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Inhibitory Effects of Antisense RNA on Expression of Cholesteryl Ester Transfer Protein in Vaccinia Virus Expression System

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Abstract: Cholesteryl ester transfer protein (CETP), a hydrophobic glycoprotein promoting transfer of cholesteryl esters (CE) from high-density lipoproteins (HDL) to lower-density lipoproteins in the plasma, has been recognized a potent atherogenic factor during the development of coronary artery diseases. This study demonstrated a possible utilization of antisense RNA to inhibit expression of the CETP gene using vaccinia virus as an expression system. The CETP cDNA was inserted into a transfer vector (pSC11) in sense and antisense orientations and used to generate recombinant viruses. Recombinants containing sense or antisense orientations of the CETP cDNA were isolated by TK^- selection and X-gal test. The inserted CETP cDNAs in the recombinants were identified by Southern blot analysis and allowed to transcribe in host cells (CV-1). Expressions of the exogenous CETP mRNA, extracted from the CV-1 cells coinfected with viruses containing sense and antisense DNAs, were monitored by Northern blot analysis using the CETP cDNA probe, by Western blot analysis using monoclonal antibody against the C-terminal active region of the CETP and by the CETP assay. Decreased expressions of the exogenous CETP cDNA were clearly evident in the Northern and Western blot analyses as the dose of antisense expression increased. In the CETP assay, the CETP activities decreased compared to the activity obtained from the cell extracts infected with sense construct only.

Key words: antisense nucleotide, cholesteryl ester transfer protein (CETP), vaccinia virus expression system.

Cholesteryl ester transfer protein (CETP), a hydrophobic plasma glycoprotein of Mw 74 kDa, promotes a net mass transfer of cholesteryl esters (CE) from high-density lipoproteins (HDL) to triglyceride (TG)-rich lipoproteins, very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL), with a reciprocal transfer of TG in the reverse direction (Hesler *et al.*, 1987). Direct evidences that the CETP regulates the rate of delivery of CE from plasma to the liver (Whitlock *et al.*, 1989) suggest a major role of the CETP in a reverse cholesterol transport pathway, a centripetal movement of cholesterol from peripheral tissues to the liver via the plasma lipoproteins. In the initial step of the pathway, excessive cholesterols in peripheral tissues are taken up by HDL acting in conjunction with lecithin:cholesterol acyltransferase (LCAT), which esterifies cholesterol in the HDL (Glomset, 1968). When a high cholesterol diet is ingested, the CETP activity increases due to increased CETP expression in the peripheries and liver (Martin *et al.*, 1993). The increment of CETP activity results in an increased rate of CE transfer from HDL to lower-density lipoproteins, leading to a reduced concentration of CE in HDL and an increased concentration in LDL. As the reverse cholesterol transport increases, LDL receptors are down-regulated due to the excessive deposition of cholesterol in the liver as a result of the CETP activity. Consequently, CE-enriched VLDL remnants and LDL accumulate in the plasma. The LDL are susceptible to oxidation and in turn the oxidized LDL are taken up by macrophages resulting in the formation of foam cells, a participant in the development of arterial plaques (Henrickson *et al.*, 1981; Sparrow *et al.*, 1989). The increased incidences of coronary heart diseases positively related to the rise of the LDL/HDL cholesterol ratio imply that the CETP is a risk factor for atherogenesis. There has been a report that transgenic mice expressing CETP have much worse atherosclerosis than non-expressing mice (Marotti *et al.*, 1993), which supports this supposition.

Thus, we focused on reduction of CETP activity and took advantage of antisense RNA to inhibit CETP ex-

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pression in a recombinant vaccinia virus expression system. Antisense RNA has been successfully used to suppress target genes in eukaryotic cells in a sequence-specific manner (Knecht *et al.*, 1987; Moxham *et al.*, 1993). This study shows an inhibitory effect of antisense RNA on the expression of exogenous CETP and the possibility of utilization of antisense RNA for the inhibition of endogenous CETP gene expression.

Materials and Methods

Cell culture

CV-1 cells (ATCC CCL 70) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), HeLa S3 cells (ATCC CCL 2.2) in Eagle's minimal essential medium (MEM) with 5% FBS, and HuTK$^-$ 143B cells (ATCC CRL 8303) were maintained in MEM containing 10% FBS and 25 μg/ml of 5-bromodeoxyuridine (BUdR). Cells were incubated in CO_2 incubator adjusted with 5% CO_2 at 37℃ and were maintained in the logarithmic growth phase. CV-1 cells were used for transfection, HeLa S3 cells for amplification of viruses and HuTK$^-$ 143B cells for TK$^-$ selection.

Viruses and insertion vector

A wild-type vaccinia virus (strain WR) was used to make all recombinants and propagated in HeLa S3 cells. After titration, the virus stocks were stored at −70℃ until use. pSC11, a general transfer vector for insertion of foreign DNA into the thymidine kinase (TK) gene of vaccinia virus (Chakrabarti *et al.*, 1985), was used to obtain recombinant vaccinia viruses.

Construction of insertion vector and transfection

Plasmid pSC11 was chosen as a viral insertion vec-

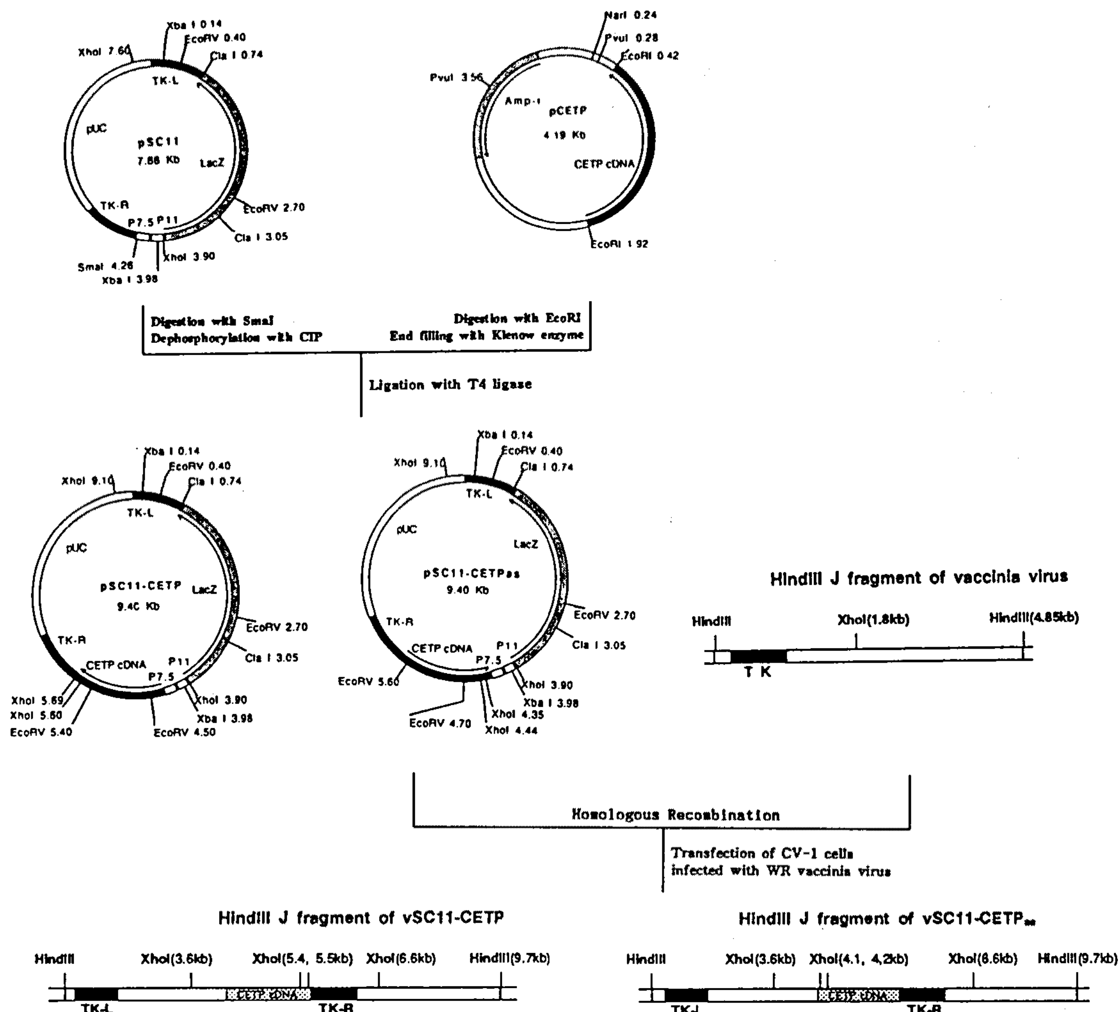

Fig. 1. Strategy for generation of recombinant vaccinia viruses. The CETP cDNA obtained from pCETP was inserted into pSC11 in the sense (pSC11-CETP) and the antisense orientations (pSC11-CETP$_{as}$). Reconstructed insertion vectors were transfected into CV-1 cells previously infected with wild-type vaccinia viruses (WR strain). The recombinant viruses were selected by the plaque assay overlaid with low-melting-point agarose containing BUdR and X-gal and designated vSC11-CETP and vSC11-CETP$_{as}$.

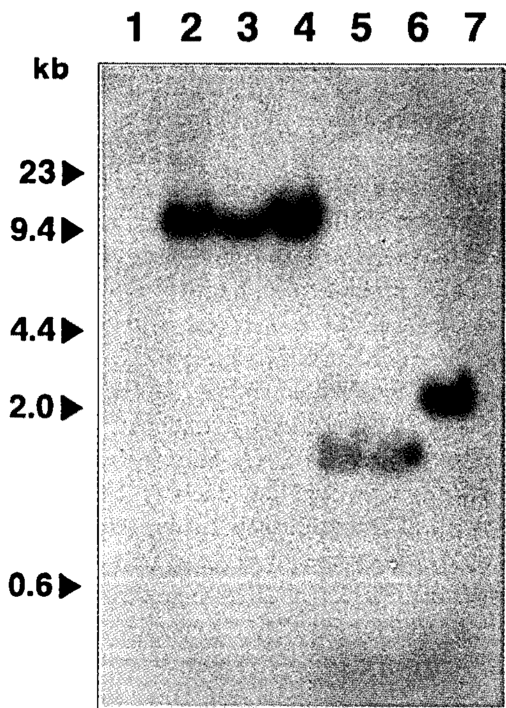

Fig. 2. Southern blot analysis of genomic DNA isolated from recombinant vaccinia viruses. The DNA extracted from the CV-1 cells infected with recombinant viruses, was electrophoresed, transferred onto nylon membrane and hybridized with a [^{32}P]-labeled probe synthesized from double-stranded CETP cDNA. Lane 1: WR (*Hin*dIII); lanes 2~3 : vSC11-CETP (*Hin*dIII); lane 4 : vSC11-CETP$_{as}$ (*Hin*dIII); lanes 5~6 : vSC11-CETP (*Hin*dIII+*Xho*I) and lane 7 : vSC11-CETP$_{as}$ (*Hin*dIII+*Xho*I).

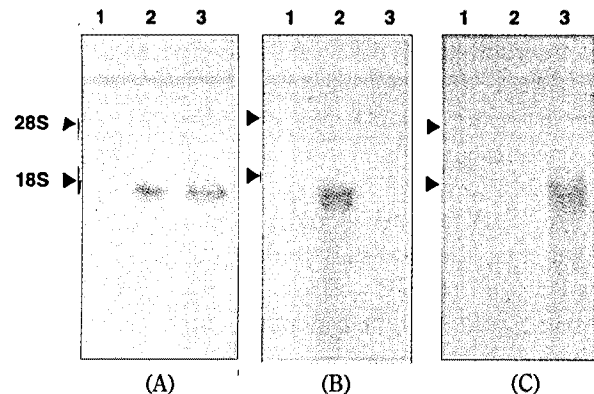

Fig. 3. Northern blot analysis of RNA isolated from recombinant vaccinia viruses. The RNA extracted from CV-1 cells infected with wild-type virus, vSC11-CETP and vSC11-CETPas were electrophoresed and hybridized with a [^{32}P]-labeled probe synthesized from double-stranded (A), single-stranded sense (B) and single-stranded antisense (C) CETP cDNA template. Lane 1 : WR; lane 2 : vSC11-CETP and lane 3 : vSC11-CETP$_{as}$.

tor, since it contains a single convenient *Sma*I site just downstream of the vaccinia viral early/late promoter (P7.5). The plasmid also contains an *lacZ* gene under the control of the vaccinia viral late promoter (P11), which allows an easy selection of recombinants through an X-gal test. The pSC11 was reconstructed to transfect CV-1 cells infected with wild-type vaccinia virus as follows (Fig. 1): the CETP cDNA (1.5 kb) was separated from pCETP, pUC18 containing CETP cDNA in EcoRI site, end-filled to get blunt ends, and inserted into pSC 11 previously digested with *Sma*I. After transformation into *E. coli*, the colonies containing the antisense orientation of inserted CETP cDNA were isolated from those of sense oriention by restriction enzyme mapping, using *Cla*I, *Eco*RV, and *Xba*I-*Xho*I digestions (Fig. 2). All DNA and plasmid manipulations were conducted as described by Sambrook *et al.* (1989).

For transfection, CV-1 cells (1×10^{6}) infected with wild-type vaccinia viruses (0.01 pfu/cell) on a culture dish were exposed to calcium phosphate-precipitated insertion vector DNA (10 μg/dish). After 24 h, the transfectants were collected for selection of recombinants.

Selection of recombinant viruses

Human TK^{-} 143B cells were infected with the transfectants at a multiplicity of infection (MOI) of 0.01 pfu/cell and incubated for 2 h in the medium containing 25 μg/ml of BUdR. The TK^{-} recombinants were isolated by plaque assays overlaid with 1% (w/v) low-melting-point agarose containing BUdR (25 μg/ml) and X-gal (300 μg/ml). Blue plaques were collected and the process was repeated three times for plaque purification. The DNAs isolated from the recombinant viruses were subjected to restriction endonuclease digestions and Southern blot analysis to confirm the predicted inserts using a [^{32}P]-labeled probe synthesized from double-stranded CETP cDNA (Fig. 3).

Stocks for recombinants were prepared by infecting the trypsinized crude stocks to HeLa S3 cells at an MOI of 0.01 pfu/cell for 2 days. The infected cells were scraped, centrifuged, and resuspended in 1 ml of MEM supplemented with 2.5% (v/v) FBS for future use.

RNA preparation and analysis

Confluent CV-1 cells were coinfected with the recombinant viruses and the wild-type viruses at a MOI of 30 pfu/cell. After 1 h, the inocula were replaced with a medium containing 5% FBS and cycloheximide (25 μg/ml) to increase and prolong the synthesis of early mRNA by the inhibition of protein synthesis. After 5 h, the cells were washed with PBS and total RNA was purified by the guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski *et al.*, 1987). The RNA was subjected to electrophoresis on formaldehyde gel, followed by blotting to a nylon membrane for the hybridization.

To demonstrate antisense CETP transcripts using Northern blot analysis, a probe synthesized from single-

stranded CETP cDNA was prepared. The CETP cDNA was inserted into pUC18 and amplified and sense and antisense orientations were selected by restriction enzyme mapping. The plasmids were digested with *Bam*HI for linearization and the resulting DNA was used as a template to make the single-stranded probe with the M13/pUC18 forward sequencing primer (17 mer, Promega). For the double-stranded probe, CETP cDNA isolated from pCETP was used as a template for probe synthesis with random primer (6 mer, Amersham).

CETP assay

Activity of the expressed CETP was analyzed by monitoring the amount of [^{3}H]-labeled cholesteryl esters transferred from HDL to unlabeled LDL (Park *et al.*, 1992). Assay mixture contained [^{3}H]-CE-HDL_R immobilized to agarose, unlabeled LDL, and CETP source in the final incubation volume of 0.4 ml. The mixture was allowed to react for 6 h at 37℃ with shaking. After brief centrifugation, the supernatant (200 μl) was subjected to liquid scintillation counting (Packard, Meriden; CT, USA).

Western blot analysis

Polyacrylamide gel electrophoresis and Western blot analysis were performed by the standard procedure (Towbin *et al.*, 1979). Monoclonal antibody against the active region of CETP was used for the immunoblot and the bands were visualized using anti-rabbit IgG conjugated with alkaline phosphatase (Vector, Burlingame, CA, USA).

Results and Discussion

Construction of insertion vector and selection of recombinant viruses

The full-length CETP cDNA obtained from pCETP was inserted into pSC11 (Fig. 1). The orientations of inserted CETP cDNA were verified by restriction enzyme mapping. In brief, double digestions of the plasmids with *Xba*I and *Xho*I produced 0.4 kb, 1.6 kb, 3.8 kb, and 4.0 kb in the sense orientation and 0.4 kb, 3.8 kb and 5.1 kb in the antisense orientation (data not shown). The verified plasmids of sense and antisense orientations were designated as pSC11-CETP and pSC11-CETPas, respectively.

Using the reconstructed plasmids, the recombinant vaccinia viruses were prepared by isolating the blue plaques. Crude DNA was isolated from the host cells infected with several different blue plaques and used for dot blot analysis using a [^{32}P]-labeled double-stranded CETP cDNA probe. Results showed that the selected recombinant vaccinia viruses contain the CETP cDNA (data not shown). The recombinant vaccinia viruses containing DNAs with the sense and antisense orientations were designated vSC11-CETP and vSC11-$CETP_{as}$, respectively.

Characterization of the recombinant viruses

The genomic DNA of the recombinant viruses was analyzed by restriction enzyme digestions and subsequent Southern blot analysis with [^{32}P]-labeled double-stranded CETP cDNA as a probe. The common region used for the insertion of foreign genes into the vaccinia virus genome has been shown to be the TK locus within the 4.85 kb *Hin*dIII J fragment (Hurby *et al.*, 1983). Thus, when the DNA isolated from the recombinant viruses was digested, the size of the *Hin*dIII J fragment should be increased by the size of the inserted DNA. The recombinant vaccinia viruses were shown to contain the 9.7 kb of DNA fragments (Fig. 2, lanes 2~4) corresponding to the expected size. When the viral DNA from the recombinant viruses was double-digested with *Hin*dIII and *Xho*I, DNA bands of 1.8 kb and 2.4 kb appeared in the vSC11-CETP (Fig. 2, lanes 5~6) and in the vSC11-CETPas (Fig. 2, lane 7), respectively. However, the viral DNA from the wild-type virus used as a negative control did not show such bands (Fig. 2, lane 1).

Transcription of the inserted CETP cDNA was examined by Northern blot analysis using single-stranded sense and antisense CETP cDNA probes. The CV-1 cells infected with the vSC11-CETP and the vSC11-$CETP_{as}$ produced RNA species of approximately 1.8 kb that are hybridized with sense and antisense probes (Fig. 3). The observed RNA species were larger than the expected 1.5 kb probably due to a delayed termination of transcription occurring in the transcriptional termination signal of thymidine kinase gene (Pensiero *et al.*, 1988).

Decrement of CETP mRNA

To examine effects of antisense RNA on the amount of CETP mRNA, CV-1 cells were coinfected with vSC 11-CETP and different dosages of vSC11-$CETP_{as}$. CV-1 cells coinfected with vSC11-CETP and vSC11-CET-P_{as} at a ratio of 1 : 1 were shown to contain a decreased level of RNA transcripts for CETP compared to those infected with vSC11-CETP only, as hybridized with the double-stranded and the single-stranded sense probe (Fig. 4). The RNA transcripts from CV-1 cells coinfected with the vSC11-CETP and the wild-type virus at a ratio of 1 : 1 (a control to detect a possible dilution-effect of the coinfection) showed similar results to those infected with the vSC11-CETP only. From these observations, it was postulated that the more anti-

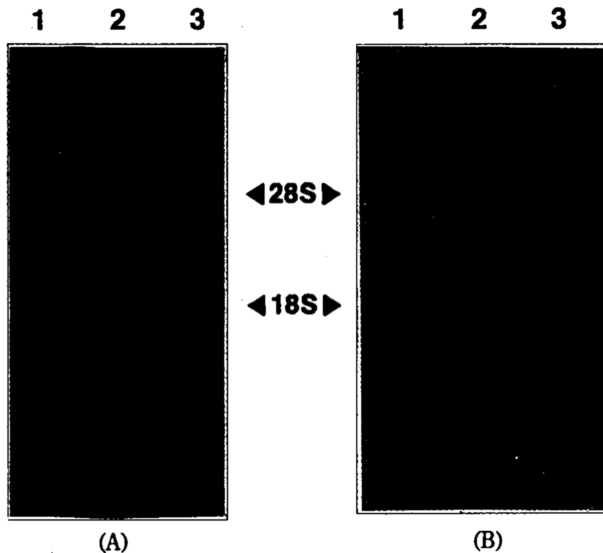

Fig. 4. Inhibitory effect of antisense RNA on sense CETP mRNA. RNA from CV-1 cells coinfected with vSC11-CETP and vSC 11-CETP$_{as}$ were hybridized using a [^{32}P]-labeled probe synthesized from double-stranded (A), single-stranded sense (B) CETP cDNA template. Lane 1 : vSC11-CETP and vSC11-CETP$_{as}$; lane 2 : vSC11-CETP and WR and lane 3 : vSC11-CETP only.

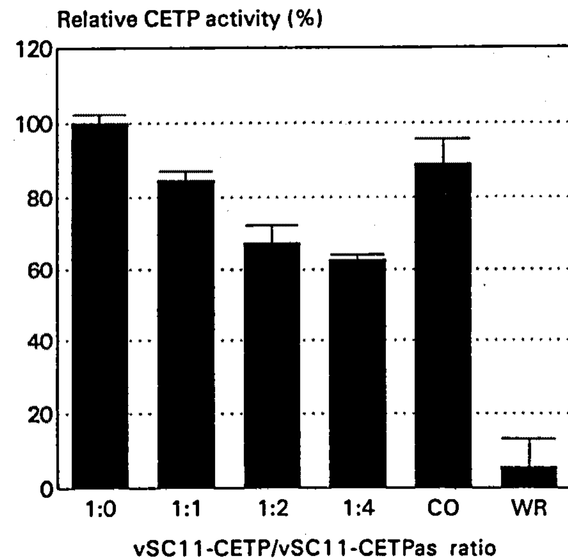

Fig. 6. Inhibition of CETP activity by antisense construct. Lysates of CV-1 cells coinfected with vSC11-CETP/vSC11-CETP$_{as}$ and with vSC11-CETP/wild-type viruses were used as CETP sources in the mixtures of CETP assay. Cell lysate obtained from CV-1 cells infected with wild-type only was used as a negative control (WR).

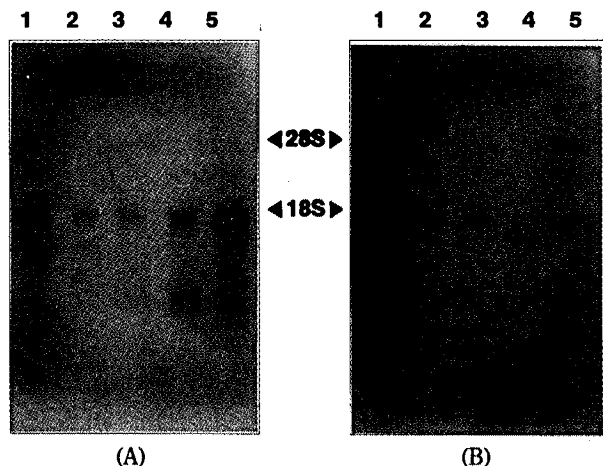

Fig. 5. Northern blot analysis using a [^{32}P]-labeled probe synthesized from double-stranded (A), single-stranded sense (B) CETP cDNA template. The CV-1 cells were coinfected with vSC11-CETP/vSC11-CETP$_{as}$ with the ratios of 1 : 1, 1 : 2 and 1 : 4 (lanes 2~4 in A and lanes 3~5 in B). Cells infected with vSC11-CETP only (lane 5 in A and lane 1 in B) and coinfected with vSC11-CETP/WR with the ratio of 1 : 4 (lane 1 in A and lane 2 in B) were used as controls.

sense RNAs are transcribed, the less CETP mRNA would be detected. To determine whether the antisense RNAs can be used to change the CETP expression in a dose-dependent manner, CV-1 cells were coinfected with vSC11-CETP/vSC11-CETP$_{as}$ at ratios of 1 : 1, 1 : 2 and 1 : 4. Results showed decreased levels of the CETP mRNA as the dose of vSC11-CETP$_{as}$ increases (Fig. 5). On the other hand, the CV-1 cells coinfected with the vSC11-CETP and the WR at a ratio of 1 : 4 did not change a level of expression of the CETP mRNA.

Although the hybrids formed between the sense and antisense RNAs were not possible to observe directly, the evidences shown in this study were consistent with several previous findings made in other expression systems (Strickland *et al.*, 1988; Wormington, 1986), suggesting that the antisense transcripts may produce hybrids with the sense mRNAs generating sense-antisense duplex RNAs with a short half-life (Korl *et al.*, 1988).

Decrement of CETP expression

Cell lysates were prepared from CV-1 cells coinfected with the recombinants containing the sense and antisense constructs. Other cell lysates were prepared from the same cells but with the antisense construct replaced by the wild-type virus. These two lysate samples were subjected to a CETP assay to compare the activities of CETP generated from the two different cells. We have shown that the CV-1 cells infected with the viral recombinants containing CETP cDNA express the biologically active CETP (Jang *et al.*, 1995). Results in this study showed that the cells containing sense/antisense constructs possessed approximately 30% less activity than those with sense/wild-type constructs (Fig. 6). To obtain further evidences on the inhibitory effects of antisense RNA on the expression of CETP, the production of CETP in the CV-1 cells was monitored by Western blot analysis. Total cell lysates were prepared

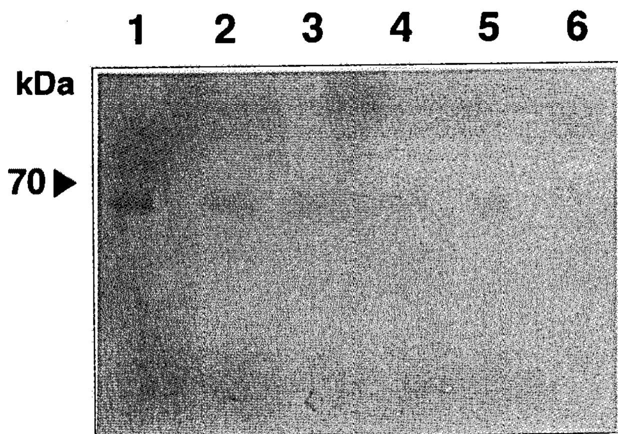

Fig. 7. Western blot analysis with the expressed CETP. The CV-1 cells coinfected with vSC11-CETP and vSC11-CETPas were lysed, electrophoresed and immunoblotted using monoclonal antibody against C-terminal active region of CETP. Lane 1 : vSC11-CETP; lanes 2~4 : vSC11-CETP/vSC11-CETPas (1 : 1, 1 : 2, 1 : 4) and lane 5 : vSC11-CETP/WR (1 : 4).

from the CV-1 cells coinfected with sense and antisense viruses. The lysates were electrophoresed and immunoblotted with monoclonal antibody against the C-terminal active region of CETP. A 70 kDa CETP band was clearly observed and the thickness of the band became thinner as the dose of the antisense construct ($vSC11\text{-}CETP_{as}$) increased (Fig. 7).

Taken all the observations made in the study together, a reduction of CETP production was consistent in the results obtained from the Northern and Western blot analysis and the CETP assays. Direct observations on the formation of duplex RNA was not possible, probably because the RNA hybrids are usually degraded soon after formation (Nellen *et al.*, 1993). The RNA transcripts for CETP were provided exogenously in this study. Cells known to detect CETP RNA transcript with ease were shown to be of hepatic origin. Accordingly, attempts were made to inhibit CETP production in HepG2 cells. Unfortunately, this generated problems in the process of viral infection, which made us choose testing the possibility of inhibiting CETP production by antisense RNA.

Since the CETP has been shown to play a negative role in the clearance of cholesterol from the blood, great efforts are being made throughout the world to reduce the production of CETP or inhibit its activity. The method shown in this study may be utilized to reduce CETP production, if it is further developed.

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