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Evidence for Targeted Gene Delivery to Hep G2 Hepatoma Cells in Vitro†

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ABSTRACT: We have developed a system for targeting foreign DNA to hepatocytes in vitro using a soluble DNA carrier that takes advantage of receptor-mediated endocytosis to achieve internalization. The idea is based on the fact that hepatocytes possess a unique receptor that binds and internalizes galactose-terminal (asialo)glycoproteins. To create a targetable carrier system that could bind DNA in a nondeforming manner, we used poly(L-lysine) to bind DNA in a strong but noncovalent interaction. An asialoglycoprotein, asialoorosomucoid (AsOR), was chemically coupled to poly(L-lysine) to form an asialoorosomucoid–poly(L-lysine) conjugate. Various proportions of conjugate to DNA were tested to determine conditions that maximized DNA content in a soluble complex and that limited solubility of complexes. To test the targetable gene delivery system, AsOR–poly(L-lysine) conjugate was complexed to the plasmid pSV2 CAT containing the gene for chloramphenicol acetyltransferase (CAT) driven by an SV-40 promoter. We tested this complex using a model system consisting of human hepatoma cell line Hep G2 [asialoglycoprotein receptor (+)], hepatoma SK-Hep 1, IMR-90 fibroblasts, and uterine smooth muscle [receptor (−)] cells. Each cell line was incubated with 0.2 μm filtered AsOR–poly(L-lysine)–DNA complex or controls consisting of DNA plus AsOR, DNA plus poly(L-lysine), or DNA alone. Cells were assayed for the presence of CAT activity as a measure of gene transformation. SK-Hep 1, IMR-90, and smooth muscle [receptor (−)] cells produced no detectable acetylated chloramphenicol derivatives under any of these conditions. However, Hep G2 [receptor (+)] cells incubated with the AsOR–poly(L-lysine)–DNA complex *were* transformed as indicated by the appearance of CAT activity (0.028 CAT unit/10^6 cells).

Foreign genes have been introduced into mammalian cells in vitro by a variety of methods in order to study gene regulation. The most popular technique employs a precipitation method in which DNA is coprecipitated with calcium phosphate to form insoluble particles (Graham & Van der Eb, 1973). A proportion of these precipitates becomes internalized within host cells by phagocytosis (Loyter et al., 1982). Following internalization, some of the DNA avoids degradation and eventually enters the nucleus, resulting in expression of new genes (Graham & Van der Eb, 1973). We wondered whether DNA, in the proper form, could similarly survive a receptor-mediated internalization. Our objective was to develop a simple, soluble DNA carrier system to target DNA specifically to hepatocytes using receptor-mediated endocytosis. The idea is based on the following concepts: (1) Hepatocytes possess *unique* receptors that bind and internalize galactose-terminal (asialo)glycoproteins (Ashwell & Morell, 1974). Coupling of DNA to an asialoglycoprotein could permit internalization of DNA via asialoglycoprotein receptors. However, chemical coupling of DNA to a carrier could alter the DNA and prevent proper expression of the genes. To circumvent this problem, we took advantage of the fact that (2) DNA can bind polycations, e.g., poly(L-lysine), in a strong, noncovalent interaction forming *soluble* complexes (Li et al., 1973). (3) Lastly, chloramphenicol acetyltransferase (CAT)[1]

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is a bacterial enzyme that catalyzes acetylation of the antibiotic chloramphenicol (Gorman et al., 1982). The gene for this enzyme is *not* present in mammalian cells.

We proposed the following hypothesis: If poly(L-lysine) were coupled to an asialoglycoprotein, subsequent addition of DNA to form a soluble asialoglycoprotein–poly(L-lysine)–DNA complex could permit targeted delivery of DNA specifically to asialoglycoprotein receptor bearing cells. Our strategy to test this system was to deliver DNA in the form of a bacterial plasmid, pSV2 CAT which contains the CAT gene. Because mammalian cells lack this gene, the appearance of CAT enzyme activity in target cells could be used as a convenient *marker* for gene transformation.

Materials and Methods

Cells and Cell Culture. Human hepatoma cell lines Hep G2 (Knowles et al., 1980) (from B. Knowles, Wistar Institute, Philadelphia, PA) and SK-Hep 1 (from D. Shafritz, Albert Einstein College of Medicine, Bronx, NY); IMR-90 human fibroblasts (NIA Aging Cell Repository, Camden, NJ), and human uterine smooth muscle cells were maintained in plastic dishes containing MEM plus 10% fetal calf serum at 37 °C and 5% CO_2 (Wu et al., 1978).

Assay for Asialoglycoprotein Uptake. Orosomucoid was prepared from pooled human serum (American Red Cross, Farmington, CT) according to the method of Whitehead et al. (1966). Sialic acid was removed to expose galactose residues using neuraminidase (Kawasaki & Ashwell, 1977). The asialoorosomucoid (AsOR) thus formed was determined to have no residual sialic acid (Warren, 1959). For studies on DNA–protein complex formation, AsOR was iodinated with carrier-free $Na^{125}I$ using a solid-phase lactoperoxidase system (Bio-Rad Laboratories, Richmond, CA) as described by the manufacturer.

Asialoglycoprotein uptake activity of the cell lines was determined according to the method of Schwartz et al. (1981). In brief, confluent dishes of cells were incubated separately at 37 °C in medium containing 2 μg/mL ^{125}I-AsOR. At regular time intervals, medium was removed, and the cells were washed with ice-cold saline. The cell layer was removed with 0.1 N NaOH, and cell-associated ^{125}I radioactivity was determined by using a γ counter. Nonspecific uptake was determined in the presence of a 100-fold excess of unlabeled AsOR. Specific uptake was calculated as the difference between total and nonspecific uptake. All points were determined in triplicate and results expressed as means ± SD.

Preparation of a Targetable DNA Carrier System. A targetable carrier system for DNA was prepared by coupling ^{125}I-AsOR to poly(L-lysine) (mean M_r 59000; Sigma Chemical Co., St. Louis, MO) in a 5:1 molar ratio using *N*-succinimidyl 3-(2-pyridyldithio)propionate (Pierce Chemical Co., Rockford, IL) as described previously by Jung et al. (1981). The conjugate was separated from noncoupled ^{125}I-AsOR and poly(L-lysine) on a Bio-Gel A1.5m column (Bio-Rad Laboratories, Richmond, CA) eluted with 0.01 M Hepes and 2 M guanidine hydrochloride, pH 7.4. To identify the conjugate peak, samples of the eluted fractions were hydrolyzed in 6 N HCl at 100 °C for 24 h. The conjugate peak was identified as that containing both ^{125}I radioactivity and a lysine content in excess of that contributed by AsOR alone as determined by amino acid analysis. On the basis of the specific activity of ^{125}I-AsOR and the lysine content of the conjugate, the molar ratio of AsOR to poly(L-lysine) in the conjugate was calculated to be 5:1. The conjugate was found to be stable at −4 °C for at least 4 months. A 10% SDS–polyacrylamide gel of the conjugate after electrophoresis (Laemmli, 1970), stained with Amido Black, revealed a single band.

Plasmid Preparation. The plasmid pSV2 CAT (Gorman et al., 1982) (from G. Carmichael, University of Connecticut, Farmington, CT) was grown in *Escherichia coli*, isolated, and purified according to the method of Birnboim and Doly (1979). Purity was confirmed by 0.8% agarose gel electrophoresis, demonstrating an absence of bacterial cellular DNA.

Conditions for Maximization of DNA in a Targetable Complex. To determine the proportion of AsOR–poly(L-lysine) conjugate that should be mixed with plasmid DNA to optimize the DNA content of a soluble complex, increasing amounts of ^{125}I-labeled conjugate in 2 M NaCl were added dropwise to form samples that contained 87 nM DNA and 0, 10, 20, 40, 60, 80, 100, 120, and 160 nM conjugate [based on poly(L-lysine) content], all in 2 M NaCl. Samples were incubated for 1 h at 25 °C and then dialyzed for 24 h against 0.15 M saline through membranes with a molecular weight limit of 3500 (Spectrum Medical Industries, Los Angeles, CA). After dialysis, all samples were filtered through 0.2-μm membranes (Millipore Corp., Bedford, MA) to ensure that complexes to be used did not contain precipitates. Samples containing equal concentrations of DNA (30 nM) were electrophoresed on a 0.8% agarose gel at 50 V for 3 h as described previously (Maniatis et al., 1982) and stained with ethidium bromide to visualize DNA. To detect the location of the ^{125}I-AsOR–poly(L-lysine) conjugate, the gel was dried and an autoradiogram obtained using XAR film (Eastman Kodak Co., Rochester, NY) at −70 °C for 8 h.

Determination of Conjugate to DNA Proportions That Limit Solubility of Targetable Complexes. To determine the proportions of conjugate to DNA that limit solubility of the complex, and to confirm the progressive increase in DNA content within complexes as ratios of conjugate to DNA in samples were increased, pSV2 CAT was labeled with ^{32}P by nick-translation according to the method of Maniatis et al. (1975). Constant quantities of labeled DNA were mixed with increasing concentrations of conjugate to form samples that contained 87 nM labeled DNA and 0, 10, 40, 80, 120, 160, 200, 240, and 280 nM unlabeled AsOR–poly(L-lysine) conjugate [based on poly(L-lysine) content]. Samples were then dialyzed and filtered as described in the previous experiment. The filters were washed with 0.15 M NaCl, then placed in vials containing aqueous counting scintillant (Packard), and counted on a β scintillation counter. Filtrates of the soluble complexes, samples with conjugate:DNA ratios up to 3.27 (containing *equal* quantities of DNA), were electrophoresed on an agarose gel, and, after drying, an autoradiogram was obtained as described before.

Targeted DNA Delivery. Hep G2, SK-Hep 1, IMR-90, and uterine smooth muscle cells were grown separately to one-quarter confluence in 100-mm plastic dishes. Growth medium was replaced with MEM, 10% fetal calf serum, 5 mM Ca^{2+} containing AsOR–poly(L-lysine)–DNA complex [1.04 nM AsOR, 200 pM poly(L-lysine), and 90 pM DNA] alone, complex plus 10.4 nM AsOR, 1.04 nM AsOR plus 90 pM DNA, 200 pM poly(L-lysine) plus 90 pM DNA, or 90 pM DNA alone. Each sample was filtered through 0.2-μm membranes prior to incubation with cells. After incubation for 48 h at 37 °C under 5% CO_2, medium was aspirated, and the cells were washed with ice-cold phosphate-buffered saline. Cells

[1] Abbreviations: CAT, chloramphenicol acetyltransferase; AsOR, asialoorosomucoid; CA, chloramphenicol; PL, poly(L-lysine); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; MEM, modified Eagle's medium; Tris, tris(hydroxymethyl)aminomethane.

were then scraped from dishes in that same buffer, centrifuged at 1500 rpm for 5 min at 4 °C, and washed twice by resuspension and centrifugation. The pellets were resuspended in 1 mL of 0.2 M Tris, pH 7.5, sonicated for 1 min, and then centrifuged at 10000 rpm for 10 min at 4 °C. Aliquots of the supernatants were assayed for protein content according to the method of Bradford (1976). For comparison, Hep G2 and SK-Hep 1 cells were also transformed by calcium phosphate precipitation (Graham & Van der Eb, 1973) using identical amounts of DNA, 10 μg/10^6 cells. CAT activity was determined as described by Gorman et al. (1982). In brief, aliquots of supernatant containing equal amounts of protein were incubated at 25 °C for 30 min with [^{14}C]chloramphenicol (New England Nuclear Co., Boston, MA) in 0.25 M Tris, pH 7.5, to which was added acetyl-CoA (Sigma Chemical Co., St. Louis, MO). The samples were extracted with ice-cold ethyl acetate, spotted onto silica gel thin-layer chromatography plates (E. Merck, Darmstadt, West Germany), and developed using a 95:5 mixture of chloroform and methanol (v/v) (Gorman et al., 1982). After being dried, the plates were autoradiographed using XAR film at −70 °C for 4 days. Standard CAT enzyme (P-L Biochemicals, Milwaukee, WI) incubated with [^{14}C]chloramphenicol was spotted onto each plate for quantitation of CAT activity in cell samples. Quantitation of the spots was accomplished by densitometric scanning of triplicate thin-layer chromatographic assays.

Time Course of Targeted Gene Expression. To determine the expression of CAT μm as a function of time after targeted gene delivery, Hep G2 cells were incubated for 48 h in MEM, 10% fetal calf serum, and 5 mM Ca^{2+} containing 0.2 μm filtered AsOR–poly(L-lysine)–DNA complex [1.04 nM AsOR, 200 pM poly(L-lysine), and 90 pM DNA] at 37 °C as described previously. Medium was then replaced with medium without complex, and cells were grown to confluence. Cells were replated at half-confluence and passaged 5 times. From each passage, cells were removed and assayed for CAT activity in triplicate. The results were expressed as means ± SD in CAT units/10^6 cells.

RESULTS

Figure 1 compares the specific uptake of ^{125}I-AsOR as a function of time for the hepatoma cell lines Hep G2 and SK-Hep 1. Hep G2 demonstrated a significant uptake rate of 140 ng h^{-1} (mg of cell protein)$^{-1}$, a figure similar to that described previously for this cell line (Schwartz et al., 1981). In contrast, the specific uptake of SK-Hep 1 cells was barely detectable [less than 10 ng h^{-1} (mg of cell protein)$^{-1}$] under the same conditions. On the basis of these data, the hepatoma cell line Hep G2 was designated receptor (+) and SK-Hep 1 designated receptor (−). IMR-90 fibroblasts and uterine smooth muscle cells were likewise found to have no significant asialoglycoprotein uptake activity (data not shown).

Increasing the proportion of ^{125}I-AsOR–poly(L-lysine) conjugate in the DNA samples affected plasmid DNA migration in an agarose gel as seen in Figure 2. Panel A shows this gel stained with ethidium bromide to visualize DNA. Panel B is an autoradiogram of the same gel to detect ^{125}I-AsOR–poly(L-lysine) conjugate. DNA alone is shown in lanes 1 and 1′; blanks containing only saline, lanes 10 and 10′. Lane 1 reveals four DNA bands corresponding to four forms of the plasmid DNA: supercoil, nicked circular, and dimers of each of these forms (Breitman et al., 1982). Lanes 11 and 11′ contain only ^{125}I-AsOR–poly(L-lysine) conjugate. Lane 11′ shows that the conjugate appeared as a single radioactive band that did *not* migrate from the top of the gel. As the proportion of ^{125}I-AsOR–poly(L-lysine) conjugate in the samples increased

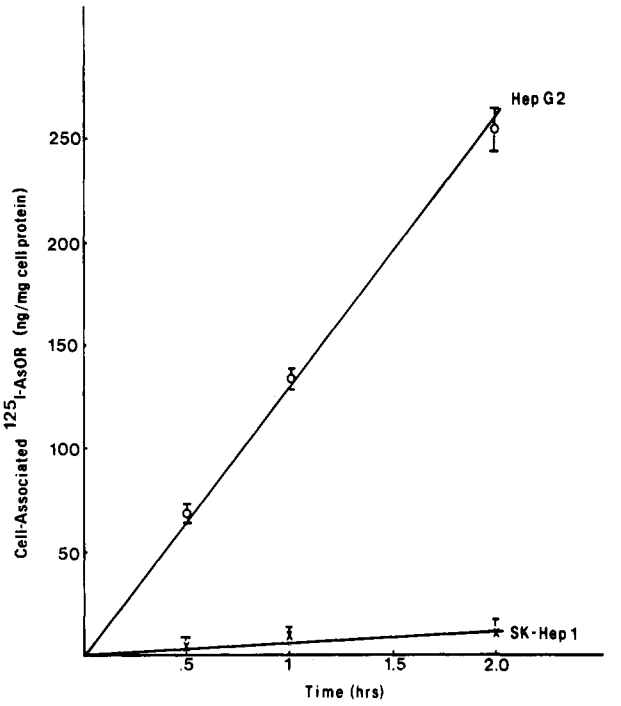

FIGURE 1: Comparison of specific ^{125}I-AsOR uptake by Hep G2 and SK-Hep 1 cells. Both cell lines were grown to confluence, then changed to medium containing 2 μg/mL ^{125}I-AsOR, and incubated at 37 °C according to the method of Schwartz et al. (1981). At regular time intervals, medium was removed and the cell layer washed with ice-cold saline. Cells were taken up in 0.1 N NaOH and counted to determine cell-associated ^{125}I radioactivity. Nonspecific uptake was determined in the presence of a 100-fold excess of unlabeled AsOR. Specific uptake was calculated as the difference between total and nonspecific uptake for the two cell lines. All points were determined in triplicate and plotted as means ± SD.

Table I

sample	conjugate:DNA (molar ratio)	filter (cpm)	filtrate[a] (cpm)
1	0	8440	79900
2	0.23	7930	85900
3	0.47	7750	88100
4	0.94	8550	85300
5	1.40	7940	84800
6	1.87	8730	82500
7	2.34	8750	85300
8	2.80	8480	82430
9	3.27	8540	85450
10	3.55	15100	78900
11	3.88	45500	50300
12	6.09	72500	18400
13	9.13	85200	6000

[a] Filtrates from samples 1 through 9 correspond to samples applied to an agarose gel, lanes 1 through 9 in Figure 3.

(evident in lanes 2′ through 9′), there was a decrease in staining intensity of DNA that entered the gel as seen in lanes 2 through 9. There was a corresponding *increase* in staining of DNA that did not migrate, remaining at the top of the gel with the conjugate. This suggested that progressively increasing amounts of DNA were retained by the AsOR–poly(L-lysine) conjugate in the wells as the proportion of conjugate to DNA was increased in the samples.

Increasing the proportion of AsOR–PL to DNA in samples affected the solubility of the complexes as shown in Table I. Conjugate to DNA ratios above 3.27:1 resulted in progressive increases in amounts of insoluble DNA retained by the filters. However, at conjugate to DNA ratios of 3.27:1 and below, counts that remained on the filters accounted for only ap-

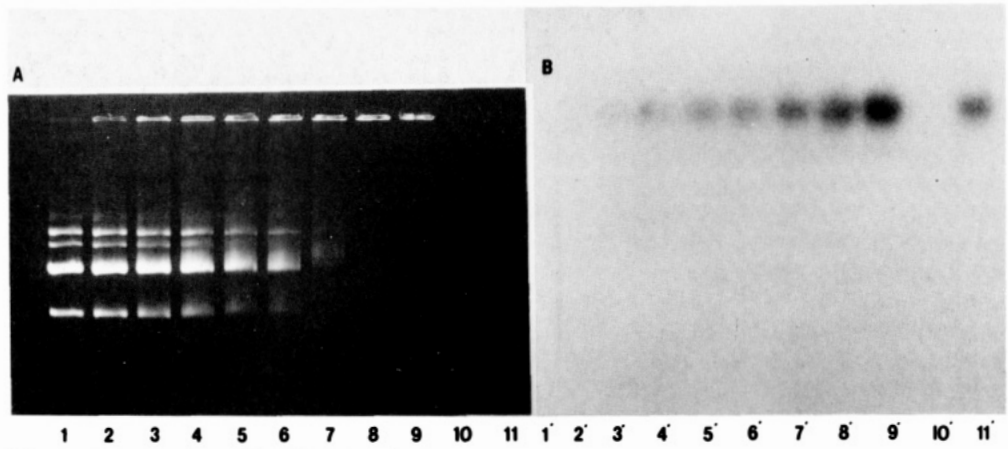

FIGURE 2: Effect of increasing proportions of AsOR–poly(L-lysine) conjugate on plasmid DNA electrophoretic migration through a 0.8% agarose gel. (Panel A) Increasing amounts of ^{125}I-AsOR–poly(L-lysine) conjugate in 2 M NaCl were added to DNA to form samples that contained 87 nM DNA and 0, 10, 20, 40, 60, 80, 100, 120, and 160 nM conjugate [based on poly(L-lysine)content] in 2 M NaCl. After incubation at 25 °C for 1 h, each sample was dialyzed and filtered as described under Materials and Methods. Samples were electrophoresed through a 0.8% agarose gel using a Tris–phosphate buffer system as described previously (Maniatis et al., 1982) and stained with ethidium bromide to visualize DNA. Lane 1, DNA alone (87 nM); lanes 2–9, DNA with progressively increasing concentrations of conjugate (10–160 nM); lane 10, saline; lane 11, ^{125}I-AsOR–poly(L-lysine) conjugate (160 nM). (Panel B) The agarose gel was dried and autoradiographed as described under Materials and Methods.

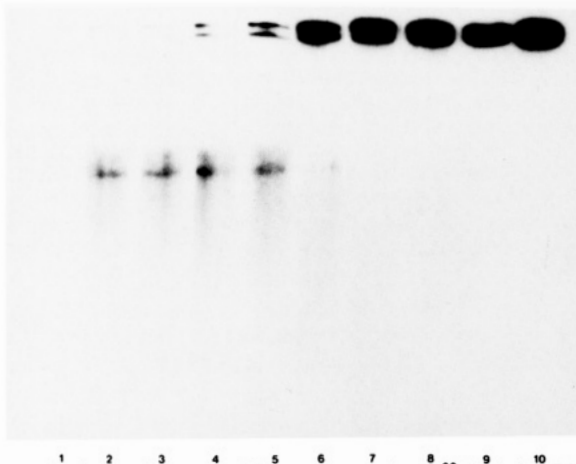

FIGURE 3: Autoradiogram of free and complexed ^{32}P-labeled DNA. The plasmid pSV2 CAT was labeled with ^{32}P by nick-translation (Maniatis & Klein, 1975). Increasing concentrations of unlabeled AsOR–poly(L-lysine) conjugate were added to labeled DNA to form samples that contained 87 nM DNA and 0, 10, 40, 80, 120, 160, 200, 240, and 280 nM conjugate, on the basis of poly(L-lysine) content, yielding conjugate:DNA molar ratios of 0, 0.23, 0.47, 0.94, 1.40, 1.87, 2.34, 2.08, and 3.27, respectively. After dialysis and filtration, as described under Materials and Methods, samples were electrophoresed through a 0.8% agarose gel (Maniatis et al., 1982). The gel was dried and autoradiographed. Lane 1, DNA alone; lanes 2–9, samples with conjugate:DNA molar ratios of 0.23–3.27, respectively.

proximately 10% of the total applied counts, indicating that these complexes remained soluble. The lack of significant variation in the DNA counts of the filtrates for all conjugate to DNA ratios up to and including 3.27:1 confirms that the filtrates applied on the agarose gels contained equal quantities of DNA.

Figure 3 shows an autoradiogram of the agarose gel of filtrates from samples with conjugate to DNA ratios ranging from 0 to 3.27:1. This gel confirms that with increasing proportions of conjugate in the samples, increasing amounts of [^{32}P]DNA were retained in complexes at the top of the gel. The DNA content of the complexes was maximized at a conjugate to DNA ratio of between 1.87:1 (lane 6) and 2.34:1 (lane 7), the latter sample representing the lowest conjugate:DNA ratio in which DNA entry into the gel was completely abolished.

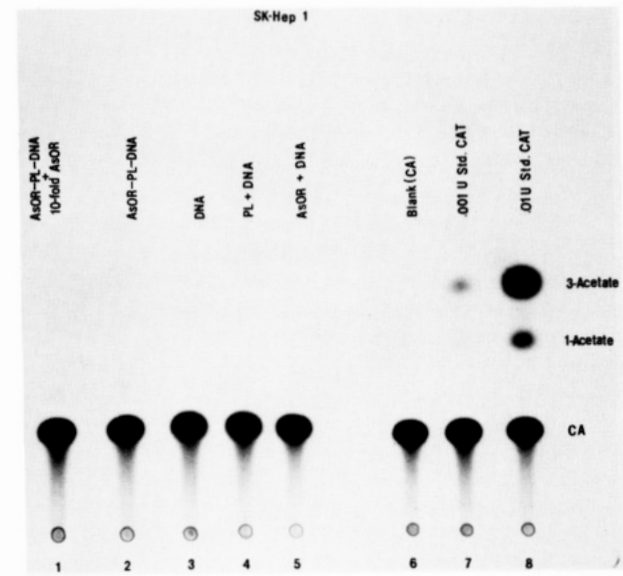

FIGURE 4: Assay for gene transformation in SK-Hep 1 cells. Cells were grown to one-quarter confluence and then incubated for 48 h at 37 °C under 5% CO_2 in the presence of filtered AsOR–poly(L-lysine) complex, or components of the complex. After harvesting and sonication, cells were assayed for CAT activity by thin-layer chromatography as described under Materials and Methods and originally described by Gorman et al. (1982). The effects of incubation with AsOR–poly(L-lysine) complex [1.04 nM AsOR, 200 pM poly(L-lysine), and 90 pM DNA] alone are shown in lane 2; complex plus 10.4 nM AsOR, lane 1; 90 pM DNA, lane 3; 200 pM poly(L-lysine) plus 90 pM DNA, lane 4; 1.04 nM AsOR plus 90 pM DNA, lane 5. [^{14}C]Chloramphenicol alone is shown in lane 6; CAT enzyme standard (0.001 unit), lane 7; CAT (0.01 unit), lane 8. [AsOR, asialoorosomucoid; PL, poly(L-lysine); CA, chloramphenicol; CAT, chloramphenicol acetyltransferase.]

The efficacy of AsOR–poly(L-lysine)–DNA complex in gene transformation of the hepatoma cell line SK-Hep 1, receptor (–) cells, is shown in Figure 4. Lanes 7 and 8 show that standard CAT enzyme activity can be detected by the presence of 1- and 3-acetylchloramphenicol derivatives. The effects of incubation of SK-Hep 1 cells with AsOR–poly(L-lysine)–DNA complex are shown in lane 2; incubation with controls consisting of components of the complex is shown: DNA alone, lane 3; poly(L-lysine) plus DNA, lane 4; AsOR plus DNA, lane 5. Control samples contained amounts of components

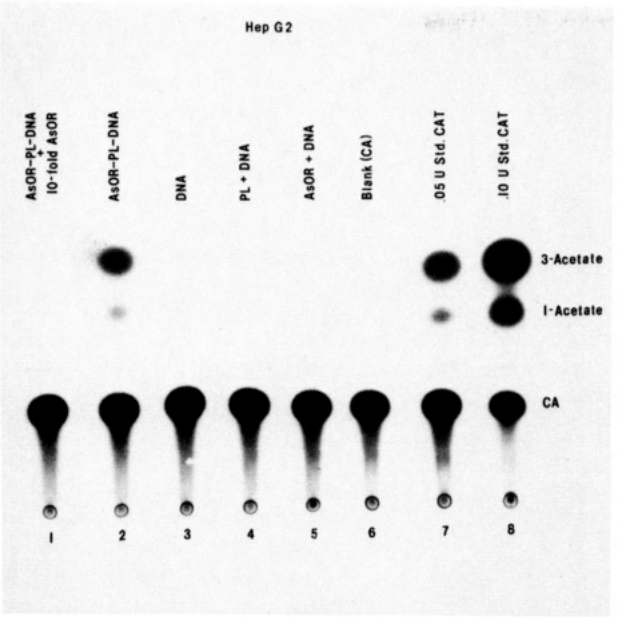

FIGURE 5: Assay for gene transformation in Hep G2 cells. Cells were grown to one-quarter confluence and then incubated for 48 h at 37 °C under 5% CO_2 in the presence of filtered AsOR–poly(L-lysine) complex, or components of the complex. After harvesting and sonication, cells were assayed for CAT activity by thin-layer chromatography as described under Materials and Methods and originally described by Gorman et al. (1982). The effects of incubation with AsOR–poly(L-lysine) complex [1.04 nM AsOR, 200 pM poly(L-lysine), and 90 pM DNA] alone are shown in lane 2; complex plus 10.4 nM AsOR, lane 1; 90 pM DNA, lane 3; 200 pM poly(L-lysine) plus 90 pM DNA, lane 4; 1.04 nM AsOR plus 90 pM, lane 5. [^{14}C]Chloramphenicol alone is shown in lane 6; CAT enzyme standard (0.05 unit), lane 7; CAT (0.1 unit), lane 8. [AsOR, asialoorosomucoid; PL, poly(L-lysine); CA, chloramphenicol; CAT, chloramphenicol acetyltransferase.]

identical with those present in the sample containing the complete AsOR–poly(L-lysine)–DNA complex. There was no detectable CAT activity for SK-Hep 1, indicating a lack of gene transformation in this cell line under any of the conditions.

However, Figure 5 shows that incubation of Hep G2, receptor (+) cells, with AsOR–poly(L-lysine)–DNA complex, lane 2, *did* result in gene transformation as shown by the formation of acetylchloramphenicol derivatives. Incubation of Hep G2 cells with controls [DNA alone, lane 3; DNA plus poly(L-lysine), lane 4; or DNA plus AsOR, lane 5] did *not* result in gene transformation. Furthermore, competition by a 10-fold excess of AsOR, lane 1, inhibited transformation and expression of CAT in the cells, indicating that recognition of the complex by Hep G2 was directed by the asialoglycoprotein component of the complex. The specificity and relative efficiency of the targeted gene delivery are further demonstrated in Table II, showing densitometric quantitation of CAT activity obtained in various cell lines. As in the case of SK-Hep 1, receptor (−) hepatoma cells, CAT activity in fibroblasts (IMR-90) and smooth muscle cells was not detectable after incubation with the complex. However, in Hep G2 cells, CAT activity was determined to be 0.028 ± 0.003 CAT unit/10^6 cells using identical amounts of DNA (10 μg/10^6 cells) and numbers of cells per dish. The transformation efficiency of Hep G2 by the complex was approximately 2-fold greater than that of the calcium phosphate precipitation method in our hands. Table II also shows that CAT activity (0.019 ± 0.002 CAT unit/10^6 cells) could be obtained in SK-Hep 1 cells by calcium phosphate precipitation, indicating that the lack of transformation of these cells by our complex was not due to an inherent inability of these cells to undergo gene transformation.

Table II: Densitometric Quantitation of CAT Activity

cells	transformation method[a]	CAT activity (units/10^6 cells)
SK-Hep 1	calcium phosphate	0.019 ± 0.002
Hep G2	calcium phosphate	0.012 ± 0.002
Hep G2	AsOR–poly(L-lysine)	0.028 ± 0.003
SK-Hep 1	AsOR–poly(L-lysine)	0
IMR-90	AsOR–poly(L-lysine)	0
uterine smooth muscle	AsOR–poly(L-lysine)	0

[a] Identical quantities of DNA (10 μg/10^6 cells) were used in each experiment.

Table III: Expression of Targeted Genes in Hep G2 Cells as a Function of Time Posttransformation

time posttransformation (days)	passage	CAT activity (units/10^6 cells)
0	0	0.028 ± 0.002
2	1	0.012 ± 0.003
4	2	0.007 ± 0.002
6	3	0.003 ± 0.002
8	4	0

The expression of CAT gene targeted to Hep G2 as a function of time posttransformation is shown in Table III. A maximum activity of 0.028 unit/10^6 cells was measured immediately subsequent to the transformation incubation period. Thereafter, the activity declined to 0.003 unit/10^6 cells by the third passage (sixth posttransformation day), and by the fourth passage, CAT activity was no longer detectable. Under these *nonselective* conditions, gene expression was transient, similar to that noted in other transfection systems (Selden et al., 1986) in the absence of selective pressure.

We conclude that asialoglycoprotein–poly(L-lysine) conjugates can be used to target genes in a *soluble* form resulting in specific delivery to cells possessing surface asialoglycoprotein receptors. Furthermore, a foreign gene thus delivered can be expressed by these cells.

Discussion

Our conjugate mixing studies in which the negative charges of DNA were titrated with the positive charges of the poly(L-lysine) component of the conjugate indicate that the proportion of conjugate to DNA in the complex cannot exceed a limiting ratio beyond which insoluble complexes will be formed. This is consistent with previous studies on DNA complex formation and the effects of poly(L-lysine) concentration on DNA solubility (Chang et al., 1973).

It has been shown previously that the presence of polycations in culture medium can increase in vitro cellular uptake of a variety of substances, e.g., albumin (Ryser, 1967) and nucleic acids (Farber et al., 1975; Amos, 1961). These effects were shown to be dependent on size (Farber et al., 1975) and concentration (Ryser, 1967; Farber et al., 1975) of the polycation. The enhancement was found to be most effective with polyornithines, but polylysines also demonstrated considerable activity. However, polylysines of a size comparable to that used in our conjugate required concentrations approximately 2000 times greater than present in our complex. Furthermore, the lack of gene transformation in our control experiments with combinations of poly(L-lysine) plus DNA in concentrations identical with that used in the complete complex indicates that the enhancement of DNA uptake by the polycation alone was not responsible for the transforming effect of the AsOR–poly(L-lysine)–DNA complex. Inhibition of transformation

of Hep G2 by a 10-fold excess of AsOR would not be expected if the uptake of the complex were dependent on the poly(L-lysine) portion of the conjugate. These data support the notion that the recognition of the complex by Hep G2 cells is directed by the asialoglycoprotein component of the complex.

The fact that all receptor (−) cells tested, regardless of the tissue source (SK-Hep 1, hepatoma; IMR-90, fibroblasts; uterine smooth muscle cells), failed to undergo gene transformation under conditions identical with those which were successful for Hep G2 [receptor (+)] cells further supports the concept that the cell specificity of this new delivery system for gene transformation is based on the presence of unique receptors on the target cells.

It has been shown that asialoglycoproteins internalized by their receptor are ultimately delivered via membrane-limited vesicles to lysosomes with subsequent glycoprotein degradation (Wall et al., 1980). Our previous work has demonstrated that this receptor-mediated endocytotic process can be used to target protective agents specifically to asialoglycoprotein receptor-bearing cells (Wu et al., 1983, 1985). Because recognition of our DNA carrier system is directed by an asialoglycoprotein, the AsOR–poly(L-lysine)–DNA complex would be expected to follow this pathway and be subject to the same fate. However, there is evidence that the efficiency of degradation of internalized asialoglycoproteins can be less than complete. For example, Chang and Kullberg (1982) demonstrated that a conjugate consisting of an asialoglycoprotein bound to the toxic polypeptide A chain of diphtheria toxin was internalized by the asialoglycoprotein receptor and killed the target cells, indicating that escape from the normal degradative pathway is possible for some proteins.

Similarly, expression of foreign DNA introduced by phagocytosis of calcium phosphate precipitates (Gorman et al., 1982), or polycation, or DEAE-dextran complexes (Farber et al., 1975) also suggests that DNA, under appropriate conditions, can enter the cytoplasm and eventually the nucleus without nuclease digestion.

Gene transformation of mammalian cells in vitro has been achieved previously by using a variety of techniques including delivery by viruses (Karlsson et al., 1985) and liposomes (Fraley & Parahadjopolos, 1982), electroporation (Potter et al., 1984), microinjection (Anderson, 1980), cell fusion (Shaffner, 1980), and DEAE-dextran (Gopal, 1985) and calcium phosphate precipitation (Graham & Van der Eb, 1973). We have demonstrated, here, that a soluble DNA complex can be used to achieve *targeted* gene transformation based on a cell-specific receptor-mediated endocytotic process. This type of DNA complex can permit targeted gene delivery that, by virtue of its solubility and specificity, may be useful in the study of gene regulation in vivo.

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