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Biochemistry

Expression of P_1-450 and P_3-450 DNA coding sequences as enzymatically active cytochromes P-450 in mammalian cells

(recombinant vaccinia viruses/aryl hydrocarbon hydroxylase/acetanilide hydroxylase/mixed-function oxygenases)

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ABSTRACT **Two cDNA clones representing the mRNA coding sequences for mouse cytochromes P_1-450 and P_3-450 were inserted into the thymidine kinase gene of the wild-type vaccinia virus under the control of the vaccinia virus promoter. Murine and human cells infected with each of the resulting infectious recombinant viruses efficiently expressed their respective P-450 proteins. The newly synthesized protein products are translocated into the microsomes, and their characterization by immunochemical analysis indicates that the sizes of the polypeptides expressed were indistinguishable from their cytochrome P-450 counterparts found in mammalian liver microsomes. Functional analysis of each of the proteins by spectral and enzymatic analysis indicates that the expressed proteins have incorporated heme, and the holoenzymes displayed catalytic activities characteristic of their respective cytochrome P-450 enzymes. Thus, this system can be used to produce properly processed and catalytically active P-450 gene products in a wide variety of cells. The remarkable fidelity of expression and processing of these enzymes suggests that the vaccinia virus recombinants can be used for a wide variety of studies, including analysis of the effects of defined mutations produced *in vitro*, and directly correlate the structure/activity relationships of the cytochrome P-450 enzymes.**

The cytochromes P-450 are a large family of hemoproteins capable of metabolizing xenobiotics, such as drugs, carcinogens, and environmental pollutants (1, 2), as well as endobiotics, such as steroids, fatty acids, and prostaglandins. Some members of the cytochrome P-450 family are inducible in animals and cultured cells, whereas other constitutive forms are noninducible (1, 3). This group of enzymes carries out beneficial metabolic activities by detoxification of xenobiotics as well as harmful metabolic conversion of xenobiotics to toxic, mutagenic, and carcinogenic forms (1). A key to understanding their role in beneficial and detrimental functions lies in the isolation of individual forms and characterization of their catalytic activities.

The multiple molecular forms of cytochromes P-450 exhibit several common properties, such as size, structure, immunologic and spectral characteristics, and overlapping substrate specificities. The multiplicity and the common properties of the cytochrome P-450 render difficult the separation of different forms of cytochrome P-450, especially the minor forms. This has prevented a full understanding of the role of individual forms of cytochromes P-450 in metabolism, detoxification, and activation of xenobiotic and endobiotic substrates.

This laboratory is using molecular biological techniques of monoclonal antibodies (4) and recombinant DNA (5, 6) to study the contribution of individual cytochrome P-450 enzymes in the metabolism of P-450 substrates, the families of P-450 enzymes, and their structural and functional relationships. Recently Gonzalez *et al.* (7) isolated and characterized by DNA sequencing full-length cDNAs representing the mRNA coding sequences for two forms of 3-methylcholanthrene-induced mouse cytochromes—namely, P_1-450 and P_3-450. Expression of functional proteins from these P-450 coding sequences in heterologous cells with minimal or no background expression would permit the isolation of these enzymes and characterization of their role in drug and carcinogen metabolism and activation and cell transformation.

The purpose of this study is to develop a system in which the cDNA copies of the mouse cytochromes P_1-450 and P_3-450 express enzymatically active proteins. Here we describe the construction of infectious recombinant vaccinia viruses containing the P_1-450 and P_3-450 cDNA sequences. Infection of a variety of cells, including murine and human cells, with the recombinant viruses resulted in efficient expression of P_1-450 and P_3-450 polypeptides. The expressed proteins have incorporated heme and are translocated into the microsomes. The newly expressed proteins displayed enzyme specificities that are characteristic of these two enzymes. The utility of the vaccinia virus as a general expression system for identification and characterization of the individual members of the cytochrome P-450 mutligene superfamily are discussed.

MATERIALS AND METHODS

Enzymes and Chemicals. Restriction endonucleases, DNA polymerase I and its Klenow fragment, and T4 DNA ligase were purchased from commercial sources and used according to the manufacturer's specifications. 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) was purchased from Boehringer Mannheim. [^{14}C]Acetanilide (31.7 mCi/mmol; 1 Ci = 37 GBq) was purchased from California Bionuclear.

Viruses and Cells. Vaccinia virus (strain WR), HeLa cells, CV-1 cells, BSC-1 cells, and human thymidine kinase-negative (TK^-) 143 cells were kindly provided by B. Moss (National Institutes of Health). The virus was grown in HeLa cells and purified from cytoplasmic extracts by sucrose density gradient centrifugation (8). All cells were grown in Dulbecco's modified Eagle's minimal essential medium containing 10% fetal bovine serum, and TK^- cells in addition had 25 μg of BrdUrd per ml.

Vectors and DNA. Coexpression insertion vector pSC-11 (9) was provided by B. Moss and S. Chakrabarti. cDNA clones of cytochrome P_1-450 and P_3-450 (10) were provided by F. Gonzalez (National Institutes of Health) and D. Nebert (National Institutes of Health). The plasmids were grown in bacteria and their DNAs were purified by two sequential centrifugations on CsCl/EtBr equilibrium density gradients. DNA fragments were separated on agarose gels and purified

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Abbreviations: X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; TK, thymidine kinase; WT-VV, wild-type vaccina virus.

by electroelution. Other recombinant DNA procedures were carried out by standard procedures (11). Vaccinia virus DNA was extracted from the purified virions as desribed (12). All recombinant DNA and virus procedures were carried out according to the guidelines of the National Institutes of Health.

Infection, Transfection, and Isolation of Recombinant Viruses. The procedures were carried out as described by Moss and colleagues (12–15). Subconfluent CV-1 monkey kidney cells infected with wild-type vaccinia virus (WT-VV) were transfected with 10 μg of recombination vector and 1 μg of wild-type vaccinia virus DNA. Two days after incubation, the recombinant TK^- viruses formed within cells were distinguished from the wild type by plaque assay on TK^- cells in the presence of BrdUrd (9). The TK^- cells with the TK^- plaques were overlaid with agar containing 400 μg of X-Gal per ml to check for the concomitant expression of β-galactosidase and also to distinguish TK^- recombinants from TK^- mutants. The TK^-, $\beta\text{-gal}^+$ recombinants were then screened for the presence of P_1-450 and P_3-450 cDNA inserts by dot blot hybridization (15), and the virus stocks were prepared in HeLa cells.

Protein Analysis by Immunoblotting. Cells were harvested by scraping, and the lysates were prepared by three freeze–thaw cycles and brief sonication in a buffer containing 0.05 M Tris·HCl (pH 7.5) and 0.25 M sucrose. Protein concentration was determined by Lowry's method (16). Electrophoresis in 7.5% polyacrylamide gels in the presence of $NaDodSO_4$ was performed as described by Laemmli (17). Prestained protein molecular weight standards (Bethesda Research Laboratories) were used to estimate the size of the polypeptides. The electrophoresed proteins were transferred to nitrocellulose membranes and the transferred proteins were detected by electrophoretic transfer blotting (18) using a mixture of rabbit antisera against P-450c and P-450d. The P-450c and the P-450d forms are the rat homologues of mouse P_1-450 and P_3-450, respectively. These proteins share a high degree of homology and their antisera cross-react with each other (10, 19, 20). The immunoblots were detected by incubating with goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (KPL Labs, Gaithersburg, MD) in conjunction with the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate/*p*-nitroblue tetrazolium chloride.

Measurement of CO-Reduced Difference Spectra. Microsomal fractions were prepared from cell lysates. The lysates were centrifuged at 700 × *g* for 10 min and the supernatant was recentrifuged at 8000 × *g* for 10 min. The resulting 8000 × *g* supernatant was centrifuged again at 100,000 × *g* for 60 min to pellet the microsomes. The microsomal fraction was suspended in 0.1 M potassium phosphate buffer (pH 7.5) containing 20% glycerol. For difference spectra, the microsomal fraction was solubilized with 0.4% Emulgen 913 (15 min) and centrifuged at 100,000 × *g* for 60 min, and the supernatant was used. The spectra were measured in an Aminco Instruments model DW-2a spectrophotometer as described (21).

Enzyme Assays. Aryl hydrocarbon hydroxylase activity was determined by measurement of the fluorescence of phenolic metabolites formed from benzo(*a*)pyrene (22). The reaction mixture contained (in 1.0 ml) 50 μmol of Tris·HCl (pH 7.5), 0.3 μmol of $MgCl_2$, 0.6 μmol of NADPH, 100 nmol of benzo(*a*)pyrene, and 400 μg of cell homogenate. Aryl hydrocarbon hydroxylase activity is expressed as pmol of product equivalent to 3-OH benzo(*a*)pyrene formed per mg of protein per min. Acetanilide hydroxylase activity was determined by measuring the conversion of [^{14}C]acetanilide to its hydroxylated derivatives. The substrate and its metabolites were separated by silica gel thin-layer chromatography (TLC). The assay was carried out in a final volume of 1.0 ml containing 50 μmol of Tris·HCl (pH 7.5), 0.3 μmol of $MgCl_2$, 0.6 μmol of NADPH, 2 μmol of [^{14}C]acetanilide at a specific activity of 1.0 mCi/mmol, and 500 μg of total cell homogenate. The enzyme activity is expressed as pmol of product formed per mg of protein per min. An aliquot of the reaction product in methanol was spotted on a 250-μm thin-layer silica gel plate (Whatman) and eluted with 95% chloroform/5% methanol. The R_f values for acetanilide and 4-hydroxyacetanilide under these conditions are 0.74 and 0.2, respectively. The gel plate was autoradiographed and the product was quantified by counting the radioactivity after scraping from plate.

RESULTS

Construction of Plasmids and Virus Recombinants. Construction of the chimeric genes containing the transcriptional regulatory signals and RNA start site of vaccinia virus genes, the translational start site of the coding sequences of the mouse P_1-450 and P_3-450, and the incorporation of these sequences into the wild-type vaccinia virus to form recombinants are diagramatically presented in Fig. 1. The starting plasmid in the construction sequence for generating the recombinant virus is the insertion vector pSC-11 (9). This coexpression insertion vector contains the *E. coli* β-galactosidase gene under the control of vaccinia promoter for the M_r 11,000 protein, a second promoter for the M_r 7500 protein for transcription of coding sequences, a unique *Sma* I site downstream of the M_r 7500 promoter for the insertion of foreign protein coding sequences, and flanking vaccinia virus TK sequences for homologous recombination inside infected

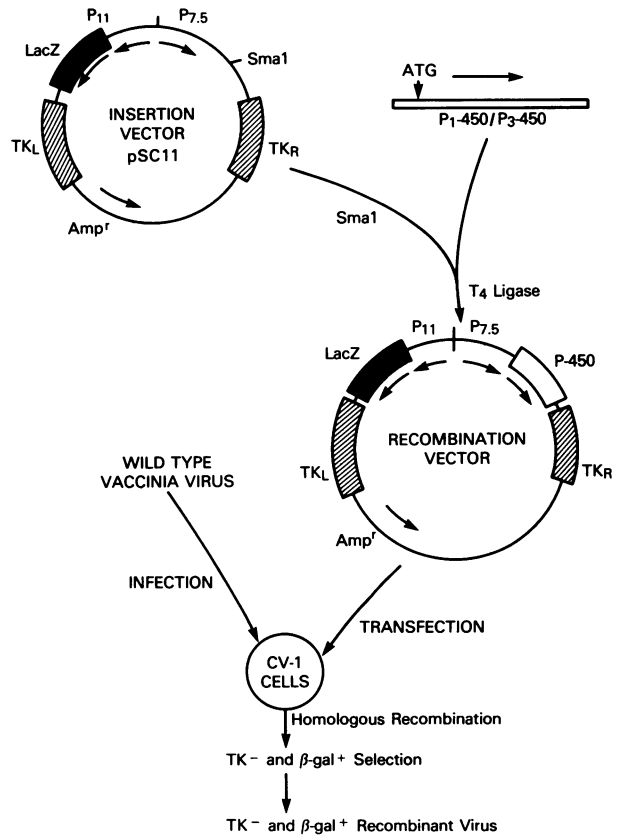

FIG. 1. Construction of recombinant vaccinia viruses for expressing mouse cytochromes P_1-450 and P_3-450. P_{11} and $P_{7.5}$, vaccinia transcriptional regulatory sequences for M_r 11,000 and M_r 7500 polypeptides, respectively; LacZ, *Escherichia coli* β-galactosidase gene; TK_L and TK_R, split segments of vaccinia virus DNA from left and right positions of *TK* gene, respectively. Amp^r is ampicillin-resistance gene.

cells (13). A 2.6-kilobase (kb) full-length cDNA for cytochrome P_1-450 and a 1.9-kb full-length cDNA for cytochrome P_3-450 (10) were inserted into the unique *Sma* I site of the insertion plasmid to form the recombination vectors containing the individual P_1-450 and P_3-450. Selection of the recombination plasmids containing the P_1-450 and P_3-450 inserts in correct orientation was accomplished by restriction enzyme mapping. The two recombination plasmids were then individually used to transfect CV-1 cells previously infected with WT-VV. Homologous recombination between vaccinia TK sequences in the recombination plasmid and the virus genome resulted in insertion of the P-450 and the β-galactosidase sequences into the vaccinia virus. The progeny viruses were then plaque assayed on TK^- cells in the presence of BrdUrd to select for TK^- virus and overlaid with agar containing X-Gal (chromogenic substrate for β-galactosidase) to select for β-gal^+ virus. The presence of the P_1-450 and P_3-450 inserts in the TK^- and β-gal^+ recombinants was confirmed by dot blot hybridization (15). After two sequential plaque purifications the recombinant virus stocks containing the P_1-450 and P_3-450 inserts were designated VV-P_1 and VV-P_3, respectively.

Analysis of P_1-450 and P_3-450 Proteins in VV-P_1- and VV-P_3-Infected Cells. Lysates of human and mouse cells infected with each of the recombinant viruses were electrophoresed on $NaDodSO_4$/polyacrylamide gels and analyzed by immunoblotting (Fig. 2*A*). Human WI-38 cells and mouse NIH 3T3 cells infected with the recombinant virus VV-P_1 showed a peptide band that cochromatographed with P_1-450 of mouse liver microsomes at M_r 55,000. These same cells infected with the recombinant virus VV-P_3 showed a slightly faster migrating protein band that cochromatographed with P_3-450 of mouse liver microsomes at M_r 54,000. In lysates of uninfected control cells or cells infected with WT-VV, neither the P_1-450 nor the P_3-450 band was detected. The time course of synthesis of P_1-450 and P_3-450 in virus-infected NIH 3T3 cells is shown in the immunoblots in Fig. 2*B*. Cytochromes P_1-450 and P_3-450 were detected as early as 2 hr after infection and the amount of expression product increased during a 15-hr time interval. Based on the relative intensities of the protein bands of P_1-450 and P_3-450 found in infected human and mouse cells and the P-450 content of the mouse liver microsomes, we estimate the specific content to be in the range of 15–90 pmol/mg of infected cells lysates. These results clearly show that the infectious vaccinia virus recombinants directed the synthesis of cytochromes P_1-450 and P_3-450. The polypeptide products formed were indistinguishable from the native P_1-450 and P_3-450 of mouse microsomes. The synthesis of the correct size P_1-450 and P_3-450 polypeptides indicates that no fusion polypeptides were formed nor were any incorrect reading frames expressed. The detection of P-450 expression products at early times after infection is consistent with the use of early vaccinia promoter (13).

Subcellular Localization of the Newly Synthesized P_1-450 and P_3-450. Native cellular P-450 apoproteins normally complex with heme to form hemoproteins, which are subsequently transported to microsomal membranes. We sought to determine whether the newly expressed P_1-450 and P_3-450 likewise are transported to the microsomes in the cell. Thus, we examined the distribution of P_1-450 and P_3-450 polypeptides in different subcellular fractions of VV-P_1- and VV-P_3-infected cells by immunoblotting. Results presented in Fig. 3 show that the newly expressed cytochromes P-450 were concentrated in the microsomal fraction (100,000 × *g* pellet). Either none or negligible amounts were detected in the 100,000 × *g* supernatant. This result indicates that the cytochromes P-450 synthesized on recombinant vaccinia viruses VV-P_1 and VV-P_3 are translocated to the microsomal membranes. By comparing the relative band intensities of infected cell lysates and microsomes, we estimate a 10-fold enrichment of the expressed P-450s in the microsomal fraction.

Spectral Characterization of P_1-450 and P_3-450. A feature characteristic of the microsomal cytochrome P-450 hemoproteins is that the native catalytically active forms exhibit absorption maxima of reduced CO complex at 450 nm, and the denatured catalytically inactive forms exhibit absorption maxima around 420 nm (21). Examination of the microsomal fraction of NIH 3T3 cells infected with VV-P_1 showed an absorption maxima of reduced CO complex at 450 nm, indicating that the newly expressed cytochrome P_1-450 in microsomes is in native configuration (Fig. 4). Similarly, the microsomal fraction of cells infected with VV-P_3 showed a 450-nm peak characteristic of native P-450. The specific content of cytochrome P_1-450 was 0.028 ng/mg and P_3-450 was 0.033 ng/mg of detergent-solubilized microsomal fraction. These results indicate that the cytochrome P_1-450 and P_3-450 proteins synthesized in virus-infected cells incorporate a heme moiety and are transported and sequestered into the microsomal fraction in a manner indistinguishable from the native *in vivo* processed cellular P-450s.

Enzyme Activities of P-450s in VV-P_1- and VV-P_3-Infected Cells. Aryl hydrocarbon hydropylase activity in lysates of NIH 3T3 cells infected with VV-P_1 (Fig. 5) was detected as early as 1 hr after infection and increased for 12 hr thereafter. However, the lysates of cells infected with VV-P_3 showed only a small fraction of activity compared to that of VV-P_1 even at 12 hr after infection. No detectable activity was found in uninfected control cells or in WT-VV-infected cells. The aryl hydrocarbon hydroxylase activity was completely inhibited by antisera against P-450c and P-450d (unpublished results). The specific aryl hydrocarbon hydroxylase activity in different experiments varied from 10 to 70 pmol/mg of cell lysates. The activity was at least 30-fold greater with P_1-450 than with P_3-450.

Lysates of NIH 3T3 cells infected with VV-P_1 and VV-P_3 for different time intervals were assayed for acetanilide

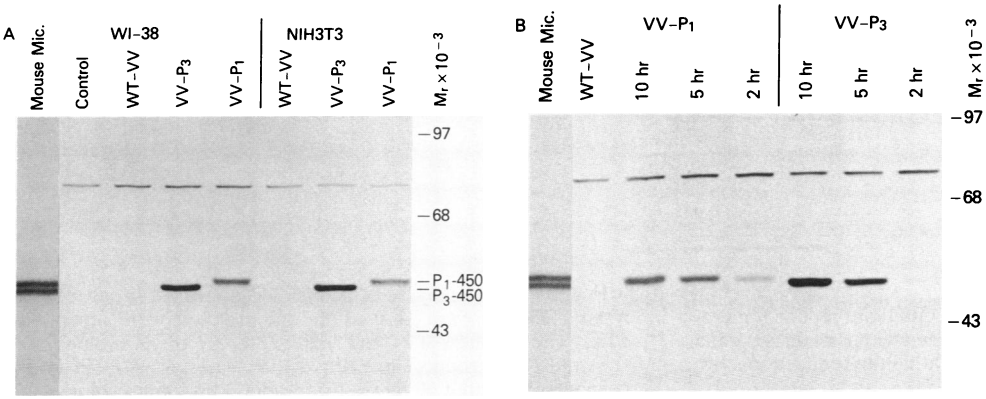

FIG. 2. Identification of cytochrome P_1-450 and P_3-450 polypeptides. Lysates (100 μg) were electrophoresed and detected by immunoblotting. Stained protein molecular weight markers are shown on the right. Mic., microsomes. (*A*) Expression in WI-38 and NIH 3T3 cells. (*B*) Time course of synthesis in NIH 3T3 cells. The minor M_r 80,000 band detected in all cell lanes was not detected when diluted antiserum was used.

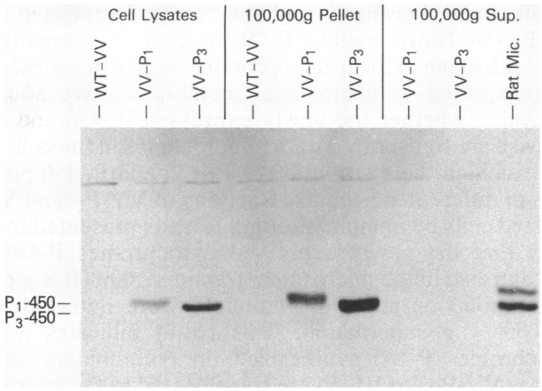

FIG. 3. Cytochrome P_1-450 and P_3-450 polypeptides in subcellular fractions. In each lane 100 μg of protein was electrophoresed and immunoblotted. Sup., supernatant. Mic., microsomes.

hydroxylase activity, and autoradiograms of TLC analysis of the products formed are presented in Fig. 6. Lysates of cells infected with VV-P_3, in addition to the intense substrate band (R_f value of 0.74), showed a slow-moving band (R_f value of 0.2) identifying the TLC migration position of the hydroxylated products of acetanilide. The formation of hydroxylated metabolites increased with time. In cells infected with VV-P_1, however, there was no detectable band at an R_f value of 0.2. No activity was detectable in the control uninfected cells or in cells infected with WT-VV. A minor band at an R_f value of 0.2 was detected in Hepa 1 cells infected with VV-P_1 after prolonged exposure (result not shown). The specific acetanilide hydroxylase activity was about 45 pmol/mg of cell lysate. The acetanilide hydroxylase activity was at least 20-fold greater with P_3-450 than with P_1-450.

The expression of enzyme activities clearly shows that the P_1-450 and P_3-450 proteins in recombinant virus-infected cells are catalytically active. Cytochrome P_1-450 has a 30- to 40-fold higher aryl hydrocarbon hydroxylase activity than P_3-450, and cytochrome P_3-450 showed a 20-fold higher acetanilide hydroxylase activity than P_1-450, a characteristic and distinguishing features of these two enzymes (23). The early detection of enzyme activity is consistent with the

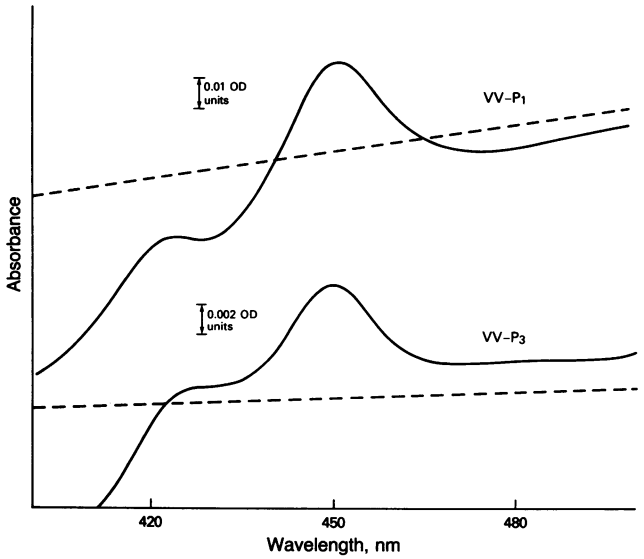

FIG. 4. The CO difference spectra of dithionite-reduced microsomal fractions. Microsomes were solubilized with Emulgen 913 and the supernatant was used for spectra. ---, Dithionite-reduced spectra; —, reduced and CO-saturated spectra.

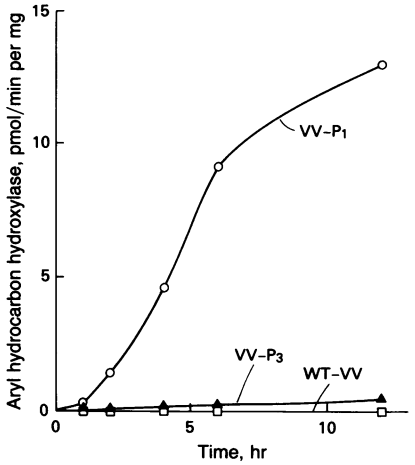

FIG. 5. Expression of aryl hydrocarbon hydroxylase activity. Infected cells were harvested at the indicated time intervals and the lysates were assayed for aryl hydrocarbon hydroxylase activity. NIH 3T3 cells were infected with VV-P_1 (○), VV-P_3 (▲), or WT-VV (□). No detectable activity was found in uninfected control cells (not shown).

utilization of early vaccinia promoter (13). Cells infected with recombinant viruses expressed 10^7–10^8 molecules of the newly synthesized cytochromes per cell, and this represents 0.1–1.0% of total cellular proteins.

DISCUSSION

In this communication we report the expression of two catalytically active mouse cytochrome P-450 enzymes in mammalian cells via a recombinant vaccinia virus expression system. Several lines of evidence presented here demonstrate the authenticity of the expression products and that they are synthesized from the exogenously introduced coding DNA sequences. The evidence is this: the P_1-450 and P_3-450 polypeptides that are synthesized comigrate with native P_1-450 and P_3-450 of mouse liver microsomes. The apoproteins expressed incorporate heme and are translocated to the microsomes. The recombinant DNA-derived P_1-450 enzyme displayed high aryl hydrocarbon hydroxylase activity and low acetanilide hydroxylase activity and the P_3-450 enzyme displayed high acetanilide hydroxylase activity and low aryl hydrocarbon hydroxylase activity, which are distinguishing

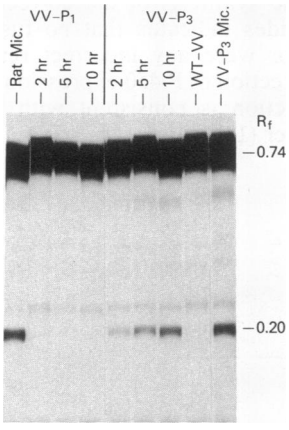

FIG. 6. TLC analysis of acetanilide hydroxylase activity. Cell lysates (500 μg) were assayed and the products were separated by TLC. The numbers 0.74 and 0.20 represent the R_f values of the substrate and product, respectively. Lysates of WT-VV-infected cells were prepared 10 hr after infection. Mic., microsomes.

and diagnostic features of these two closely related enzymes (23). The various experiments described were repeated two to four times and the results were reproducible. In control uninfected cells and in cells infected with the WT-VV, neither of these proteins nor their enzymatic activities were detected. Thus, there was no detectable P_1-450 or P_3-450 background in the recipient cells.

In their functional state, cytochromes P-450 are not catalytically self-sufficient for mixed-function oxygenase oxidations but require NADPH and NADPH cytochrome P-450 reductase. Results presented here demonstrate that the newly expressed vaccinia virus-directed and recombinant DNA-derived P_1-450 and P_3-450 apoproteins have complexed with heme and have been transported to and associated with the microsomal membranes. These processes have resulted in a complex with a configuration appropriate for functional associations with NADPH and cytochrome P-450 reductase and resulting in mixed-function oxidase activity. Thus, the cellular machinery of the host avian, murine, and human cells performs the posttranslational processing of the primary protein product in a manner indistinguishable from the process resulting in active native P-450 enzymes. The posttranslational processing machinery for cytochrome P-450 enzymes thus appears to be evolutionarily conserved in these systems.

Progress in the understanding of P-450 enzymology has been hindered by the difficulty of presently available procedures to purify the multiple forms of some enzymes, particularly the constitutive forms and the minor forms. The vaccinia virus system used in this study to express functional proteins in heterologous cells with almost no background offers a unique solution to the difficult problem of P-450 isozyme purification to obtain clean enzymes in quantities required to study their catalytic, immunologic, and structural properties. Expression of closely related forms with overlapping substrate specificities and subsequent characterization will allow analysis of the contribution of each of the enzymes to the metabolism of carcinogens and drugs in intact cells in the absence of other P-450s. This expression system can also be used as a general procedure for testing the coding potential of P-450 DNA sequences and hence to identify cDNAs of unknown function. For example, human P-450 cDNAs can be expressed and their functions analyzed. Purification and characterization of the enzyme products and analysis of their role in the metabolism of chemical carcinogens will also be served well by this prototype system.

The vaccinia virus expression system is highly efficient in that the cells infected with the recombinant viruses synthesized 10^7–10^8 molecules per cell, which constitutes 0.1–1.0% of total cell protein. The P_1-450 enzyme expressed has efficiently metabolized benzo(*a*)pyrene, and this aryl hydrocarbon hydroxylase activity is about 30-fold greater than that of the P_3-450. On the other hand, the P_3-450 enzyme expressed has efficiently metabolized acetanilide, and this acetanilide hydroxylase activity is about 20-fold greater than that of P_1-450. The high activity of the P_1-450 for aryl hydrocarbon hydroxylase and the very low activity for acetanilide hydroxylase and similarly the high activity of P_3-450 for acetanilide hydroxylase and the low activity for aryl hydrocarbon hydroxylase show the extraordinary usefulness of this system for obtaining highly pure enzymes of P-450s for studies defining substrate and product specificities. This system is likely to be considerably superior to the classical methods of enzyme purification required to obtain P-450s in states of sufficient purity necessary to define enzyme specificity.

A critical analysis of P-450 gene structure and function requires the expression of the coding sequences in functional form. Several laboratories have cloned different coding P-450 DNA sequences (20); however, there is only a single recent report (24) demonstrating the expression of functional product in COS-1 cells and one report (25) in yeast cells. The experiments presented here on the expression of the coding sequences of P_1-450 and P_3-450 into functional proteins will facilitate future studies of their structure–function relationships.

We greatly appreciate the supply of coexpression vector and cells from Drs. B. Moss, S. Chakrabarti, and colleagues. cDNA clones of cytochromes P_1-450 and P_3-450 from Drs. F. Gonzalez and D. Nebert, and antisera against P-450c and P-450d forms from K. Nagata and J. Gillette. We also thank Drs. T. Aoyama, F. Friedman, and F. Gonzalez for comments on the manuscript and G. K. Townsend for helping with experiments.

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