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Recombinant mouse cytochromes P1-450 and P3-450: enzymatic characterization of the hemoprotein expressed in human cells infected with recombinant vaccinia virus.

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Abstract

We expressed mouse cytochrome P1-450 and P3-450 using recombinant vaccinia virus gene expression system in HeLa cells that were devoid of significant basal levels of P-450. HeLa cells were infected with the recombinant vaccinia virus containing either mouse cytochrome P1-450 or P3-450 cDNA, and the cell lysates were analyzed for the kinetics of P-450 enzyme activity and protein expression at the same time. 7-Ethoxycoumarin O-deethylase and ethoxyresorufin O-deethylase activities were measured as an expression of the cytochrome P-450 enzyme activities. Both cell lines began to express these enzyme activities as early as 12h after infection. The activities increased linearly up to the 24 h time point, and were kept for 36 h. Western immunoblot analysis showed that these cytochrome P-450 proteins were detected at 16 h and reached maximum quantity at 24 h after infection. These data showed a good correlation between cytochrome P-450 enzyme activity and protein concentration throughout the process of P-450 gene expression by vaccinia virus vector, suggesting a complete formation of cytochrome P-450 holoenzyme from the early stage of the protein expression.

KEYWORDS: cytochrome P-450, vaccinia virus, kinetics

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Recombinant Mouse Cytochromes P_1-450 and P_3-450: Enzymatic Characterization of the Hemoprotein Expressed in Human Cells Infected with Recombinant Vaccinia Virus

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We expressed mouse cytochrome P_1-450 and P_3-450 using recombinant vaccinia virus gene expression system in HeLa cells that were devoid of significant basal levels of P-450. HeLa cells were infected with the recombinant vaccinia virus containing either mouse cytochrome P_1-450 or P_3-450 cDNA, and the cell lysates were analyzed for the kinetics of P-450 enzyme activity and protein expression at the same time. 7-Ethoxycoumarin *O*-deethylase and ethoxyresorufin *O*-deethylase activities were measured as an expression of the cytochrome P-450 enzyme activities. Both cell lines began to express these enzyme activities as early as 12h after infection. The activities increased linearly up to the 24h time point, and were kept for 36h. Western immunoblot analysis showed that these cytochrome P-450 protiens were detected at 16h and reached maximum quantity at 24h after infection. These data showed a good correlation between cytochrome P-450 enzyme activity and protein concentration throughout the process of P-450 gene expression by vaccinia virus vector, suggesting a complete formation of cytochrome P-450 holoenzyme from the early stage of the protein expression.

Key words : cytochrome P-450, vaccinia virus, kinetics

Cytochromes P-450 are microsomal hemoproteins which metabolize xenobiotics such as drugs, carcinogens, and environmental chemicals, as well as endobiotics such as steroids and prostaglandins (1, 2). These enzymes are involved not only in detoxification but also in the activation of xenobiotics to toxic, mutagenic, and carcinogenic forms (3, 4). Cytochromes P-450 proteins comprise a large number of individual forms, and multiple forms of cytochromes P-450 exhibited size similarities and overlapping substrate specificities (5, 6). Therefore, to study the role of cytochromes P-450, it is important to obtain a single form of cytochrome P-450.

To obtain a single pure cytochrome P-450, several transient and stable gene transfer systems have been developed to express single P-450 in cells which lack constitutive cytochrome P-450 expression (7-16). No stable gene transfer systems has successfully shown a strong cytochrome P-450 activity in cells so far, although these systems are suitable for long term experiments to study the biological consequences of cytochrome P-450-mediated metabolism, such as DNA adduct formation and mutations caused by endogenously activated carcinogens/mutagens (12, 15).

Conversely, a vaccinia virus gene expression system has produced a strong cytochrome P-450 enzyme activity as well as strong protein expression, although the system shows transient gene expression due to cell death in a few days (17).

Different kinds of parental recombinant plasmids have been constructed to introduce foreign genes into vaccinia

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virus (18, 19). Each plasmid has a different structure and promoter. The kinetics of protein expression in cells infected with recombinant virus were closely related to promoters of the recombination vector, the gene inserted into the vector, and the cells infected with that recombinant viruses. Furthermore, a multiplicity of infection has a dramatic effect on protein expression in the vaccinia virus system (16). Therfore, it is very important to define the kinetics of synthesized protein and its enzyme activities in individual cases.

Cytochromes P_1-450 and P_3-450 are two forms of 3-methylcholanthrene-induced cytochromes P-450, and both of them belong to the cytochrome P450IA gene subfamily (20). Cytochrome P_1-450 is known to metabolize benzo (a) pyrene and P_3-450 metabolize acetanilide and several food derived mutagens including 2-amino-3-methylimidazo [4, 5-*f*] quinoline and 2-amino-3, 8-dimetylimidazo [4, 5-*f*] quinoxaline which are among the most potent mutagens yet tested in the Ames Salmonella mutagenicity assay (21, 22).

In this report, HeLa cells derived from humans were infected with two different kinds of vaccinia virus pSC-11 recombinants containing either cytochrome P_1-450 or P_3-450 cDNA, and the kinetics of expressed cytochrome P-450 was analyzed by comparing the protein expression and the enzyme activities during vaccinia virus mediated cytochrome P-450 expression.

Meterials and Methods

Chemicals and antibodies. Chemicals were purchased from the following sources: Ethoxycoumarin and 7-hydroxycoumarin, Aldrich; ethoxyresorufin, resorufin, and NADPH, Sigma. Goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase was purchased from KPL Labs, Gaithersburg, MD, USA. Antibody against mouse cytochrome P_3-450 was raised in rabbit.

Viruses and cells. Vaccinia virus (Strain WR), HeLa cells, CV-1 cells were kindly provided by B. Moss (National Institutes of Health, MD, USA). Coexpression insertion vector pSC-11 (18) was provided by B. Moss and S. Chakarabarti. cDNA clones of mouse cytochrome P_1-450 and P_3-450 (23) were provided by F. Gonzalez (National Institutes of Health) and D. Nebert. HeLa cells were obtained from the Amemican Type Culture Collection. 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.

Construction of recombinant vaccinia virus. The recombinant vaccinia viruses were constructed as previously described (9). The recombinant vaccinia virus containing cytochrome P_1-450 cDNA and P_3-450 cDNA were designated VV-Pl and VV-P3, respectively.

Infection of HeLa cells with recombinant vaccinia virus. Subcofluent HeLa cells (2.5×10^7 cells/150 mm dish) were infected with recombinant vaccinia viruses at a multiplicity of infection of 2. HeLa cell was chosen because of its rapid growth and lack of constitutive cytochrome P-450 expression. The infected cells were incubated at 37 °C for indicated times, washed with 6 ml of phosphate buffer saline (PBS) 3 times, and harvested by scraping. The cells were centrifuged at $700 \times g$ for 5 min. The pellet was suspended in the same volume of buffer (0.1 M potassium phosphate buffer pH 7.5, 20 % glycerol) and saved as infected samples. The lysates of the samples were prepared by 3 freeze-thaw cycles and subsequent brief sonication. Protein concentrations were determined by Lowry's method (24).

Western immunoblot analysis. The samples were subjected to SDS-PAGE according to the mothod of Laemmli (25). The electrophoresed proteins were transferred from the polyacrylamide gel to nitrocellulose membrane and the proteins were detected using rabbit antisera to mouse cytochrome P_3-450. Cytochrome P_1-450 and P_3-450 proteins share a high degree of homology, and this antibody against P_3-450 crossreacts with P_1-450. The immunoblots were detected by incubating with alkaline phosphatase-conjugated goat anti rabbit immunoglobulin G in conjunction with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium phosphatase substrate system (KPL Labs, Geithersburg, MD). The intensity of the bands were measured densitometrically (Molecular Dynamics Scanning Imager 300 SX), and expressed as a ratio to band intensity of the 32 h sample.

Ethoxycoumarin O-deethylase (ECD). ECD assay was carried out as previously described by Greenlee (26). The sample proteins (0.3 mg in 100 μl 0.1 M sodium phosphate buffer, pH 7.4) were mixed with 0.9 ml cofactor (9.3 mg NADPH in 18 ml 0.01 M $MgCl_2$, 0.1 M sodium phosphate buffer, pH 7.4) and 4 μl substrate (50 mM 7-ethoxycoumarin), and incubated at 37 °C for 30 min. The reaction was terminated with 125 μl of 15 % trichloroacetic acid. The reaction product was extracted with chloroform and subsequently with 0.01 N NaOH containing 1 M NaCl. The concentration of 7-hydroxycoumarin in the alkaline phase was measured spectorofluorometrically (Perkin-Elmer 650-10S fluorescence spectrophotometer), using an excitation maximum at 368 nm and an emission maximum at 456 nm. The assays were done in duplicate.

Ethoxyresorufin O-deethylase (EROD). EROD assay performed by measuring the fluorescence of metabolites (27). The sample proteins (0.3 mg) were mixed with 1.0 ml cofactor (8.3 mg NADPH and 20 mg bovine serum albumin in 10 ml 0.1 M potassium phosphate buffer, pH 7.5) and water was added to the total volume of 1.25 ml. The reaction was performed at 37 °C for 10 min by addition of the substrate (5 μl, 400 μM ethoxyresorufin) to the sample mixture, and was terminated with 2.0 ml of methanol. The supernatant of the reaction mixture was obtained following centrifugation at $2,000 \times g$ for 5 min. The concentration of reaction product resorufin were measured spectrofluorometrically, using an excitation maximum at 550 nm and an emission maximum at 585

nm.

Distribution of newly expressed cytochrome P-450. To analyze the localization of cytochrome P-450 in the cells infected with the recombinant vaccinia virus, HeLa cells were infected with VV-P3, incubated for 24 h, and the sample was prepared according to the method described above. The obtained sample was homogenized with a teflon homogenizer, centrifuged at 700 × *g* for 10 min and the supernatant was recentrifuged at 8,000 × *g* for 15 min. The recovered supernatant was centrifuged at 105,000 × *g* for 60 min. The supernatant after 8,000 × *g* and 105,000 × *g* centrifugation, and the pellet after 105,000 × *g* centrifugation were subjected to ECD analysis. All procedures were performed at 4 °C.

Results and Discussion

The recombination vector comprised of LacZ gene driven by P_{11}(vaccinia virus promoter for Mr. 11,000 polypeptide) for X-gal selection and cytochrome P-450 cDNA driven by $P_{7.5}$ (vaccinia virus promoter for Mr. 7,500 polypeptide) between the two halves of the TK gene (Fig. 1). CV1-cells were transfected with this recombination vector following infection with the wild type vaccinia virus. The plasmid then used its TK sequence for induction of homologous recombination with wild type vaccinia virus. As a result, DNA between the two halves of the TK gene in the recombination vector was integrated into the wild type vaccinia virus. This integration also inactivated the TK gene in the wild type vaccinia virus and made TK selection possible.

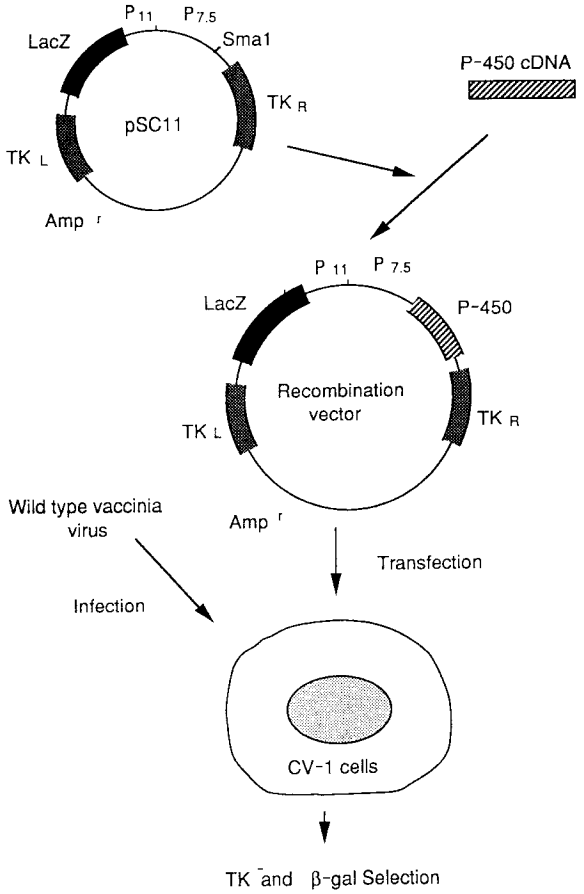

Fig. 1 Construction of recombinant vaccinia virus. $P_{7.5}$, vaccinia virus promoter for Mr. 7,500 polypeptide; P_{11}, vaccinia virus promoter for Mr. 11,000 polypeptide; TK_R and TK_L, split segments of vaccinia virus TK gene; LacZ, *Escherichia coli* β-galactosidase gene; Amp^r, ampicillin resistance gene.

The localization of induced cytochrome P-450 was analyzed using HeLa cells infected with VV-P3. Table 1 shows that ECD activity was concentrated in the 105,000 × *g* pellet, that is the microsomal fraction. These data indicate that the cytochrome P-450 induced by the recombinant vaccinia virus is located in the microsomal fraction.

The expression of cytochrome P_1-450 and P_3-450 in recombinant vaccinia virus-infected HeLa cells were analyzed by Western immunoblot analysis. The result in Fig. 2a shows that cytochrome P-450 protein was not detcted in parental HeLa cells. Cytochrome P_1-450 protein in VV-P1-infected cells could not be detected until 8 h. The protein band appeared at 16 h (48 % intensity), the intensity reached almost maximum (85 % intensity) at the 24 h time point, and it stayed at maximum until the 32 h time point (100 % intensity). Cytochrome P_3-450 protein in HeLa cells infected with VV-P3 showed a similar pattern (Fig. 2b). The band intensities of the protein were 13 %,

Table 1 Localization of newly expressed cytochrome P-450

Fraction	Total protein (mg)	ECD activity (pmol product/min/mg protein)	ECD activity (pmol product/min /fraction)
8,000 × *g* supernatant	18.8	0.83	15.6
105,000 × *g* supernatant	13.7	0.11	1.5
105,000 × *g* pellet	2.44	3.70	9.0

HeLa cells infected with vaccinia virus containing cytochrome P_3-450 cDNA were homogenized with teflon homogenizer and centrifuged consecutively at 700 × *g*, 8,000 × *g*, 105,000 × *g*. The supernatant after 8,000 × *g* and 105,000 × *g* centrifugation, and the pellet after 105,000 × *g* centrifugation were subjected to ethoxycoumarin *O*-deethylase (ECD) analysis. The specific ECD activity (pmol/product/min/mg protein) and the recovery of the ECD activity (pmol/product/fraction) were demonstrated.

65 %, and 100 % at 16, 24, and 32 h, respectively. HeLa cells infected with VV-Pl and VV-P3 showed protein bands comigrated with 3-methylcholanthrene-induced mouse microsomal protein that was known to express mainly cytochrome P_1-450 and P_3-450 (23) (data not shown).

Cytochrome P-450 enzyme activities were measured by ECD and EROD assays concomitantly. These assays are a sensitive and convenient way to examine whether the induced proteins express specific and active enzymes. In Fig. 3a, parental HeLa cells (0 h time point) showed neither detectable ECD nor EROD activity. Both ECD and EROD activity of VV-P1 infected cells which could not be detected at the 4 h time point, linearly increased from 8 h to 20 h, and was kept until 36 h. These enzyme activities were parallel to the protein expression. VV-P3-infected HeLa cells expressed almost the same ECD activities as VV-P1-infected cells at all time points examined (Fig. 3b). EROD activity of VV-P3-infected HeLa cells was much lower than that of VV-P1-infected HeLa cells. These enzyme activities also showed tendencies parallel to the protein expression. These experiments were done in duplicate. The variation of these experiments were < 30 %. To obtain the cytochrome P-450 enzyme activity by the vaccinia virus expession system, the virus must infect cells, the protein must be translated in the infected cells and form a complex with hem. The data in Fig. 3 showed that cytochrome P-450 proteins expressed in both VV-P1 and VV-P3 infected cells accompanied their enzyme activities. These data

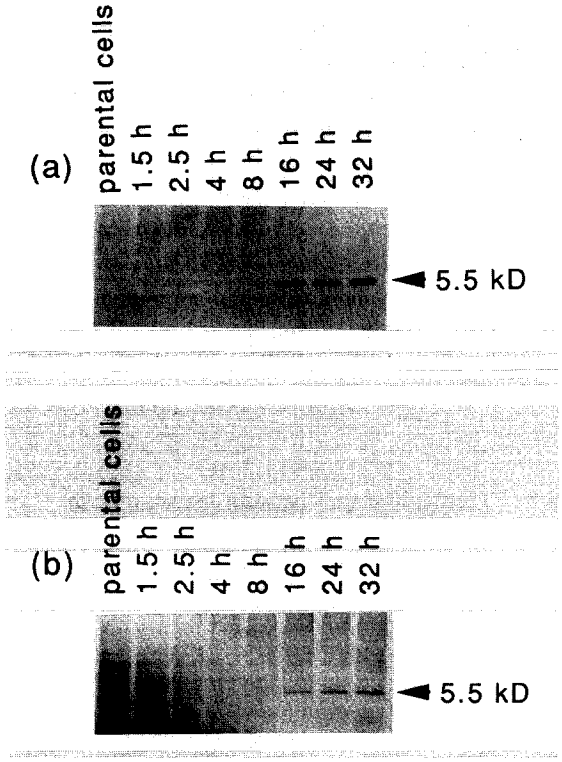

Fig.2 Expression of cytochrome P-450 protein by recombinant vaccinia viruses. HeLa cells infected with vaccinia virus containing either cytochrome P_1-450 (**a**) or cytochrome P_3-450 cDNA (**b**) were incubated for indicated times. The lysates (4 μg protein) were electrophoresed, transferred to nitrocellulose membrane, and proteins were detected by Western immunoblotting analysis. Parental cells, uninfected HeLa cells.

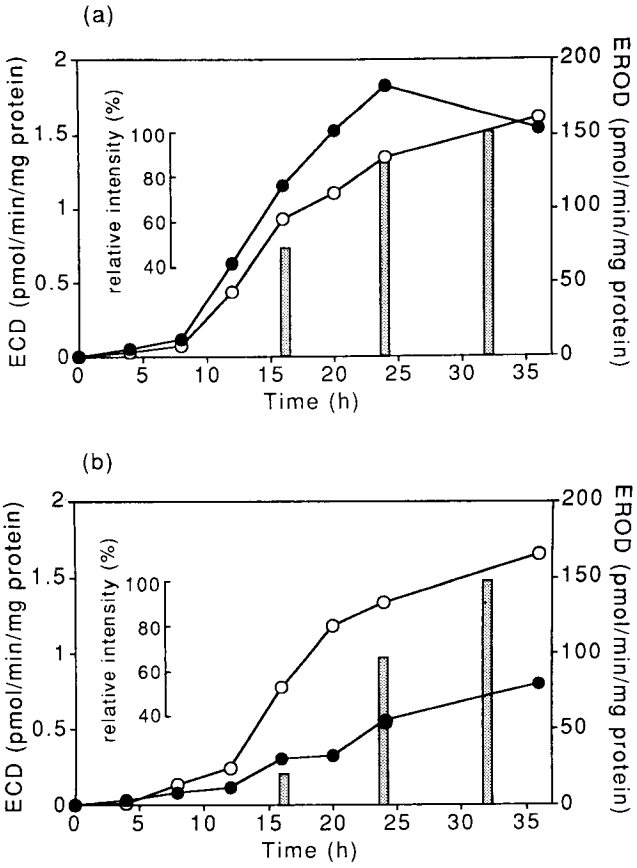

Fig.3 Relationship between protein expressions and enzyme activities. Cell lysates were assayed for ethoxycoumarin *O*-deethylase (ECD), ethoxyresorufin *O*-deeothylase (EROD) and the protein expression. Open circles, ECD activity; closed circles, EROD activity; shaded bars, relative protein expression. Vaccinia virus containing cytochrome P_1-450 cDNA (**a**), P_3-450 cDNA (**b**).

suggest that newly synthesized proteins were incorporated with hem soon after translation and not many P-450 existed as apoprotein at early stage after infection, although the sensitivity of immunoblots might affect the detection of P-450 protein. The good correlation between protein expression and enzyme activity indicates the formation of holoenzyme throughout this infection for 36 h.

Since cytochrome P-450 cDNAs were cloned, many cDNA expression systems have been examined to express cytochrome P-450 in cells (8-16). The COS cell gene expression system is widely used to analyze cytochrome P-450 (8). This system is the easiest way found to date, but the expression of P-450 is very low. The strongest P-450 protein expression can be obtained by baculovirus system among transient P-450 experssion systems (11). However, this system shows a large amount of cytochrome P-450 apoprotein which lacks hem incorporation and has no enzyme activity, and can be applied to insect cells only.

The vaccinia virus system is the most powerful and reproducible transient P-450 expression system so far developed. The vector pSC-11 that we examined here is widely used to express cytochrome P-450 in cells. In this study, we demonstrated the formation of enzymatically active holoenzyme soon after translation and provided a basic kinetics of vaccinia virus mediated cytochrome P-450 expression system.

Recently, a new vector which has the T7 RNA polymerase gene was developed (16, 19). This system uses two recombinant viruses: one virus containing the bacteriophage T7 polymerase gene driven by the vaccinia virus promoter, and the second virus containing the cDNA driven by the T7 RNA polymerase promoter. Coinfection of both viruses causes a production of T7 polymerase and subsequent transcription of the cDNA, results in a 5-fold increase in P-450 expression.

Additional basic experiments with this new vaccinia virus vector need to be done in the future.

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