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Psoralen Treatment of Adenovirus Particles Eliminates Virus Replication and Transcription While Maintaining the Endosomolytic Activity of the Virus Capsid

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Adenovirus entry into its host cell transiently permeabilizes the cell allowing the coentry of reagents such as DNA. We compare here adenovirus inactivation with β-propiolactone and several psoralen derivatives, seeking reagents that disrupt the viral genome without impairing the viral entry functions. No virus replication can be detected after 8-methoxypsoralen (8-MOP) modification. Viral transcription is not detectable by Northern analysis, and reverse transcriptase/PCR analysis demonstrates at least a 1000-fold decrease in viral transcription after 8-MOP treatment. Using [^{3}H]8-MOP, the psoralen is found to enter the virus capsid and react throughout the viral genome, with approximately one psoralen modification per 100 bp of viral DNA. This inactivated adenovirus allows us to deliver DNA to target cells without interference from adenovirus gene expression or replication. Furthermore, we can now study the host cell response to adenovirus entry without the complications of adenovirus gene expression.

INTRODUCTION

The adenovirus capsid performs several functions. It packages and protects the virus genome against physical shearing and enzymatic damage. It also delivers the genome to the target cell, serving both a ligand function to trigger receptor-mediated endocytosis as well as an endosome disruption function that allows passage of the viral genome through the endosomal membrane into the cytoplasm of the target cell (Greber *et al.*, 1993). The ability of adenovirus particles to enhance the cellular entry of polylysine–ligand packaged DNA is well documented (Curiel *et al.*, 1991; Wagner *et al.*, 1992; Cotten *et al.*, 1992, 1993a; Cristiano *et al.*, 1993a,b; Baatz *et al.*, 1994; Fisher and Wilson, 1994; reviewed in Cotten and Wagner, 1993). The entry enhancement is thought to be a function of the viral capsid; viral gene expression or viral DNA replication is not required for the gene delivery enhancement. The utility and safety of this gene delivery system increases if we ensure the absence of viral gene expression or replication. One simple solution would be to use empty adenovirus capsids devoid of viral DNA. Unfortunately, empty adenovirus capsids have not undergone proteolytic maturation and are inactive for cytoplasmic entry (Defer *et al.*, 1990; M.C. unpublished data). As an alternative to empty capsids, we have studied methods of inactivating the viral genome of mature adenovirus particles while maintaining the protein capsid ligand and endosome-disruption functions.

We have previously reported that 8-methoxypsoralen (8-MOP)/uv treatment of viral particles produces a 5 log decline in the replication ability of adenovirus without disrupting the DNA delivery and cellular entry functions of virus particles (Cotten *et al.*, 1992). We describe here a detailed analysis of this inactivation process, a comparison of several psoralen derivatives (8-MOP, 4′-aminomethyl-4,5′,8-trimethylpsoralen AMT, and 4,5′,8-trimethylpsoralen TMP) as well as a comparison to inactivation by β-propiolactone (βPL, Morgeaux *et al.*, 1993; Shu de *et al.*, 1986; Budowsky and Zalesskaya, 1991). Psoralens are reported to enter a number of different types of viral particles and, upon 365 nm uv irradiation, form covalent inter- and intrastrand adducts with the viral DNA or RNA (Hanson *et al.*, 1978; Hanson, 1992). These adducts block both virus transcription and replication (Hanson, 1992). We find that no replication-competent adenovirus can be detected in 8-MOP or AMT-inactivated preparations. Treatment of adenovirus with βPL generates a decline in virus titer comparable to the inactivation obtained with 8-MOP or AMT. However, a more sensitive plaque assay reveals that following βPL treatment, a small amount of virus genome remains intact and replication competent virus can be detected at a low frequency. Northern analysis of gene expression from the inactivated virus demonstrates that both 8-MOP and βPL block virus gene expression (E1a and E3) to the same extent. Analysis of virus gene expression by reverse transcriptase–polymerase chain reaction (RT-PCR) demonstrates that RNA synthesis from 8-MOP-inactivated virus is inhibited at least 1000-fold relative to noninactivated virus. Labeling adenovirus with [^{3}H]8-MOP shows that

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the psoralen adducts are distributed throughout the virus genome at a frequency of one adduct per 100 bp of DNA.

This development of an entry-competent adenovirus with no detectable transcription and replication provides a valuable tool for studying the mechanism of adenovirus entry. We can now analyze the cellular response to virus receptor binding and internalization in the absence of virus gene expression. Furthermore, this inactive virus is useful for gene delivery applications which require only the virus entry functions.

MATERIALS AND METHODS

Virus procurement

Adenovirus 5 dl1014 (defective for most of the E4 region; Bridge and Ketner, 1989) was grown on the E4-complementing cell line W162 (Weinberg and Ketner, 1983). The E1a-defective adenovirus 5 dl312 (Jones and Shenk, 1979) was grown on the E1 complementing cell line 293 (Graham *et al.*, 1977). Both viruses were purified by CsCl gradient as previously described (Cotten *et al.*, 1994). The purified virus (0.3 to 1 mg/ml protein corresponding to 1 to 3.4 $\times$ 10^{12} virus particles/ml) was biotinylated with NHS-LC–biotin (Wagner *et al.*, 1992) and dialyzed extensively against HBS/40% glycerol before treatment with inactivating agents.

Treatment with 8-MOP

Biotinylated adenovirus preparations (Wagner *et al.*, 1992) were treated with 8-MOP using a modification of previously published methods (Cotten *et al.*, 1992). Virus samples (in HBS/40% glycerol) were placed in four-well tissue cell culture dish (NUNC No. 176740) with 300 μl virus per well. Aliquots of 33 mg/ml 8-MOP (Sigma No. M-3501, dissolved in DMSO) were added to the virus to generate the desired final concentration. The samples were placed on ice (with cover on), 3 cm below the filter of 365-nm light source (UVP model TL-33, 6 $\times$ 15 W bulbs producing 12,000–13,000 μW/cm^2 at 3 cm) and irradiated for 25 min; the plate was repositioned every 10 min to avoid shadows. Unreacted psoralen was removed by gel filtration: the virus/psoralen sample (2 ml) was applied to a Pharmacia PD-10 gel filtration column (preequilibrated with 30 ml of HBS/40% glycerol. The sample was washed into the column with 0.5 ml HBS/40% glycerol and the virus was eluted with HBS/40% glycerol: the first 400 μl was discarded, and the next 4 ml was collected in 0.5-ml fractions. A Bradford protein assay was performed to locate virus fractions, which were pooled, the protein concentration was measured, and the virus was frozen in aliquots at −70°C.

Virus samples were treated with AMT (from HRI Research Inc., Concord, CA), stock solution dissolved in HBS at 5 mg/ml) or TMP (also from HRI, stock solution dissolved in DMSO at 1 mg/ml) exactly as described for 8-MOP, using the concentrations of reagents indicated in Fig. 1.

Treatment with βPL

Virus samples were adjusted to 0.3 *M* HEPES, pH 7.9, before addition of 10$\times$ concentrated βPL solutions. Concentrated βPL solutions were prepared by diluting β-propiolactone (Sigma, No. P5648) with HBS just prior to use. Control experiments were performed to demonstrate that 0.3 *M* HEPES, pH 7.9, was sufficient to buffer βPL treatment at 1%. Aliquots of βPL were added to the buffered virus solutions at room temperature and the virus samples were incubated for 4 hr at room temperature before either storage at −70°C or use in DNA transfection experiments.

Analysis of DNA Delivery

Adenovirus/DNA transfection complexes were prepared as previously described (Wagner *et al.*, 1992). Briefly, 10^{10} particles of either noninactivated, or variously inactivated, biotinylated adenovirus dl1014 was diluted into 150 μl of HBS (20 m*M* HEPES, 150 m*M* NaCl, pH 7.4) and mixed with 150 μl of HBS containing 800 ng of streptavidin–polylysine (Wagner *et al.*, 1992). After 30 min at room temperature, 100 μl of HBS containing 6 μg of the CMV-driven luciferase construct pCLuc DNA was added, after a further 30 min, 100 μl of HBS containing 5 μg of transferrin–polylysine was added (Wagner *et al.*, 1991). The complexes were supplied to K562 cells (500,000 cells, grown in RPMI/10% fetal calf serum (FCS)), Cloudman S91, clone M3 cells (M3) (250,000 cells/25 cm^2 flask, grown in Ham's F-12/5% FCS/5% horse serum (HS)), or primary human skin fibroblasts cultured as previously described (Cotten *et al.*, 1994). After 2 hr, the medium was replaced and 24 hr later the cells were analyzed for luciferase activity (Cotten *et al.*, 1993b). K562 cells were pretreated with desferrioxamine (50 μM) for 18 hr prior to transfection to upregulate transferrin receptors (Cotten *et al.*, 1990).

Analysis of relative viral titer by CPE assay

Cytopathic effect (CPE) assays were performed using W162 cells plated at 50,000 cells/well of 24-well plates. Serial dilutions of samples were prepared in DMEM/2% HS and the diluted virus was supplied to the cells in 500 μl of DMEM/2%. After a 2-hr incubation at 37°C the medium was replaced with fresh DMEM/10% FCS. After 5 days at 37°C the cells were fixed with formaldehyde and stained with crystal violet (Cotten *et al.*, 1993a). A dilution of virus was scored positive for CPE when <20% of the cell monolayer remained at 5 days postinfection.

Plaque assay of adenovirus titer

Plaque assays were performed on W162 cells plated at 500,000 cells per well in six-well plates. After exposing

the cells to virus samples (in 2% HS/DMEM), the cells were overlayed (5 ml per well) with 1% SeaPlaque agarose (low gelling temperature, FMC No. 50103) in DMEM/10% FCS/2.5 m*M* HEPES, pH 7.4. Six days after adding the virus, each well was overlayed with an additional 2–3 ml of overlay solution. Under normal conditions, plaques become apparent at Day 7–10. Plaques were counted at Day 14–18 postinfection.

Gene expression from inactivated virus

A. Northern analysis. RNA Northern analysis of adenovirus gene delivery was performed as described (Paeratakul *et al.,* 1988). Forty-eight hours after a K562 cell transfection with combination complexes containing various noninactivated or inactivated virus preparations, the cells were washed three times in HBS and dilutions representing 10,000 or 3000 cells were applied to a nitrocellulose filter using a 96-well slot blot template. The filter was then processed as described (Paeratakul *et al.,* 1988), hybridized to labeled DNA probes, and processed by standard Northern procedures (Sambrook *et al.,* 1989). The radioactive pattern was visualized by exposure to a phosphoimager screen for 18 hr.

Labeled DNA probes (^{32}P) from the E1A and E3 region were prepared from PCR products of adenovirus dl1014 sequences. The following primers were used for the PCR reactions:

E1a.1:5′ GGA GGC GGT TTC GCA G 3′
(Ad 5: 736–751)

E1a.2: 5′ CAC TTA CTG TAG ACA AAC A 3′
(Ad 5: 1119–1101)

E3.a: 5′ GTC GCC ACC CAA GAT G 3′
(Ad 5: 28722–28737)

E3.b: 5′ GGT ACA GAC CAA AGC G 3′
(Ad 5: 29157–29142) .

Note that the target for the E1A primer is part of the region deleted in adenovirus dl312. Following PCR, the reaction products were purified and labeled using a random primer labeling reaction (Feinberg and Vogelstein, 1984).

B. RT-PCR analysis. RNA purification was performed with 1×10^6 transfected M3 cells harvested 24 hours post-transfection by scraping into PBS and pelleting briefly in an Eppendorf centrifuge. RNA from the cell pellets was purified using Trisolv (Biotecx) following the manufacturer's protocol. Contaminating DNA in RNA samples was removed by digestion for 60 min at 37°C with RNase-free DNase 1 (Boehringer Mannheim). The DNase was subsequently inactivated by further incubation at 95°C for 5 min.

Reverse transcription reactions contained 10 μl of RNA solution, 2 μl of 10 m*M* dNTPs, 4 μl 25 m*M* $MgCl_2$, 2 μl of 10× RT buffer (100 m*M* Tris–HCl, 900 m*M* KCl, pH 8.3), 1 μl of 50 μ*M* oligo (d)$(T)_{16}$, 1 μl of 20 U/μl RNase inhibitor (Perkin Elmer), and 1 μl of MuLV reverse transcriptase (Perkin Elmer). Reverse transcription reactions were carried out for 15 min at 42°C, 45 min 37°C followed by 5 min at 95°C to inactivate the reverse transcriptase. The following primers were used:

E1a up: 5′ GGT CCT GTG TCT GAA CCT GAG 3′
(Ad 5: 1228–1248)

E1a dn: 5′ TTA TGG CCT GGG GCG TTT ACA 3′
(Ad 5: 1545–1526)

E3 up: 5′ GTC GCC ACC CAA GAT GAT TAG G 3′
(Ad 5: 28722–28743)

E3 dn: 5′ GGT ACA GAC CAA AGC GAG CAC 3′
(Ad 5: 29157–29137)

human β-actin up:
5′ TGA AGT CTG ACG TGG ACA TC 3′

human β-actin dn:
5′ ACT CGT CAT ACT CCT GCT TG 3′.

The polymerase chain reactions contained 35 μl of water, 5 μl of 10 PCR buffer (100 m*M* Tris–HCl, pH 8.9, 1 *M* KCl, 15 m*M* $MgCl_2$), 1 μl of 10 m*M* dNTPs, 1 μl of each of the up and dn primers (25 m*M*), 0.5 μl of *Taq* polymerase (5 U/μl, Boehringer Mannheim) and 6.5 μl of the diluted cDNA probes (RNA as control reaction). Samples were denatured for 90 sec at 95°C, followed by 35 cycles of 30 sec 95°C, 60 sec 60°C, 30 sec 72°C, with a final extension for 3 min at 72°C. The amplified DNA products were resolved on a 2% agarose TAE gel and visualized by ethidium bromide staining.

Analysis of [^{3}H]8-MOP binding to the adenovirus genome

Tritiated 8-methoxy psoralen (HRI), 0.8 mCi/ml, 0.41 ml, was dried, dissolved in 20 μl DMSO, and mixed with 8.7 μl of unlabeled 8-MOP (33 μg/μl in DMSO). This 8-MOP mixture was added to 1.8 ml of biotinylated adenovirus dl1014. Ultraviolet irradiation and purification by PD-10 gel exclusion chromatography were performed as described above. The incorporation of [^{3}H]8-MOP was determined by counting aliquots of the purified virus in scintillation fluid.

DNA was purified from [^{3}H]8-MOP-labeled virus by incubating the virus with 0.4% SDS/0.4 mg/ml proteinase K for 45 min at 56°C, followed by phenol/chloroform extraction and isopropanol precipitation. Aliquots of noninactivated adenovirus or [^{3}H]8-MOP-labeled adenovirus DNA were digested with the restriction enzymes *Hin*dIII or *Asp*718, purified, and resolved on a 0.9% agarose/TAE gel in the presence of ethidium bromide (Sambrook *et al.,* 1989). The gel was dried, impregnated with scintilla-

tion fluor (Enhance, Amersham), and exposed to X-ray film.

RESULTS

Gene delivery vs CPE (8-MOP, TMT, AMT, βPL)

We have performed a titration analysis of 8-MOP, AMT and TMP, and βPL inactivation of adenovirus. The assay was designed to detect inactivation methods that do not perturb the virus entry mechanism but block virus gene expression (as measured by the virus preparation's ability to augment luciferase DNA delivery and by CPE assay). Adenovirus dl1014 samples (biotinylated) were treated with the indicated concentrations of 8-MOP, AMT, or TMP, irradiated with 365-nm light, and purified by gel filtration as described above. Alternately, adenovirus was exposed to various concentrations of βPL for 4 hr at room temperature. Aliquots of the treated virus were tested for their ability to delivery a luciferase reporter gene to K562 cells when assembled into complexes with streptavidin–polylysine/DNA/transferrin–polylysine (expressed as the resulting luciferase light units in Fig. 1). The relative titer of each virus preparation was also determined by CPE assay on W162 cells (relative titer in Fig. 1).

Treatment of virus with both 0.11 and 0.33 mg/ml 8-MOP generated a 5-log reduction in titer while DNA delivery activity was maintained at these concentrations. An 8-MOP concentration of 0.033 mg gave only partial adenovirus inactivation. The charged, soluble psoralen derivative AMT generated a 5-log decline in titer by CPE (0.3 and 1 mg/ml AMT; Fig. 1) while maintaining DNA delivery capacity. Lower concentrations of AMT (<0.1 mg/ml) generated only a modest decline in viral titer (Fig. 1). In contrast, a more hydrophobic derivative of psoralen, TMP, did not alter either the DNA delivery activity nor the CPE of adenovirus in the range of aqueous soluble concentrations (Fig. 1) and was not studied in any further detail.

We compared psoralen inactivation to inactivation by βPL (Morgeaux *et al.*, 1993; Shu de *et al.*, 1986; Budowsky and Zalesskaya, 1991). βPL inactivation has the advantage that the inactivation agent is unstable in aqueous solutions so that the removal of unreacted agent is not necessary. Furthermore, the inactivation process requires no uv exposure. Treatment with 0.3% βPL generated a nearly 5-log decline in virus titer, while DNA delivery activity was maintained (Fig. 1); βPL at concentrations of 1% and higher generated an even greater decrease in titer but DNA delivery deteriorated severely (Fig. 1), suggesting that capsid protein modification was occurring at the higher concentrations.

Plaque assay comparison of adenovirus dl1014 inactivation

A more sensitive analysis of the viral replication capacity after chemical inactivation was performed using a plaque assay. The CPE assay used in Fig. 1 measures the ability of chemically inactivated virus to mount an initial cytopathic viral infections but this requires infection of at least 10% of the target population in the 4-day assay period. If 10–50 particles per cell are required to initiate an infection and 50,000 cells are the target population, one can readily detect 50,000 virus particles. The plaque assay, under optimum conditions, can detect a single plaque generated from the entry of 10–50 virus particles, hence, it is approximately 1000-fold more sensitive than the CPE assay.

When the various inactivated virus preparations were tested by plaque assay, no plaques were observed with either the 8-MOP-treated adenovirus (0.33 or 0.11 mg/ml) or the AMT-treated adenovirus (0.28 or 0.83 mg/ml; Table 1). Given the 7 log dilution factor between noninactivated dl1014 (with ca. 7×10^8 PFU/ml) and the psoralen-inactivated samples, this demonstrates that there must be less than 10^2 plaque-forming units present in the psoralen-inactivated samples. In contrast, the various βPL-treated samples all generated detectable plaques, revealing inactivations from ca. 2 logs (0.3% βPL) to 5 logs ($2 \times 0.3\%$ βPL). Although 1% βPL generated a severe drop in DNA delivery capacity (see Fig. 1), it generated only an intermediate drop in viral titer, supporting the idea that the higher βPL concentration favors protein modification over DNA modification and thus interferes more strongly with virus endosome disruption (and hence, virus entry) than with virus replication. The important observation is that βPL-inactivated virus generated plaques at a detectable frequency. Thus, although the βPL inactivation appeared equivalent to the psoralen by CPE assay, the plaque assay revealed a significant difference between the two types of compounds.

Gene expression from inactivated virus

A. Northern analysis. Transfection complexes containing optimal quantities of dl312, dl312/8-MOP-inactivated, dl1014, dl1014/8-MOP-inactivated, and dl1014 βPL-inactivated were prepared, supplied to K562 cells and 48 hours later the cells were harvested for either luciferase activity measurement or subjected to an RNA analysis (see Materials and Methods) for selected adenovirus genes using the pooled material from three separate transfections.

Luciferase activity measurements demonstrated that all transfections yielded high luciferase gene expression within a factor of 3. RNA analysis was performed with either an adenovirus 5 E1A probe that recognizes a portion of the E1A gene that is deleted in adenovirus dl312 (Jones and Shenk, 1979) or an adenovirus 5 E3 probe that recognizes a portion of the E3 message that gives rise to the abundant E3 19K glycoprotein (Wold and Gooding, 1991).

The RNA signal detected by the E1A probe should be absent from the dl312 samples but present in the dl1014

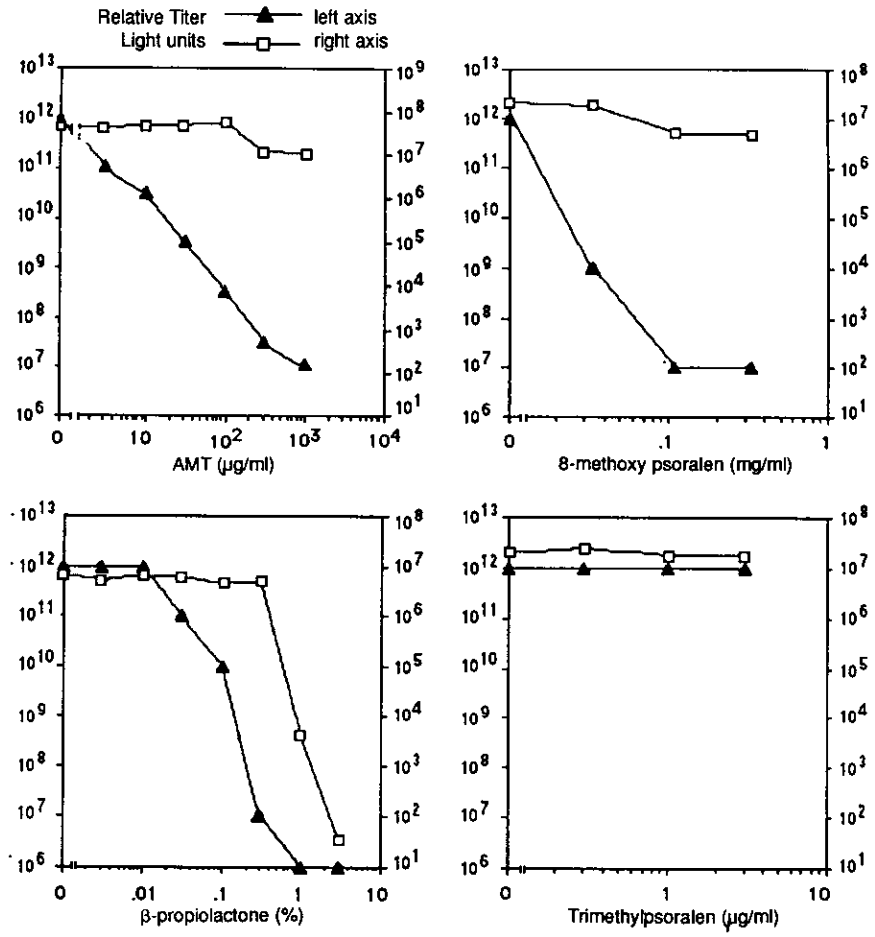

FIG. 1. DNA delivery activity versus viral titer for four different inactivation methods. Preparations of purified, biotinylated adenovirus dl1014 were inactivated with this indicated reagents as described under Materials and Methods. Duplicate transfection complexes were prepared and used to transfect K562 cells and the resulting luciferase activity (as Light units, normalized for protein content) is displayed. In parallel, duplicate CPE assays were performed on 80% confluent monolayers of W162 cells and the decline in titer (relative to noninactivated dl1014) is displayed (Relative titer).

TABLE 1

PLAQUE-FORMING ABILITY OF ADENOVIRUS AFTER INACTIVATION

Virus preparation	Titer (PFU/ml)
Noninactivated (starting virus stock)	6.7×10^8
8-MOP (0.33 μg/ml)	$<1 \times 10^2$
8-MOP (0.11 μg/ml)	$<1 \times 10^2$
AMT (0.83 mg/ml)	$<1 \times 10^2$
AMT (0.28 mg/ml)	$<1 \times 10^2$
βPL (2 × 0.3%)	5.3×10^3
βPL (3 × 0.2%)	8×10^3
βPL (4 × 0.15%)	1.3×10^4
βPL (1%)	3.6×10^5

Note. PFU/ml calculated from the number of plaques obtained (the average of two to four independent dilution series) multiplied by the dilution factor. Only dilution samples yielding less than 30 plaques per 2.5 cm well were used in the calculations. The starting virus was biotinylated adenovirus dl1014 and plaque assays were performed on W162 cells.

samples as this virus possesses a wildtype E1 region. The expression of E3 may play an important role in the immune response to transfected cells, with at least two of the E3 genes modulating surface expression of MHC class I molecules and TNF receptor molecules on the surface of infected cells. Because we are using dl1014 in gene therapy applications to elicit systemic immune responses to tumor cells (Zatloukal *et al.*, 1993), E3 expression in this context might interfere with the immune response to transfected cells.

We found that (as expected) E1A expression is absent from dl312 samples, regardless of the inactivation (Fig. 2). Expression from E1A was abundant in non-inactivated dl1014 but was completely absent in both 8-MOP-inactivated or 2 × 0.3% βPL-inactivated virus (Fig. 2). E3 expression was less abundantly expressed in noninactivated dl312 compared to dl1014. This is consistent with the E1A function as a positive modulator of E3 expression (Nevins, 1991, 1992). Both 8-MOP and βPL inactivation lowered RNA synthesis from these genes to levels that were not detectable by this analysis.

B. RT-PCR analysis. A more sensitive analysis of viral gene expression was performed using reverse transcriptase PCR. Gene delivery into primary human skin fibroblasts was performed with standard adenovirus/StrpI/DNA/TfpL complexes using either noninactivated dl1014 or 8-MOP-inactivated dl1014. Both complexes contained identical quantities of virus and delivered the luciferase reporter gene with efficiencies within one order of magnitude (3.56 million light units with noninactivated virus, 1.32 million light units with psoralen-inactivated virus, a 2.7-fold difference). At 24 hr post-transfection, cells were harvested, RNA was prepared and subjected to reverse transcription, and PCR performed using primers specific for either the E1A or the E3 region.

Using the non-inactivated adenovirus, we observed the expected PCR products from both the E1A and the E3 region. The E1A signal is also detectable at dilution of 1:1000 of the target nucleic acids but not detectable after a 1:10,000 dilution of the target nucleic acid (Fig. 3, top). The less abundant E3 message is detectable after a 1:10 dilution but not after a 1:100 dilution. In contrast, no signal was detectable in the samples derived from the 8-MOP-inactivated virus (Fig. 3, lower panel). The absence of PCR signal with the omission of the reverse transcriptase step confirmed that the signals observed were due to amplification of RNA and not due to a contamination of adenovirus DNA in the RNA preparation. The presence of a β-actin signal in both noninactivated and psoralen-inactivated virus preparations serves as a positive control. The detection of an E1A signal from noninactivatedvirus at a 1:1000 dilution of the starting nucleic acid demonstrates that the 8-MOP inactivation produced at least a 1000-fold decline in adenovirus E3 and E1A expression.

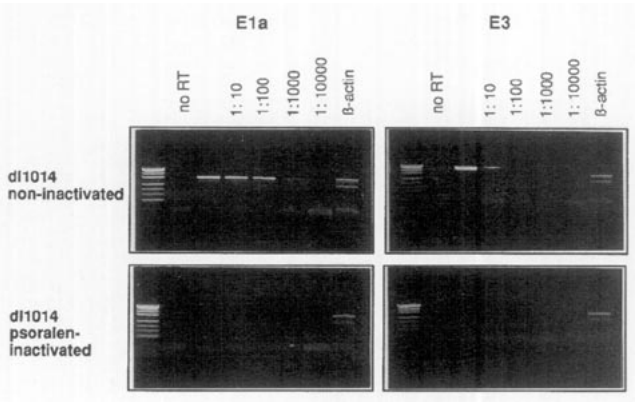

FIG. 3. RT-PCR analysis of virus gene expression before and after 8-MOP inactivation. The RNA from transduced primary human skin fibroblasts was prepared, serially diluted as indicated, and subjected to reverse transcription followed by PCR analysis using primers for either the E1A region, the E3 region, or the human β-actin as positive control. The reaction products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The values 1:10, 1:100, 1:1000, and 1:10,000 indicate the dilution of starting nucleic acid before the PCR reaction.

Analysis of [^{3}H]8-MOP binding to the adenovirus genome

To quantitate the 8-MOP bound to the adenovirus genome under our standard inactivation conditions, we included ^{3}H-labeled 8-MOP in an inactivation reaction (incubation with virus followed by 365 nm light irradiation), purified the virus from unreacted 8-MOP and determined the virus-associated radioactivity. Calculations based on the quantity of radioactivity incorporated into DNA demonstrated approximately one 8-MOP molecule per 100 bp of virus (results not shown). Furthermore, as judged by TLC analysis (silica gel, dichloromethane solvent), no free, unreacted 8-MOP remained in the virus sample (results not shown).

We determined if the 8-MOP cross-links were distributed throughout the viral genome or concentrated at certain accessible sites in the virus DNA. Viral DNA was purified from the virions, cleaved with either *Asp*718 or *Hin*dIII to yield 10 or 11 DNA fragments. The cleaved DNA was resolved on an agarose gel, which was subsequently dried, impregnated with fluor, and exposed to X-ray film. The resulting labeling pattern demonstrated [^{3}H]8-MOP distribution throughout the genome (Fig. 4) although the *Hin*dIII end fragments (HB and HC) appear to have an enhanced labeling.

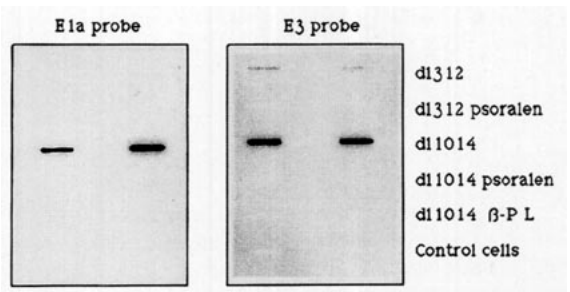

FIG. 2. Northern blot analysis of virus gene expression before and after 8-MOP or βPL inactivation. Transduced K562 cells were transferred to nitrocellulose and processed for Northern analysis using E1A or E3 probes as described under Materials and Methods. Left lanes, material derived from 10,000 cells; right lanes, material derived from 3000 cells.

DISCUSSION

We have analyzed several methods of chemical inactivation of the adenovirus genome to block virus replication and transcription functions. The desired inactivation would yield adenovirus particles that still possess endosomolytic activity useful for delivering DNA (a function of the virus capsid proteins) yet lack virus gene expression or replication which may generate undesired cellular changes.

We find that treatment of adenovirus with 8-MOP and AMT (followed by 25-min 365-nm light irradiation) as well

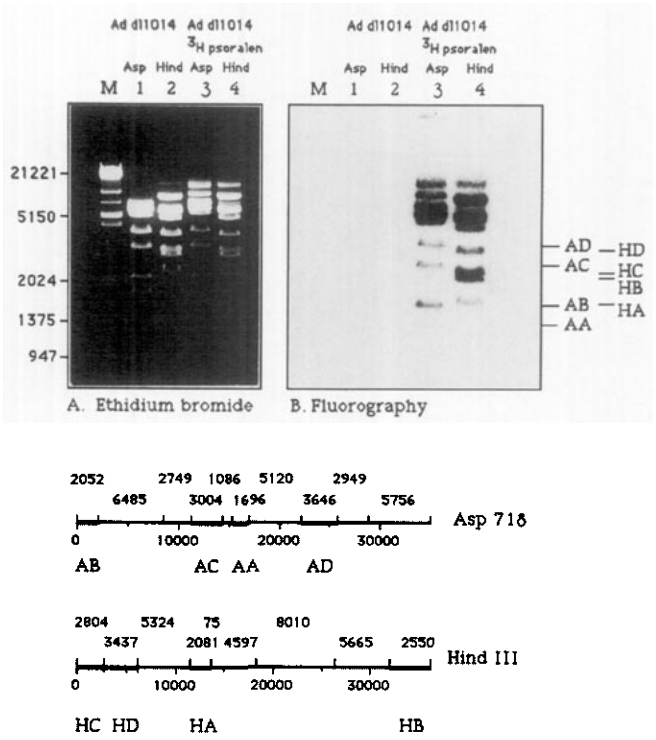

FIG. 4. Restriction analysis and fluorography of DNA from [^{3}H]8-MOP-modified adenovirus. The DNA from [^{3}H]8-MOP-modified adenovirus was purified, cleaved with *Asp*718 or *Hin*dIII, and resolved by electrophoresis through a 1% agarose gel in the presence of ethidium bromide. After photography, the gel was dried on Whatman DEAE paper, the dried gel/paper was impregnated with fluor (Enhance, Amersham) and the radioactive pattern was revealed by exposure to X-ray film at −80°C. The relative sizes and positions of the smaller restriction fragments are indicated. M, *Eco*RI/*Hin*dIII-cut λDNA. Lanes 1 and 2, DNA from control adenovirus dl1014. Lanes 3 and 4, DNA from [^{3}H]8-MOP-modified adenovirus dl1014. *Asp* 718 and *Hin*dIII treatment are indicated.

as treatment with 2 × 0.3% βPL yield virus particles that maintain efficient DNA delivery augmentation. By CPE assay, all three treatments generate a comparable decline in virus titer. However, a more sensitive plaque assay reveals that β-PL-treated virus preparations can replicate when sufficient quantities of virus are applied to the complementing cell line. Treatment of adenovirus with either of the psoralen derivatives completely blocks virus replication with no plaques detectable with this assay.

Transcription from the viral genome is also undetectable by Northern analysis after treatment with either 8-MOP or βPL (Fig. 2). Analysis of transcription by RT-PCR demonstrates a block in viral RNA production with at least a 1000-fold reduction obtained with 8-MOP treatment (Fig. 3).

Fractionation of [^{3}H]8-MOP-inactivated viral particles demonstrates that psoralen–DNA cross-links are formed and restriction analysis of [^{3}H]8-MOP-modified DNA demonstrates that these modifications are distributed throughout the viral genome (Fig. 4), consistent with our failure to detect viral transcription from the E1 and the E3 regions, which are located at distal sites in the viral genome. It is perhaps surprising that psoralen molecules can readily penetrate the viral capsid. One might argue, however, that a small molecule-permeable virus capsid is not subjected to the osmotic and temperature stresses that might disrupt membraned viruses. Furthermore, the major threat to the virus genome integrity might be nuclease attack and the penetration of a nuclease (MW > 10,000) is a far different matter than the penetration of a small psoralen molecule (MW ca. 200).

We have now tested a wide variety of adenovirus inactivation methods. We previously demonstrated that 260-nm uv irradiation provides only a minor decline in viral CPE (Cotten *et al.*, 1992). We have also found formaldehyde inactivation to give excessive damage to the DNA delivery activity and bromouridine incorporation/uv irradiation to give poor inactivation of the adenovirus (unpublished observations). The 8-MOP inactivation described here provides the most secure inactivation. However, for many applications, βPL inactivation is sufficient and the method requires only a simple addition of reagent with no subsequent purification.

Upon further consideration of the adenovirus life cycle, it becomes apparent that certain viral functions that are frequently discarded in recombinant adenoviruses might be useful in modulating the immune reaction or inflammatory response in gene therapy applications (reviewed in Gooding, 1992). We are making transfection complexes (inactivated adenovirus particles linked to polylysine-condensed DNA plasmids) that will provide both the expression of useful adenovirus genes (e.g. E3, Wold and Gooding, 1991; or E1B, Rao *et al.*, 1992; Debbas and White, 1993; or VA1, Mathews and Shenk, 1991) and the expression of therapeutic gene (e.g., Factor VIII, Zatloukal *et al.*, 1994). Our approach is to begin with a transcription and replication-free (*tabula rasa*) adenovirus particle whose only function is to enhance gene delivery. We can then add back the useful adenoviral gene functions in a controlled fashion.

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