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S R Cai, ... , M W Flye, K P Ponder

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Research Article

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Therapeutic Levels of Human Protein C in Rats after Retroviral Vector-mediated Hepatic Gene Therapy

Shi-Rong Cai,^{*†§} Susan C. Kennedy,^{*†§} William M. Bowling,^{||} M. Wayne Flye,^{||} and Katherine Parker Ponder^{*†§}

^{*}Department of Internal Medicine, [†]Department of Biochemistry and Molecular Biophysics, and ^{||}Department of Surgery, Washington University School of Medicine, St. Louis, Missouri 63110

Abstract

Protein C deficiency results in a thrombotic disorder that might be treated by expressing a normal human protein C (hPC) gene in patients. An amphotropic retroviral vector with a liver-specific promoter and the hPC cDNA was delivered to rat hepatocytes in vivo during liver regeneration. Expression of hPC varied from 55 to 203 ng/ml (1.3–5.0% of normal) for 2 wk after transduction. Expression increased to an average of 900 ng/ml (22% of normal) in some rats and was maintained at stable levels for 1 yr. All of these rats developed anti-hPC antibodies and exhibited a prolonged hPC half-life in vivo. The hPC was functional as determined by a chromogenic substrate assay after immunoprecipitation. We conclude that most rats achieved hPC levels that would prevent purpura fulminans, and that hepatic gene therapy might become a viable treatment for patients with severe homozygous hPC deficiency. Anti-hPC antibodies increased the hPC half-life and plasma levels in some rats, but did not interfere with its functional activity. Thus, the development of antibodies against a plasma protein does not necessarily abrogate its biological effect in gene therapy experiments. (*J. Clin. Invest.* 1998. 101:2831–2841.) Key words: protein C • gene therapy • retroviral vector • liver • antibody

Introduction

Human protein C (hPC)¹ is a 62-kD vitamin K-dependent protease that inhibits coagulation. It circulates in blood as a zymogen that is activated by a thrombin:thrombomodulin complex (1). Activated hPC inhibits coagulation by cleaving and inactivating coagulation factors Va and VIIIa (1). As of 1995, 331 mutations of the hPC gene have been described that affect

the antigenic and/or functional levels (2). Heterozygous hPC deficiency occurs in 1 in 200 and results in ~ 50% of normal functional levels of hPC. There is an increased risk of venous thrombosis in adults in some families, although most will never experience a thrombotic episode (3, 4). Homozygous hPC deficiency occurs in 1 in 500,000 and results in purpura fulminans, disseminated intravascular coagulation (DIC), or massive venous thrombosis (5–18).

Treatment for homozygous hPC deficiency involves parenteral administration of hPC concentrates (10–14) or anticoagulation with coumadin (15–18). Treatment with hPC concentrates usually requires an indwelling catheter, which carries the risk of infection. The short half-life of hPC, ~ 10 h in humans (11, 19–20), necessitates administration of concentrates daily or every other day, which will probably be very expensive once they are approved for commercial use. Coumadin therapy requires frequent monitoring and has resulted in thrombosis or hemorrhage during periods of under- or overanticoagulation, respectively (9, 11). Thus, although existing treatments for homozygous hPC deficiency can prolong life, they are expensive, require close medical attention, and the patients are still at risk for severe adverse effects.

Gene therapy involves transfer of a gene that encodes a functional protein into the appropriate cells of a patient with a genetic deficiency. Gene therapy might permanently correct the clinical manifestations of hPC deficiency by achieving continuous production of hPC. Liver is the optimal target organ because it is the major site of hPC synthesis and it has direct contact with the blood. Liver transplantation resulted in resolution of the thrombotic tendency in one patient with severe hPC deficiency (9). Gene therapy for other proteins involved in blood coagulation has been attempted by delivering plasmid DNA or viral vectors to various cell types using ex vivo or in vivo methods (21–24). Delivery of adenoviral vectors or plasmid DNA to the livers of mice, rats, or dogs has resulted in therapeutic levels of factor VIII (25–27) or factor IX (28–32), but levels declined rapidly in normal animals. Immunosuppression (28) or tolerization by injection of adenoviral vector at birth (31) slowed but did not prevent the decline in expression in most animals. Although integrated plasmid DNA resulted in therapeutic levels of expression of factor IX from myoblasts in mice, the procedure led to the development of a fibrosarcoma in one animal (33). Retroviral vectors have resulted in long-term but subtherapeutic levels of expression of factor IX from hepatocytes (34) or myoblasts (35) in vivo. In contrast, utilization of the strong liver-specific human α_1 -antitrypsin (hAAT) promoter led to therapeutic levels of expression of functional human factor X (hFX) from the liver in rats for at least 1 yr (36). More recently, adenovirus associated virus (AAV) vectors have resulted in therapeutic levels of expression of factor IX from muscle (37) or liver (38) in mice, although it takes 2 mo after gene transfer for these levels to be achieved.

We demonstrate here that a retroviral vector with an hPC

Address correspondence to Katherine Ponder, Department of Internal Medicine, Box 8125, 660 S. Euclid Avenue, St. Louis, MO 63110. Phone: 314-362-5188; FAX: 314-362-8826; E-mail: kponder@imgate.wustl.edu

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1. Abbreviations used in this paper: DHFR, dihydrofolate reductase; DIC, disseminated intravascular coagulation; hAAT, human α_1 -antitrypsin; hFX, human factor X; hPC, human protein C; HRP, horseradish peroxidase; IRES, internal ribosome entry site; LFABP, liver fatty acid-binding protein; LTR, long terminal repeat; nt, nucleotide.

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cDNA and the strong liver-specific hAAT promoter can result in therapeutic levels of expression of hPC in rats. We also demonstrate that some normal rats develop antibodies against hPC that prolong the half-life of the protein in blood, but do not interfere with its functional activity.

Methods

Construction of retroviral vectors. A complete hPC cDNA (39) was modified to contain a consensus translational initiation sequence by digestion with NotI (cuts 5' to hPC in the vector) and BclI (cuts 35 nucleotides [nt] downstream of the translational initiation site) and ligation with the following annealed oligonucleotides: 5'-GGCC-GCAAGCTTGCCGCCACCATGTGGCAGCTCACAAGCCTCC-TGCTGTTCGTGG-3' (top strand) and 5'-CCACGAACAGCAG-GAGGCTTGTGAGCTGCCACATGGTGGCGCAAGCTTGC-3' (bottom strand). This results in a NotI site that is 21 nt upstream of the translational initiation site. The DNA was sequenced to confirm that it contained the correct sequence. The PstI site located 14 nt downstream of the stop codon was converted to an EagI site. The plasmid containing the modified 1.4-kb hPC cDNA was designated hPC-K-280.

hPC-701 contains the ApoE enhancer, the hAAT promoter, the hPC cDNA, and the internal ribosome entry site (IRES)-dihydrofolate reductase (DHFR) cassette. It was created by digesting ApoE-hAAT-LTR (40) with NotI, and replacing the hAAT cDNA with the modified hPC cDNA obtained from hPC-K-280. hPC-702 contains the ApoE enhancer, hAAT promoter, and the hPC cDNA without the IRES-DHFR cassette. It was created by digestion of LTR-FX (36) with XhoI, which cuts just downstream of the DHFR gene (position 2937 of LNL-6 [41]), followed by blunt-end formation with T4 DNA polymerase, and ligation with XbaI linkers (CTCTAGAG). After XbaI digestion (cuts just upstream of the internal ribosome entry site sequence and in the middle of the 3' long terminal repeat [LTR]) the 6-kb fragment (containing the 5' LTR and the hFX gene) and the 550-bp fragment (containing the 3' LTR) were isolated and religated to generate hFX-515. The hAAT promoter (-347 to +56) was cloned into the BglII site of hFX-515 as described (42) to create hFX-517. A 650-bp BamHI/BglII fragment containing four copies of the 156-bp apolipoprotein E enhancer was cloned upstream of the hAAT promoter as described (40) and the factor X cDNA was removed by NotI restriction and replaced with the 1.4-kb modified hPC cDNA obtained after NotI/EagI restriction of hPC-K-280 to create hPC-702.

Generation of the retroviral vector packaging cell lines. The aphotropic GP+envAM12 (43) and NIH 3T3 murine fibroblast (44) cells were maintained as described previously (45) in high glucose DMEM containing 10% heat-inactivated supplemented calf serum (Hyclone Laboratories, Logan, UT), penicillin (10^5 U/liter), streptomycin (10^5 µg/liter), and 10 µg/ml vitamin K (Sigma Chemical Co., St. Louis, MO). 9 µg of hPC-701 or hPC-702 was cotransfected with 1 µg of PGK-neo (46) into GP+envAM12 cells using calcium phosphate-mediated transfection (41), and clones were selected with 0.5 µg/ml of G418 (Gibco/BRL, Rockville, MD). Colonies were picked, and three rounds of screening were performed to identify four colonies that conferred the highest level of expression of hPC upon NIH 3T3 cells. To quantitate the expression of hPC in singly transduced cells, NIH 3T3 cells were transduced with hPC-701 at a low multiplicity of infection, and methotrexate-resistant (Mtx^R) cells were selected with 250 nM Mtx. A pool of 50–100 colonies was grown to confluence, washed three times with PBS, then incubated with 5 ml of DMEM with 10 mM Hepes pH 7.4 and vitamin K, but without serum, for 6 h. The supernatant was collected and assayed for hPC functional and antigenic levels.

hPC ELISA. ELISA plates were coated for 2 h at 37°C with 100 µl of a 1:100 dilution of polyclonal goat anti-hPC antibody (catalog #241; American Diagnostica Inc., Greenwich, CT) as described (47),

washed three times with 200 µl of 100 mM NaCl, 50 mM Tris[hydroxymethyl]amino-methane-HCl pH 7.5 (TBS), and blocked overnight at 4°C with 200 µl of TBS with 5% dry nonfat milk (Schnuck's Grocery, St. Louis, MO) (TBS-milk). Samples or standards containing 100 µl were incubated at 37°C for 2 h and washed six times with TBS-0.05% Tween 20. The same anti-hPC antibody was coupled to horseradish peroxidase (HRP) with *N*-succinimidyl *S*-acetylthioacetate using a kit from Pierce (Rockford, IL), and 100 µl of TBS-milk containing this HRP-coupled antibody at a 1:500 dilution was incubated at 37°C for 2 h. The plates were washed six times with 200 µl of TBS-0.05% Tween 20 and the OD at 450 nm observed after the addition of 3,3',5,5'-tetramethylbenzidine dihydrochloride (47). hPC standards were generated by diluting a pool of normal human plasma and assuming a concentration of 4 µg/ml. Experimental samples were diluted to give a value that fell within the linear range of the standard curve. All rat samples obtained at 10 mo or earlier after transduction were analyzed in the same ELISA. Samples obtained at later time-points were analyzed in a separate assay using different aliquots from the same set of standards.

Protein C chromogenic substrate assay. To determine the hPC functional activity, 150 µl of the conditioned medium from singly transduced NIH 3T3 cells was mixed with 50 µl of TBS in an ELISA plate well and the hPC was activated at 25°C for 10 min with 2 U of Protac (American Diagnostica, Inc.), which is an activator of protein C derived from the venom of the copperhead snake *Agkistrodon contortrix*. hPC chromogenic substrate (Spectrozyme PCa; American Diagnostica, Inc.) was added at 100 µM final concentration, and the OD measured 1–2 h later at 405 nm; the OD observed immediately after the addition of substrate was subtracted from each sample. Blanks contained 50 µl of TBS and 150 µl of medium. Conditioned medium from nontransduced NIH 3T3 cells did not have any hPC activity. A standard curve was created by adding 20 to 1.25 µl of human plasma (George King Biomedicals, Overland Park, KS), 30–48.75 µl of TBS, and 150 µl of medium, and observing the change in OD after the addition of Protac and hPC chromogenic substrate.

In vivo transduction of rat hepatocytes. Adult male Lewis rats (Sprague Dawley, Indianapolis, IN) weighing ~200 grams received standard institutional care. 12 ml of conditioned medium was collected from each of 24 confluent 15-cm plates of packaging cells grown at 32°C (48) and concentrated by ultracentrifugation (45). The pellets were suspended in medium with 8 µg/ml polybrene, and injected without inflow occlusion into the portal vein of a rat that received a 70% hepatectomy 24 h previously (45). Retroorbital blood was anticoagulated with a 1/10 volume of 3.8% trisodium citrate.

hPC half-life study. Purified hPC obtained from human plasma was generously provided by Dr. J. Miletic. 16 µg of hPC was iodinated with 250 µCi of I¹²⁵ and IODO-GEN (Pierce, Rockford, IL) to a specific activity of 3.7×10^7 cpm/µg according to the instructions of the manufacturer. 6×10^7 cpm of radiolabeled hPC was injected into the penile vein of each rat, and plasma was collected at 1 h or later. 0.5 µl of plasma was electrophoresed on a nonreducing sodium dodecylsulphate 8% polyacrylamide gel (49), the gel was dried, and the hPC band was quantitated by using a STORM PhosphorImager.

ELISA for anti-hPC antibodies. ELISA plates were coated with 100 µl containing 1 µg/ml of hPC in 0.1 M sodium bicarbonate pH 9.5, then blocked with TBS-milk. Plasma samples were diluted at $\geq 1:100$ in TBS-milk and incubated on the plates for 2 h at 25°C and washed six times with TBS-0.05% Tween 20. An affinity purified goat anti-rat IgG antibody (Organon Teknica Corp., Durham, NC) coupled to HRP was added at a 1:1,000 dilution, the washed plate was developed with 3,3',5,5'-tetramethylbenzidine dihydrochloride, and the OD was read at 450 nm (47). Initial experiments demonstrated that all pretransduction plasma samples had a low activity in the anti-hPC antibody assay when tested at a 1:100 dilution, while most samples had detectable activity above background when assayed at a 1:50 dilution. We therefore tested all posttransduction samples at a 1:100 dilution, and performed serial dilutions if the initial value was positive. Samples were considered positive if the OD was at least twice as high

as that observed in the 1:100 dilution of the pretreatment sample for that rat.

hPC immunoprecipitation/functional assay. For each sample, 20 μ l of Protein G Sepharose 4 Fast Flow (Pharmacia LKB Biotechnology, Piscataway, NJ) was incubated for 2 h at 25°C with 5 μ l of goat anti-hPC antibody. The beads were spun for 2 min at 500 g and washed six times with 1 ml of TBS-0.05% Tween 20 per sample, once with 1 ml of TBS, resuspended in 50 μ l of TBS per sample, and aliquoted into 1.5-ml tubes. Human plasma (2.5–40 μ l), TBS (blank), or rat plasma (5–40 μ l) were added and the total volume in each tube was increased to 500 μ l by adding TBS. The samples were incubated for 2 h at 25°C on a nutator, then spun for 30 s in a microfuge and washed six times with 1 ml of TBS-0.05% Tween 20, once with 1 ml of TBS, and resuspended in 300 μ l of TBS. 2 U of Protac was added for 15 min at 25°C, then chromogenic hPC substrate was added at a final concentration of 83 μ M. After 12 h at 25°C, the samples were spun in a microfuge for 1 min and the OD of the supernatant read at 405 nm in an ELISA reader. Blanks did not receive any plasma, and were subtracted from all samples. The activity observed using an equal volume of control rat (nontransduced) plasma was 1% of the activity observed with human plasma, and was subtracted from the calculated activity for each sample.

DNA analysis. DNA was isolated as described previously (45). To quantitate the copy number of the hPC-containing retroviral vector in the liver, a multiplex PCR technique involving primer sets that amplified both proviral (hPC) and rat genomic DNA (liver fatty acid binding protein promoter; LFABP) sequences was used. Primers hPC-502 (5'-GAGCTTCCTCAATTGCTCGCTGGAC-3'; top strand for nt 502–526) and hPC-1000 (5'-TGCGATGTCATTGTCGGTG-GTGCTC-3'; bottom strand of nt 1000–976) amplified a 498-bp fragment. Primers LFABP-1 (5'-GCATTGCTAGAGATGTGATTCA-CATGTC-3'; top strand for nt 270–298) and LFABP-2 (5'-CCA-CAGCTGACCACAACAGCTCTG-3'; bottom strand for nt 623–599) amplified a 353-bp fragment (50). Each 50- μ l PCR reaction included 50 ng of DNA, 20 mM Tris-HCl (pH 8.55), 16 mM (NH₄)₂SO₄, 3.3 mM MgCl₂, 150 μ g/ml bovine serum albumin, 2 mM deoxynucleotide triphosphates, 25 pmol of each oligonucleotide primer, and 1.25 U *Thermus aquaticus* DNA polymerase (Amplitaq; Perkin-Elmer, Norwalk, CT). 30 cycles were performed with denaturation at 94°C for 90 s, annealing at 65°C for 90 s, and extension at 72°C for 90 s. Amplified DNA sequences were electrophoresed through a 2% agarose/1X Tris ammonium acetate gel, blotted to GeneScreen nitrocellulose membranes, and hybridized with a 1.4-kb hPC cDNA (39) probe labeled to a specific activity of 3 \times 10⁹ cpm/ μ g DNA with random primer labeling (49). After quantitation with a PhosphorImager, the membrane was stripped and rehybridized with a radiolabeled 620-bp LFABP (50) probe.

To quantitate the copy number of the IRES-containing retroviral vectors, PCR followed by Southern blot was performed using a primer set specific for the IRES and the LFABP primer set, as described previously (45).

RNAse protection assay. RNA was isolated from rat liver using guanididium isothiocyanate as described previously (45). A plasmid containing the encephalomyocarditis IRES in pSP72 (Promega Corp., Madison, WI) was digested with HindIII to remove nt 236 to 570 of the IRES and religated to generate p746- Δ IRES. p746- Δ IRES was digested with BglII and transcribed with SP6 RNA polymerase, α -[³²P]UTP and unlabeled ATP, CTP, and GTP (49) to generate an antisense probe with a specific activity of 3 \times 10⁹ cpm/ μ g. 40 μ g of RNA from rat liver or from a pool of NIH 3T3 cells that were transduced with a single copy of hPC-701 was heated to 85°C for 10 min then hybridized for 5 h with 1 \times 10⁵ cpm of radiolabeled probe in 30 μ l containing 80% formamide, 0.4 M NaCl, 1 mM EDTA, and 40 mM Pipes pH 6.4. Samples were digested at 37°C with 300 μ l of buffer containing 0.3 M NaCl, 10 mM Tris-HCl, pH 7.4 and 5 mM EDTA with 30 U/ml of RNase T1 (Calbiochem Corp., La Jolla, CA) and 1 μ g/ml of RNase A (Sigma Chemical Co.), extracted with phenol, and precipitated with ethanol. Samples were electrophoresed on an 8% polyacrylamide gel with 8 M urea and 1/2 \times TBE (45 mM Tris base, 45 mM boric acid, 1.25 mM EDTA). The IRES-specific band was quantitated with a PhosphorImager.

Results

Production of a retroviral packaging cell line. The retroviral vectors designated as hPC-701 and hPC-702 are shown in Fig. 1. hPC-701 contains a modified hPC cDNA downstream of the ApoE enhancer-hAAT promoter, and the IRES-DHFR cassette that confers methotrexate resistance (Mtx^R). The ApoE-hAAT transcriptional elements were chosen because they directed high-level and long-term expression of the hAAT cDNA from a retroviral vector in rat liver in vivo (40). The sequence immediately upstream of the hPC translational initiation signal is GCCTCCAGA for the wild-type hPC cDNA. Since this sequence deviates from the consensus translational initiation signal described by Kozak (51), it was modified to contain the translational consensus sequence GCCGCCACC. hPC-702 is identical to hPC-701 except that it does not contain a selectable marker gene. The IRES-**DHFR* was deleted to test the possibility that these sequences affected expression levels in vivo. The effect of the alteration in the 5' untranslated

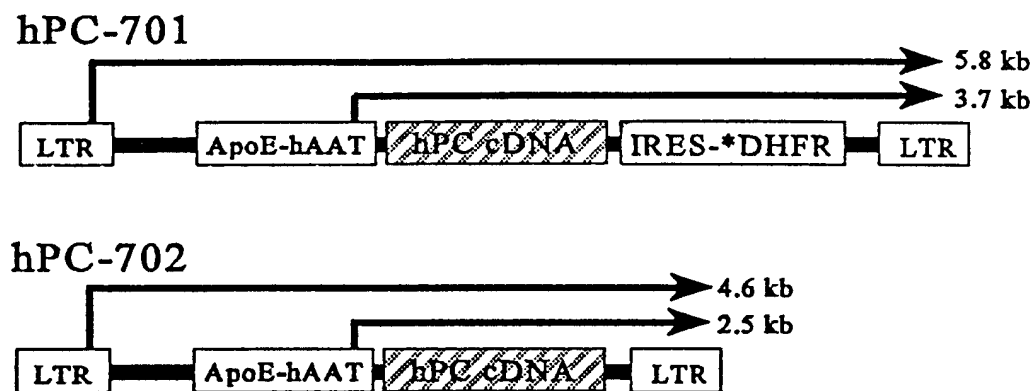


Figure 1. Retroviral vectors hPC-701 and hPC-702. hPC-701 contains retroviral long terminal repeats (LTR) at both ends, the apolipoprotein E (ApoE) enhancer:human α ₁-antitrypsin (hAAT) promoter, the human protein C (hPC) cDNA, and the 1.2-kb internal ribosome entry site (IRES)-mutant dihydrofolate reductase (**DHFR*) cassette. The size of the mRNAs that initiate from the LTR or the hAAT promoter is indicated. hPC-702 is similar except it does not contain the IRES-**DHFR* cassette.

sequence upon the production of hPC protein was determined. A plasmid containing the wild-type hPC cDNA or the hPC cDNA with the altered 5' untranslated sequence was placed downstream of the cytomegalovirus (CMV) immediate early promoter and was cotransfected into NIH 3T3 cells along with a separate plasmid expressing the human α_1 -antitrypsin (hAAT) cDNA. Both hPC constructs gave similar levels of expression after normalization for the hAAT protein in the supernatant, suggesting that the alteration of the 5' untranslated sequence did not improve expression of hPC (data not shown).

These retroviral vector plasmids were used to create retroviral vector packaging cell lines, as described in the Methods section. The Mtx^R titer of hPC-701 was 2×10^5 colony forming units (cfu)/ml on NIH 3T3 cells. Southern blot of DNA from transduced NIH 3T3 cells confirmed that hPC-701 had a titer of $\sim 2 \times 10^5$ infectious U/ml, and demonstrated that hPC-702 had a titer of $\sim 1 \times 10^6$ infectious U/ml (data not shown). Pools of NIH 3T3 cells that were transduced with one copy of hPC-701 per cell produced 251 ± 42 (SEM) ng of hPC/ 10^6 cells/24 h. The hPC was $82 \pm 5.3\%$ (SEM) functional, as determined by comparing the chromogenic substrate activity in serum-free conditioned medium from transduced NIH 3T3 cells with the antigen levels observed by immunoassay of the same samples. The amount of hPC produced by NIH 3T3 cells that were

transduced with hPC-702 was consistently about fivefold higher than the amount of hPC produced by NIH 3T3 cells that were transduced with an equal volume of conditioned medium containing the hPC-701 vector. We conclude that the modified hPC cDNA used in this study results in the production of functional hPC. The hPC-702 retroviral vector confers higher expression of hPC upon transduced cells than hPC-701 *in vitro*, and this difference appears to be due primarily to its fivefold higher titer.

In vivo expression of hPC. hPC-701 and hPC-702 retroviral vectors were delivered to regenerating rat hepatocytes *in vivo*. Plasma obtained at various times after transduction was tested for hPC by immunoassay, as shown in Fig. 2, A and B. For the first 2 wk after transduction, average expression of hPC in four hPC-701-transduced rats varied from 16–40 ng/ml (0.4–1.0% of the level found in pooled normal human plasma), while expression in seven hPC-702-transduced rats was approximately fivefold higher at 55–203 ng/ml (1.3–5.0% of normal). Expression fell to lower levels (< 50% of the average level observed during the first 2 wk for that rat) thereafter in two of the hPC-701-transduced rats, and two of the hPC-702-transduced rats. Two of the hPC-701-transduced rats exhibited stable expression (50–200% of the initial levels for that rat) for 145 d after transduction, and one of the hPC-702-

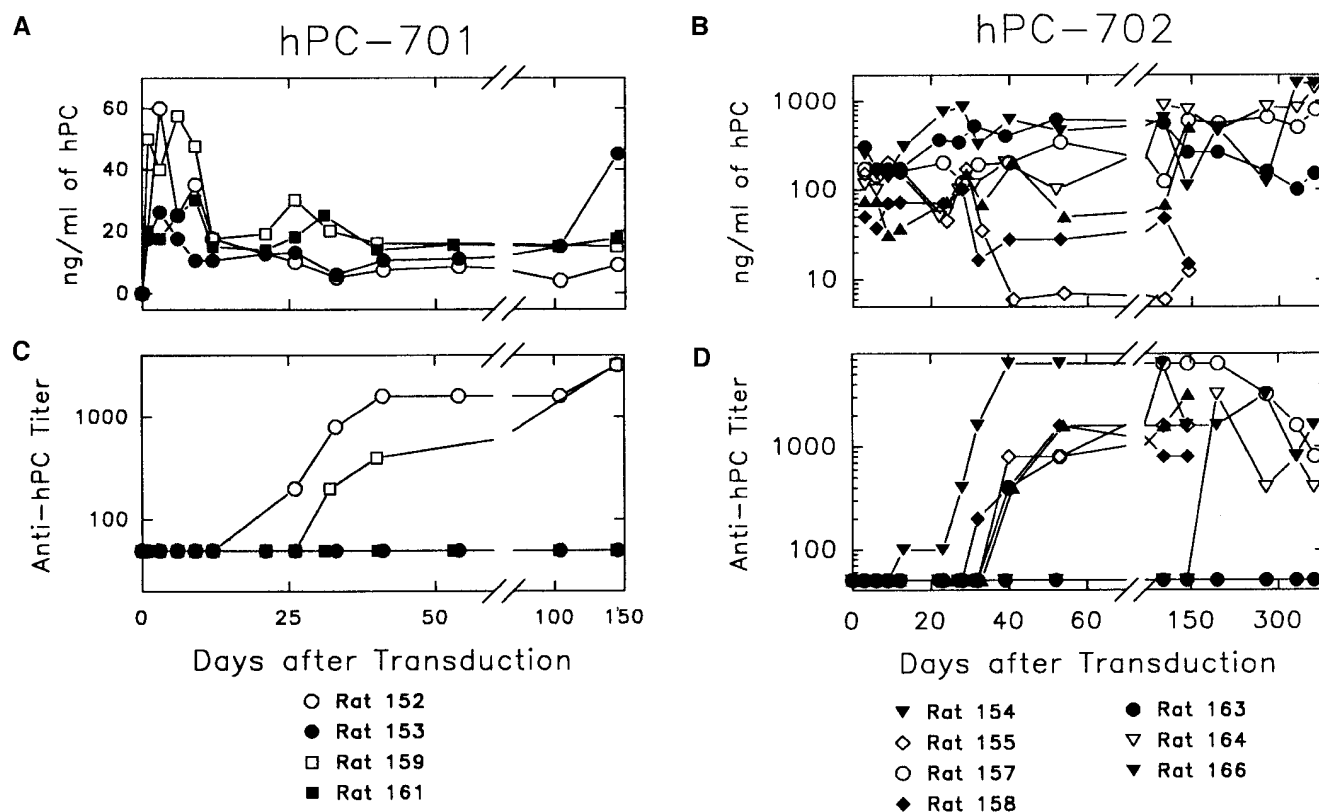


Figure 2. Expression of hPC-containing retroviral vectors in rats *in vivo* and quantitation of anti-hPC antibodies. Rats were treated with a 70% partial hepatectomy. 24 h later, 4 ml containing ultracentrifuge-concentrated retroviral vector was injected into the portal vein. (A) hPC levels in hPC-701-transduced rats. Rats were transduced with 4×10^6 cfu of hPC-701. Plasma was analyzed for hPC by ELISA. (B) hPC levels in hPC-702-transduced rats. Rats were transduced with 2×10^7 infectious particles of hPC-702. (C) Anti-hPC antibody titer in hPC-701-transduced rats. Plasma was tested for anti-hPC antibodies at a 1:100 dilution as described in the Methods section. If the assay was negative, it was reported as having a titer of 1:50. If the assay was positive, it was serially diluted until a negative result was obtained, and the titer was reported as the highest dilution that was positive. (D) Anti-hPC antibody titer in hPC-702-transduced rats.

transduced rats exhibited stable expression for 361 d. The four remaining hPC-702-transduced rats had a fourfold to ninefold increase in hPC levels at late time points as compared with the values obtained during the first 2 wk for that rat.

Anti-hPC antibody levels in retroviral vector-transduced rats. One possible explanation for the increase in hPC levels in some rats and the fall in others is that antibodies developed that led to increased or decreased clearance, respectively, of the human protein. We therefore tested rat plasma for the presence of anti-hPC antibodies. Both of the hPC-701-transduced rats that maintained stable levels of hPC for 145 d after transduction had no detectable anti-hPC antibodies for the duration of the experiment, as shown in Fig. 2 C. Rats 152 and 159, hPC-701-transduced rats whose hPC levels fell to 22 and 42%, respectively, of their initial value, had anti-hPC antibodies that appeared at 1 mo after transduction and were present at a titer as high as 1:3,200. We conclude that two rats that had a fall in their hPC levels at 3 wk or later after transduction had anti-hPC antibodies, while two rats that exhibited stable expression did not.

hPC-702-transduced rats were also tested for the presence of anti-hPC antibodies in their plasma, as shown in Fig. 2 D. Rat 163, who exhibited stable expression of hPC for 1 yr after transduction, did not develop anti-hPC antibodies. Rats 155 and 158, hPC-702-transduced rats whose hPC levels fell to 26 and 4% of their initial values, developed anti-hPC antibodies with a peak titer of 1:1,600 and 1:800, respectively. Rats 154, 157, 164, and 166, which exhibited a fourfold to ninefold increase in hPC levels at 3 wk or later after transduction, had anti-hPC antibodies with a peak titer of 1:3,200 to 1:6,400. We conclude that anti-hPC antibodies were present in hPC-702-

transduced rats that exhibited an increase in hPC levels over time as well as in rats that had a fall in hPC protein levels.

hPC half-life in retroviral vector-transduced rats. It is possible that rats that developed anti-hPC antibodies and an increase in their hPC plasma levels over time had an increase in the hPC half-life in their blood. To measure the half-life of the protein, radiolabeled hPC was injected into the penile vein, and plasma samples were obtained from the eye at various times thereafter. Plasma samples were electrophoresed on nonreducing SDS-PAGE and the amount of hPC was quantitated by PhosphorImaging. Controls consisted of one rat that was never transduced with any retroviral vector, and two rats that were transduced with a retroviral vector that directs expression of another protein, hAAT. The amount of hPC present at the initial timepoint of 1 h was 1.5- to 2.8-fold higher in rats that had high-titer anti-hPC antibodies and an increase in the hPC levels over time (Fig. 3, lanes 6 and 16), than in a control rat (Fig. 3, lane 1) or a transduced rat that did not have anti-hPC antibodies (Fig. 3, lane 11). This suggests that the initial clearance of hPC was slower in rats with anti-hPC antibodies than in rats without anti-hPC antibodies.

The plasma half-life was determined by quantitating the amount of hPC present at 1 h or later after transduction and is listed in Table I. The three control rats had an average hPC half-life of 2.6 ± 0.2 h. This was similar to the half-life of nonlabeled hPC in four different control rats, in which the hPC levels in plasma were quantitated by ELISA (data not shown). Rats 157, 164, and 166 (transduced with hPC-702; developed high-titer anti-hPC antibodies and an increase in hPC levels over time) all had half-lives that were two- to threefold higher than controls. Rat 163, which was transduced with hPC-702 but did not develop antibodies, had a half-life of 2.75 h, which did not differ from the controls. We conclude that the anti-hPC antibodies probably decrease the rate of clearance of hPC in the plasma of some rats, and may be responsible for the increase in hPC levels that was observed in these animals.

There were four rats that had a decline in hPC levels concurrent with the development of anti-hPC antibodies. We believe that the hPC half-life would have been shorter in these animals than in controls. Unfortunately, however, these rats were killed before the half-life study was performed so it is impossible to confirm this hypothesis.

hPC functional activity in rat plasma. The above studies led us to conclude that the hPC antigenic levels might be increased in some rats because of antibodies that lead to decreased clearance. It is possible that such an antibody might inhibit the functional activity of the hPC protein. Functional assays for protein C activity are complicated by the presence of rat protein C in normal rat plasma. To overcome this difficulty, rat plasma was immunoprecipitated with a human specific anti-hPC antibody, the hPC was activated with Protac, and a chromogenic substrate specific for protein C was added. The results of this assay are shown in Table I. The functional activity in the immunoprecipitation/chromogenic substrate assay correlated reasonably well with the levels of antigen that were detected by ELISA for most rats. Discrepancies could be due to interference from the rat antibodies in either assay, or to the inaccuracies of the multistep immunoprecipitation/chromogenic substrate assay. We conclude that the hPC was functional despite the presence of hPC antibodies, as it could be activated by Protac and could cleave a small chromogenic substrate.

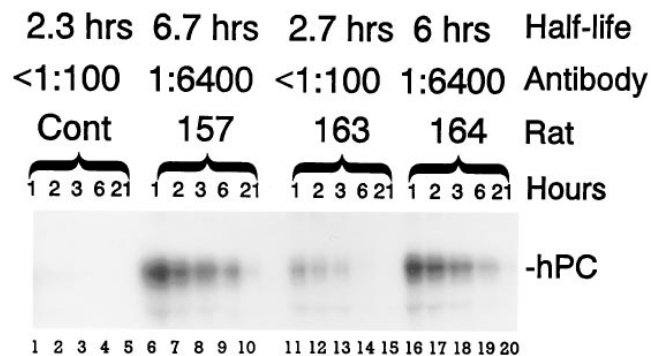


Figure 3. hPC half-life in rats. hPC-702-transduced rats were injected with 6×10^7 cpm of [125 I]-hPC at 360 d after transduction and plasma was obtained from a different site at 1–24 h later. 0.5 μ l of plasma was electrophoresed on nonreducing SDS-PAGE, and an autoradiogram was obtained. The number above each lane represents the hours after injection of label that the plasma sample was collected. Lanes 1–5 (Cont) represent a Lewis rat of similar weight that was never injected with any retroviral vector. Lanes 6–10 (Rat 157) and lanes 16–20 (Rat 164) represent rats that received the hPC-702 retroviral vector, developed anti-hPC antibodies, and exhibited an increase in hPC levels over time. Lanes 11–15 (Rat 163) represent a rat that received the hPC-702 retroviral vector, but did not develop anti-hPC antibodies or exhibit an increase in hPC levels over time. The peak anti-hPC antibody titer is indicated for each rat. The calculated hPC half-life in hours, which was based upon PhosphorImaging of the gel, is also shown.

Quantitation of DNA copy number in rat livers in vivo. It is important to determine the DNA copy number of the retroviral vectors in the liver for two reasons. First, the expression levels in vivo will be a reflection of the expression per copy and the number of copies delivered. Second, some animals exhibited an increase in their hPC levels over time. One explanation for these increased levels is that ongoing retroviral vector replication occurred that increased the DNA copy number in the liver, resulting in increased expression.

The retroviral vector copy number in the livers of hPC-transduced rats was determined by using a multiplex PCR reaction following by Southern blot, as shown in Fig. 4. Primers specific for the hPC gene were used to identify the retroviral vector sequences in either hPC-701- or hPC-702-transduced rats, and primers specific for rat genomic DNA (LFABP) were used as a control for the efficiency of PCR amplification. A

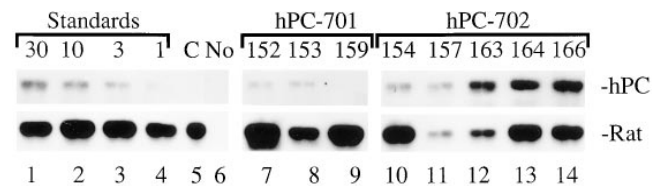


Figure 4. DNA analysis of retroviral vector-transduced rat livers. Rat livers were obtained at the time of death, and DNA was isolated. PCR was performed using primers specific for the hPC gene of the retroviral vector and rat genomic DNA. Southern blot was performed using an hPC probe (*hPC*) followed by stripping and hybridization with an LFABP probe (*Rat*). Standards (lanes 1–4) contain DNA from NIH 3T3 cells with one copy of hPC-701 per cell diluted with DNA from a nontransduced rat liver. The number above each lane represents the percentage of DNA that was derived from the singly transduced NIH 3T3 cells. Lane 5 (C) shows DNA from a nontransduced rat, while lane 6 (No) shows a sample that had no DNA added. Lanes 7–9 show DNA from hPC-701-transduced rats, while lanes 10–14 show DNA from hPC-702-transduced rats. The animal number is listed above each lane.

Table I. Summary of Results in Rats

Rat	Days after transduction	hPC assays		hPC half-life [§]	Anti-hPC antibody titer	Retroviral vector DNA copy number [¶]
		Antigen % nt*	IP assay % nt [‡]			
Controls						
		0	0	2.6±0.2 h	< 1:100	0
hPC-701-transduced rats						
152	145	0.2	0	NT	1:3200	1.0±0.7%
153	145	1.1	0	NT	< 1:100	3.2±2%
159	144	0.4	0	NT	1:3200	0.2±0.1%
161	145	0.4	0	NT	< 1:100	NT
hPC-702-transduced rats						
154	145	12.5	35±4	NT	1:3200	11.3±5%
155	145	0.4	0	NT	1:1600	NT
157	332	12.5	21.9±0.1	NT	1:1600	NT
	362	20	20.6±3	6.7±2.6 h	1:1600	15.8±4%
158	145	0.3	1±0.3	NT	1:800	NT
163	331	2.5	5.0±1	NT	< 1:100	NT
	361	3.75	1.8±0.7	2.7±1.1 h	< 1:100	32.5±10%
164	9	0.3	3±0.5	NT	< 1:100	NT
	330	47	182±13	NT	1:400	NT
	360	35	60.1±2	6.0±2.3 h	1:400	8.8±3%
166	9	4	4.1±0.3	NT	< 1:100	NT
	330	75	87.5±4	NT	1:1600	NT
	360	40	49.6±2	7.5±2.9 h	1:1600	20.8±5%

Plasma was obtained at various times after transduction with hPC-701 or hPC-702. Controls consisted of nontransduced rats and rats that were transduced with a retroviral vector that expressed hAAT. For all transduced rats, the rat number and the time after transduction that the sample was analyzed are shown. *The hPC antigenic activity as a percentage of normal is shown. [‡]The hPC functional activity was determined using the immunoprecipitation/chromogenic substrate (IP) assay, and is reported as the percentage of normal human activity. [§]The hPC half-life in hours was determined after injection of radiolabeled hPC, as shown in Fig. 3. ^{||}The anti-hPC IgG antibody titer was determined as shown in Fig. 2, C and D. [¶]The transduction efficiency was determined by using the PCR-based assay shown in Fig. 4. The percentage of liver cells that were transduced is shown. This was determined by performing the PCR-based assay for DNA copy number three times and averaging the results. For all columns, the result±the standard deviation is shown. NT, not tested.

sample without any DNA added (Fig. 4, lane 6) had no signal with either probe, as expected. Rat liver DNA (Fig. 4, lane 5) that was isolated in parallel with the experimental rat samples had a signal for LFABP but no signal for hPC, demonstrating that the primers are specific for the human protein C gene and that contamination of the rat DNA samples with hPC-containing sequences had not occurred. The standards shown in Fig. 4, lanes 1–4 were used to estimate the retroviral vector transduction efficiency for transduced rats.

The percentage of hepatocytes from hPC-701-transduced rats that contained the retroviral vector varied from 1 to 4%, with an average of 1.5%. The retroviral vector DNA copy number in hPC-702-transduced rats varied from 9 to 33%, with an average of 18%. This difference in transduction efficiency is consistent with the fact that the titer of hPC-702 was fivefold higher than that of hPC-701. Discrepancies in the ratio of the titer to the in vivo transduction efficiency are likely due to inherent inaccuracies in determination of titer by Southern blot and determination of the in vivo transduction efficiency by the PCR-based method. Previous studies demonstrated a direct relationship between the number of retroviral vector particles injected and the transduction efficiency (45, 52). These data demonstrate that the higher expression of hPC observed in the hPC-702-transduced rats as compared with the hPC-701-transduced rats was primarily due to the fact that more copies of the hPC-702 retroviral vector were delivered. The DNA copy number in hPC-702-transduced rats that exhibited an increase in hPC protein in the blood at late time-points (rats 157, 164, and 166) was similar to the DNA copy number in a rat that did not have this increase (rat 163). We conclude that it is unlikely that the increase in hPC levels in these rats was due to an increase in the retroviral vector copy number in the liver because of ongoing infection.

Analysis of DNA and RNA from rats transduced with similar retroviral vectors containing different cDNAs. The level of expression of hPC in the hPC-701-transduced rats (27±4.7 [SEM] ng/ml, or 0.44 nM) was lower than what we had observed previously after delivery of similar high-titer retroviral vectors containing other cDNAs, as summarized in Table II.

Table II. Comparison of Protein Levels, DNA Copy Number, and RNA Levels in Rats That Were Transduced with Retroviral Vectors with the Human α_1 -antitrypsin Promoter and the IRES-DHFR* Cassette but Expressed Different cDNAs**

Retroviral vector	Protein level in blood*	DNA copy number	RNA levels [§]
% transduction [‡]			
hFX-514	1747±387 ng/ml	1.6±0.6	585±102%
hAAT-540B	475±141 ng/ml	0.5±0.1	252±67%
hPC-701	27±4.7 ng/ml	1.6±0.7	Weakly positive

*Protein levels in blood were determined by testing serum (hAAT) or plasma (hFX and hPC) for the appropriate antigen level using a human-specific ELISA. [‡]DNA copy number was determined by multiplex PCR using primers specific for the retrovirus (IRES) and rat genomic DNA (LFABP), as shown in Fig. 5 A. [§]RNA levels were determined by using an RNase protection assay with a probe specific for the IRES, as shown in Fig. 5, B and C. The level of RNA relative to a pool of NIH 3T3 cells that were transduced with a single copy of hPC-701 is shown. RNA levels from the hPC-701-transduced rats were difficult to quantitate as the signal was just barely above the background level. Averages±SEM are shown.

hFX-514 is a retroviral vector that is identical to hPC-701 except it contains the hFX cDNA instead of the hAAT cDNA, and does not contain the ApoE enhancer. hAAT-540B is a retroviral vector that is identical to hPC-701 except it contains the hAAT cDNA instead of the hPC cDNA. The hFX level in hFX-514-transduced rats was 1,747±387 ng/ml, or 28 nM (36). The hAAT level in our initial report in hAAT-540B-transduced rats was 5,385±2,408 ng/ml, or 108 nM (40). However, the hAAT-540B-transduced rats whose DNA and RNA are analyzed here received 10-fold fewer retroviral particles and the average hAAT level was correspondingly lower at 475±141 ng/ml (36).

Possible explanations for decreased expression of hPC in this study as compared with expression of other cDNAs in previous studies include delivery of fewer retroviral vector particles, decreased levels of transcription, decreased mRNA stability, decreased protein translation or secretion, or decreased half-life of the protein in blood. To try to identify which of these mechanisms might contribute to the lower levels of hPC obtained in vivo, we quantitated the retroviral vector DNA and RNA levels in livers from rats that were transduced with each of these retroviral vectors. These assays used probes specific for the IRES sequences that are present on all three vectors.

Quantitation of the DNA copy number in rats that were transduced with different retroviral vectors is shown in Fig. 5 A and summarized in Table II. In this assay, the hFX-514-transduced rats had an average retroviral vector DNA copy number of 1.6±0.6% (SEM), the hAAT-transduced rats had an average DNA copy number of 0.5±0.1%, and the hPC-701-transduced rats had an average copy number of 1.6±0.7%, as summarized in Table II. These data are consistent with the DNA copy number determined previously for hFX-514- and hAAT-540B-transduced rats using the IRES primers, and for the hPC-701-transduced rats using the hPC-

specific PCR-based assay. We conclude that differences in transduction efficiency are minor and cannot account for the > 20-fold lower expression that was observed with hPC-701-transduced rats as compared with rats that were transduced with hFX or hAAT.

RNA levels were determined using an RNase protection assay specific for the IRES sequences that are present on all three retroviral vectors, as shown in Fig. 5, B and C. RNA obtained from the hFX-514-transduced rats (Fig. 5, lanes 2–5) gave a signal that was sixfold stronger than the signal from hPC-701-transduced NIH 3T3 cells. hAAT-540B-transduced rats (Fig. 5, lanes 6 and 7) had a signal that was 43% of that observed in hFX-514-transduced rats. The signal from hPC-701-transduced rats (Fig. 5, lanes 8–10) was only slightly higher than the signal observed in the control (nontransduced) rat shown in Fig. 5, lane 1, and could not be accurately quantitated. The RNase protection assay was also performed using other digestion conditions, and we consistently obtained a much fainter signal with RNA from hPC-701-transduced rats than with RNA from rats transduced with the other retroviral vectors (not shown). We conclude that hPC-701-transduced rats have less retroviral vector RNA in their liver than rats that were transduced with retroviral vectors containing other cDNAs, although the DNA copy number was similar or higher for the hPC-701-transduced rats as compared with the other rats. This suggests that the hPC coding sequences affect either transcription or mRNA stability.

Discussion

Severe homozygous hPC deficiency is a serious disorder that results in spontaneous thrombosis and DIC if untreated. Although treatment with frequent parenteral infusions of purified hPC is effective in preventing these clinical manifestations, the high cost and the need for close medical supervision will likely limit this approach in humans once hPC concentrates are approved for clinical use. The alternative treatment, life-long anticoagulation with coumadin, is complicated by bleeding or thrombosis during periods of over- or anticoagulation, respectively. Thus, successful gene therapy for severe hPC deficiency would dramatically improve the quality of life of patients with this disorder.

Therapeutic levels of expression of hPC were achieved in some rats. In this study, two different retroviral vectors were delivered to the liver in vivo during hepatocyte regeneration. During the first 2 wk after transduction, hPC-701-transduced rats had an average hPC level of 27.5±4.7 (SEM) ng/ml (0.7% of normal human levels), while hPC-702-transduced rats had an average hPC level of 134.1±22.1 ng/ml (3.3% of normal). The higher expression in the hPC-702-transduced rats was probably due to the fact that the transduction efficiency (18% of hepatocytes) was higher than that of the hPC-701-transduced rats (1.5%). We consider the level of expression that was achieved during these 2 wk to be indicative of what would have been maintained long-term in the absence of anti-hPC antibody formation.

The hPC was functional as determined by using a chromogenic substrate assay after immunoprecipitation with human-specific hPC antibodies and activation with Protac. We also activated the protein C present in rat plasma with Protac, and tested its anticoagulation activity in an APTT assay with hPC-deficient human plasma. Addition of 5 μ l of plasma from

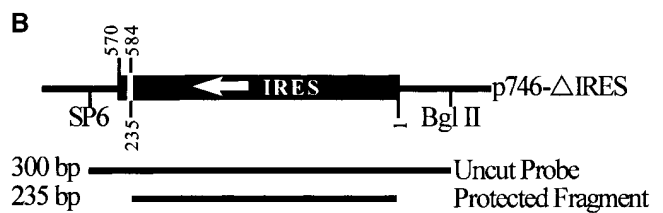
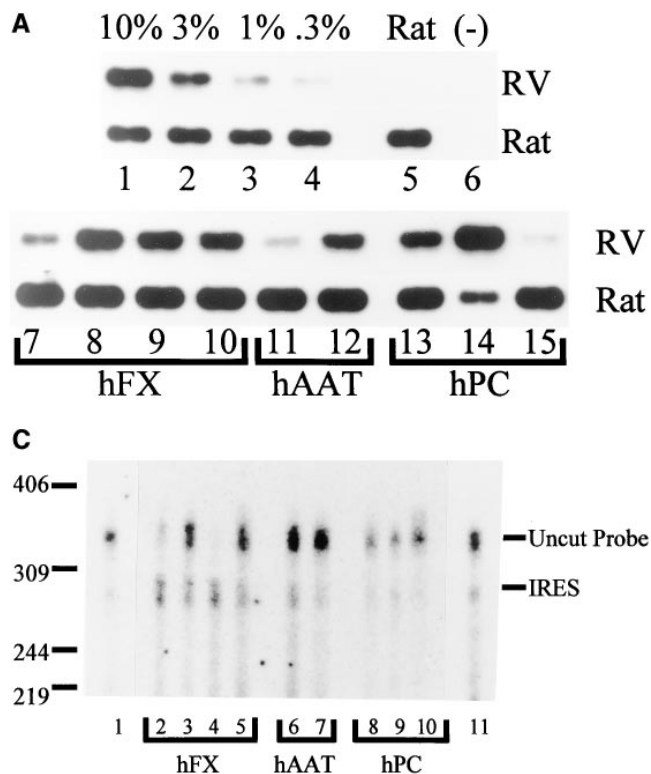


Figure 5. DNA and RNA levels in rat liver in vivo for animals that were transduced with retroviral vectors expressing different cDNAs. DNA and RNA were obtained previously from the livers of rats that were transduced with human factor X (*hFX*) or human α_1 -antitrypsin cDNAs (36) on a retroviral vector that contained the IRES-*DHFR cassette and the human α_1 -antitrypsin promoter. The *hFX*-514-transduced rats analyzed here had an average *hFX* level of 1,700 ng/ml in plasma, while the *hAAT*-transduced rats had an average of 475 ng/ml of *hAAT* in serum. DNA and RNA were obtained from this study from *hPC*-701-transduced rat livers, the retroviral vector that contains the IRES-*DHFR cassette. (A) Retroviral vector DNA copy number. DNA was amplified in a PCR reaction using primers specific for the internal ribosome entry site sequence (*RV*) and the rat LFABP promoter (*Rat*). The PCR reactions were electrophoresed, transferred to a membrane, and hybridized with an IRES-specific probe, then stripped and hybridized with the LFABP probe. DNA standards shown in lanes 1–4 represent DNA from NIH 3T3 cells that were transduced with a single copy per cell of *hPC*-701. This was mixed with rat genomic DNA to give samples that represent a retroviral vector copy number of 10 to 0.3%, as shown above each sample. The ratio of signal in these samples was used to generate a standard curve to estimate the retroviral vector

DNA copy number for the rat liver samples. DNA from a control nontransduced animal (*Rat*) is shown in lane 5, while lane 6 (–) did not have any DNA added. The samples shown in lanes 7–10 represent DNA obtained from *hFX*-514-transduced rats, lanes 11 and 12 represent DNA from *hAAT*-540B-transduced rats, and lanes 13–15 represent *hPC*-701-transduced rats. (B) Antisense RNA used in an RNase protection assay to determine the amount of retroviral vector RNA. A portion of p746- Δ IRES, a plasmid that contains the IRES, is shown. This contains IRES sequences from nucleotide 1 to 235, and 570 to 585. After linearization with *Bgl*II, transcription with SP6 RNA polymerase results in a 300-bp RNA probe which is complementary to the IRES present in all three retroviral vectors. When this probe is hybridized with IRES-containing retroviral RNA and digested with single-strand specific, a 235-nt band is protected. (C) RNase protection assay using rat liver RNA and the antisense IRES probe. RNA was hybridized with the antisense IRES probe shown in panel B, digested with single-stranded RNA-specific RNases, and electrophoresed on a denaturing polyacrylamide gel. The position and size of end-labeled pBR322 *Msp*I DNA markers is shown on the left. The position of the uncut 300-nt probe (*Uncut probe*) and the 235-nt band that is protected from digestion in the presence of IRES-containing RNA (*IRES*) are shown on the right. Note that RNA migrates \sim 10% slower than DNA with the same number of nucleotides because of the extra hydroxyl group. Lane 1 shows RNA from a control (nontransduced rat). Lanes 2–5 represent RNA from *hFX*-514-transduced rats, lanes 6 and 7 represent RNA from *hAAT*-540B-transduced rats, and lanes 8–10 represent RNA from *hPC*-701-transduced rats. Lane 11 contains RNA from *hPC*-701-transduced NIH 3T3 cells.

all of the animals with high *hPC* levels to 75 μ l of *hPC*-deficient plasma resulted in a prolongation of the APTT, as compared with the APTT observed using an equal amount of plasma from a variety of normal rat controls (data not shown). Although this suggests that the *hPC* was functionally active at inhibiting coagulation, quantitative of the *hPC* levels was complicated by the fact that addition of rat plasma to *hPC*-deficient human plasma markedly blunted the prolongation of the APTT that is otherwise observed when Protac-activated *hPC* is added to *hPC*-deficient human plasma. *hPC* activity is rapidly inhibited by rat plasma (53; data not shown), which is likely due to interaction of *hPC* with known inhibitors of protein C such as protein C inhibitor or α_1 -antitrypsin (54–56). In addition, species differences in the interaction of PC with its cofactor protein S and its substrates factor Va and VIIIa (53) may also complicate determination of *hPC* activity in a coagulation-based assay. Thus, although the prolongation of the APTT with Protac-activated plasma from transduced rats with high *hPC* antigen levels suggests that the *hPC* functioned to

inhibit coagulation, the problems of this heterologous system precluded precise quantitation of the amount of functional *hPC* present. We therefore consider the antigen levels achieved to be most predictive of whether or not a therapeutic effect would be observed in humans.

Homozygous *hPC*-deficient patients with 5–20% of normal *hPC* levels usually do not experience purpura fulminans or DIC, although they may present with thrombosis as young adults (57–60). Similarly, purpura fulminans and DIC can be prevented in patients with severe *hPC* deficiency by achieving > 10% of normal human levels by injection of *hPC* concentrates (11). Thus, a minimal therapeutic goal would be to achieve 5–10% of normal *hPC* levels, although it would be preferable to achieve 100% of normal human levels. Most of the *hPC*-702-transduced rats achieved a level of expression that would have a significant effect upon the clinical manifestations of severe *hPC* deficiency. We plan to determine if the *hPC*-expressing retroviral vectors will prevent the spontaneous thrombosis and early death that occurs in homozygous mice

whose protein C gene has been knocked out by homologous recombination (61).

The level of hPC achieved in rats was 20-fold lower than the levels of two other plasma proteins, hAAT and hFX, that were expressed from a similar retroviral vector using similar delivery methods, as summarized in Table II. The production of hPC protein in hPC-701-transduced NIH 3T3 (fibroblast) cells in vitro in this study was similar to the in vitro expression of hFX or hAAT by hFX-514- (36) or hAAT-540B-transduced (40) cells, respectively, in previous studies, suggesting that differences in expression only occurred in the liver in vivo. It is unlikely that differences in protein half-life can account for the discrepancy in protein expression. Although the half-life of hAAT is considerably longer in mice at ~ 24 h (62) than the half-life of hPC in rats (2.6 h), the half-life of hFX in blood at 3.5 h in rats (Le, M., and K. Ponder, unpublished data) is similar to that of hPC in normal rats. Differences in transduction efficiency cannot account for the discrepancy in protein expression, as the DNA copy number in the liver in vivo was similar for the different retroviral vectors.

The levels of retroviral vector-derived transcripts were lower for the hPC-701-transduced rats than they were for the hFX-514- or hAAT-540B-transduced rats, as determined by using an RNase protection assay. This suggests that the presence of the hPC cDNA resulted in decreased transcription or decreased mRNA stability. Attempts to quantitate the transcription rate of the various constructs in the liver using a run-on transcription assay have not been successful to date but are being continued. These data demonstrate that expression of different genes from a retroviral vector may vary considerably in vivo, and that gene sequences may alter the transcription and/or half-life of an mRNA in vivo.

Antibodies probably prolonged the hPC half-life and increased the hPC levels in some rats. The immunological response to a foreign protein will be an important consideration for gene therapy in human patients, which usually lack some or all of the epitopes of a protein. In this study, four rats developed anti-hPC antibodies and exhibited a four- to ninefold increase in the hPC levels. Three of these four rats were studied to determine the hPC half-life, and all had a two- to threefold longer half-life than a variety of controls that did not have anti-hPC antibodies. Despite these antibodies, the hPC was biologically active in an immunoprecipitation/chromogenic substrate assay. We believe that the anti-hPC antibodies resulted in the prolonged hPC half-life. Alternative explanations, such as the possibility that animals with a longer hPC half-life are more likely to develop antibodies, appear to be unlikely in these inbred rats, but cannot be excluded by this study. Three other rats had no significant antibodies and stable expression of hPC, while four rats had anti-hPC antibodies and hPC plasma levels that fell to 4, 22, 26, or 42% of their initial levels. Although we hypothesize that the latter rats would have exhibited a decrease in the hPC half-life commensurate with the decrease in hPC levels, these animals were killed before the hPC half-life study was performed.

Some investigators working in the field of gene therapy have assumed that the production of antibodies will necessarily result in increased clearance and decreased levels of a plasma protein in animals (63, 64). In this study, although three rats had a moderate decline and one had a severe decline in hPC levels coincidentally with the development of anti-hPC antibodies, four rats had an increase in functionally active hPC

levels and a prolongation of the hPC half-life that was presumably due to antibodies. We conclude that the development of antibodies does not necessarily lead to increased clearance or inactivation of the biological effect of the protein. Our data are consistent with the fact that a large fraction of patients that are treated with porcine insulin (65–68), growth hormone (69), factor VIII (70), or glucocerebrosidase (71, 72) develop antibodies, and the majority of these antibodies do not have an adverse effect upon the biological response to the protein. Indeed, most patients that were treated with porcine insulin developed antibodies that prolonged the half-life of radiolabeled insulin in vivo without blocking its effects upon glucose metabolism (65–68). Although antibodies that block the effect of a protein can pose a serious clinical problem for the treatment of diabetes, growth hormone deficiency, hemophilia, or Gaucher disease (65–70; 73), we conclude that not all antibodies are deleterious. It is likely that the immunological response to a protein that is delivered by gene therapy procedures will not differ significantly from that of infused protein, although it will be necessary to assess the immunological response to proteins after gene therapy procedures are performed in humans. In a series of nine patients who received intermittent or continuous injection of hPC concentrates, none have developed antibodies to date (11).

Implications for human gene therapy. Retroviral-mediated hepatic gene therapy has been limited in humans because of subtherapeutic protein expression and the surgical risks associated with the genetic modification of hepatocytes. The first problem, that of low-level expression, can be overcome by using a strong liver-specific promoter and delivering a large number of retroviral vector particles in vivo, as was done in this study.

The second major obstacle is that in vivo and ex vivo approaches (24) require a major surgical procedure such as the 70% partial hepatectomy that was performed in this study to induce hepatocyte replication. We have used 70% portal branch occlusion to induce apoptosis of the occluded liver and compensatory replication of the nonoccluded liver in rats (74) and pigs (75) with little morbidity. Alternatively, efficient in vivo retroviral vector transduction was achieved in mice after hepatocyte replication was induced by the administration of keratinocyte growth factor (76) or hepatocyte growth factor (77). There are therefore a variety of less toxic avenues for facilitating in vivo retroviral vector delivery to the liver that are currently being explored. The inefficiency of retroviral vector-mediated gene transfer in large animals (34) is likely due to the failure to deliver a sufficient number of retroviral vector particles to the liver during the peak period of hepatocyte replication. Improvements in production of higher-titer retroviral vector particles and identification of the time period during which hepatocyte replication occurs should make it possible to improve the transduction efficiency in large animals. If these approaches are successful and safe, in vivo retroviral vector-mediated gene therapy will be a very attractive method for permanently correcting the clinical manifestations of patients with severe hPC deficiency.

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