Chain Length of the Polysine in Receptor-targeted Gene Transfer Complexes Affects Duration of Reporter Gene Expression Both in Vitro and in Vivo*

(Received for publication, July 30, 1998, and in revised form, October 29, 1998)

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Complexes composed of peptide ligand for the serpin enzyme complex receptor covalently coupled to poly-l-lysine condensed by charge interaction with plasmid DNA direct gene transfer into receptorbearing cells. We compared intensity and duration of reporter gene expression in vitro and in vivo from serpin-enzyme receptor-directed gene transfer complexes prepared with poly-l-lysine of different chain lengths. When substituted with linker and ligand to comparable extents, DNA complexes containing short chain poly-l-lysine were larger and gave higher peak expression but significantly shorter duration of expression than those containing long chain poly-l-lysine. Both peak expression and duration of expression exceeded that observed with Lipofectin. Neither naked DNA nor DNA complexed with unsubstituted polylysine was effective in gene transfer. For in vivo experiments, complexes containing optimal ligand and degree of substitution (based on in vitro data, peptide C105Y, 11 ligands/plasmid DNA molecule) were prepared with either short chain or long chain polylysine and a β-galactosidase expression plasmid. Following injection into the tail veins of mice, longer chain complexes gave significantly higher expression of reporter gene in lung and spleen that lasted for a significantly longer period of time than the shorter chain complexes. The short chain poly-l-lysine-DNA complexes were larger in diameter, as assessed by electron microscopy or atomic force microscopy, and gave less protection against DNase digestion in vitro than longer chain complexes. Thus, for gene transfer complexes directed at the serpin enzyme complex receptor, longer chain poly-l-lysine gave a much longer duration of expression both in vitro and in vivo. We speculate that this may be due to protection against degradation afforded the plasmid DNA by the tighter compaction produced by long chain poly-l-lysine.

Receptor-mediated gene transfer has great appeal as a strategy for gene therapy because of its specificity and low toxicity in vivo but has drawbacks, including low level and transient gene expression (1–9). Targeting the serpin (serine protease inhibitor) enzyme complex receptor (SEC-R) might transfect many cell types that are potentially interesting for gene therapy, including hepatocytes, macrophages, and neurons (10–14). Ligand-conjugated poly-l-lysine (poly K)-DNA complexes directed at this receptor deliver reporter genes, specifically, to receptor-bearing cells. The synthetic ligands are based in sequence on amino acids 346–374 of human α1-antitrypsin (9). We undertook a systematic study of the contribution of the protein portion of the complex to intensity and duration of gene expression.

Prior studies of the composition of the protein portion of receptor-directed gene transfer complexes have focused on the degree of substitution (6, 15). One study did not find a consistent relationship between chain length and gene expression in vitro (6). No study has examined the relation between the chain length of poly K used to condense DNA and duration of expression. Moreover, it is not clear whether modifications worked out in an in vitro model can be applied to the in vivo situation (16, 17).

Our system has several advantages for studies of this type, including the ability to determine the extent of lysine substitution by linker and ligand, even at very low levels, with NMR. Furthermore, the same ligands bind to the receptors of human, mouse, and rat, thus allowing parallel examination in vitro in human cells and in vivo in animal models. We used this system to investigate the influence of chain length of poly K used on the intensity and duration of gene expression in vitro and in vivo. Using our in vitro model, we compared the optimally substituted poly K molecules of average chain length 36 with those of average chain length 256. Longer chain poly K molecules gave significantly longer duration of expression both in vitro and in vivo than shorter chain poly K molecules for a given ligand and degree of substitution.

Manipulation of the protein portion of receptor-targeted DNA complexes strongly influenced the duration and intensity of gene expression. This demonstration, in vitro and in vivo, addresses some of the limitations of the system and provides a new basis for improvement of this molecular conjugate for therapeutic purposes.

EXPERIMENTAL PROCEDURES

Materials—DNA-modifying enzymes, nucleotides, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were purchased from Boeh-

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*This work was supported by National Institute of Health Grants DK 49138 (to P. B. D.), DK 43999 (to P. B. D.), P30 DK27651 (to P. B. D.), T32 HL07653 (to A.-G. Z.), HL37784 (to D. H. P.), and AG11577 (to D. H. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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†The abbreviations used are: SEC-R, serpin enzyme complex receptor; poly K, poly-l-lysine; AFM, atomic force microscopy; sulfo-LC SPDP, sulfo succinimidyl 6-[3(2-pyridyldithio)-propionamido] hexanooate; kb, kilobases; CMV, cytomegalovirus.
ringer Mannheim. β-Galactosidase luminescent activity was measured using reagents obtained from Tropix (Bedford, MA). Pol K was obtained from Sigma, and LC sulfo-N-succinimidyl-3-(2-pyridyldithio) propionate (sulfo-LC SPDP) was purchased from Pierce. Luciferase activity was measured using Promega (Madison, WI) assay reagents. Sodium dodecyl sulfate (SDS) gel electrophoresis of plasmid DNA was performed on a Criterion Cell (Bio-Rad). Peptides C105Y (CS IPPEF KKFKP FYVYL) and C1315 (CFLE AIPS IPPEP KKFKP FVFYL IHDD) were synthesized by solid phase method, purified, and subjected to amino acid composition and sequence analysis as described previously (10). The 5-amino acid binding motif, FYVFL, shown in bold type, is sufficient for SEC-R binding (12).

**Cell Culture**—HuH7 cells were maintained as described previously (9).

**Construction of the Protein Portion of DNA Complexes**—Peptides C1315 and C105Y were covalently linked to different size poly Ks (average poly K length, 36 amino acids (9.7 kDa) and 256 amino acids (53.7 kDa)) using the heterobifunctional cross-linking reagent sulfo-LC SPDP as described previously (18). To account for the differences in the size of the poly Ks, reaction concentrations were normalized to moles of lysine residues present in each poly K. Aliquots of each of the poly Ks were mixed with sulfo-LC SPDP in 0.1 M phosphate-buffered saline, pH 7.4, at room temperature (about 22 °C) for 30 min. The reaction mixtures were then dialyzed exhaustively to remove unreacted sulfo-LC SPDP, the remaining mass reaction products, and samples were reserved for NMR analysis. Pol K linker conjugates were then mixed with C1315 or C105Y at 22 °C for 24 h. Aliquots were set aside for NMR analysis.

Two approaches were taken to conjugate construction. In one, all the pol K used to condense DNA was substituted randomly with sulfo-LC SPDP and then with ligand. These are termed “random substitution” experiments and produced poly Ks substituted on either 3.5 or 7.8% of lysines with sulfo-LC SPDP. Each of these constructs was then substituted with ligand on 0.017, 0.097, or 0.27% of the lysine residues. These values were determined by NMR. When these substituted poly K molecules were complexed to charge neutrality with the pGL3 plasmid, the complexes contained ~2, ~10, or ~35 ligands.

The second approach was to adjust conditions to substitute only one lysine on each poly K molecule with sulfo-LC SPDP-ligand. Substituted poly K molecules were mixed with unsubstituted poly K of the same chain length to give mixtures that, when complexed to charge neutrality to the pGL3 plasmid, had a specified number of ligands/DNA molecule, depending on the relative proportions of substituted and unsubstituted poly K in the mixture. This approach was termed the “ligand dilution” method. Optimal ligand concentration was then chosen to construct CMV lacZ II complexes for animal experiments.

**NMR Spectroscopy**—To verify the rates of substitution of the different poly Ks as well as examine the structure of each of the peptides, we used NMR. An aliquot (5–10 mg) of the conjugate was exhaustively dialyzed against water, lyophilized from water and subsequently from D2O, and then resuspended in 0.75 ml of 99.99% D2O (Aldrich) or 90% Me2SO–10% D2O. Proton NMR spectra were obtained at 600 MHz on a Varian Unity Plus 600 NMR spectrometer using standard proton parameter, 7.4, at room temperature (about 22 °C) for 30 min. The reaction mixtures were also lyophilized and examined by NMR to verify the absence of the HDO resonance at 4.8 ppm or the Me2SO– resonance at 2.5 ppm.

**Construction of the Protein Portion of DNA Complexes**—Electron micrographs were prepared as described previously (3, 5). Aliquots of each reaction mixture were examined by electron microscopy to assay condensation. SEC-R directed DNA complexes containing the optimal conjugate for DNA (5). Aliquots of each reaction mixture were scanned in water for 1 h in a sealed chamber, and scanned. Images were transferred in binary format to a SPARC 10 Sun Microsystems workstation, converted to grayscale, and analyzed.

**Transfection of Cells in Culture**—HuH7 cells were transfected as described previously (9). Controls included: (a) HuH7 cells transfected with 1.2 pmol of pGL3 control condensated with unconjugated poly K in the presence of free C1315 or C105Y peptides and free sulfo-LC SPDP linker (comparable with peptide and linker content in the conjugated complexes); (b) HuH7 cells transfected with 1.0 pmol of pGL3 control DNA by Lipofectin; and (c) HuH7 cells transfected with 1.11 pmol of unconjugated poly K condensed DNA by Lipofectin. Control (a) was designed to test for nonspecific uptake; controls (b) and (c) confirmed that target cells could express the transgene. After addition of the complex and/or Lipofectin, all cells were incubated at 37 °C for 3 h. Cells were then rinsed with Ca2+/Mg2+ phosphate-buffered saline, and fresh growth medium was added and incubated at 37 °C (with a change of medium every 2 days) until the functional assay was performed. All transfections were done in triplicate. No excess cell death was observed, and in any of the wells transfected with the DNA-conjugated poly K complexes throughout the incubation. Luciferase expression was measured at 2, 4, 10, and 16 days after transfection. Number of samples (N) denotes transfections done on different days with different cells with different complexes.

**Assay for Luciferase Expression**—Harvested cells were homogenized in a homogenizer (Microdismembrator S, B. Braun Melsungen AG), centrifuged at 50,000 × g for 10 min, and the supernatants were collected for assay. Aliquots (20 μl) were analyzed for luciferase activity as described previously (23) and normalized for protein content by the Bradford method (Bio-Rad). Results, expressed as the integrated light units/mg protein, are the averages of duplicate samples.

**Animals**—SEC-R ligands were based on the binding sequence of human α1-antitrypsin but bind to receptors of mice and rats as well as humans. SEC-R-directed DNA complexes containing the optimal C105Y conjugates examined in vitro (11 ligands/DNA molecule) were injected into the tail vein of anesthetized adult C57BL/6 (Charles River Inc.) mice (5 weeks old, ~25 g). Complexes contained 15 μg (50–60 μl) of the plasmid CMV lacZ II bound to the optimal 9.7- or 53.7-kDa poly K-ligand conjugate (11 ligands/DNA plasmid molecule). Preliminary experiments showed no advantage to increasing the dose to 25 μg DNA. Controls included animals injected with a 1 m NaCl solution alone or with the plasmid CMV lacZ II condensed with unconjugated poly K (9.7 or 53.7 kDa). Immediately following injection, there was 12% mortality in all groups of animals injected with solutions containing 1 m NaCl due to apnea. Animals were sacrificed 2, 4, 16, and 30 days after treatment, and tissues were removed for analysis. Preliminary experiments (including the lung, liver, spleen, brain, and kidney) showed that the highest activity was observed in lung and spleen, so we examined these organs in this study. Examination of the cellular distribution of lacZ activity in these tissues showed that activity was evident only in macrophages. No lacZ activity was detected with 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside staining in the lungs or spleens of animals in-
jected either with NaCl only or with unconjugated poly K-DNA complexes. The animal research protocol was reviewed and approved by the Case Western Reserve University Institutional Animal Care Committee.

Assay for β-Galactosidase Activity—Lungs and spleens of animals were immediately frozen in liquid nitrogen and stored at −80 °C until homogenized in lysis solutions containing 12 mM leupeptin, 20 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100 in a phosphate-buffered saline solution. Homogenates were incubated at 48 °C for 1 h to inactivate endogenous β-galactosidase activity. Triplicate aliquots were added to 200-μl reaction buffer (Tropix, Bedford, MA) and incubated at room temperature for 1 h. 300 μl of light emission accelerator (Tropix, Bedford, MA) was added, and the reaction was monitored for 10 s in a luminometer.

DNase Protection Assay—10-μg samples of pGL3 DNA were condensed with either the 9.7- or 53.7-kDa optimal conjugates (11 ligands/DNA plasmid molecule) and incubated with DNase I (Boehringer Mannheim) for 10 or 30 min in phosphate buffer containing Mg²⁺. Samples were heated for 10 min at 80 °C to inactivate the enzyme. The DNA was precipitated in a 75% ethanol/10% NaCl solution, dried, and solubilized in water. Free DNA, in the presence of ligand and sulfo-LC SPDP, was also digested for 10 and 30 min. Treated DNA in all treatment groups was subjected to agarose gel electrophoresis to examine DNA integrity.

Statistical Analysis—Data are expressed as the mean ± S.E. Statistical analyses of treatment groups were assessed using a general linear modeling utilizing standard multiple regression techniques (24). Residual analyses were conducted to assess validity of model assumptions, including normality and homoscedasticity. Logarithmic transformation (24) of the data was used to achieve satisfactory conformity with model requirements. This modeling approach was utilized for all analyses, including those related to the evaluation of random substitution, where the data were considered as a whole, and for specific experimental modalities to assess the relationship between poly K size and the time of evaluation of response. The same approach was applied to the subset of the data from the dilution experiments that corresponded to concentrations of ligand to DNA molecule common to the two ligands and also to modified poly K. This procedure enabled us to calculate the ratio of poly K to linker to peptide. Key to the analysis is the presence of unique aromatic proton resonances for both the sulfo-LC SPDP conjugated to poly K and for the peptides conjugated to SPDP-poly K. Proton NMR of sulfo-LC SPDP conjugated to poly K (10% sulfo-LC SPDP mole ratio) produced three resonances that appear at 7.4, 7.9, and 8.5 ppm representing the four aromatic protons (H3, H4+H5, and H6, respectively) of the sulfo-LC SPDP 2-sulphhydryl pyridine group (26). Based on the molar ratios of poly Ks to sulfo-LC SPDP during the coupling reaction calculated from five experiments on eight conjugates, we estimate that this reaction was, on average, 77% efficient, independent of poly K chain length.

¹H NMR analysis of the C1315-poly K and C105Y-poly K conjugates confirmed conjugation with the peptides has occurred by the disappearance of the sulfo-LC SPDP aromatic protons and by the appearance of new aromatic proton resonances in the region of 7–9 ppm from aromatic residues in the ligand peptides.

Integration of conjugate proton resonances provided actual ratios of poly K to linker to peptide. Both peptides had similar coupling characteristics. For reactions where 0.1, 0.5, and 1.5% peptide was added to poly K, 0.017% (1 in 5882 lysine residues modified), 0.097% (1 in 1039 residues modified), and 0.27% (1 in 370 residues modified) actually reacted, respectively. Modification with peptide was, on average, 20% efficient. In eight different conjugates of each of the poly Ks used, the chain length of poly K did not affect this efficiency of coupling. Thus, we were able to obtain conjugates substituted to the same extent with either linker or ligand on a per lysine residue basis that differed only in poly K chain length.

Structure of Gene Transfer Complexes by Electron Microscopy and AFM—We examined the complexes by electron microscopy and AFM. Solutions used to make complexes contained no structures, although DNA was seen as rope-like strands. We measured electron microscopy photographs of 50 particles from each of eight complexes for each poly K size and found DNA

![Fig. 2. Atomic force microscopy images of dried DNA complexes on mica chips.](image)

Immediately after formation of DNA complexes, a drop of the solution (1:100 dilution) was applied to the surface of a 2 × 2-cm Mica wafer. These samples were then dried for 3 h and scanned by a Nanoscope III atomic force microscope. DNA was SV40 pGL3 control plasmid (5.22 kb), and condensation proceeded appropriately. The protein portion of the complex was: 53.7-kDa poly K containing 3.5% sulfo-LC SPDP and 0.017% C105Y ligand (A) and 9.7-kDa poly K containing 3.5% sulfo-LC SPDP and 0.017% C105Y ligand (B). Gray scale indicates height of complexes. Bar, 100 nm.
condensed with shorter average length poly K (9.7 kDa) formed complexes significantly larger than those constructed with longer poly K (53.7 kDa) with the same substitution rate (24 ± 0.8-nm versus 17 ± 0.9-nm diameter, p < 0.01) (Fig. 1). The complexes appeared spheroidal by rotary shadowing.

The same conjugates were studied by AFM as well, which allowed us to analyze both dry and hydrated complexes and also to evaluate their three dimensional structure. In dried samples (50 particles), longer length poly K (53.7 kDa, Fig. 2A) condensed DNA to particles averaging 25 ± 1.9 nm in diameter, whereas the shorter chain poly K condensed DNA into particles 39 ± 2.5 nm in diameter (9.7 kDa, p < 0.05; Fig. 2B). All complexes were similar in height (16 ± 0.4 nm; Fig. 2). Images of hydrated complexes were less distinct, and complexes appeared larger (~50–60 nm in diameter and ~20 nm in height), but complexes made with long chain poly K appeared smaller than those with short chain poly K (data not shown).

**Effect of Chain Length on DNA Protection**—As examined by gel electrophoresis, the pGL3 plasmid was more protected from DNase degradation when condensed with longer poly K (up to 10 min at 37 °C) than DNA condensed with shorter poly K or

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**Fig. 3. Effect of poly K size on reporter gene expression.** HuH7 cells were transfected with 1.11 pmol of complexed DNA, and luciferase activity was assayed at 2, 4, 10, and 16 days later. Nine experiments were conducted with each of the constructed conjugates. In all 36 conjugates were examined, 12 with each of the different size poly Ks (6 with each peptide). Representative figures for 18 of those conjugates are demonstrated above. Graphed values represent averages of the experiments conducted for each of the conjugates (n = 9), and error bars show S.E. A, luciferase activity obtained from cells transfected with two different size poly Ks substituted with 7.8% linker and 0.27% C105Y ligand. B, luciferase activity obtained from cells transfected with two different size poly K substituted with 3.5% linker and 0.017% C105Y ligand. C, luciferase activity obtained from cells transfected with various control mixtures lacking receptor ligand. In all nine experiments with each of the conjugates displayed above, luciferase activity was significantly greater (p < 0.01) than cells transfected with DNA alone or unmodified poly K condensed DNA.
free DNA (data not shown). Both long and short poly Ks failed to protect DNA against degradation after 30 min of treatment with DNase I.

**Experimental Strategy**—To test the contribution of the chain length of the poly K molecule to the intensity and duration of gene expression, we used two different approaches, random substitution and ligand dilution, which generally gave concordant results. To focus this comparison on poly K length, we first report on the selection of the better ligand and the best ratio of ligands to DNA.

**Comparison of Receptor-directed Complexes and Controls**—All transfections with complexes containing any of the poly K linked to ligand produced significantly greater luciferase expression \( (p < 0.01) \) than transfections with DNA alone \( (n = 18) \), DNA condensed with unmodified poly K (all sizes) in the presence of linker \( (n = 36) \), and DNA condensed with linker modified poly K \( (n = 24) \). Transfections with Lipofectin-DNA complexes, used as a positive control, resulted in peak activity 8–10-fold lower than the average peak luciferase activity obtained with ligand-containing complexes \( (10^7 \text{ integrated light units/mg protein}; \text{Fig. 3}) \). Expression from Lipofectin transfection was indistinguishable from background by 10 days.

**Random Substitution Experiment**—Complexes constructed with the 53.7-kDa poly K produced peak expression at 4–10 days post transfection, whereas complexes containing the 9.7-kDa poly K peaked at 2 days but declined rapidly. Two days post transfection, complexes with the shorter poly K produced, on average, 10-fold higher expression than the longer poly K. At 4–10 days, the longer poly K complexes produced greater expression. At 16 days, the longer poly K resulted in significantly higher expression than the shorter poly K \( (C105Y, p = 0.0001 \text{ and } C1315, p = 0.0162) \). Fig. 3 shows a representative example for the most and least substituted complexes. We compared the effects of C1315 and C105Y on intensity of duration of reporter gene expression. Both peptides gave comparable intensity of expression, but the C105Y peptide containing complexes produced longer lasting expression when compared with the C1315 peptide over a broad range of substitution patterns \( (p < 0.01) \).

To further examine this phenomenon, we performed five experiments with each of the 53.7-kDa poly K constructs and extended the time course to 40 days (Fig. 4). Although in these experiments there is no significant difference between the two peptides at day 2 or 18, all 53.7-kDa constructs containing the C105Y peptide expressed higher levels of luciferase at days 25 and 40 post transfection than constructs with C1315 \( (n = 60, p < 0.01) \). No excess toxicity (as assessed by whole cell protein measurements) was observed for either peptide.

**Ligand Dilution Experiment**—Due to the complexity of the random substitution results, we designed a simpler experiment to address the optimal ligand to DNA ratio. We assumed that all of the conjugate added to DNA during the condensation step complexes with the plasmid. The size of the plasmid (5.22 kb) gives the number of negative charges. Because we know the ligand to lysine residue ratio, we can estimate the number of ligands/DNA molecule once the lysines have completely neutralized the 10440 negatively charged phosphate groups on each molecule of DNA. For the 53.7-kDa poly K constructs substituted once with ligand, a maximum of –43 ligands would interact with each DNA molecule; for the 9.7-kDa poly K substituted once, a maximum of –290 ligands would interact. This ligand number could be reduced by dilution with unmodified poly K.

Initial experiments with each of the poly K sizes included complexes containing 5–40 ligands \( (C105Y \text{ and } C1315)/\text{DNA molecule} \). For the C105Y ligand, optimal complex preparations contained 8–14 ligands/DNA molecule (Fig. 5). Six separate experiments conducted with the 53.7-kDa poly K constructs demonstrated that all complexes except those containing 40 ligands/plasmid produced expression, which peaked at 4 days.
post transfection and remained higher \((p < 0.01)\) than background for the duration of the experiments \((16\, \text{days})\). For the C1315 ligand, \(20–35\) ligands/DNA were required for optimal expression, but complexes with \(20–25\) ligands/DNA molecule retained expression best.

Complexes constructed with the 9.7-kDa poly K \((\text{Fig. 6})\) produced expression that peaked at 2 days and then rapidly declined. The optimal ligand ratio to DNA molecule was similar to long chain poly K, for C105Y \((8–14\, \text{ligands})\) or C1315 ligand \((20–35\, \text{ligands})\). Thus, for these experiments, as for the random substitution experiments, short length poly K complexes had high initial expression with rapid decline, and longer chain poly K complexes had expression that peaked later and persisted longer.

Like the random substitution experiments, the ligand dilution experiments showed that C105Y gave better duration and no less intensity than C1315. Sixteen days post transfection, conjugates containing 5–20 C105Y ligands/DNA molecule and the longer or shorter poly K produced higher expression \((p = 0.0001)\) than those containing the C1315 ligand. When comparing the effect of poly K length on duration of expression \((16\, \text{days})\), we found that complexes containing the 53.7-kDa poly K produced longer duration of expression than those containing the 9.7-kDa poly K \((p = 0.0005)\). Furthermore, ligand dilutions between 5 and 20 ligands/DNA molecule produced longer duration of expression than complexes containing 40 ligands/DNA molecule \((p = 0.0002)\). Conversely, at 2 days, complexes containing the shorter poly K produced higher expression than those containing the longer poly K \((p = 0.0001)\).

**Effect of Poly K Length on in Vivo Gene Transfer**—To test whether our \textit{in vitro} model for SEC-R-mediated gene transfer predicts, in principle, the \textit{in vivo} response, we injected via the tail vein C57/BL6 mice with DNA complexes containing the CMV \textit{lacZ} II plasmid \((10.8\, \text{kb})\) bound to C105Y conjugated to either the 53.7- or 9.7-kDa poly K \((11\, \text{ligands/DNA plasmid molecule})\). C105Y and the number of ligands/DNA molecule were chosen to maximize long term expression based on \textit{in vitro} data. \(\beta\)-Galactosidase activity in the lung and spleen of these animals \((n = 4–5\, \text{animals for each treatment group at each time point})\) injected with DNA complexes containing the C105Y/9.7-kDa poly K peaked 4 days after injection \((\text{different from salt, } p < 0.01, \text{ and unsubstituted poly K complex injected control animals; } 53,751 \, \text{integrated light units/mg protein} \pm 9,006 \, \text{in lung and } 42,474 \, \text{integrated light units/mg protein} \pm 6,323 \, \text{in spleen; } p < 0.001)\), was declining by 16 days post treatment, and no longer differed from background. Animals injected with the C105Y-53.7-kDa complexes had peak expression at 16 days, which had disappeared by 30 days \((\text{Fig. 7})\). Gene expression in animal groups treated with the C105Y-53.7-kDa complexes differed statistically from control animals treated with 1M salt \((p < 0.001)\) or complexes formed with unsubstituted poly K and DNA at both 4 and 16 days \((p < 0.01)\). At 16 days, expression from the C105Y-53.7-kDa poly K complexes also significantly exceeded that from complexes containing the C105Y-9.7-kDa poly K. Fig. 7 demonstrates \textit{in vivo} gene expression using both poly K lengths and the controls. Animals treated with complexes containing DNA
and unconjugated poly K did not differ from salt injected animals at any time point.

**DISCUSSION**

The composition of the protein portion of receptor-targeted gene transfer protein-DNA complexes profoundly affects the intensity and duration of gene expression, both *in vitro* and *in vivo.* In *vitro* the number of ligands/DNA molecule, the identity of the ligand, and the chain length of poly K all have strong effects on intensity and duration of gene expression, but the most striking effect was that of poly K chain length on the duration of expression. Using the ligand and number of ligands/DNA molecule found to be optimal in the cell culture system, we tested the effect of poly K chain length on in *vivo* expression. Using the ligand and number of ligands/DNA molecule found to be optimal in the cell culture system, we tested the effect of poly K chain length on in *vivo* expression. Complexes made with long chain poly K produced much more protracted gene expression *in vitro* than complexes prepared with short chain poly K.

Optimization of the SEC-R-directed gene transfer for in HuH7 cells resulted in greatly increased reporter gene expression (relative to Lipofectin control) compared with our prior report (9). Activity in the best complexes tested in the present study was about 20-fold higher than activity produced by the same amount of plasmid DNA delivered with the Lipofectin reagent; in our previous report maximal activity was less than the Lipofectin control. Increased peak activity indicates increased efficiency, which has been a problem for most nonviral strategies. At the same time, duration of activity could be greatly prolonged *in vitro* by informed selection of the specific receptor ligand (C105Y versus C1315), the number of ligands/plasmid DNA molecule (over 2–40 ligands), and the chain length of poly K used to condense the DNA. It is likely that the number of ligands and the identity of the ligand entrains routing within the cell, with the best complexes promoting routing into the endosomal compartment rather than to the lysosomal degradative pathway. For another receptor, the epidermal growth factor receptor, high number of ligands promotes lysosomal trafficking, whereas fewer ligands promote endosomal recycling (27), so fewer ligands might well entrain a more favorable trafficking pattern, delaying the destruction of delivered DNA. In addition, different ligands for some receptors, such as the transferrin receptor, may be trafficked differently (28). A similar mechanism may account for the differences in duration of expression for complexes containing the two SEC-R ligands, which have very similar affinity for the receptor and very similar initial transgene expression.
In vitro, the expression of the firefly luciferase reporter gene persisted (within a log maximum) in HuH7 cells for at least 40 days for some complexes that contained the long chain poly K-ligand conjugates. Because the half-life of the luciferase protein in mammalian cells is 3 h (29) and we observed rapid decline in luciferase activity in HuH7 cells transfected using Lipofectin (thus, HuH7 cells do not retain high levels of luciferase activity, however the gene is delivered), protracted luciferase expression from complexes made with long chain poly K probably represents continuing transcription of plasmid DNA. In an in vitro assay, we did not observe transcription from plasmid DNA compacted with either short or long chain poly K, so it is likely that in vivo, plasmid DNA separates from poly K before transcription. These considerations suggest that the prolonged expression results from retention of plasmid DNA complexed with poly K for a longer period of time for complexes containing long chain poly K than for the short chain. Condensation with poly K protects DNA against degradation (4), and better protection is afforded by long chain poly K than short chain in in vitro assays. Thus, we speculate that plasmid DNA is retained within the cell for both complexes (because both have better persistence of expression than Lipofectin) with gradual degradation, which is resisted longer by complexes containing long chain poly K. Whether the complexes reside in membrane-bound cytoplasmic compartments, the cytoplasm, or the nucleus is not yet clear. It is also possible that the less tightly compacted complexes with the shorter poly K allow better access for the RNA polymerase to the DNA and accounts for the higher initial expression. In such a scenario, transgene expression would depend on a dynamic relationship between the availability of the DNA for transcription and the rate of its subsequent degradation.

Transgene activity persists better in vitro (40 days) than in vivo (less than 30 days), even for genes delivered using complexes containing long chain poly K. There are probably several reasons for this difference. In vitro, only processes within the transfected cell account for decay of transgene activity, whereas in vivo, immune processes can be recruited to destroy cells expressing proteins not recognized as “self.” Bacterial β-galactosidase has been shown, in and of itself, to incite cytotoxic lymphocyte responses in vivo, and therefore, cells expressing this protein are preferentially targeted and destroyed (27). This is probably one mechanism of extinguishing β-galactosidase activity. In addition, the HuH7 cells transfected in vitro are
immortal but fail to grow and divide after about 12–14 days under our culture conditions if they are not subcultured. In vivo, normal cell turnover of transfected cells may limit duration of expression.

Complexes containing a minimal number of ligands that specifically deliver exogenous DNA to receptor bearing cells might be less immunogenic than more heavily substituted complexes. DNA (30) and poly-L-amino acids (31) have been found to be relatively nonimmunogenic. Thus, possible immunogenicity of receptor-targeted complexes in vivo may depend on the ligand portion and, in part, its abundance. The abundance of SEC-R in lung, liver, and brain (14), all of which might be potential target tissues for therapeutic gene transfer in common inherited (e.g., α1-antitrypsin deficiency) or acquired (e.g., Alzheimer’s disease) disorders, has made it a desirable target for receptor-mediated gene therapy. The development of optimal complexes that produce high level gene expression for short (9.7-kDa poly K conjugates) or longer (53.7-kDa poly K conjugates) periods of time will be useful in achieving optimal therapeutic effects.

Acknowledgments—We thank John Kim for crucial assistance with the in vivo studies, Dr. Thomas Gerken for assistance with NMR analysis, Helga Beegen for technical assistance with electron microscopy, and Dr. Roger Marchant for assistance with AFM microscopy.

REFERENCES

1. Cotten, M., Wagner, K., Zatleukal, K., Phillips, D. T., Curiel, S., and Birnstiel, M. L. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6094–6098
2. Ferkol, T., Perales, J. C., Mulato, F., and Hanson, R. W. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 101–105
3. Ferkol, T., Kaetzel, C. S., and Davis, P. B. (1992) J. Clin. Invest. 92, 2394–2400
4. Michael, S. I., and Curiel, D. T. (1994) Gene Ther. 1, 223–232
5. Perales, J. C., Ferkol, T., Beegen, H., Ratnoff, O. D., and Hanson, R. W. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4086–4090
6. Wagner, E., Cotten, M., Faisner, R., and Birnstiel, M. L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4255–4259
7. Wagner, E., Zenke, M., Cotten, M., Beug, H., and Birnstiel, M. L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3410–3414
8. Wu, G. Y., and Wu, C. H. (1987) J. Biol. Chem. 262, 4299–4432
9. Zaidy, A. G. Perales, J. C., Ferkol, T., Gerken, T., Beegen, H., Birnretter, D. H., and Davis, P. B. (1997) Am. J. Phys. 273, G545–G552
10. Bu, G., Morton, P. A., and Schwartz, A. L. (1992) J. Biol. Chem. 267, 15995–15999
11. Perales, J. C., Ferkol, T., Kaetzel, C. S., Schaseen, C. S., and Fallon, R. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3753–3757
12. Perales, J. C., Joslin, G., Nelson, P., Schaseen, C., Adams, S. P., and Fallon, R. J. (1990) J. Biol. Chem. 265, 16713–16716
13. Joslin, G., Fallon, R., Bullock, J., Adams, S. P., and Perales, D. H. (1991) J. Biol. Chem. 266, 11282–11288
14. Perales, D. H. (1994) Pediatr. Res. 36, 271–277
15. Erbacher, P., Roche, A. C., Monsigney, M., and Midoux, P. (1995) Bioconjugate Chem. 6, 401–410
16. Ferkol, T., Lindberg, G. L., Perales, J. C., Chen, J., Ratnoff, O. D., and Hanson, R. W. (1993) FASEB J. 7, 1081–1091
17. Ferkol, T., Perales, J. C., Kaetzel, C. S., Eckman, E., Hanson, R. W., and Davis, P. B. (1995) J. Clin. Invest. 95, 493–502
18. Jung, G., Kohnlein, W., and Luders, G. (1981) Biochem. Biophys. Res. Commun. 101, 599–606
19. Lin, W. C., and Culp, L. A. (1991) BioTechniques 11, 344–351
20. Maniatis, T., Frinsch, E. F., and Stambrook, J. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 1.7–1.52, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Wilson, D. L., Kump, K. S., Eppell, S. J., and Marchant, R. E. (1995) Langmuir: Amer. Chem. Soc. Surf. Colloids 11, 265–272
22. Wolfert, M. A., and Seymour, L. W. (1996) Gene Ther. 3, 269–273
23. Bruker, A. R., Tate, J. E., and Habener, J. F. (1989) BioTechniques 7, 1116–1122
24. Kleinaush, D. G., Kupper, L. L., and Muller, K. E. (1988) Applied Regression Analysis and Other Multivariable Methods, 2nd Ed., Duxbury Press, Duxbury, MA
25. Glantz, S. A. (1987) Biostatistics, pp. 287–355, McGraw-Hill Company, New York
26. Thompson, J. P., Hayes, L. S., and Lloyd, D. B. (1991) Gene (Amst.) 103, 171–177
27. Lai, W. H., Cameron, P. H., Doherty, I. W. J.-J., H. Kay, D. G., Posner, B. I., and Bergeron, J. J. M. (1989) J. Cell Biol. 109, 2741–2749
28. Scidmore, M. A., Fisher, E. R., and Hackstadt, T. (1996) J. Cell Biol. 134, 363–374
29. Brubaker, J. O., Thompson, C. M., Morrison, L. A., Knipe, D. M., Siber, G. R., and Finberg, R. W. (1996) J. Immunol. 157, 1596–1604
30. Ehrner, R., and Derynck, R. (1991) Cell Regul. 2, 599–612
31. Maurer, P. H. (1962) J. Immunol. 88, 330–338