*Journal of Biochemistry and Molecular Biology*, Vol. 35, No. 2, March 2002, pp. 172-177

JOURNAL OF
Biochemistry and Molecular Biology



# *In Vivo* Effects of CETP Inhibitory Peptides in Hypercholesterolemic Rabbit and Cholesteryl Ester Transfer Protein-Transgenic Mice

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*Received 25 September 2001, Accepted 1 November 2001*

**We previously reported that cholesteryl ester transfer protein (CETP) inhibitory peptides (designated $P_{28}$ and $P_{10}$) have anti-atherogenic effects in hypercholesterolemic rabbits (*Biochim. Biophys. Acta* (1998) 1391, 133-144). To further investigate those effects, we studied rabbit plasma that was collected after 30 h of a $P_{28}$ or $P_{10}$ injection. We found that there is a strong correlation between the *in vivo* CETP inhibition effects and alterations of lipoprotein particle size distribution in rabbit plasma, as determined on an agarose gel electrophoresis and gel filtration column chromatography. *In vivo* effects of the peptide were observed again in C57BL/6 mice that expressed simian CETP. The $P_{28}$ or $P_{10}$ peptide (7 μg/g of body weight) that was dissolved in saline was injected subcutaneously into the mice. The $P_{28}$ injection caused the partial inhibition of plasma CETP activity up to 50%, decreasing the total plasma cholesterol concentration by 30%, and increasing the ratio of HDL/ total-cholesterol concentration by 150% in the CETP-transgenic (tg) mice. The CETP inhibition by the $P_{28}$ or $P_{10}$ made alterations that modulated the size re-distribution of the lipoproteins in the blood stream. Particle size of the very low (VLDL) and low density lipoproteins (LDL) from the peptide-injected group was highly decreased compared to the saline-injected group (determined on the gel filtration column chromatography). In contrast, The HDL particle size of the $P_{28}$-injected group increased compared to the control group (saline-injected). The expression level of the CETP mRNA of the $P_{28}$-injected CETP-tg mouse appeared lower than the saline-injected CETP-tg mouse. These results suggest that the injection of the CETP inhibitory peptide could affect the CETP expression level in the liver by influencing lipoprotein metabolism.**

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**Keywords:** Cholesteryl ester transfer protein (CETP), Peptide inhibitor, Cholesteryl ester (CE), Lipoprotein, Cholesterol, Metabolism

## Introduction

The cholesteryl ester transfer protein (CETP) plays an important role in the reverse cholesterol transport pathway which is involved in the redistribution of the lipoprotein particle size and composition (Albers *et al.*, 1984). CETP, a 74 kDa of hydrophobic glycoprotein (Hesler *et al.*, 1987, Jarnagin *et al.*, 1987), promotes the transfer of cholesteryl ester (CE) from high-density lipoproteins (HDL) to apo-B that contain lipoproteins and a reciprocal transfer of triglycerides (TG) to HDL (Abbey *et al.*, 1985). Studies with CETP-deficient patients in Japan (Koizumi *et al.*, 1985, Inazu *et al.*, 1990), the inhibition of CETP activity in rabbits (Abbey *et al.*, 1989; Whitlock *et al.*, 1989) and hamsters (Evans *et al.*, 1994), and the introduction of human CETP into rats (Ha *et al.*, 1985; Quig *et al.*, 1986) and mice (Agellon *et al.*, 1991; Hayek *et al.*, 1992) suggest that CETP plays a major role in determining plasma lipoprotein composition, particularly the CE and TG composition of HDL.

The CETP-transgenic (tg) mouse that expressed the simian CETP gene (Marotti *et al.*, 1992) is a valuable model for the development of a lipid-lowering drug since their lipid metabolism is very similar to that of humans. Since the mouse had a high level of plasma CETP activity with an increased LDL particle size compared to a normal C57BL/6 mouse, it promptly absorbed dietary cholesterol (Marotti *et al.*, 1993).

The CETP inhibitory peptide was isolated from the hog plasma by ultracentrifugation, sequential column chromatographies (including Sephadex G-200, DEAE-Sephadex A-25 columns), and finally electroelution from gels (Cho *et al.*, 1998). A peptide that was synthesized chemically, according to the amino acid sequence of the peptide

(designated $P_{28}$ and $P_{10}$), showed approximately the same degree of CETP inhibitory activity as the isolated peptide. The sequence of the $P_{28}$ was as follows: N-Glu-Asp-Thr-Ser-Pro-Glu-Asp-Lys-Met-Gln-Asp-Tyr-Val-Lys-Gln-Ala-Thr-Arg-Thr-Ala-Gln-Asp-Ala-Leu-Thr-Ser-Val-Lys-C. The $P_{10}$ peptide had ten amino acids from the amino terminal of the $P_{28}$. We reported that the CETP inhibition effects of the $P_{28}$ and $P_{10}$ in hypercholesterolemic rabbit plasma, approximately 75% CETP activity, disappeared from the plasma within 1 h after the injection. The effect lasted up to 30 h. The inhibition of CETP *in vivo* led to a concomitant decrease in the total plasma cholesterol level up to 30%, and an increase in the level of HDL-cholesterol up to 32% (Cho *et al.*, 1998).

In this report, we investigated the further inhibitory effects at the point of the lipoprotein size distribution in the rabbit plasma. We also examined the *in vivo* effects of the CETP inhibitory peptides using CETP-transgenic mice at the point of the CETP inhibition, as well as its influential change of lipoprotein elution profile on the gel filtration column chromatography.

## Materials and Methods

**Animals** Male CETP-transgenic C57BL6 mice-originally from Upjohn Lab (Marotti *et al.*, 1993) (Kalamazoo, USA), 10 weeks post-weaning and weighing 25-30 g-were maintained in colony cages (7 mice/cage) on 12 h-light/dark cycles. After 1 week of lab-chow feeding, the mice were allowed free access to water and a powdered semi-synthetic diet that contained high cholesterol and high fat (HCHF) for 12 weeks. The diet contained dairy butter (15%), sucrose (50%), casein (20%), cholesterol (2%), and corn oil (5%). It also contained cellulose, vitamins, and minerals (Nishina *et al.*, 1990). After feeding the HCHF diet, the mice were randomly divided into experimental and control groups (Table 1). For the mice of the experimental groups, the $P_{28}$ or $P_{10}$ peptides (200 mg/200 ml, approximately final conc.: 6 μM in body weight) were injected subcutaneously. Saline was injected in the control groups. The injection was carried out twice at 0 and 12 h after the initial injection.

**Sample collection and analyses** Blood samples were collected from the periorbital sinus into heparinized microhematocrit capillary tubes (Sigma 02-668-66) from the non-fasted male mice at designated intervals (12 h intervals). Plasma was obtained by low-speed centrifugation (5,000 *g*) at 4°C for 5 min and stored at −20°C until analyzed. The collected mice plasma were subjected to a CE transfer assay and cholesterol concentration determination.

To determine the size distribution of lipoproteins, the mouse plasma, which had been bled 24 h post-injection, was pooled and accessed according to the method described previously (Chen *et al.*, 1982). One-milliliter of the pooled plasma was labeled with 1 μCi of [1α,2α-(n)-[$^3$H]-cholesterol (specific activity = 50 Ci/mmol, Amersham, U.K.) by incubation at 37°C for 24 h in order to allow the free cholesterol to diffuse into all of the lipoprotein particles. The [$^3$H]-cholesterol labeled plasma was loaded onto a Bio-Gel A-5 m column (1.5 × 90 cm), which had been previously equilibrated with tris-buffered saline (TBS: 10 mM Tris-HCl/140 mM NaCl/5 mM EDTA, pH 7.4). After the column application, the sample was eluted with the TBS buffer at a flow rate of 10 ml/h on gravity. The fractions were collected in 1 ml per tubes. Each 100 μl aliquot of all of the column fractions was subjected to scintillation counting using a liquid scintillation analyzer (Packard Tricarb 1600TR) in order to visualize the eluted lipoprotein peak.

**Northern blot hybridization** To compare the expression level of CETP between the peptide and saline-injected groups, the RNA sample was isolated from the mouse liver, according to the method described by Sambrook *et al.* (Sambrook *et al.*, 1989). Denatured total RNA was (20 μg) electrophoresed on 1% agarose gel that contained formaldehyde and blotted onto a Nytran-Plus membrane (Schleicher & Schull, Dassel, Germany) according to the standard protocol (Sambrook *et al.*, 1989). The membrane was pre-hybridized and hybridized in 50% formaldehyde, 5 × SSPE (sodium chloride/sodium phosphate/EDTA), 5 × Denhardts solution, 1% SDS, and 100 μg/ml denatured salmon sperm DNA at 42°C. The CETP cDNA was labeled with [α-$^{32}$P]-dCTP using the Prime-a-Gene labeling system (Promega, Madison, USA). The boiled and isotope labeled CETP cDNA probe was added to the hybridization buffer for an additional 16 h. To exclude nonspecific binding, the membrane was washed at 42°C with 2 × SSC (sodium chloride/sodium citrate)/0.1% SDS (sodium dodecyl sulfate) for 1 h, and again at 65°C with 0.2 × SSC/0.1% SDS. After the washings, the membrane was exposed to Curix RPI film (Agfa, Germany) in order to visualize the band signal.

**Lipoprotein isolation and analysis with rabbit plasma** Lipoproteins were isolated from the rabbit plasma according to the

**Table 1.** Experimental design of *in vivo* test with $P_{28}$ and $P_{10}$ in CETP-transgenic mice

| Group | Diet | Injected material | Mice* | Initial cholesterol conc (mg/dl) | n |
|---|---|---|---|---|---|
| A | HCHF** | $P_{28}$ (200 μg) | CETP-tg | 118.8 ± 3.5 | 7 |
| B | HCHF | $P_{10}$ (200 μg) | CETP-tg | 113.0 ± 5.2 | 7 |
| C | HCHF | Saline | CETP-tg | 83.8 ± 3.5 | 7 |
| D | Lab-chow | Saline | C57BL/6 | 41.8 ± 6.5 | 7 |

*The mice were CETP-transgenic (CETP-tg) mice that expressed cynomolgus monkey CETP (Marotti *et al.*, 1992).
**HCHF was high cholesterol, high fatty acid. The diet was a semi-synthetic diet which contained dairy butter (15%), sucrose (50%), casein (20%), cholesterol (2%), and corn oil (5%), as well as cellulose, vitamins, and minerals (Nishina *et al.*, 1990).

standard method described by Havel *et al.* (Havel *et al.*, 1955) with a slight modification. Briefly, pooled rabbit plasmas were brought to density 1.225 g/ml with dried solid KBr and centrifuged for 48 h at 54,000 rpm using the Ti 70.1 rotor of Beckman XL-70 centrifuge (Beckman Inc., Palo Alto, USA). After the ultracentrifugation, the lipoproteins were floated at the top of the ultracentrifuge tubes and collected. The collected lipoproteins were subjected to a Bio-Gel A-5m column (1.5 × 90 cm) chromatography to separate the VLDL, LDL, and HDL according to their particle size (Chen *et al.*, 1982). Electrophoretic mobility of intact rabbit lipoproteins were examined by electrophoresis in agarose gel, as described by Noble (Noble, 1968). After gel fixation and drying, the gel strip was stained for 2 h at room temperature in 50% methanol with 0.125% Coomassie brilliant blue R-250.

**Other methods** The cholesteryl ester transfer activity of plasma was determined as previously described (Park *et al.*, 1992; Cho *et al.*, 1998) using artificial substrates. Normal mouse plasma was used for determination of the non-specific CE transfer in this assay system since minimum CETP activity is reported to be present in the mouse plasma (Ha *et al.*, 1982, Jiang *et al.*, 1991).

Total serum cholesterol and HDL-cholesterol concentration were determined according to Allain *et al.* (Allain *et al.*, 1974) using a Sigma diagnostic kit (Sigma 352-20 and 352-3, respectively).

Concentration of the protein was determined using a bicinchroninic acid (BCA) protein assay reagent (Pierce, Rockford, IL), as described by Smith *et al.* (Smith *et al.*, 1985), with bovine serum albumin as a standard.

## Results and Discussion

**Changes of lipoprotein size distribution in the rabbit plasma by the peptide injection** Lipoproteins of the pooled rabbit plasma from each group were isolated by ultracentrifugation and Bio-Gel A-5 m column chromatography. The Bio-Gel A-5m column elution profile indicated that the VLDL and LDL protein peaks of the $P_{28}$ and $P_{10}$-injected groups were smaller than those of the saline-injected group (Fig. 1); whereas the HDL protein peak of the $P_{28}$ and $P_{10}$-injected groups were larger than those of the saline-injected group. When electrophoretic mobility of the lipoprotein fractions was measured using agarose electrophoresis, the VLDL and LDL (2 μg of protein per each lane) of the $P_{28}$ and $P_{10}$-injected groups migrated farther than those of the saline-injected group; whereas the HDL (6 μg of protein per each lane) of the $P_{28}$ and $P_{10}$-injected groups showed lower electromobility than those of the saline-injected group (Fig. 2). These results indicate that the reduced CETP activity by the $P_{28}$ and $P_{10}$ might cause concomitant change in size distribution of the lipoproteins in both hypercholesterolemic and normocholesterolemic rabbits.

**Changes of CETP activity in CETP-transgenic mice** When the peptides ($P_{28}$ or $P_{10}$) were subcutaneous injected into the mice, the CETP activity of group A (HCHF-fed, $P_{28}$-injected) decreased by 50% compared to the initial activity at

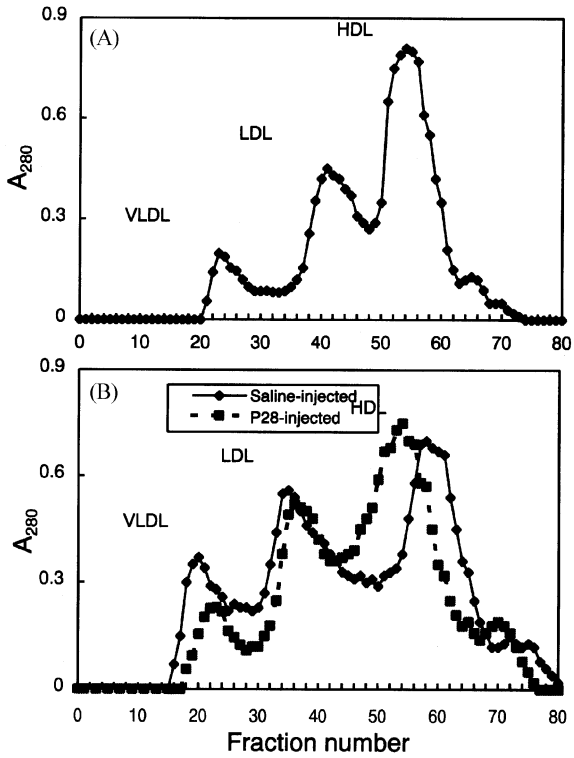


**Fig. 1.** Elution profile of isolated lipoproteins from peptides injected-rabbit plasma in Bio-Gel A-5m column (1.5 × 90 cm) chromatography. The lipoproteins of each group were isolated by ultracentrifugation (Havel *et al.*, 1955). The plasma was brought to density 1.225 g/ml with solid KBr and centrifuged for 48 h at 54,000 rpm using the Ti 70.1 rotor of the Beckman XL-70 centrifuge. After ultracentrifugation, lipoproteins floating at the top of the ultracentrifuge tubes were collected and dialyzed against the column application buffer. The column was equilibrated with 10 mM Tris/140 mM NaCl that contained 1 mM EDTA (pH 7.4) (Tris buffered saline, TBS) and eluted with the same buffer. Two-ml fractions were collected with a flow rate of 10 ml/h. Panel A: saline-injected, lab-chow fed group, panel B: saline-injected (◆) and $P_{28}$ (7-9 mg/kg body weight) injected (■) groups, 0.3% cholesterol containing diet-fed. Each lipoprotein peak was assigned after confirming on a 10% SDS-PAGE and marked on the panel.

24 h post-injection. The CETP activity of group B (HCHF-fed, $P_{10}$-injected) decreased by 23% compared to the initial activity at 36 h post-injection; whereas the activity of group C (HCHF-fed, saline injected, CETP-tg mice) increased by 53% compared to the initial activity at 36 h post-injection (Fig. 3). The CE-transfer activity of group C showed a slight increasing tendency with progress of bleeding time. Since normal mice do not have CETP activity (Ha *et al.*, 1982, Jiang *et al.*, 1991), the CETP activity of group D (chow-fed, saline-injected, normal mice) was not determined.

**Changes of total serum cholesterol level and HDL-cholesterol ratio** After feeding a semi-synthetic diet that contained 2% cholesterol for 12 weeks, the CETP-tg mice

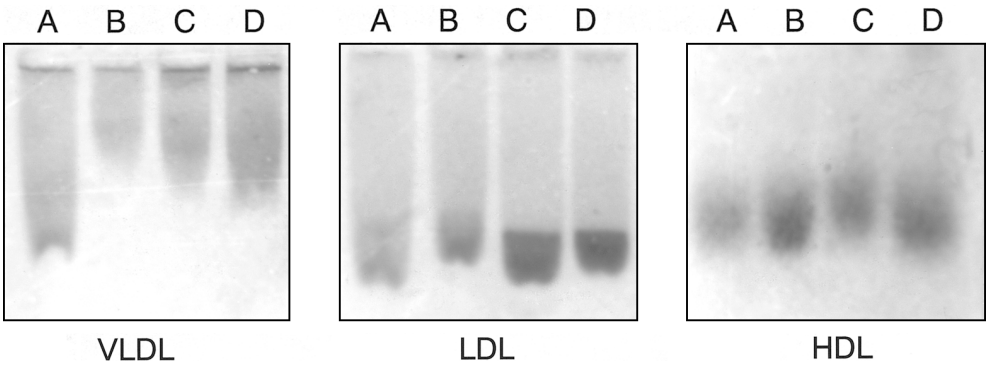


**Fig. 2.** Electrophoretic mobility of intact VLDL, LDL, and HDL isolated from rabbit plasma on 0.5%, 1.0%, and 1.5% agarose gel, correspondingly. Each plasma sample was collected 30 h post-injection. The lipoproteins were isolated by the method described previously (Chen *et al.*, 1982). Approximately 2 μg of VLDL and LDL, and 6 μg of HDL, were applied on each gel. Lane A: saline-injected, chow diet-fed group, Lane B: saline-injected, 0.3% cholesterol containing diet-fed, Lane C: $P_{28}$ (7-9 mg/kg body weight) injected, 0.3% cholesterol containing diet-fed, Lane D: $P_{10}$ injected (7-9 mg/kg body weight). The lipoprotein bands were visualized by 0.125% Coomassie brilliant blue R-250 in 50% methanol.

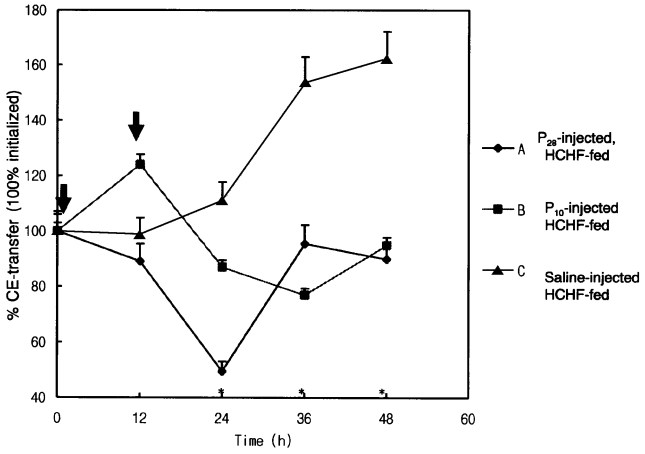


**Fig. 3.** The change of CETP activity in hypercholesterolemic CETP-transgenic (tg) mice. Synthetic peptides ($P_{28}$ and $P_{10}$; 7-9 mg/kg of body weight) were injected into the CETP-tg mice subcutaneously at 0 and 12 h. All of the CETP-tg mice were fasted for 16 h before the injection, and the arrows indicate the time of injection. The CETP-tg mice in each group (n=7) were bled, and the CETP activity determined using an artificial substrate system, as described in "Materials and Methods". Data were expressed as a percent of the initial value. Line A, the $P_{28}$-injected, high cholesterol high fat (HCHF)-fed; line B, $P_{10}$-injected, HCHF-fed; line C, saline-injected, HCHF-fed; line D, saline-injected, Lab-chow-fed. Asterisk symbols (*) on the x-axis indicate a statistically significant difference between the saline-injected and at least one of peptide-injected groups at the corresponding time point by a one-way analysis of the variance and Duncan's multiple range test ($p<0.05$).

became hypercholesterolemic mice. They have 80-120 mg/dl of total serum cholesterol, in contrast to 40-50 mg/dl before the HCHF feeding.

The total cholesterol concentration of group A ($P_{28}$-injected) decreased by 30% compared to its initial concentration at 24 h post-injection. Group B ($P_{10}$-injected) decreased by 18% compared to the initial value at 36 h post-injection. The total cholesterol concentration of group C (saline-injected) increased by 116% compared to its initial value. Group D (saline-injected) showed no notable change in total cholesterol concentration (Fig. 4, panel A). The ratio of the HDL-cholesterol/total-cholesterol (HDL-C/Total-C) of Group A increased by 170% and 250% in group B compared to its initial value 24 h after the initial injection. The ratio of HDL-C/Total-C of Group C decreased by 17% to the initial value. Group D showed no significant change (panel B of Fig. 4). These results indicate that the blocking of the CETP activity in plasma could cause decreased total cholesterol and increased HDL-cholesterol concentration.

**Changes of lipoprotein size distribution in the blood of mice** As shown in panels C and D of Fig. 5, the Bio-Gel A-5m column elution profile of mice plasma showed typical patterns of hypercholesterolemic CETP-tg mouse and normal C57BL/6 mice, respectively, as previously reported (Marotti *et al.*, 1993). However, the CETP inhibition by the $P_{28}$ or $P_{10}$ might cause modulation of size distribution of LDL and HDL in the bloodstream. As shown in Fig. 5, 24 h post-injection, VLDL and LDL peak size of the peptide-injected group (panel A, B) were smaller than the saline-injected group (panel D). In contrast, the peak size of HDL from the peptide-injected group (panels A, B) was larger than that of the saline-injected group (panel D).

**Difference of CETP expressional level in liver** As shown in Fig. 6, the CETP band did not appear in normal C57BL/6 mice (lane 4), as previously reported (Ha *et al.*, 1982, Jiang *et al.*, 1991), but the saline-injected CETP-tg mouse represented a high expression level of CETP (lane 3), as previously reported by Marotti *et al.* (1993). The CETP mRNA expression level of the $P_{28}$-injected CETP-tg mouse (lane 1)

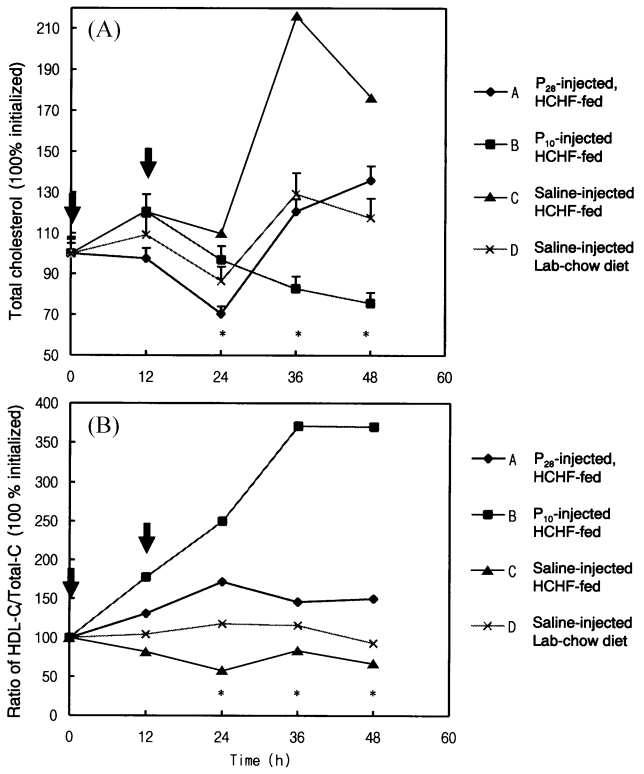


**Fig. 4.** Effect of peptide on total cholesterol (A) and HDL-C/ Total-C ratio (B) in hypercholesterolemic CETP-transgenic mice. All of the mice were fasted for 16 h before injection. Arrows indicate the time of injection. The mice in each group (n=7) were bled, and HDL-C and Total-C determined using Sigma diagnostic kits (Sigma 352-3 and 352-20, respectively). Every data are expressed as a percent of the initial value. HCHF was the abbreviation of the high cholesterol high fat-diet described in Table 10. Asterisk symbols (*) on the x-axis indicate a statistically significant difference between the saline-injected and at least one of peptide-injected groups at the corresponding time point by a one-way analysis of the variance and Duncan's multiple range test ($p<0.05$).

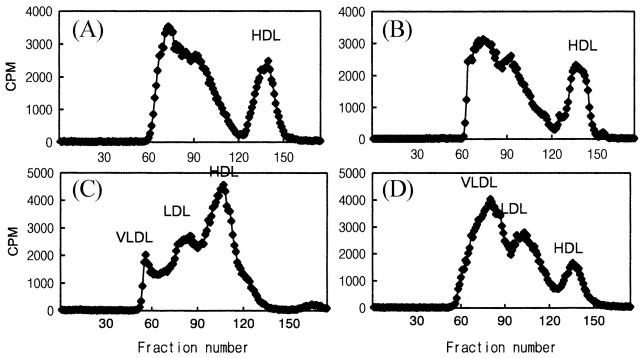


**Fig. 5.** Elution profile of Bio-Gel A-5 m column chromatography (1.5 × 90 cm) with tritiated mouse plasma. The plasma was radiolabeled with 1 μCi of [1α,2α-(n)-$^3$H]-cholesterol at 37°C for 24 h as previously described (Chen *et al.*, 1982). The column was equilibrated with 10 mM Tris/140 mM NaCl that contained 1 mM EDTA, pH 7.4, (Tris buffered saline, TBS) eluted with the same buffer. One-ml fraction was collected with a flow rate of 10 ml/hr. An aliquot (100 μl) of the column fractions was subjected to liquid scintillation counting to visualize lipoprotein peak. Panel A, the $P_{28}$-injected, high cholesterol high fat (HCHF)-fed; panel B, $P_{10}$-injected, HCHF-fed; panel C, saline-injected, HCHF-fed; panel D, saline-injected, Lab-chow-fed.

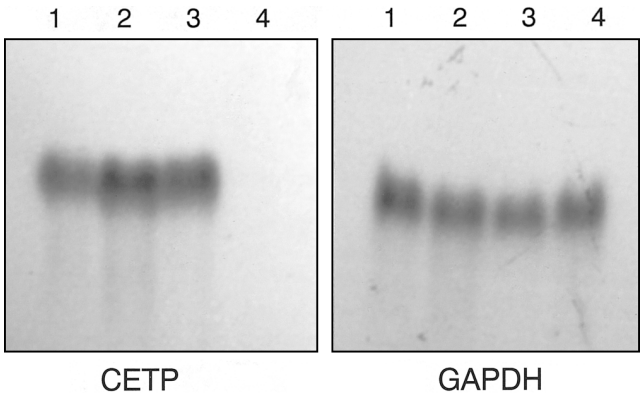


**Fig. 6.** Northern blot analysis of the CETP gene. Total RNA (20 μg) of each group from the liver was loaded on each lane and hybridized with α-[$^{32}$P]-labeled CETP cDNA as detailed in "Materials and Methods". Control hybridization was done with a probe for the GAPDH gene after washing the blot in 0.1% SDS at 65°C. Lane 1, group A ($P_{28}$-injected, CETP-tg mice); lane 2, group B ($P_{10}$-injected, CETP-tg mice); lane 3, group C (saline-injected, CETP-tg mice); lane 4, group D (saline-injected, C57BL/6 normal mice).

was lower than that of the saline-injected CETP-tg mouse. This result suggests that the $P_{28}$ might affect the CETP expression level in the liver of the hypercholesterolemic CETP-tg mouse.

It can be concluded that the $P_{28}$ or $P_{10}$ injection might cause partial inhibition of plasma CETP activity by decreasing the total plasma cholesterol concentration and increasing the ratio of the HDL/total-cholesterol concentration in hypercholesterolemic CETP-tg mice. These results agree with the *in vivo* observations, which revealed clear relationships between plasma CE transfer activity and size distribution of both HDL and LDL (Agellon *et al.*, 1991; Lagrost, 1993).

**Acknowledgment** This research was supported by a grant (#1999-2-220-005-5) from the Korea Science and Engineering Foundation (KOSEF).

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