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Expression of Cholesteryl Ester Transfer Protein cDNA using Recombinant Vaccinia Viruses

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Abstract: cDNA for human cholesteryl ester transfer protein (CETP), a potent atherogenic plasma protein that redistributes the neutral lipids among lipoproteins, was expressed in recombinant vaccinia virus-infected cells (CV-1). Two insertion vectors regulated by different promoters were constructed. The vectors were introduced into human thymidine kinase-negative (TK^-) 143B cells infected with wild-type vaccinia virus (WR strain). Recombinant viruses were selected with 5-bromodeoxyuridine (BUdR) and X-gal and identified with DNA dot blot analysis (vSC11-CETP and vTM1-CETP). The CETP cDNA insert in the recombinant vaccinia virus genome was identified by Southern blot analysis. Transcription of CETP cDNA in CV-1 cells infected with recombinant vaccinia virus was monitored by Northern blot analysis using the CETP cDNA as a probe. Positive signals were detected at 1.8 kb in cells infected with vSC11-CETP and at 2.3 kb in cells infected with vTM1-CETP. The recombinant vaccinia virus-infected CV-1 cells were shown to produce functional CETP when the culture medium was subjected to the CETP assay.

Key words: Cholesteryl Ester Transfer Protein (CETP), Lipoprotein Metabolism, Vaccinia Virus Expression System, Gene Expression.

Cholesteryl ester transfer protein (CETP) transfers cholesteryl esters (CEs) and other nonpolar lipids between the major plasma lipoproteins (Quig *et al.*, 1990). Its activity plays an important role in the reverse cholesterol transport pathway by which plasma cholesterols are returned through the plasma to the liver for catabolism. Animals with a high CETP activity tend to have high levels of very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL), whereas those with a minimum CETP activity tend to have a high level of high-density lipoproteins (HDL) (Agellon *et al.*, 1991). Consequently, CETP has been recognized as a potent atherogenic plasma protein.

CETP has been purified to homogeneity (Hesler *et al.*, 1987; Jarnagin *et al.*, 1987) and its cDNA has been cloned and sequenced (Drayna *et al.*, 1987). The sequence indicates that it is a highly hydrophobic protein with a molecular mass of 62~74 kDa and a single polypeptide of 476 amino acids with a large carbohydrate content (Jarnagin *et al.*, 1987; Hesler *et al.*, 1987).

In this communication, results from initial attempts for the production of functional human CETP using the vaccinia virus as an expression vector are reported. Vaccinia virus was chosen since recombinant vaccinia virus-infected cells are recognized to express high levels of the recombinant proteins with proper processing, glycosylation and cellular transport.

Materials and Methods

Viruses and cells

Vaccinia viruses, strain WR and TF 7-3 (Fuerst *et al.*, 1986) were obtained from Dr. Byung-Yoon Ahn (Department of Genetic Engineering, Korea University), replicated in HeLa S3 cells, and purified as reported previously (Mackett *et al.*, 1985). HeLa S3 cells were grown in Eagle's minimal essential medium supplemented with 5% fetal bovine serum (FBS). Human TK^- 143B cells were grown in Eagle's medium containing 10% FBS and 25 μg/ml of 5-bromodeoxyuridine (BUdR). CV-1 monkey kidney cells were grown in Dulbecco's modified Eagle's medium containing 10% FBS.

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Plasmids

The pCETP, pUC18 containing CETP cDNA, was obtained from Judith A. K. Harmony (Department of Pharmacology and Cell Biophysics, University of Cincinnati, USA) and Shizuya Yamashita (Second Department of Internal Medicine, Osaka University, Japan). The vaccinia virus insertion vectors, pSC11 and pTM1, were generously provided by Pann-Gill Suh (Department of Life Science, Pohang Institute of Science and Technology). The construction of pSC11-CETP and pTM1-CETP, recombinant plasmids containing the entire CETP cDNA, is described in the legend to Fig. 1.

Construction of vaccinia virus recombinants

All DNA and plasmid manipulations were carried out as described by Sambrook *et al.* (1989). Recombinant vaccinia virus was prepared by transfecting wild-type vaccinia virus-infected CV-1 monolayers with recombinant plasmids by the calcium phosphate method. TK^- recombinants were then isolated by a plaque assay of transfected cell lysate on TK^- 143B cells overlaid with 1% low-melting-point agarose overlay containing 25 μg of BUdR and 300 μg of X-gal per ml. To isolate recombinants for pTM1-CETP (has no LacZ gene), DNA dot blot analysis was performed. Blue plaques or dot blot-positive plaques were isolated and subjected to three rounds of plaque purification. The DNA isolated from the recombinant virus was examined by restriction enzyme digestion and Southern blot analysis to confirm the predicted insert as described previously (Rice *et al.*, 1985).

RNA preparation and analysis

Confluent monolayers of CV-1 cells were infected

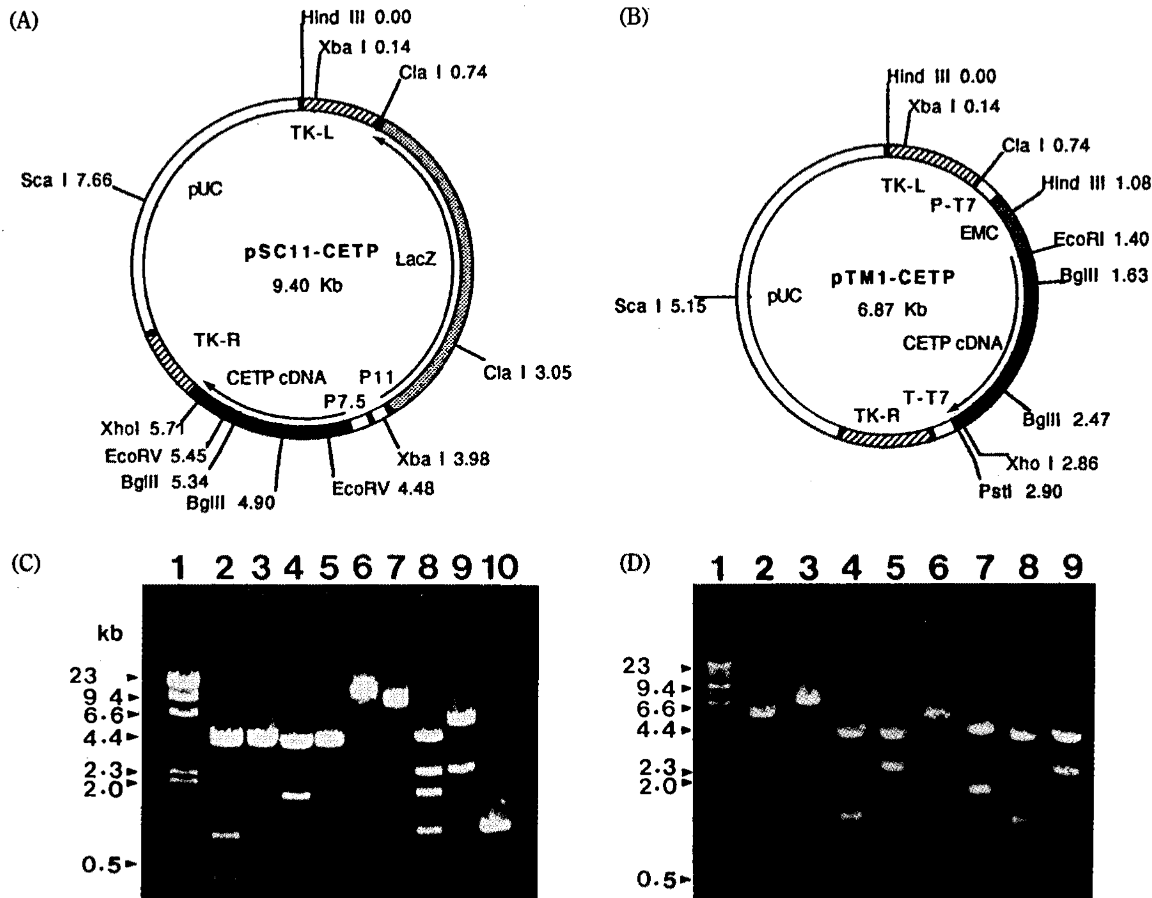

Fig. 1. Construction of recombinant human CETP cDNA for the expression in vaccinia virus-infected cells. (A) pSC11-CETP; pSC11 digested with *Sma*I was ligated with CETP cDNA end-filled with Klenow fragment and (B) pTM1-CETP; pTM1 digested with *Sma*I was ligated with CETP cDNA trimed with T4 DNA polymerase. (C) Restriction enzyme analysis of pSC11-CETP. Lane 1 : λDNA digested with *Hin*dIII; Lane 2, 4, 6, 8 : pSC11-CETP digested with *Xba*I+*Bgl*II, *Xba*I+*Xho*I, *Hin*dIII, *Eco*RV, respectively; Lane 3, 5, 7, 9 : pSC11 digested with *Xba*I+*Bgl*II, *Xba*I+*Xho*I, *Hin*dIII, *Eco*RV, respectively; Lane 10, complete CETP cDNA. (D) Restriction enzyme analysis of pTM1-CETP. Lane 1 : λDNA digested with *Hin*dIII; Lane 2, 4, 6, 8 : pTM1 digested with *Eco*RI, *Xba*I+*Xho*I, *Xba*I+*Bgl*II, *Xba*I+*Pst*I, respectively; Lane 3, 5, 7, 9 : pTM1-CETP digested with *Eco*RI, *Xba*I+*Xho*I, *Xba*I+*Bgl*II, *Xba*I+*Pst*I, respectively. P7.5; early/late vaccinia promoter, P11; late vaccinia promoter, TK^-R or L; segments of thymidine kinase gene, EMC; encephalomyocarditis virus untranslated region, P-T7; bacteriophage T7 promoter and T-T7; bacteriophage T7 terminator.

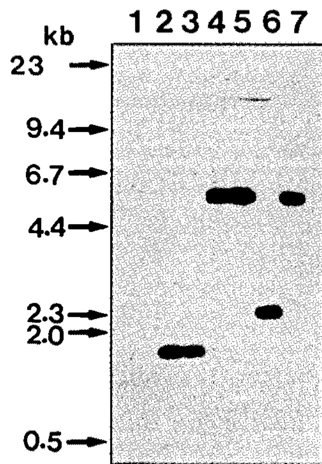

Fig. 2. Southern blot analysis of the CV-1 cells infected by the recombinant vaccinia viruses. Viral DNA (500 ng) isolated from CV-1 cells infected by wild-type virus (WR strain, Lane 1), vTM1-CETP (Lane 2~3) and vSC11-CETP (Lane 4, 5, 7: pSC11-CETP was inserted into genome of recombinant vaccinia virus with forward orientation and Lane 6: with reverse orientation) were digested with restriction enzymes (wild-type virus; *Hin*dIII+*Xba*I, vTM1-CETP; *Eco*RI+*Sal*I, vSC11-CETP; *Hin*dIII+*Xho*I), electrophoresed and transferred to nylon membranes. Hybridizations were carried out with random primed [^{32}P]-labeled CETP cDNA probe.

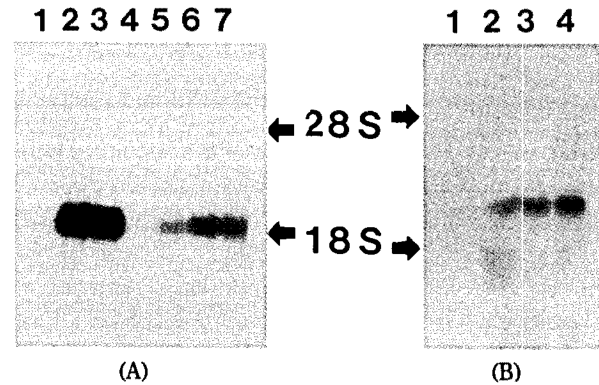

Fig. 3. Northern blot analysis of the CV-1 cells infected by the recombinant vaccinia viruses. Total RNAs (10 μg) isolated from CV-1 cells infected with wild-type vaccinia virus, vSC11-CETP or vTM1-CETP were electrophoresed and transferred to nylon membranes. Hybridizations were carried out with random primed [^{32}P]-labeled CETP cDNA probe. (A) Lane 1: wild-type virus; Lane 2~7: clones of recombinant vaccinia virus, vSC11-CETP and (B) Lane 1: wild-type vaccinia virus, Lane 2~4: clones of recombinant vaccinia virus, vTM1-CETP.

with vSC11-CETP at a MOI (multiplicity of infection) of 10 in the presence of 100 μg/ml cycloheximide. After incubation for 4 h, cells were harvested and RNA was isolated from cells as previously described (Chomckzynski *et al.*, 1987). RNAs were electrophoretically separated on a 1.0% agarose gel in the presence of 2.2 M formaldehyde, and were transferred to nylon membranes. RNAs on the membranes were then hybridized with [^{32}P]-labeled CETP cDNA as a probe.

Polypeptide analysis

[^{35}S]-methionine labeling of expressed proteins was carried out as described by Elroy-Stein *et al.* (1989). Confluent monolayers of CV-1 cells in 35-mm dish were infected with wild-type vaccinia virus (MOI of 20) or coinfected with vTF7-3 (MOI of 10) and vTM1-CETP (MOI of 10). At 24 h after infection, cells were starved for methionine for 20 min and then were pulse-labeled with 30 μCi of [^{35}S]-methionine for 30 min in hypertonic condition (190 mM NaCl). Cell lysates were prepared and equal volumes (corresponding to 2×10 cells) were loaded and subjected to the 10% SDS/polyacrylamide gel. After electrophoresis, the gel was fixed, dried, and exposed to X-ray film.

CETP assay

Cholesteryl ester transfer activity of CETP was monitored as a rate of transfer of cholesteryl-[^{14}C]-oleate from reconstituted human plasma HDL (HDL_R) covalently bound to agarose beads to human plasma LDL by the modified method of Park *et al.* (1992). At 24 h after infection with the recombinant vaccinia viruses, the cultured medium was used as a CETP source. To measure CE transfer activities, 50 μl of HDL_R, 300 μl of LDL and 50 μl of the medium were mixed and placed in a shaking incubator (260 rpm) at 37℃. The HDL_R-agarose was then removed by centrifugation. Radioactivity in the supernatant LDL fraction was determined by liquid scintillation counting.

Results and Discussion

Construction and characterization of vaccinia virus recombinants

We chose plasmid pSC11 and pTM1 for the construction of viral recombinants, since pSC11 has a single convenient *Sma*I site as well as a β-galactosidase gene for ease of selection and since pTM1 is used with the vaccinia-T7-EMCV hybrid system in which the level of expression is higher than that obtained with conventional vaccinia virus vectors. We inserted CETP cDNA into insertion vectors such that the 5′ end of the cDNA was proximal to the P7.5 promoter of pSC11 or the P-T7 promoter of pTM1. The recombinant plasmids, pSC11-CETP and pTM1-CETP, were isolated and used for *in vivo* recombination experiments.

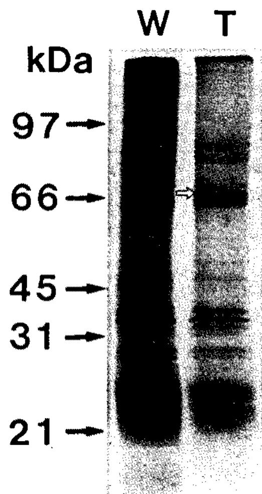

Fig. 4. [^{35}S]-methionine labeling of expressed CETP. After CV-1 cells were coinfected by vTF7-3 and vTM1-CETP and metabolically labeled with [^{35}S]-methionine, lysate of the CV-1 cells was applied on the 10% polyacrylamide gel. Lane W: wild-type vaccinia virus-infected cells and Lane T: vTM1-CETP infected cells. Molecular weight markers are shown on the left (expressed as $Mr \times 10^{-3}$).

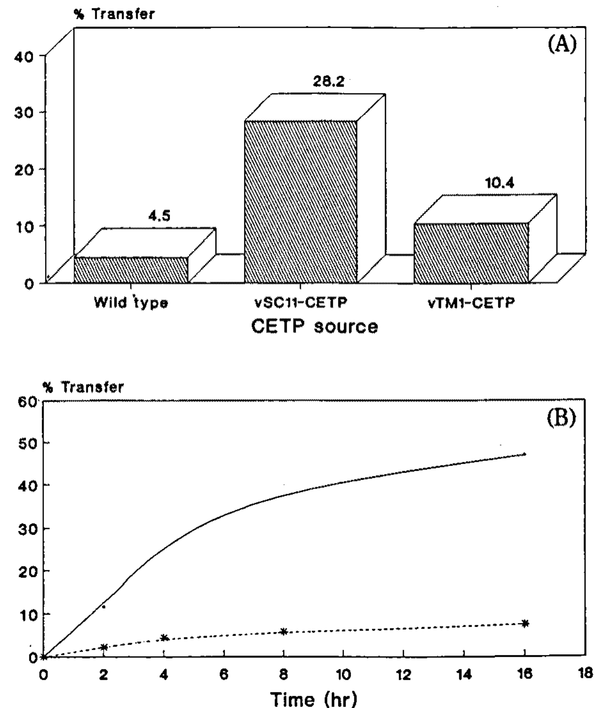

Fig. 5. Cholesteryl ester transfer activities in cultured medium of CV-1 cells infected with wild-type and recombinant vaccinia viruses. HDL_R (50 μl) and LDL (300 μl) were resuspended with cultured medium from wild-type and recombinant vaccinia viruses (A) for 4 h or (B) according to time (0, 2, 4, 8, 16 h) at 37℃. ●-●: vSC11-CETP, *---*: wild-type.

Cells infected with wild-type vaccinia virus were transfected with calcium phosphate-precipitated derivatives of pSC11-CETP and pTM1-CETP. The cells were subsequently harvested and TK^- recombinant viruses were selected by plaque assay on TK^- cells in the presence of BUdR, and the recombinant viruses were further screened by dot-blot hybridization to characterize the presence of the CETP cDNA. Viral plaques positive for CETP cDNA were then purified once more in TK^- cells with BUdR selection, and large stocks were prepared under nonselective conditions in HeLa S3 cells.

To characterize the recombinants, DNA from the above recombinants as well as from wild-type vaccinia virus was digested with *Hin*dIII and *Xba*I for the pSC11-CETP and *Eco*RI and *Sal*I for the pTM1-CETP. The resulting fragments were separated by electrophoresis on 0.8% agarose gel. The gel-separated DNA fragments were blotted onto a nylon membrane and hybridized to [^{32}P]-labeled CETP cDNA. Autoradiography revealed that the 5.9 kb *Hin*dIII-*Xba*I fragment for the pSC11-CETP and the 1.5 kb *Eco*RI-*Sal*I fragment for the pTM1-CETP were hybridized with the probe (Fig. 2). The recombinant viruses selected as above were designated as vSC11-CETP and vTM1-CETP, respectively.

Transcription of CETP cDNA in cells infected with recombinant vaccinia virus

The first of the two recombinant vaccinia viruses, vSC11-CETP, contains the entire CETP gene under the control of a vaccinia early/late promoter inserted into the TK locus of the vaccinia virus genome. The viral recombinants were designed to be constructed so that the first ATG initiation codon of CETP cDNA would be the authentic translation initiation codon. The transcriptional termination signal of the vaccinia virus (Yuen *et al.*, 1987) is located approximately 300 bp downstream of the CETP sequence, at the 3′ end of the viral TK gene. The second recombinant virus, vTM 1-CETP, contains the entire CETP gene between the bacteriophage T7 promoter and the terminator.

Transcription of the CETP gene in CV-1 cells infected with the recombinant vaccinia viruses was monitored by Northern blot analysis. Cycloheximide, inhibitor of protein synthesis, was used to increase and prolong the synthesis of early RNA. CV-1 cells infected with vSC11-CETP and vTM1-CETP produced the expected full-size RNA species of approximately 1.8 kb and 2.0 kb, respectively, and these are hybridized with CETP cDNA probe (Fig. 3).

Metabolic labeling and enzymic activities of expressed proteins

Confluent CV-1 cells were coinfected with vTM1-CETP and vTF7-3 to provide T7 RNA polymerase.

Protein synthesis was monitored by SDS-PAGE with cytoplasmic proteins from the cells labeled with [^{35}S]-methionine at 24 h after infection in hypertonic (190 mM NaCl medium). A 70 kDa band, corresponding in size to the CETP polypeptide, was observed among the proteins from the cells infected with vTM1-CETP, and this band was absent in the proteins obtained from control cells infected with the wild-type vaccinia virus, WR strain (Fig. 4).

In CETP assay, the cultured supernatants from cells infected by vSC11-CETP showed high CE transfer activities. However, the media from cells infected by vTM1-CETP showed low CE transfer activities. The *Nco*I site of plasmid pTM1 contains the translation initiation codon, which might cause the vTM1-CETP to be unable to secrete the CETP into the culture media (Fig. 5).

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