

High-efficiency receptor-mediated delivery of small and large (48 kilobase gene constructs using the endosome-disruption activity of defective or chemically inactivated adenovirus particles

(gene therapy/endocytosis/transferrin/asialoglycoprotein/cosmid)

MATT COTTEN*, ERNST WAGNER*, KURT ZATLOUKAL*, STEPHEN PHILLIPS*, DAVID T. CUIEL†, AND MAX L. BIRNSTIEL*

*Research Institute for Molecular Pathology, Dr. Bohr-gasse 7, 1030 Vienna, Austria; and †University of North Carolina, Chapel Hill, NC 27599-7020

Communicated by Max L. Birnstiel, March 12, 1992

ABSTRACT One limit to successful receptor-mediated gene delivery is the exit of the endocytosed material from the endosome. We demonstrate here the delivery of marker genes to tissue culture cells using a modification of the receptor-mediated gene delivery technique that exploits the endosomolytic activity of defective adenovirus particles. In particular, >90% of the transfected-cell population is found to express a β -galactosidase gene, and, most importantly, this high level of expression can be obtained with psoralen-inactivated virus particles. Furthermore, because the delivered gene is not carried within the genome of the adenovirus particle, the size constraints are relieved, and we can, therefore, show the delivery of a 48-kilobase cosmid DNA molecule.

We are developing methods of introducing DNA into mammalian cells with the goal of generating a safe and reliable method that introduces both small and large DNA into a high percentage of the target cells. Our strategy has been to exploit the receptor-mediated endocytosis route to deliver DNA. This approach uses various polycation–ligand chemical conjugates, such as transferrin–polylysine (1–5), which serve to condense the DNA to be transferred into compact, ligand-coated toroids, comparable in size to a typical DNA virus (6). These ligand-coated DNA particles can then bind to receptors on the surface of target cells, and upon endocytosis, the DNA finds its way into the nucleus.

We have demonstrated both transient and stable gene delivery into a variety of cell types. In certain cell lines, such as K-562 cells, we can show transient gene expression in >90% of the target cells (5). Stable expression of introduced genes can also be demonstrated, with up to 0.5–1% of the initial transfected-cell population recoverable as stable expressing clones (unpublished results).

We suspected that one of the major limits to gene delivery by endocytosis in many cell types may be the exit of the endocytosed material from the endosome. However, many viruses are known to enter cells via a similar receptor-mediated endocytosis route and possess molecular skills to mediate disruption of the endosomes to enter the cytoplasm of host cells. We have previously found that this endosomolytic activity of a virus can function in trans to facilitate the coentry of receptor-bound DNA particles (7). Plasmid DNA containing a marker gene is complexed and condensed into a compact parcel with polylysine covalently linked to a cell-binding ligand, such as transferrin. When these ligand-coated DNA donuts are supplied to the appropriate cells in the presence of a replication-defective adenovirus, both the DNA complex and the virus are endocytosed, and the low-pH-triggered adenoviral endosome disruption allows ef-

ficient cellular entry of the DNA (Fig. 1). This maneuver enhances delivered gene expression 100- to 1000-fold in a variety of cell types (7, 8).

We present evidence here that when the target cell displays receptors for both the DNA-bound ligand (transferrin) and adenovirus, a very high percentage (>90%) of the target cells take up and express the marker gene. This trans application of virus solves many of the safety and practical problems associated with using recombinant viruses to deliver genes. In our system, the virus is functioning merely as an endosomolytic agent, and because virus gene expression is not required, we can use a transcription- and replication-defective virus. Most importantly, because the endosomolytic activity of the virus is probably a function of the virus protein capsid, we find that we can treat the virus with various agents that damage the nucleic acid of the virus while sparing the protein component, such as short-wave UV radiation or the DNA intercalator psoralen plus long-wave UV, and the DNA-delivery activity of the virus is largely retained. These inactivation protocols further block virus gene expression and replication. Because the virus particle is functioning in trans and the DNA to be delivered is not incorporated within the virus capsid, this system allows great size and sequence variety in the DNA to be delivered; in contrast to the 6- to 8-kilobase (kb) size limit of the standard recombinant adenovirus vectors (9), we routinely deliver 15-kb plasmids, and we demonstrate here the functional delivery of a 48-kb luciferase-encoding cosmid. Furthermore, the fact that the cargo DNA need not participate in the replication steps of the virus (as is required with the sequences carried by recombinant virus vectors) relieves a further constraint on the length and types of DNA sequences that a given virus can transport.

This use of a defective-virus particle in trans represents another class of gene-delivery techniques. It combines the entry efficiency of a virus with the flexibility and safety of a number of nonviral methods. We believe that this approach provides another way of thinking about DNA delivery and facilitates a variety of possibilities for molecular biology and gene-therapy applications.

METHODS

General Methods. Human and mouse transferrin–polylysine (hTfpL, mTfpL), asialofetuin–polylysine (AfpL), and galactose–polylysine (gal)4pL, were prepared and characterized as described (1, 4, 5; E.W., unpublished results). All DNA preparations (plasmid or cosmid) were subjected to two

Abbreviations: FCS, fetal calf serum; CosLuc, luciferase cosmid; AfpL, asialofetuin–polylysine; hTfpL and mTfpL, human and mouse transferrin–polylysine, respectively; (gal)4pL, galactose–polylysine conjugate; DMEM, Dulbecco's modified Eagle's medium; DMEM + 10%FCS, DMEM/2 mM glutamine/streptomycin at 10 μ g/ml/penicillin at 100 units/ml/10% FCS.

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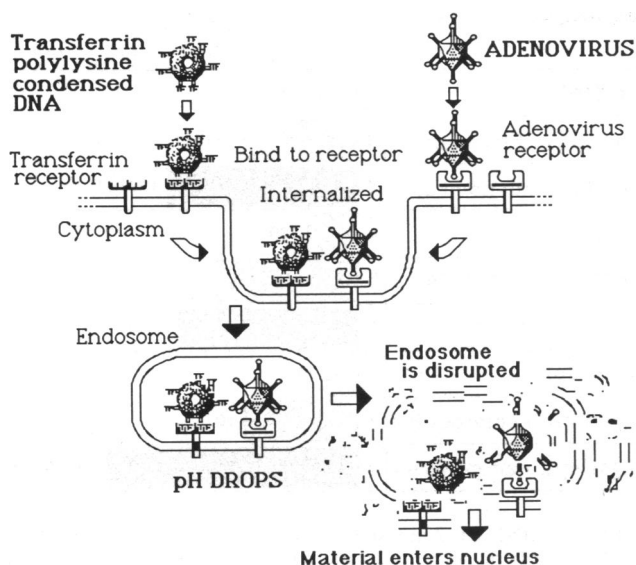


FIG. 1. Summary of adenovirus-enhanced receptor-mediated gene delivery. DNA condensed with transferrin-polylysine conjugate is supplied to cells in the presence of adenovirus particles. Upon coendocytosis, a low-pH-triggered change of the virus capsid is thought to generate endosome disruption, allowing release of coendocytosed transferrin-polylysine/DNA.

rounds of isopycnic banding on CsCl, as described (5). HeLa, mouse hepatocyte BNL CL.2, and human neuroblastoma GI-ME-N cells (10) were grown in Dulbecco's modified Eagle's medium (DMEM)/2 mM glutamine/streptomycin at 10 μ g/ml/penicillin at 100 units/ml/10% fetal calf serum (FCS) (DMEM+10%FCS).

Preparation of Adenovirus dl312. Adenovirus dl312, a replication-defective strain of human adenovirus 5 lacking functional E1a sequences (11), was propagated on the complementing cell line 293 (12) as described (7). Virus was isolated by two rounds of CsCl density-gradient purification, diluted with an equal volume of glycerol, and stored at -70°C .

Viral titers were determined by end-point dilution, cytopathic effect (CPE) assay on 293 cells (13), by UV-absorbance measurement of extracted viral DNA [using the relationship 1 absorbance unit at 260 nm (A_{260}) equals 10^{12} viral particles per ml] (14) and by protein content (using the relationship 1 mg of viral protein = $3.4\text{--}3.5 \times 10^{12}$ virus particles) (15).

Preparation of Luciferase Cosmid (CosLuc). A 3.0-kb *Sal* I fragment, containing a single *Photinus pyralis* luciferase-coding sequence under the control of the Rous sarcoma virus promoter, was isolated from the plasmid p220RSVLuca and ligated into the unique *Sal* I site of the cosmid clone C1-7aA1 (provided by A. Weith, Vienna) to form concatemers. C1-7aA1 comprises a 37-kb human genomic DNA *Sau*3A fragment (partial digest), encoding no apparent genes, cloned into the *Bam*HI site of the cosmid vector pWE15 (Stratagene). The ligation reaction was then packaged *in vitro*, and an aliquot of the resulting phage particles were infected into *Escherichia coli* NM554 and plated on LB ampicillin plates. The recombinants were screened by colony hybridization using the 3.0-kb *Sal* I fragment (^{32}P labeled by random priming) as a hybridization probe, and a number of positives were analyzed by restriction mapping. A cosmid construct (CosLuc) containing a single copy of the *Sal* I insert was grown and purified on a CsCl gradient (total size = 48 kb).

The small control cosmid pWELuc (12 kb) was prepared by digesting CosLuc with *Not* I, religating, transforming bacteria, and isolating a clone containing the appropriate plasmid.

This procedure resulted in a 12-kb DNA molecule lacking the human DNA insert and part of the polylinker of CosLuc 1.

The plasmid clone pSPNeoLuc (8 kb) was obtained by ligating the 3.0-kb *Sal* I fragment from p220RSVLuca described above into the *Sal* I site of pSPNeo, a vector prepared by ligating a *Xba* I-*Asp*-718/Klenow blunt-ended fragment containing the polylinker of Bluescript M13+ into the *Xba* I and *Bam*HI/S1 nuclease blunt-ended sites of pSSTneoA. The plasmid pSSTneoA is the large *Sst* I fragment from pUC μ locus (16).

Transfection Protocols. In general, 6 μ g of DNA was diluted in 330 μ l of HBS (20 mM Hepes/150 mM NaCl, pH 7.4) and a sample of hTfpL, mTfpL, AfpL, or (gal)4pL (equivalent to 4 μ g of polylysine) was diluted with 170 μ l of HBS. The two solutions were mixed and allowed to incubate at room temperature for 30 min. One ml of DMEM/2%FCS was then added to the mixture followed by a sample of the adenovirus (quantities are specified in the figure legends). The medium was removed from a 6-cm dish of cells (grown to $\approx 80\%$ confluence) and replaced with the DNA/hTfpL/adenovirus mixture. After a 1-hr incubation at 37°C , 4 ml of DMEM+10%FCS was added to the dish. Transfections using chloroquine contained 100 μ M chloroquine during a 4-hr exposure to the DNA/conjugates, after which the cells were washed into fresh 10% FCS-containing medium. After a 24-hr incubation at 37°C , the cells were harvested for luciferase assay (5).

Psoralen Inactivation. Samples of purified virus were adjusted to 0.33 μ g/ μ l of 8-methoxypsoralen solution (stock concentration 33 μ g/ μ l 8-methoxypsoralen dissolved in dimethyl sulfoxide), and exposed to a 365-nm UV light source (UVP model TL-33) on ice, at 4 cm from the lamp filter; exposure to the UV light was for 15–30 min, as indicated in the figure legends. The virus samples were then passed over a Sephadex G-25 column (Pharmacia, PD-10) equilibrated with HBS/40% (vol/vol) glycerol.

RESULTS

What Percentage of Transfected Cells Express the Gene? We have previously demonstrated that transient DNA delivery into HeLa cells is augmented by a factor of 1000 or more when cells are simultaneously exposed to transferrin-polylysine/DNA complexes and replication-defective adenovirus particles (7). Our hypothesis has been that the transferrin-polylysine-complexed DNA is efficiently endocytosed into a large percentage of the transfected cell population (8) but that the exit from the endosome into the cytoplasm occurs in only a limited number of cells. This endosomal exit is promoted either by agents that induce endosomal or lysosomal swelling, such as chloroquine (2, 3), or by the endosomal disruption induced by adenovirus (17–21). We wanted to know what percentage of cells transiently express genes introduced by adenovirus/transferrinfection. We exposed HeLa cells to hTfpL/DNA complexes containing a plasmid construct of β -galactosidase under control of the cytomegalovirus immediate-early promoter/enhancer (22) and then added either 100 μ M chloroquine or defective-adenovirus particles. After staining of transfected cells 48 hr later with the chromogenic substrate 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-Gal) (23), we find $<0.1\%$ of a transfected HeLa cell population stains positive (Fig. 2A), and there is only a slight improvement when transfection is done with 100 μ M chloroquine (Fig. 2B). However, $>90\%$ of the cells stain positive when the transferrinfection is done with adenovirus (Fig. 2C). Furthermore, high-level transient expression can be obtained with a number of other cell types, including the mouse hepatocyte line BNL CL.2 with (gal)4pL (Fig. 2D) or mTF pL (Fig. 2E) and a neuroblastoma line GI-ME-N (data not shown). Psoralen-inactivated adenovirus (see below) provides DNA-delivery augmentation similar to that obtained with nontreated adenovirus dl312 (Fig. 2F). Note,

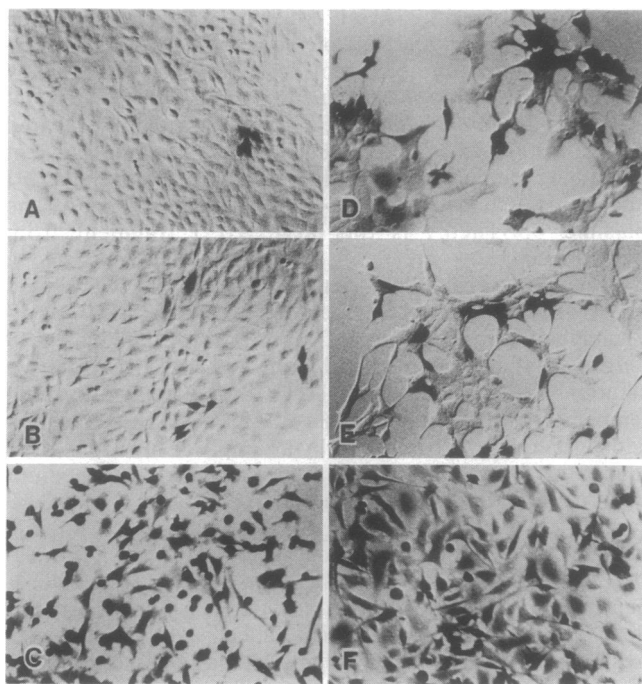


FIG. 2. β -Galactosidase staining patterns of various transfected-cell populations. (A) HeLa cells transfected with hTfpL. (B) HeLa cells transfected with hTfpL in the presence of 100 μ M chloroquine. (C) HeLa cells transfected with hTfpL in the presence of adenovirus dl312. (D) BNL CL.2 cells transfected with (gal)4pL+adenovirus dl312. (E) BNL CL.2 cells transfected with mTfpL+adenovirus dl312. (F) HeLa cells transfected with hTfpL+psoralen-inactivated adenovirus dl312. In all samples the virus particle-to-cell ratio was 5,000–10,000:1.

however, that a certain cell toxicity (the presence of small, dark, rounded cells) is associated with high-level DNA delivery in the HeLa cells (Fig. 2C), and this toxicity can be partially relieved by psoralen treatment of the virus (Fig. 2F).

Augmentation of Delivery Involving Other Receptor–Ligand Pairs. Does the adenovirus augmentation occur with other receptor/ligand systems? Successful receptor-mediated gene delivery to hepatocytes has been obtained using asialoglycoprotein–polylysine conjugates that bind to the liver-specific asialoglycoprotein receptor (24, 25). We have developed a completely synthetic, galactose-containing, polylysine conjugate that presents as the receptor-recognition signal a cluster of four lactose moieties attached to a synthetic, branched linker peptide (E.W., unpublished results), which serves to target DNA delivery to hepatocytes. We tested the adenovirus-delivery enhancement of DNA delivered with this synthetic ligand [(gal)4pL] as well as a natural asialoglycoprotein receptor ligand (AfpL) and mTfpL.

These three different ligand polylysine conjugates were tested for DNA delivery into the mouse hepatocyte line BNL CL.2 (Fig. 3). Delivery was tested in the absence of an endosomolytic agent (bars 1, 4, 7), in the presence of 100 μ M chloroquine (bars 2, 5, 8), and in the presence of adenovirus dl312 particles (bars 3, 6, 9). We find very little delivery with any conjugate in the absence of chloroquine, modest delivery in the presence of chloroquine, and a very robust enhancement with all three conjugates in the presence of adenovirus.

Hence, the DNA-delivery enhancement observed with DNA delivered with transferrin–polylysine conjugates is not unique for the transferrin receptor. Adenovirus can also function to augment delivery into hepatocytes by using both synthetic [(gal)4pL] and natural (AfpL) asialoglycoprotein-receptor ligands. In this hepatocyte line, the liver-specific

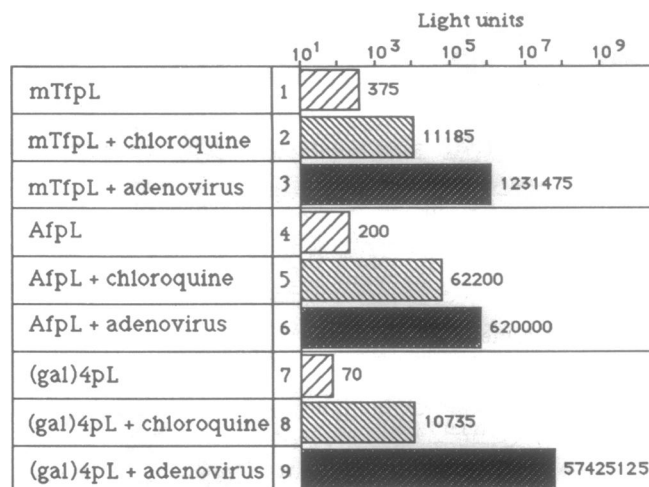


FIG. 3. BNL CL.2 mouse hepatocytes transfected with mTfpL, AfpL, or (gal)4pL alone, with 100 μ M chloroquine or with 10 μ l of adenovirus dl312 containing 5×10^{11} particles per ml.

asialoglycoprotein conjugates function with efficiencies comparable to the transferrin conjugates.

Physical and Chemical Inactivation of Adenovirus Without Disrupting DNA-Delivery Activity. Previous studies have demonstrated that heat-inactivated (45°C) adenovirus dl312 no longer supports DNA delivery (7), consistent with the idea that the endosome-disruption activity is a property of the protein capsid of the virus and, thus, denaturing this protein component interferes with the delivery. The nucleic acid of the virus apparently plays no role in the entry event. Although we are using a deletion mutant of adenovirus reported to be replication defective, at some of the high-input doses of virus this block has a certain leakiness with both early gene expression and viral replication occurring (ref. 11 and our own observations). This leakiness may be accompanied by toxicity, which interferes with the long-term viability of the transfected cells. Therefore, we sought additional physical and chemical methods of inactivating the viral nucleic acid without damaging the endosome-disruption activity of the protein capsid. We initially attempted short-wave UV irradiation, hoping to introduce lesions in the DNA of the virus while sparing the protein components. UV-inactivated adenovirus has been reported to function in augmenting epidermal growth factor-toxin entry into target cells (18).

Preparations of adenovirus were irradiated with 260-nm UV light for various times and then tested for their ability to support DNA delivery (as measured by luciferase-DNA delivery into HeLa cells) and also tested for their ability to replicate on 293 cells. We find that a 300-sec exposure generates a 4-logarithm drop in viral titer that is accompanied by a 1-logarithm drop in the ability of the virus preparation to augment DNA delivery (Fig. 4A). Thus, it is possible to inactivate viral-replication functions without seriously impairing the endosome disruption activity.

We sought a greater decrement in viral titer with less damage to the protein component. The psoralens are a class of small, hydrophobic molecules that can readily diffuse into intact virus particles and intercalate into the nucleic acid. Upon irradiation at 365 nm, the compounds form a covalently crosslinked structure that interferes with both transcription and replication reactions (26). Psoralen treatment followed by 365-nm UV irradiation has proven to be an effective means of inactivating a variety of both enveloped and nonenveloped virus contaminations in various blood protein preparations while generating only a modest inactivation of the active protein component in the preparation (27–29). The DNA in

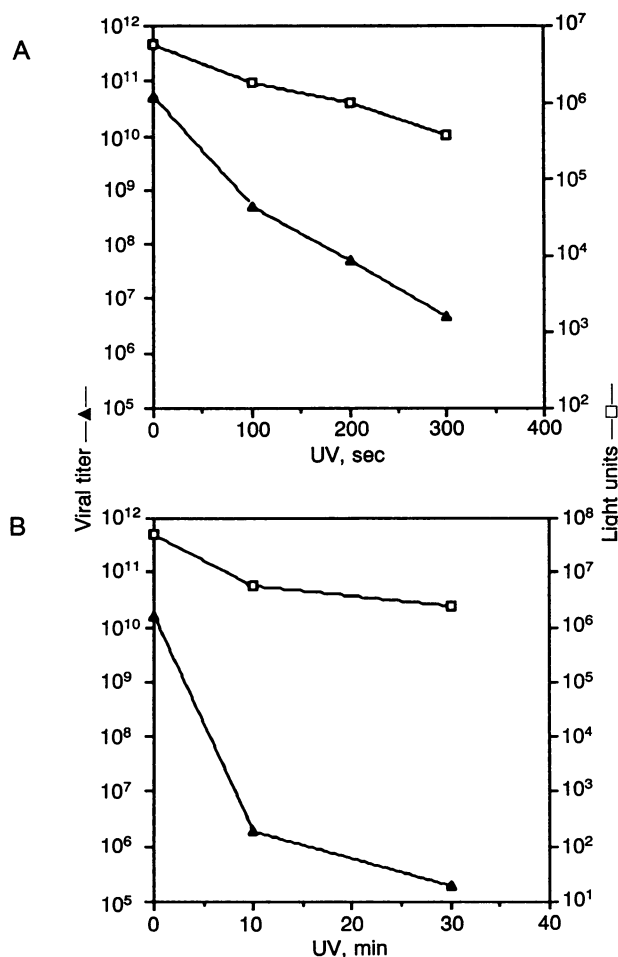


FIG. 4. Virus inactivation with either short-wave UV irradiation (A) or with long-wave UV plus 8-methoxypsoralen treatment (B). In A and B, viral preparations were tested either for their activity in augmenting pCLuc-hTfPL conjugate delivery into HeLa cells by using a virus-to-cell ratio of 1000:1 (as evidenced by the resulting light units of luciferase activity, right ordinates) or for the ability to replicate in 293 cells (viral titer, left ordinates).

intact adenovirus virions has been shown to be accessible to and capable of being crosslinked with psoralen (30).

We treated purified adenoviral samples with 8-methoxypsoralen followed by 10 or 30 min of 365-nm UV light. The unbound 8-methoxypsoralen was then removed by gel filtration, and the adenovirus preparations were assayed for viral titer (by cytopathic effect assay on 293 cells) and for their ability to support luciferase-DNA delivery. We find 8-methoxypsoralen exposure coupled with 10 min of 365-nm UV generates a 4-logarithm drop in virus titer; when 8-methoxypsoralen exposure is coupled with 30-min UV exposure, a >5-logarithm drop is generated, and this is accompanied by only a 2- to 3-fold drop in luciferase-DNA-delivery activity (Fig. 4B).

Delivery of 48-kb DNA. Although the use of recombinant viruses to deliver genes to eukaryotic cells has become routine in many laboratories, these vectors are generally limited in the size and type of DNA sequences that can be carried within the viral genome. The commonly used Moloney retroviral constructs have a size limit in the range of 5 kb (31, 32). Recombinant adenovirus constructs allow delivery of 8 kb of additional sequences (9, 33-35). The parvovirus constructs have a strict 5-kb capacity (36-38).

The size of polycation-condensed DNA particles shows a curious feature, in that the final size of the particle is relatively insensitive to the size of the DNA molecule used

(39-41). Therefore, the delivery of large DNA molecules into cells may be possible with the adenovirus augmentation if we perform the condensation correctly. Certainly we have no clear indication of the size of the lesions generated within the endosome by the adenovirus particle, if, in fact, these are discrete holes and not a general fragmentation of the vesicle. The major limit may be the size of a particle that can be engulfed in a single endocytic invagination; EM studies have shown coated pits to have diameters of 100-200 nm (42, 43).

With this in mind, we have tested the delivery efficiency of a Rous sarcoma virus-luciferase gene present on either an 8-kb circular DNA plasmid or on a 48-kb circular DNA cosmid. We find very little DNA delivery using the transferrin-polylysine system in the absence of adenovirus and in the presence of chloroquine with HeLa cells (Fig. 5A, bar 2), whereas the small plasmid is delivered successfully into these cells (Fig. 5A, bar 1). However, we find that with HeLa cells in the presence of the adenovirus, the resulting luciferase activity (as an indication of productive delivery of DNA into the target cells) is virtually the same for the 8-kb and the 48-kb DNA molecules testing DNA quantities from 6 to 0.5 μ g (Fig. 5A, rows 3-12). With a neuroblastoma cell line GI-ME-M, these difficult-to-transfect cells show very little activity with either a small 12-kb luciferase plasmid or the 48-kb luciferase cosmid when the DNA complexes are delivered alone (Fig. 5B, bars 1 and 2) or in the presence of chloroquine (bars 3 and 4). Again we find that in the presence of adenovirus, high levels of luciferase activity are obtained (Fig. 5B, bars 5 and 6) with a 6-fold difference in activity between the small and the large DNA molecules. Note that these results are expressed in terms of mass of DNA delivered; the cosmid has one luciferase gene per 48 kb, whereas the plasmid has one luciferase gene per 12 kb; therefore, the 6-fold difference is equivalent to a 1.5-fold difference on a molar basis.

DISCUSSION

We have previously demonstrated that the endosome-permeabilization activity of defective-adenovirus particles can be exploited to enhance receptor-mediated gene delivery (7, 8). We have explored here some of the additional features of the adenovirus augmentation. We find that transient transfection efficiencies approaching 100% can be obtained when a chromogenic marker gene is introduced with this technique.

We have explored the variety of receptors that can be cointernalized with the adenovirus receptor and are, thus, amenable to the augmentation effect. We also find that DNA delivery with conjugates that bind the asialoglycoprotein receptor (Fig. 3) and conjugates that bind CD4 can also be enhanced with adenovirus treatment (M.C., unpublished results). Although this is only a test sample of three different receptors, this work suggests that internalized receptors reach a common endosomal pool. The limit will now be the expression of the adenovirus receptor of the target cells. However, additional strategies linking the adenovirus particle to the polylysine-ligand/condensed DNA have been developed (44, 45). These strategies allow adenovirus entry via the specific ligand attached to the polylysine and should, in principle, relieve the need for adenovirus-receptor levels on target cells.

The UV and psoralen-inactivation results further show that the active principle in the endosomolysis is a portion of the virus capsid. Agents specific for DNA disruption can interfere with viral replication without seriously impairing the DNA-delivery capacity of the virus, suggesting that the protein component of the virus triggers the endosome disruption. This gives us hope that we can mimic the endosomolytic properties of the intact virus by using purified viral components and completely dispense with the viral genome.

The delivery of large DNA molecules suggests many applications of this technique. We must caution, however, that the data in this paper demonstrate the successful delivery

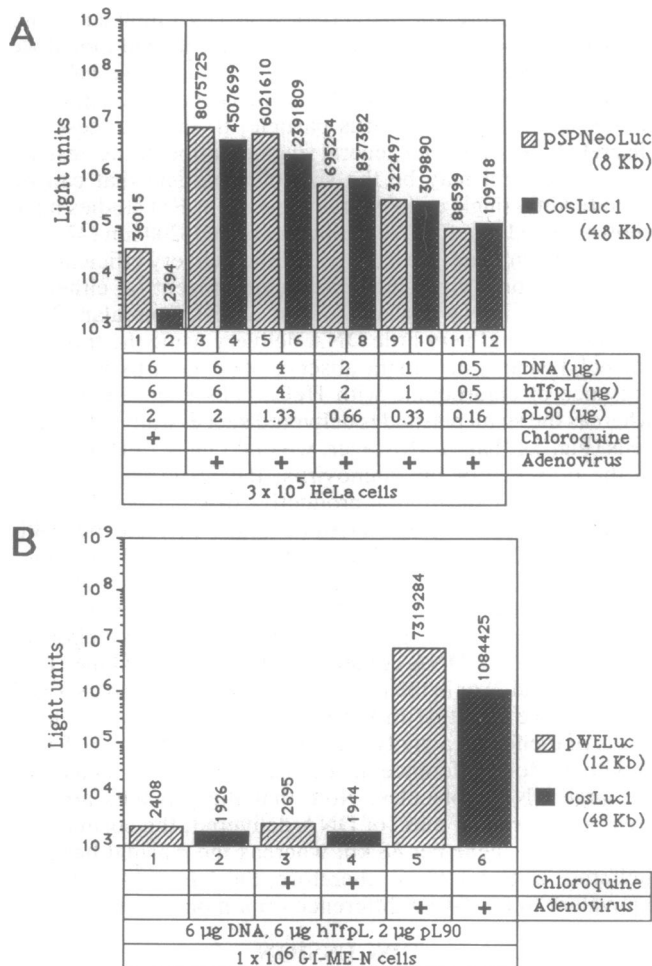


FIG. 5. Delivery of 48-kb DNA. (A) HeLa cells (3×10^5 cells per 6-cm dish) covered with 1 ml of DMEM/2% FCS were incubated with hTfpL/DNA complexes prepared as described, containing the indicated quantities of hTfpL, free polylysine, and DNA. Cell incubation mixtures included, in addition, either 100 µM chloroquine (bars 1 and 2) or 10 µl of adenovirus dl312 containing 5×10^{11} particles per ml (bars 3–12). After a 2-hr incubation at 37°C, 4 ml of DMEM+10%FCS was added to each dish; 24 hr later cells were harvested, and luciferase activity was measured. (B) GI-ME-N cells (1×10^6 cells per 6-cm dish) covered with 1 ml of DMEM/2% FCS were incubated with hTfpL/DNA complexes prepared as described, containing the indicated quantities of hTfpL, free polylysine, and DNA. Cell incubation mixtures included, in addition, either 100 µM chloroquine (bars 3 and 4) or 10 µl of adenovirus dl312 containing 5×10^{11} particles per ml (bars 5 and 6). After a 2-hr incubation at 37°C, 4 ml of DMEM+10%FCS was added to each dish; 24 hr later cells were harvested, and luciferase activity was measured.

of a small gene on a large DNA molecule. It is a slightly different task to deliver a large gene, intact, on a large DNA molecule. Whether fragmentation of the large molecule occurs and to what extent is, of course, an important question if this technique is to be used for gene-mapping studies.

We are grateful to Dr. Andreas Weith for both his reagents and his advice in the construction of the large luciferase DNA molecules. We thank Dr. Martin Zenke and Dr. Andreas Weith for critical reading of the manuscript, and we appreciate the technical help of Helen Kirlappos, Karl Mechtler, Karin Kos, and Mediyha Saltik.

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