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EVIDENCE FOR TARGETED GENE TRANSFER BY RECEPTOR-MEDIATED ENDOCYTOSIS

STABLE EXPRESSION FOLLOWING INSULIN-DIRECTED ENTRY OF *NEO* INTO HepG2 CELLS

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Abstract—Evidence is presented for targeted gene delivery to HepG2 cells via the endocytotic pathway under the direction of insulin. Serum albumin was treated with the water-soluble carbodiimide *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride and the resultant positively charged *N*-acylurea albumin covalently conjugated to insulin by glutaraldehyde cross-linkage. The conjugated protein is shown by nitrocellulose filter binding and gel band shift assays to bind DNA, and by competitive displacement of [^{125}I]insulin to bind to the insulin receptor. When the expression vectors ptkNEO and pAL-8 which incorporate the *neo* gene were complexed to the conjugate in an *in vitro* system of transfection, G418 resistant clones developed at frequencies of 2.0–5.5 × 10^{-5}. Subsequently, a 923bp PstI fragment within the *neo* sequence was identified by Southern transfer in genomic DNA from transfected cell populations which had been maintained on a G418 regime for 44 days.

The deliberate insertion and integration of functionally active genes into mammalian cells is now a commonplace procedure which has manifold application in molecular biology and medicine. The transfer of specific genes permits: study of the functional domains of proteins [1], fine dissection of the mechanisms by which gene expression is regulated [2], elucidation of cellular differentiation hierarchies by the introduction of clonal markers into embryonic cells or primitive stem cells [3], analysis of oncogene and putative oncogene function in carcinogenesis [4], the production of transgenic animals [5] and the correction of genetic disorders [6].

A variety of methodologies exist for the necessary transfer of exogenous DNA to the cell interior. The most straightforward and commonly used are chemical methods such as those involving treatment with calcium phosphate [7] or a cationic facilitator [8] which induce indiscriminate DNA attachment to the membrane and thereby give rise to endocytotic uptake. It has been shown recently, however, that the calcium phosphate transfection protocol has been implicated in perturbations of gene expressions [9, 10]. Other popular methods of gene transfer involve active injection of DNA during physical puncture [11] or passive uptake during poration or abrasion of the membrane in the presence of DNA [12, 13] but, while moderately efficient, these are open to some criticism on the grounds that they are intrinsically aggressive. Also, as with the chemical techniques, they are applicable only to *in vitro* cell systems. A further category of techniques relies upon the mediation of DNA transfer via membrane-bound vesicles such as intact protoplasts [14], erythrocyte ghosts [15], reconstituted viral envelopes [16] and liposomes [17], the last of these having the design capacity to be adapted for targeting to specific cell types both *in vitro* and *in vivo* [18]. In a class of their own are viral vectors: reconstructed viruses, especially retroviruses, disarmed and expropriated for the carriage, insertion and precisely controlled genomic integration of DNA sequences [19]. Such vectors constitute the most efficient means of gene transfer known at the present time, but the technicalities are too intricate for many straightforward applications and there are numerous difficulties still existing which must be resolved before this approach can be widely advocated [20].

In this communication we present a procedure for gene transfer which exploits the efficiency and specificity of internalization afforded by the process of receptor-mediated endocytosis [21]. DNA in the naked state is carried into the cell reversibly bound to a soluble protein ligand recognizable by its cognate receptor at the plasma membrane surface. The non-covalent complexing of DNA to ligand is achieved by modifying the protein with a water-soluble carbodiimide, *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (CDI‡) under controlled conditions which allow the formation of basic *N*-acylurea moieties at the carboxyl groups of available aspartate and glutamate residues [22]. The resultant electropositive *N*-acylurea protein readily interacts with ionized phosphodiester backbone regions of DNA and tenaceous salt bridges are

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‡ Abbreviations: BSA, bovine serum albumin; CDI, *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride; DMSO, dimethyl sulphoxide; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid; SSC, 0.15 M NaCl, 0.015 M trisodium citrate.

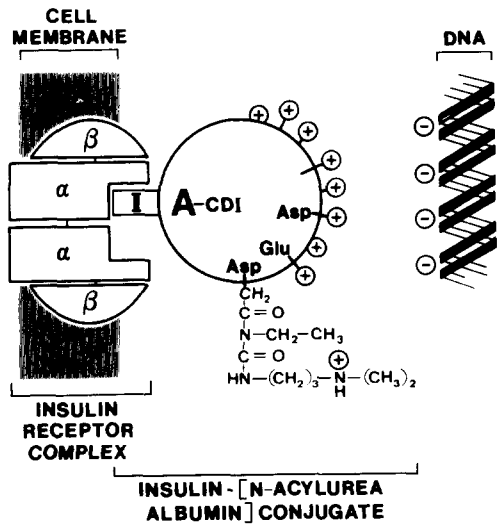

Fig. 1. Binding interactions of a conjugate consisting of a small polypeptide ligand such as insulin covalently cross-linked to *N*-acylurea albumin. The ligand component retains its facility for specific receptor binding. The *N*-acylurea albumin component, produced by carbodiimide modification of serum albumin, bears numerous positively charged *N*-acylurea moieties (⊕) in place of acidic amino acid side chain carboxyl groups (aspartate and glutamate residues) and readily interacts electrostatically with negatively charged DNA phosphodiester backbone (⊖). I, insulin; A-CDI, *N*-acylurea albumin; α and β, insulin receptor subunits

formed [23]. In the case of small polypeptide ligands, modification of the ligand itself is minimized by covalently attaching it in the unmodified state to *N*-acylurea serum albumin, thereby creating a conjugate which relegates the receptor binding and DNA binding functions to separate protein surfaces on one macromolecule (Fig. 1).

Since the modified ligands are extensively purified prior to use in transfection, and are applied together with unmodified DNA to recipient cells at low concentration in medium which requires no special adjuvants, they mimic the behaviour of their native counterparts and undergo natural endocytotic uptake in a highly directed and non-inimical manner. Prudent choice of ligand could possibly make this method of gene transfer applicable to a variety of cell lines in culture. Furthermore, the potential exists to develop the concept for targeting genes to specific tissue types in whole animal investigations.

In this study we have modelled the approach using insulin as the ligand of choice. We describe the preparation of insulin-[*N*-acylurea albumin] conjugates, their binding to insulin receptors on cultured HepG2 cells, and their application in the uptake into and expression in HepG2 cells of the *neo* gene from the bacterial transposon Tn5 incorporated into the expression vectors ptkNEO and pAL-8.

MATERIALS AND METHODS

Materials. Bacteriological culture media and ingredients were obtained from Difco Labs (Detroit, MI), cell culture media from Gibco (Grand Island, NY) and sterile plasticware from CelCult. Filter sterilization units and nitrocellulose discs were from Millipore. Monocomponent porcine insulin was purchased from Novo Biolabs; bovine serum albumin (fraction V), DNA polymerase I (EC 2.7.7.7), DNA polymerase I Klenow fragment, restriction endonucleases and plasmid pBR322 from Boehringer-Mannheim (Mannheim, F.R.G.); DNase I (EC 3.1.21.1), lysozyme (EC 3.2.1.17), ethidium bromide, ampicillin, chloramphenicol and Geneticin (G418) from the Sigma Chemical Co (St Louis, MO). [*Methyl*,1′,2′-^{3}H]Thymidine 5′-triphosphate (sp. act. 93 Ci/mmol), thymidine 5′-[α-^{32}P] triphosphate (sp. act. 3000 Ci/mmol), [^{125}I-tyrosine A14] human insulin (sp. act. 2000 Ci/mmol), Hybond C-extra and Hyper-film MP were obtained from Amersham (Bucks, U.K.). Anti-porcine insulin was purchased from Bio-Yeda. *N*-Ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride and caesium chloride were from Merck (Darmstadt, F.R.G.). Agarose (ultra pure grade and low melting temperature grade), acrylamide, bis-acrylamide, nitrocellulose sheets and Immun-Blot kit were obtained from BioRad (Richmond, CA). Sephadex G-50 and G-100 were from Pharmacia (Uppsala, Sweden). All other reagents were of analytical grade.

Maintenance of HepG2 cells. HepG2 cells [24] were grown at 37° in flasks containing Eagle's minimal essential medium supplemented with 10 mM $NaHCO_3$ and buffered with 20 mM Na-HEPES, pH 7.3 (MEM) containing 10% (v/v) foetal calf serum (MEM+S). Cells at semi-confluence (4–7 days growth) were trypsinized with a solution containing 0.25% (w/v) trypsin, 0.1% (w/v) EDTA, and the suspensions subcultured at 1/6 or 1/3 dilutions.

Generation of expression vectors. The expression vector plasmids ptkNEO and pAL-8 were each propagated in *Escherichia coli* strain HB101. Initial transformation was achieved using a simple calcium method [25] and stocks of the respectively transformed host strain kept on ampicillin media as both anaerobic stab cultures and frozen glycerol-broth cultures. Large scale plasmid production followed conventional protocols [26], extraction of plasmids was by the boiling method [26] or the lysozyme–Triton X-100 method of Katz *et al.* [27] and purification was by centrifugation in caesium chloride–ethidium bromide (CsCl–EB) density gradients [28]. After EB removal from the plasmid fractions and subsequent dialysis against TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) the final preparations were stored over chloroform (5% v/v) at −15°.

Quantification of DNA. DNA was estimated spectrophotometrically by UV absorption.

Agarose gel electrophoresis. Plasmids and restriction digests of plasmids were analysed on 1.2% agarose gels run at pH 7.5 and stained with EB using conventional methodology [26]. Preparative electrophoresis for the separation and excision of restriction fragments for subsequent labelling as probes was performed in a similar manner on gels of low melting temperature agarose. For the analysis of DNA-protein binding by band shift assay, plasmids were bound to proteins prior to electrophoresis as

described in the legend to Fig. 5. For the analysis of the charge properties of proteins, protein samples were electrophoresed as above but subsequently fixed in 10% (w/v) TCA, stained with 0.025% (w/v) aqueous Coomassie Brilliant Blue in 10% (w/v) TCA, and destained in 10% (w/v) TCA, 5% (v/v) acetic acid.

Labelling of pBR322. The plasmid pBR322 was labelled by the nick translation protocol [29] in the presence of [*methyl*,1′,2′-^{3}H]thymidine 5′-triphosphate and subsequently purified by the spun column method using Sephadex G-50 [26]. The product was stored at −15°. Specific activity was 6.0–8.5 × 10^6 cpm/μg TCA-precipitated DNA.

Labelling of neo *probe.* A 923bp PstI fragment of ptkNEO representing the majority of the *neo* gene sequence and no extraneous sequences was labelled with thymidine 5′-[α-^{32}P] triphosphate by the method of Feinberg and Vogelstein [30]. The desired fragment was excised from an electrophoretic gel of low melting temperature agarose, water added (3 μL/mg gel), and the sample boiled for 7 min immediately prior to use in the labelling reaction. The reaction product was subjected to spun column purification using Sephadex G-50 [25] then stored at −15°. Specific activity was 6.0 × 10^8–2.2 × 10^9 cpm/μg TCA-precipitated DNA.

Preparation of N-*acylurea albumins.* The *N*-acylurea derivative of bovine serum albumin (BSA) was prepared as previously described [23] by reaction of the protein with *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide HCl (CDI), at a 1:500 mole ratio. The product was dialysed exhaustively against saline-EDTA (50 mM NaCl, 0.3 mM EDTA, pH 7.0) at 5° and aliquots stored at −15°.

Conjugation of insulin to albumins. Insulin was cross-linked to BSA at a 10:1 mole ratio by a development of the method of Poznansky *et al.* [31]. Insulin (4.2 mg, 7.37 × 10^{-7} moles, dissolved in 600 μL DMSO at 37°) was mixed with unmodified BSA or its *N*-acylurea derivative (5 mg, 7.35 × 10^{-8} moles, in 1 mL saline-EDTA) at 20° then diluted with 4.0 mL 50 mM acetate buffer, pH 3.6, and the solution cooled to 5°. Glutaraldehyde (90 μL, 25%) was added and the reaction mixture maintained in the cold overnight. Thereafter, crystalline glycine (75 mg) was added, dissolved, and allowed to react with excess glutaraldehyde for 2.5 hr at 20°. The reaction mixture was then dialysed exhaustively against 10 mM acetate buffer, pH 4.0, containing 5% (v/v) DMSO at 5°. The resultant clear, pale straw-coloured solutions were stored in aliquots at −15°.

Fractionation of conjugates. Conjugates were purified by gel filtration through Sephadex G-100. Fractions (1 mL) were stored at −15° for up to 8 weeks. Conjugates for use in transfection experiments were fractionated under sterile conditions.

Quantification of protein. Protein was estimated by the method of Lowry *et al.* [32] using BSA as standard.

Identification of insulin in the conjugates. Insulin was detected semi-quantitatively in the conjugate fractions by immuno-dot-blotting. Each fraction tested was diluted in elution buffer to a concentration of 66 ng protein/μL. These starting solutions, together with a range of double dilutions derived from them, were applied in 1-μL aliquots to nitrocellulose and processed according to the BioRad Immun-Blot procedure using anti-insulin (1/333) as first binding agent, protein A-horseradish peroxidase (1/2500) as second binding agent, and a mixture of 4-chloro-1-naphthol (dissolved in methanol) and hydrogen peroxide (aqueous) as colour reagent.

Molecular size determination. Protein conjugates were analysed by SDS–polyacrylamide gel electrophoresis according to the method of Laemmli [33].

DNA binding assay. The binding of DNA to protein conjugate fractions was estimated by filtration through nitrocellulose in a variation of the method previously described [23]. Mixtures containing 50 mM NaCl, 20 mM Tris–HCl (pH 7.0), 1 mM EDTA, [^{3}H]pBR322 (4.5 ng, 3.3 × 10^4 cpm), unlabelled pBR322 (5.5 ng) and protein as indicated in the figure legends in a final volume of 200 μL were incubated at 20° for 30 min, then filtered through nitrocellulose discs (Millipore Type HA, 25 mm diameter, 0.45 μm pore size) and rinsed with 2.0 mL of buffer consisting of 50 mM NaCl, 20 mM Tris–HCl (pH 7.0) and 1 mM EDTA. The nitrocellulose filters were wetted in rinse buffer prior to use. Finally, filters were air-dried and counted for radioactivity in scintillation fluid.

Receptor binding studies. HepG2 cells were heavily seeded (1/3 splits) into 35-mm diameter well plates and grown to semi-confluence. Cells were washed twice with MEM (no serum) at 37° (2 mL per well) then incubated at 37° for 1 hr in the presence of a further 2 mL MEM, which was subsequently removed. Plates were placed on ice and MEM at 5° added (1 mL per well) together with [^{125}I]insulin and unlabelled insulin or insulin conjugate as indicated in the figure legends. Additives were thoroughly mixed into the binding medium and plates incubated at 10°. Cells were subsequently washed three times with cold phosphate buffered saline (PBS) (4 mL per well) and drained. After addition of water (1 mL per well), cells were loosened into suspension with a rubber policeman, 200 μL removed for protein determination, and the remainder fully dispersed by the addition of 1 mL lysis buffer (0.5% SDS, 100 mM NaCl, 40 mM Tris–HCl, 20 mM EDTA, pH 7.0) [34]. The lysates were transferred to tubes for gamma counting.

Transfection procedure. HepG2 cells were heavily seeded (1/3 splits) in 25 cm^2 flasks and grown to semi-confluence. Cells were washed three times with 4 mL MEM (no serum), then incubated at 37° for 1 hr in the presence of a further 4 mL MEM, which was subsequently removed. Flasks were placed on ice and 2 mL MEM at 5° added together with I–[A-CDI] or control protein as indicated in the legend to Fig. 8. After overnight incubation at 10°, flasks were again placed on ice, the medium removed, and 1 mL MEM at 5° added together with expression vector as indicated. The vector samples used comprised superhelical and nicked circular forms of plasmid DNA in approximately equal proportions. Flasks were transferred from ice to incubation at 10° (30 min), 20° (30 min), then 37° (5.0 hr). At this stage 3 mL MEM + S (plus serum) was added and

incubation continued for 24 hr. Flasks were trypsinized and duplicate subcultures established containing 10^6 cells per flask. Growth was continued for a further 24 hr before the first addition of G418.

Stable expression of NEO*: G418 selection.* Following the trypsinization of post-transfection cells, G418 stock solution (24 mg total antibiotic/mL H_2O adjusted to pH 7.4 with NaOH and filter sterilized) was added to the medium to give a final concentration of 1200 μg/mL (biological activity = 50% of this) and incubation continued for 5 days, after which the medium was changed and fresh G418 added at a final concentration of 600 μg/mL. Medium changes were effected at approximately 5 day intervals thereafter, the G418 level being lowered further to and maintained at 500 μg/mL. Following the unambiguous appearance of resistant clones (14–21 days) and the death of most non-resistant cells (21–28 days), one set of flasks was stained for the recording of clone distribution. Surviving cells in the other set were trypsinized for subculture and propagation in a G418 maintenance regime and ultimately subjected to genomic DNA extraction.

Extraction of genomic DNA from HepG2 cells. Duplicate 75 cm^2 flasks of cells were used for each DNA preparation. Extraction was according to the method of Shih and Weinberg [34]. Final DNA solutions were dialysed exhaustively against 0.1 SSC at 5° then stored at −15°.

Detection of the integrated neo *sequence in transfected HepG2 DNA.* This was carried out by Southern hybridization [35]. Blots were probed with a 923bp *neo* fragment and washed under conditions of high stringency. Bands were visualized by autoradiography.

RESULTS

Preparation of N*-acylurea albumin*

N-Acylurea albumin (A-CDI) was prepared by treating BSA with the water-soluble carbodiimide *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (CDI), under conditions previously described, in which 27 out of a total of 97 side chain carboxyl groups per BSA molecule become substituted by *N*-acylurea moieties leading to significantly increased electropositivity as shown by agarose gel electrophoresis at pH 7.5 [23]. During the reaction the carbodiimide induces a degree of polymerization of albumin as shown by the behaviour of A-CDI on gel filtration (Fig. 2).

Preparation of insulin–albumin conjugates

Monocomponent porcine insulin (I) was covalently cross-linked to BSA (A) and *N*-acylurea BSA (A-CDI) by reaction with glutaraldehyde to give rise to the conjugates I–A and I–[A-CDI] respectively (Fig. 1). In order to ensure the attachment of several molecules of insulin (M_r 5700) to each molecule of albumin (M_r 68,000) reaction mixtures contained an insulin to BSA mole ratio of 10:1 (mole calculations based on unmodified BSA input in both cases). Reaction and subsequent purification was conducted at low pH (3.6–4.0) in the presence of DMSO (5–10% v/v) in order to maintain insulin solubility at the required protein concentration. Gel filtration of the

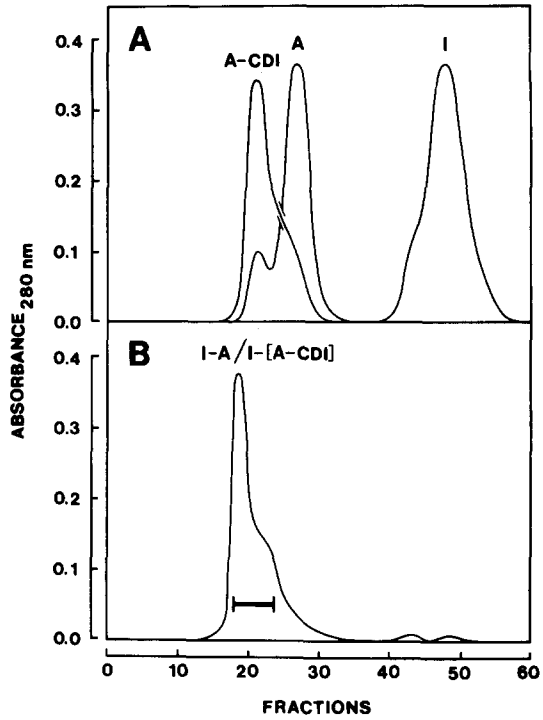

Fig. 2. Gel filtration chromatography of insulin, albumin and derivatives. Proteins were purified and characterized by gel filtration through Sephadex G-100 in a 7 × 1500 mm column using 10 mM acetate buffer, pH 4.0, containing 5% (v/v) DMSO as eluant at a flow rate of 6.0 mL/hr. Absorption at 280 nm was determined as a continuous trace, and fractions of 1 mL collected. A: Unmodified insulin (I), unmodified albumin (A) and *N*-acylurea albumin (A-CDI). B: Insulin–albumin (I–A) and insulin–*N*-acylurea albumin (I–[A-CDI]). Reaction mixtures were dialysed exhaustively prior to loading on to the column; reaction mixtures containing insulin were dialysed in low molecular weight cut-off dialysis tubing. The horizontal bar shows those conjugate fractions containing the highest proportions of insulin to total protein as estimated by immuno-dot-blot analysis.

dialysed reaction mixtures through Sephadex G-100 showed the two reaction products to have identical profiles representing a series of high molecular weight complexes with only insignificant amounts of protein evident at the position of unconjugated insulin elution (Fig. 2). The incorporation of insulin into the conjugate fraction was confirmed by SDS–polyacrylamide gel electrophoresis (results not shown). Furthermore, presence of insulin in the high molecular weight product was demonstrated unambiguously by immuno-dot-blot analysis, with the highest ratios of insulin to total protein being apparent in fractions 18–24 (Fig. 2). Results (not shown) of agarose gel electrophoresis at pH 7.5 of the conjugate fractions bore out the anticipated charge differences between I–A and I–[A-CDI], the latter showing considerably increased electropositivity due to the presence of basic *N*-acylurea moieties attached to surface aspartate and glutamate residues.

Binding of DNA to insulin–albumin conjugates

Purified conjugates obtained by gel filtration

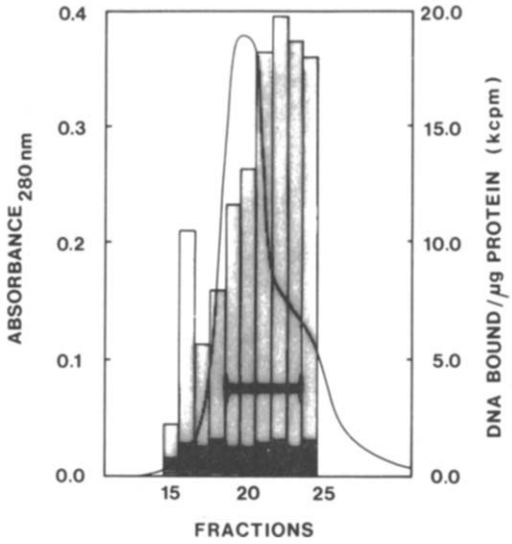

Fig. 3. DNA binding capacity of insulin–albumin conjugates fractionated by gel filtration. DNA binding was assayed by the nitrocellulose filtration technique. Each assay mixture contained 4.5 ng [^{3}H]pBR322, 5.5 ng unlabelled pBR322 and 1.5 μg protein conjugate. Absorption at 280 nm of I–A and I–[A-CDI] (——); DNA bound by I–A (heavy hatching); DNA bound by I–[A-CDI] (light hatching). The horizontal bar indicates those fractions pooled for use in subsequent binding and transfection experiments.

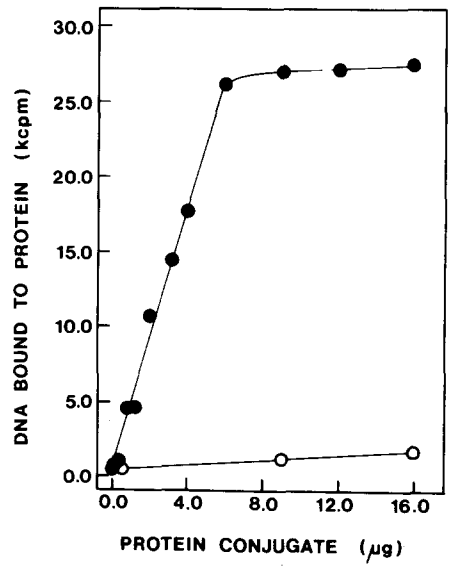

Fig. 4. Binding of DNA to insulin-[*N*-acylurea albumin] at varying protein concentration. DNA binding was assayed by the nitrocellulose filtration technique. Each assay mixture contained 4.5 ng [^{3}H]pBR322 and 5.5 ng unlabelled pBR322. Each protein conjugate was added in the form of a pooled gel filtration peak component as indicated in Fig. 3. I–[A-CDI] (●); I–A control (○).

through Sephadex G-100 were assayed for DNA binding activity by the nitrocellulose filtration technique. The binding of DNA to *N*-acylurea proteins has been shown to be salt concentration dependent [23]; consequently the ionic strength of the incubation and washing medium must be carefully controlled. In addition, both protein–DNA binding and protein–nitrocellulose binding are pH dependent, and it was found to be important to conduct these assays between pH 6.0 and 7.5 (results not shown). Accordingly, for these experiments, an assay buffer was devised which maintained suitable ionic strength and pH despite the addition of variable volumes of conjugate dissolved in 10 mM acetate buffer, pH 4.0. The assay buffer used was 50 mM NaCl, 20 mM Tris–HCL (pH 7.0), 1 mM EDTA.

Figure 3 illustrates typical binding interactions of pBR322 DNA with the various size species of I–A and I–[A-CDI] obtained by gel filtration. Each assay mixture contained 1.5 μg conjugate. At this level of protein the DNA present in the assay mixture (10 ng) is not limiting (refer to Fig. 4). The capacity to bind DNA is demonstrated by I–[A-CDI] but not I–A. While all fractions of I–[A-CDI] undergo significant binding, binding is lowest for the very large polymers and there is a reproducible trend for the binding capacity of the larger species of conjugate to be inversely proportional to molecular size (fractions 15–20). This is likely to be a reflection of the reduction in the ratio of surface area to weight that accompanies polymerization. Maximal binding is achieved and maintained over a range of the more moderately sized species (fractions 21–24).

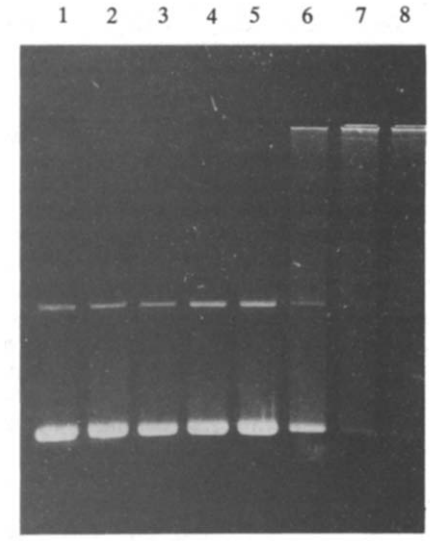

Fig. 5. Band shift assay of binding interactions between insulin–albumin conjugates and DNA. pBR322 was incubated with protein conjugate at 20° for 30 min prior to agarose gel electrophoresis. Each incubation mixture contained 50 mM NaCl, 20 mM Tris–HCl (pH 7.0), 1 mM EDTA, 0.45 μg pBR322, and a variable amount of protein conjugate as indicated, in a final volume of 10 μL. 1, pBR322 control (no protein); 2 and 3, pBR322 plus I–A (2.0 and 3.3 μg, respectively); 4–8, pBR322 plus I–[A-CDI] (0.4, 1.0, 1.66, 2.66, and 3.3 μg, respectively).

On the basis of (i) DNA binding capacity, (ii) insulin content, and (iii) concentration, I–[A-CDI] fractions 19 to 23 inclusive were pooled to provide an I–[A-CDI] stock solution for use in all subsequent

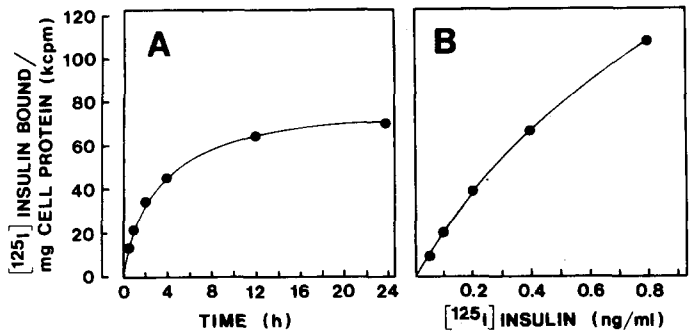

Fig. 6. Binding of human insulin to HepG2 insulin receptors. Semi-confluent monolayers of HepG2 cells were grown and prepared for binding as described in Materials and Methods. A: [^{125}I-Tyr^{A14}] human insulin (2 μL, 0.4 ng, 2.36×10^5 cpm) was added to each well and the incubation period at 10° varied. B: Variable amounts of [^{125}I-Tyr^{A14}] human insulin were added to the wells and incubation at 10° allowed to continue for 18 hr. After incubation each well was processed as described in Materials and Methods.

experiments. Fractions of I–A were pooled in an equivalent manner. Stock solution concentrations ranged from 0.38 to 0.42 μg protein/μL.

The interaction of pBR322 DNA with insulin–albumin conjugates at varying concentrations of protein is shown in Fig. 4. Results confirm that only I–[A-CDI] undergoes binding. Total DNA per reaction mixture is 10 ng. The amount of DNA bound is seen to increase linearly as the input of I–[A-CDI] rises to 6 μg; maximum binding is reached at 8 μg I–[A-CDI].

DNA binding by I–[A-CDI] is illustrated by alternative means in Fig. 5 which shows the result of DNA gel electrophoresis following incubation of pBR322 samples (0.45 μg) with varying amounts of insulin–albumin conjugates. Retardation of migration resulting from the complexing of DNA to protein starts to become visible at 1.0 μg I–[A-CDI], particularly in relation to the supercoil band, and at 1.66 μg I–[A-CDI] significant retardation of both supercoil and nicked circular forms is seen as continuous smudging of the DNA. At higher levels of I–[A-CDI] a proportion of the complex is virtually unable to enter the gel. In contrast, I–A is shown to bring about no significant retardation of migration. Unmodified albumin and the basic protein cytochrome *c* are without effect on pBR322 migration under the same electrophoretic conditions [23]. Insulin is similarly without effect (result not shown).

Binding of insulin and insulin–albumin conjugates to the insulin receptor

Since laboratory iodination of insulin with ^{125}I incurs oxidation damage and brings about indiscriminate labelling of tyrosine residues leading to decreased biological activity including aberrant receptor binding [36], commercially produced human insulin [^{125}I]-labelled only at the A14 tyrosine was employed in these investigations. Monoiodo[Tyr^{A14}]insulin is fully active in receptor binding assays [36]. The binding to insulin receptors of unlabelled porcine insulin and insulin–albumin conjugate derivatives was studied by determining their ability to compete with free radioactively labelled human insulin for receptor sites on HepG2 cells. Preliminary experiments were carried out, therefore, to establish the normal binding characteristics of [^{125}I-Tyr^{A14}] human insulin to HepG2 cells.

Prior to all binding procedures cells were washed to remove extracellular ligand, incubated at 37° for 1 hr in serum-free medium in order to clear the receptors of bound ligand, then cooled. Temperatures below 16° are known to inhibit absorptive endocytosis of insulin [37]. Accordingly, binding incubations were set up in serum-free medium at 10°.

Figure 6A presents the time course of binding of human insulin to HepG2 cells at 10°. Binding takes place rapidly for the first 2 hr, after which it slows down, reaching a maximum at approximately 16 hr. For all subsequent binding experiments incubation was for an 18 hr period at 10°. The effect of ligand concentration on the binding of human insulin to HepG2 cells is shown in Fig. 6B. Binding increases with insulin input, and at an insulin concentration of 0.8 ng/mL, saturation of the receptor population in the system is not yet reached.

Porcine insulin as well as insulin–albumin conjugates derived from porcine insulin compete effectively with human insulin for specific receptor sites on HepG2 cells (Fig. 7). Binding resulting from the addition of 0.4 ng [^{125}I-Tyr^{A14}] human insulin alone is reduced to 50% in the presence of 50 ng porcine insulin (Fig. 7A), 300 ng I–A and 200 ng I–[A-CDI] (Fig. 7B). By contrast, albumin unconjugated to insulin does not compete for binding. Furthermore, Fig. 7C shows that when I–[A-CDI] is prebound to DNA its receptor binding facility is not affected. DNA itself has no affinity for the binding site.

Insulin–[N-acylurea albumin] mediated transfection

DNA transfer to HepG2 cells was tested using the expression vectors ptkNEO and pAL-8, both of which carry the bacterial sequence *neo* from transposon Tn5 which encodes aminoglycoside 3′-phosphotransferase II. This gene is normally absent in mammalian cells but if inserted and expressed confers upon them resistance to the lethal effects of the 2-deoxystreptamine antibiotic G418 (Geneticin), thus acting as a dominant selective marker [38]. Viral promoter sequences allow expression of *neo* on

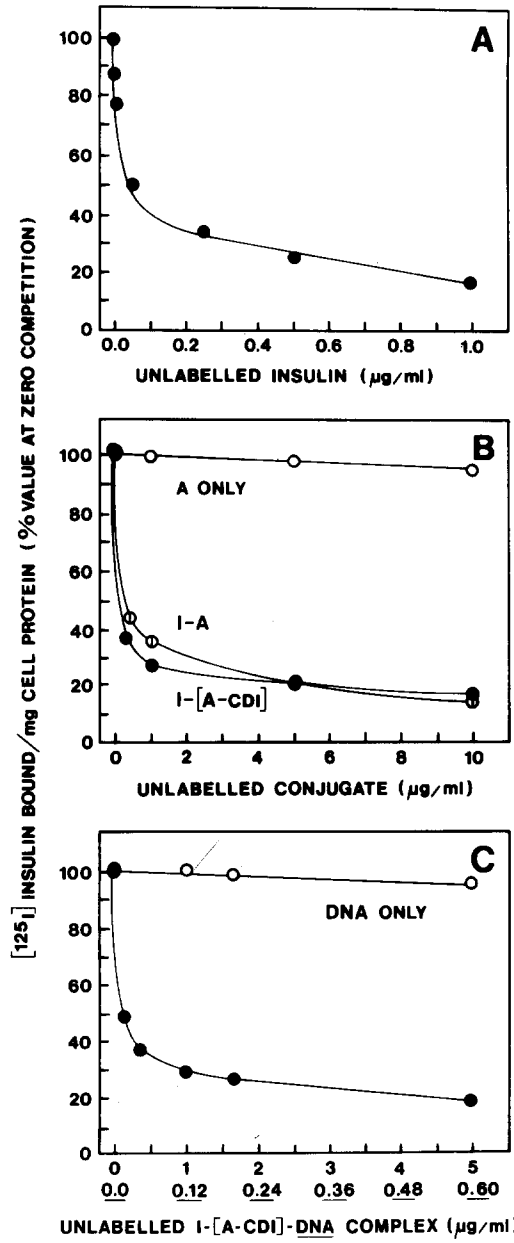

Fig. 7. Competitive displacement of human insulin from HepG2 insulin receptors by porcine insulin and its conjugated derivatives. Semi-confluent monolayers of HepG2 cells were grown and prepared for binding as described in Materials and Methods. [^{125}I-Tyr^{A14}] human insulin (2 μL, 0.4 ng, 2.36×10^5 cpm) was added to each well together with variable amounts of unlabelled competitor protein as shown. A: Porcine insulin. B: (Porcine) insulin–albumin conjugates I–A (Ⓘ) and I–[A-CDI] (●), plus albumin controls (○). C: I–[A-CDI] which had been incubated with pBR322 DNA in binding buffer at 20° for 30 min prior to addition (●), plus DNA controls (○); DNA values underlined. Incubation was at 10° for 18 hr. Subsequent processing was as described in Materials and Methods.

transfer: in ptkNEO the gene is linked on either side to HSV thymidine kinase regulatory sequences; in pAL-8 the flanking transcriptional control regions are derived from SV40.

Figure 8 illustrates clonal colonies which have arisen over a period of 28 days in the presence of G418, indicating reception of the *neo* gene and its stable expression. The presence of numerous clones in flasks E1–E4 together with the absence of surviving cells in control flasks C1–C5 provides phenotypic evidence that DNA transfer takes place only by means of I–[A-CDI] and not as a result of any effects of I–A or unconjugated protein constituents. Frequencies of stable transfection resulting from ptkNEO transfer (mean = 2.0×10^{-5}) are lower than for pAL-8 (mean = 5.5×10^{-5}).

Genotypic evidence for *neo* transfer is provided in Fig. 9, which shows the results of Southern hybridization of genomic DNA samples extracted from the HepG2 cell line and from transfected populations derived from it. Figure 9A illustrates the absence of *neo* in two mammalian cell lines including HepG2. Figure 9B shows presence of the *neo* sequence in the transfected HepG2 populations E1–E4, grown on G418 to high cell number through several passages, over a total period of 44 days, from duplicate flasks of those shown in Fig. 8.

DISCUSSION

The carbodiimide modification of proteins to produce *N*-acylurea derivatives with DNA-binding properties and their potential in gene transfer has been reported previously [23, 39]. Here, we demonstrate that cross-linking insulin to carbodiimide-modified albumin produces a soluble macromolecule capable of both specific recognition of the insulin receptor and DNA transport, thereby affording a possible means of targeted transfection from solution without the involvement of particulate intermediates, excessive foreign chemicals, cell disruption or infective agents. We provide evidence which suggests that, by means of this DNA carrier system, the exogenous gene *neo* from the bacterial transposon Tn5 has been inserted through the insulin receptor endocytotic pathway into the HepG2 hepatoma cell line and stably expressed.

In keeping with our proposed model (Fig. 1), the gel filtration fraction of insulin–[*N*-acylurea albumin] used for transfection was selected in order to achieve an optimal combination of protein concentration, DNA binding capacity and insulin content (Figs 2 and 3). Measuring DNA binding reactions by means of nitrocellulose filter and band shift assays, and using an equivalent fraction of insulin–albumin conjugate as control, we have shown that binding takes place exclusively as a result of interaction of DNA with *N*-acylurea moieties belonging to the modified albumin component of the conjugate (Figs 3, 4 and 5). In addition, we have shown that insulin–albumin conjugates bind specifically to insulin receptor sites on HepG2 cells, recognition being due to the insulin component of the conjugate, and that the presence of *N*-acylurea substituents does not adversely affect conjugate–receptor binding characteristics (Fig. 7B). Furthermore, we have demonstrated that conjugate–DNA binding does not interfere with conjugate–receptor binding (Fig. 7C).

The complex which arises when DNA is mixed with insulin–[*N*-acylurea albumin] is soluble under

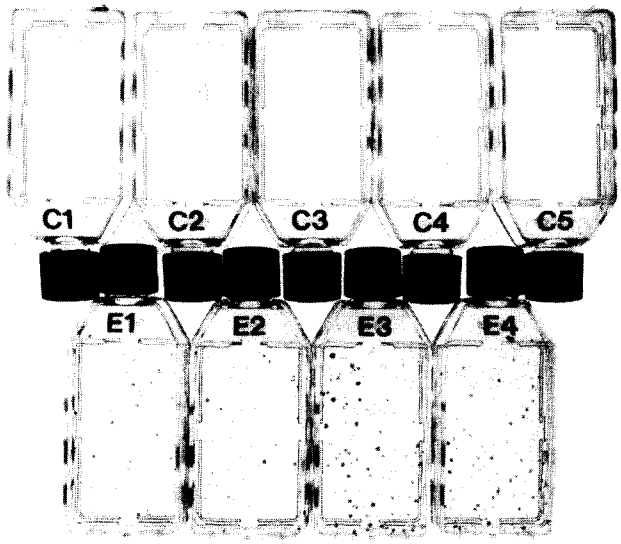

Fig. 8. Appearance of clones resistant to G418 following insulin–[*N*-acylurea albumin] mediated transfer of the expression vectors ptkNEO and pAL-8 to HepG2 cells. Cells were subjected to transfection and G418 selection as described in Materials and Methods. After 28 days of G418 treatment, one complete experimental set of flasks was stained for the visualization of clones as follows: medium was removed, cells rinsed twice with PBS (5 mL), fixed with redistilled methanol (5 mL, 2 min), stained with a 1/10 aqueous dilution of standard Giemsa (5 mL, 2–5 min), then rinsed generously with water several times. Flasks were drained and air-dried. Transfection flasks: E1 and E2, I–[A-CDI] (40 μg) plus ptkNEO (4 μg); E3 and E4, I–[A-CDI] (40 μg) plus pAL-8 (4 μg). Transfection control flasks: C1, no protein, no vector; C2, no protein, pAL-8 (4 μg); C3, unconjugated I (18.25 μg) and A (21.75 μg) plus pAL-8 (4 μg); C4, unconjugated I (18.25 μg) and A-CDI (21.75 μg) plus pAL-8 (4 μg); C5, I–A (40 μg) plus pAL-8 (4μg).

certain titration conditions as suggested by (i) reversibility of binding in the presence of high monovalent ion concentration (result not shown), and (ii) electrophoretic mobility of the complex (Fig. 5, wells 5 and 6). However, as the ratio of conjugate to DNA in the mixture is raised, large electrophoretically inert complexes which may be insoluble are formed (Fig. 5, wells 7 and 8). In our transfection protocol, therefore, we were careful to avoid the possibility of insoluble material being precipitated in the medium. We achieved this by establishing conjugate–receptor binding during a pre-incubation period, following this with removal of unbound conjugate, and only then adding expression vector DNA for attachment to the receptor-bound carrier. Receptor cycling was inhibited by temperature control until the DNA binding step was complete. This approach to binding allows the addition of excess amounts of conjugate and DNA at each step, maximizes binding and possibly increases transfection efficiency. The alternative would be to ensure removal of particulate material by membrane filtration of the complex before addition to cells. This might be a suitable approach for *in vivo* gene transfer studies, but futher investigation into the interactions of soluble I–[A-CDI]–DNA complex and components of the medium would be a desirable prerequisite.

A clear demonstration of stable expression of the *neo* gene following ptkNEO and pAL-8 transfer to HepG2 cells is provided by (i) the appearance of distinct clones among I–[A-CDI]–vector treated cells grown for 28 days on a G418 regime lethal to untreated cells (Fig. 8), and (ii) identification of the exogenous *neo* sequence in DNA extracted after clone populations produced in parallel to those shown in Fig. 8 had been grown for 44 days, through a number of subculture steps, on the antibiotic (Fig. 9). Complete lack of survivors in control flasks in which cells were provided with vector in the presence of no protein, I + A, I + A-CDI and I–A, respectively (Fig. 8) constitutes evidence that these gene transfer events depended on an agent capable of binding to both DNA and insulin receptor. Furthermore, free A-CDI, present at a concentration equivalent to that in the I–[A-CDI] conjugate, does not bring about transformation, indicating that non-specific cation-induced uptake is not involved. On these premises we suggest that gene transfer has taken place under the direction of insulin via the endocytotic pathway.

The size of the G418-resistant clones developed (Fig. 8) varied greatly within each treated population regardless of vector. The phenomenon has measurable parallels in plant genetics: individual *neo*-positive plantlets grown up from Ti plasmid-transformed tissue show wide quantitative difference in resistance to kanamycin; this is generally thought to indicate that expression of inserted genes is enhanced or

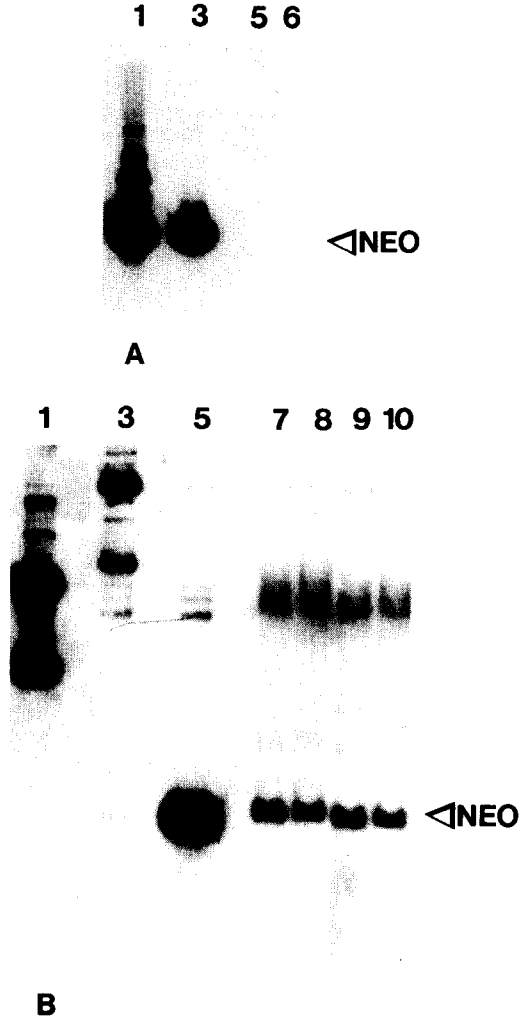

Fig. 9. Genomic Southern hybridization: detection of the *neo* sequence in stably G418 resistant HepG2 cells following insulin–[*N*-acylurea albumin] mediated transfection. Cells were subjected to transfection and G418 as described in Materials and Methods. After 28 days of G418 treatment, one complete experimental set of flasks was stained for the visualization of clones (Fig. 8). Flasks of the duplicate set containing surviving cells (E1–E4) were trypsinized and each of the cell populations propagated through several passages on a G418 maintenance regime for a further 16 days until there were sufficient cells for genomic DNA extraction. DNA was analysed for the *neo* sequence using as probe a 923bp PstI fragment of ptkNEO within the *neo* gene. A: Mini gel blot: 1, PstI digested ptkNEO (2 ng) plus salmon sperm carrier DNA (3 μg); 3, *neo* fragment excised from PstI digested ptkNEO (923bp) (2 ng) plus carrier DNA (3 μg); 5, PstI digested HepG2 cell line DNA (12 μg); 6, PstI digested HeLa cell line DNA (12 μg). B: Large gel blot: 1, undigested ptkNEO (4913bp) (1 ng) plus carrier DNA (7 μg); 3, undigested pAL-8 (>10,000bp) (1 ng) plus carrier DNA (7 μg); 5, *neo* fragment excised from PstI digested ptkNEO (923bp) (1 ng) plus carrier DNA (7 μg); 7–10, PstI digested DNAs from G418 resistant HepG2 cell populations E1–E4, respectively (28 μg per well).

suppressed according to genomic position, which varies as a result of random integration [40]. In enumerating clones we ignored those which were particularly small, thereby estimating transformation frequencies conservatively. Our results show I–[A-CDI]-induced stable gene transfer to be approximately 20 times (ptkNEO) and 55 times (pAL-8) greater than typical stable transformation of HepG2 cells brought about by the calcium phosphate procedure [41].

Ligand-directed passage of DNA across the plasma membrane is likely to be efficient. During subsequent intracellular processing, however, the route of DNA is less controlled, and numerous hazards in the form of enzymatic or membrane barriers are encountered. Ultimate delivery to the nucleus must still be a rare event. The use of insulin as ligand might offer an advantage in this respect. In contrast with some ligand proteins which are delivered via membrane-bound vesicles to lysosomes, it is increasingly evident that insulin degradation inside the cell takes place in endosomal rather than lysosomal compartments [42]; thus DNA still associated with the I–[A-CDI] complex after entry may not be directed actively towards lysosomal nucleases.

Exploitation of the efficiency and specificity of cell entry afforded by receptor-mediated endocytosis has been suggested in various ways previously. For example, Poznansky *et al.* [31] have achieved delivery of α-1,4-glucosidase to muscle cells and hepatocytes using a covalently conjugated enzyme–albumin–insulin complex, and Cheng *et al.* [43] have devised a mechanism of covalent attachment between nucleic acid and protein with the purpose in view of genetic correction via endocytosis. The method of gene transfer presented here combines the elegance of ligand-directed carrier insertion with non-covalent reversible binding of the DNA to the carrier [23], a possible advantage in the cell interior. Recent work by Wu and Wu published during the course of our investigations illustrates use of a parallel approach: by employing as carrier a conjugate of asialoorosomucoid and high molecular weight cationic poly-L-lysine, which binds DNA electrostatically in much the same manner as *N*-acylurea protein, those workers have demonstrated transient expression of the bacterial *cat* gene in HepG2 hepatoma cells in culture [44, 45] and in rat liver *in vivo* [46]. The work we describe in the present communication adds to the growing body of evidence that receptor mediated endocytosis provides a pathway by which exogenous molecules, including DNA, may be directed into the interior of target cells, and in particular shows that stable gene transformation may result from the use of this facility.

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