3$450, at a vOR/vP$_3$450 ratio of 0.75. When lysates from cells infected with vOR were mixed with lysates from cells infected with vP$_3$450, no augmentation of ethoxycoumarin *O*-deethylase activities was observed. These results establish that both proteins must be incorporated into the same membrane bilayer in order for the OR to interact with P$_3$450.

The low level of P$_3$450 activities seen in the cells infected with vP$_3$450 alone may be due to a low level of endogenous OR activity in the TK⁻ cells, even though only trace enzymatic activity, based on cytochrome *c* reduction, was detectable (Table 2). This low level of endogenous OR was confirmed by immunoblotting. When the OR immunoblots were developed with alkaline phosphatase for a longer period of time, a 78,000-Da protein was detected that comigrated with the vaccinia-expressed OR, yet its level was at least 10-fold less than that found in human liver microsomes and in TK⁻ cells infected with vOR (data not shown). The optimum ratio of OR/P$_3$450 for maximal P$_3$450 activity level was greater than 1 for both substrates. These data are in agreement with the reconstitution studies of Kaminsky and Guengerich (47). The vaccinia system appears to be ideal for determining the amount of OR needed for full catalytic activity of P450s *in situ*.

Conclusion

The human OR cDNA has been cloned, sequenced, and inserted into vaccinia virus. The cDNA-expressed enzyme was active when vOR was used to infect human cells in culture and was also able to enhance the activity of cDNA-expressed P$_3$450. The rat OR cDNA has also been expressed in yeast (7). In this system, coexpression of OR with the P450MC resulted in a 2-fold enhancement of 7-ethoxycoumarin *O*-deethylase activity. In contrast, in the present study, we achieved about a 5- to 12-fold increase in human P$_3$450-mediated activities, depending on the substrate. This may be due to the lower level of endogenous OR in human TK⁻ cells, compared with yeast cells. Because the yeast cell expression system involves stable transformation of the cells with autonomously replicating cDNA-containing plasmids, the ratios of levels of OR to P450 cannot be regulated. The vaccinia system, however, can be used to alter the ratios of OR to P450 by simply changing the ratios of infecting virus particles.

We could detect only trace levels of OR activity and protein in TK⁻ cells that had been infected with vP$_3$450 alone, even though these cells were able to support P$_3$450 activities. These data suggest either that electron transfer can occur in conditions of large P450 excess over OR or that an alternative electron transport system is active in the absence of OR. Coexpression of OR with P$_3$450 markedly elevated P$_3$450 activities, indicating that a more favorable stoichiometry of these enzymes is required for maximal P450 activities. Using the data in Table 2, we estimated that the optimal OR/P$_3$450 ratio in vaccinia-infected cells was about 1.5 for both aryl hydrocarbon hydroxylase and ethoxycoummarin *O*-deethylase activities. These data support the possibility that the vaccinia system can be used to determine whether levels of OR modulate the activities and specificities of various P450s in native endoplasmic reticulum membrane. Interestingly, the average ratio of OR/P$_3$450 in human microsomes was determined to range from 4 to 12 (48). This suggests that the OR/P450 ratio in native hepatocytes is not optimum; however, the possibility remains that other forms of P450 may have a higher affinity for OR. This can be examined by use of the vaccinia system and other human P450 cDNA clones (49).

The OR gene was localized to human chromosome 7 between 7p15 and q35. The *CYP3A* P450 locus was also been found on 7q21.3–7q22.1 (41) and both of these gene families were localized to mouse chromosome 6 (42). It is tempting to speculate that these two gene families may be linked. This association might imply that they evolved as a functional unit, perhaps from a bacterial operon. It must be emphasized, however, that our present data only indicate that the OR and *CYP3A* genes are syntenic yet they still may be quite far apart on chromosome 7. In order to firmly establish close association of the OR gene with the *CYP3A* family, the former needs to be more precisely

localized. This can potentially be accomplished either by multilinkage analysis in CEPH families (41) or by *in situ* gene mapping on chromosome spreads.

Acknowledgments

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