JOURNAL OF VIROLOGY, Oct. 1995, p. 6518–6524 Vol. 69, No. 10
0022-538X/95/$04.00+0

Lack of Evidence of Phenotypic Complementation of E1A/E1B-Deleted Adenovirus Type 5 upon Superinfection by Wild-Type Virus in the Cotton Rat

WAHIBA OUALIKENE, PATRICK GONIN, AND MARC ELOIT*

Unité de Génétique Moléculaire, Génétique Virale, Institut National de la Recherche Agronomique, Ecole Nationale Vétérinaire d'Alfort, 94704 Maisons-Alfort, France

Received 27 March 1995/Accepted 19 July 1995

The safety of replication-defective viruses used as vectors is based on the deletion of essential gene(s). Adenovirus vector safety relies on the deletion of the E1A/E1B region. This region encodes the immediate-early proteins that *trans* activate all other early regions, so DNA replication in these deletion mutants is dramatically reduced. We have previously shown that E1A deletion is efficient in vivo and significantly reduces the dissemination of adenovirus in mice and cotton rats. However, the pattern of dissemination of E1A-deleted and wild-type viruses showed that both could be localized in the same tissues, thus involving a theoretical risk of phenotypic complementation if a recipient of E1A-deleted adenovirus is infected after adenovirus-mediated gene therapy by a wild-type adenovirus. In this report, we show that complementation can be evidenced in vitro in Vero cells infected with E1A/E1B-defective adenovirus vectors expressing reporter genes (either β-galactosidase or luciferase), passaged three times until no infectious virus can be recovered by plating on 293 cells, and then infected with wild-type adenovirus 5. A mixed virus population was maintained at a stable state for at least 10 passages. In contrast, no evidence of complementation was found in cotton rats inoculated intravenously or intramuscularly with Ad-β-gal-nls and Ad-luc and infected 24 h later intranasally with wild-type adenovirus 5. No increase in the level of luciferase expression was found in these animals, compared with that in controls, nor was any viral population expressing β-galactosidase or luciferase isolated from various organs or any animal excretion or secretion.

Several DNA viruses, such as adenoviruses, herpesviruses, and parvoviruses, are currently used as vectors for gene therapy or vaccination purposes (for a review, see reference 2). Their genomes are modified in such a way that at least one essential gene is deleted, ensuring the lack of replication of the vector. Nevertheless, there is a lack of information about the behavior of such strains if superinfection by a wild-type (wt) virus should occur, which could provide in *trans* the deleted gene products. As adenoviruses have been used for gene transfer in a broad range of organs, such as the liver (14), neuronal cells (5, 16), blood monocyte-derived macrophages (13), myocardium (12), skeletal muscle (21), and airway epithelium (22), we have chosen to explore such an event in a relevant in vivo model. The biosafety of replication-defective adenoviruses relies on the deletion of the E1A/E1B region. In the first step of investigating the behavior of replication-defective viruses in vivo, we previously studied (18) the dissemination of various deletion mutants (E3-deleted or E3/E1A-deleted viruses) compared with that of the wt human adenovirus type 5 (Ad5) in mice and cotton rats (permissive of Ad5 infection). We demonstrated that E1A deletion reduces the dissemination significantly but showed that several organs, such as lymph nodes, are potential sites for *trans* complementation if superinfection by a wt virus should occur via the nasal route. However, the possibilities of phenotypic complementation remained to be studied. Two marker genes, firefly luciferase and *Escherichia coli* β-galactosidase, were chosen and cloned in an E1A/E1B/E3-deleted Ad5. Firefly luciferase has been used for monitoring promoter activity (17) and is 30- to 1,000-fold more sensitive than the chloramphenicol acetyltransferase assay (7). Van Trung et al. (25) have shown that as little as 0.02 pg of enzyme (250,000 molecules) can be detected in a quantitative assay. *E. coli* β-galactosidase has been widely used as a reporter gene (for a review, see reference 1) and is far less sensitive (limit of detection, 3×10^9 molecules) (23). As a first step, we show in this report that in the case of wt Ad5 infection of Vero cells harboring E1A/E1B-deleted adenovirus DNA in an extrachromosomal state, a mixed population was easily isolated and remained stable through passage. For in vivo assays, the mouse is not a good model as it is either not permissive or only weakly permissive of Ad5. On the contrary, like humans, the cotton rat (*Sigmodon hispidus*) is a species permissive of adenovirus infection (19, 20); therefore, it is a good model for in vivo study of the biosafety of adenovirus-mediated gene therapy in humans. The following two routes for E1A/E1B-deleted virus inoculations were tested: intravenous, because it might be used in gene therapy and to maximize complementation possibilities (better viral spread in the body), and intramuscular, as it is commonly used for gene therapy and vaccination. wt virus was administered by the nasal route, a natural route of infection. In this study, no evidence of the complementation of E1A/E1B-deleted DNA by wt virus was found, as no increases in the amounts of luciferase in a broad range of organs were found. Similarly, no mixed viral populations were isolated from various organs and any animal secretion or excretion.

MATERIALS AND METHODS

Cell lines. The 293 cell line, an adenovirus-transformed human embryonal cell line containing the E1 region of adenovirus integrated into its genome (10), was used for adenovirus transfection, amplification, and titration. These cells were

* Corresponding author. Phone: (1) 43 68 73 34. Fax: (1) 43 96 71 31.

maintained in minimum essential medium (MEM) with 10% fetal calf serum. African green monkey kidney (Vero) cells were used for viral isolation, marker gene expression assays, and titration of organ extracts and nasal swabs. These cells were maintained in Dulbecco MEM with 10% fetal calf serum. For all inoculations of cells, virus or extract was left for 1 h in contact with cells at 37°C in a small volume of medium. Cells were then rinsed and incubated at 37°C.

Plasmids. Plasmid pMLP10CAT has previously been described (3). This plasmid contains the extreme left end of the Ad5 genome (first 455 bp, carrying the inverted terminal repeat, encapsidation signal sequences, and enhancer of the E1A promoter), with the major late promoter of Ad2 (nucleotides 5780 to 6040) joined to its tripartite leader sequence (nucleotides 6041 to 6080, 7100 to 7170, and 9635 to 9725) derived from a cDNA of late adenovirus transcripts. In this plasmid, the chloramphenicol acetyltransferase gene is followed by splicing and polyadenylation signals from simian virus 40. Plasmid pRSVL was described by de Wet et al. (7), together with the firefly luciferase gene (from *Photinus pyralis*), and contains the full-length intronless gene under the control of the Rous sarcoma virus long terminal repeat promoter. Plasmid pCH-nls and details of the β-galactosidase hybrid protein targeted to the nucleus (via the nuclear localization signal) have already been described (4).

Viruses. wt human Ad5 and two recombinant vectors, Ad-β-gal-nls (which contains *E. coli lacZ*) and Ad-luc (which expresses the firefly luciferase gene) were used (see Results for the constructions of recombinants). These recombinants are E3 and E1A/E1B deletion mutants and thus replication defective. Stocks of adenovirus were prepared and titrated by the infection of 293 cells. Cells and media were harvested about 3 days after infection, and virus was released by three cycles of freezing and thawing. Sediments and cellular fragments were then removed by low-speed centrifugation. Recombinant Ad-β-gal-nls and Ad-luc viruses were purified by being banded twice in cesium chloride.

Generation of recombinant adenoviruses. Recombinant adenoviruses were constructed by in vivo homologous recombination in 293 cells between plasmid pMLPA5-β-gal or plasmid pMLPA5-luc and Ad-gp50 (8) genomic DNA. Briefly, Ad-gp50 is an E1A and E3 deletion mutant harboring the gp50 gene of pseudorabies virus and possesses a unique *Cla*I site at bp 914. 293 cells were cotransfected with 5 μg of linearized pMLPA5-β-gal or 5 μg of pMLPA5-luc and 5 μg of the large *Cla*I fragment (2.6 to 100 map units) of Ad5 DNA by calcium phosphate precipitation (11). After being overlaid with agar and incubated for 7 days at 37°C, plaques containing recombinant adenoviruses were picked and screened for nuclear β-galactosidase or luciferase activity. Viruses were cloned twice by plaque isolation under agar, and to ensure that no Ad-gp50 remained, a PCR with primers designed to amplify the gp50 gene was performed on the DNAs of isolates. Recombinant viruses were propagated in 293 cells and purified by cesium chloride density centrifugation.

Animal studies. Cotton rats (*S. hispidus*), 4 weeks of age on the day of inoculation, were obtained from IFFA-CREDO. Intramuscular inoculations were made in the back of the thigh, while intravenous injections were performed in the saphenous vein after epidermal incision, with the skin stitched thereafter. For intravenous inoculations, cotton rats were anesthetized with ketamine. All inoculations were performed after diazepam tranquilization.

Isolation of adenovirus from organs. Various organs (spleens, livers, lungs, kidneys, brains, ilea, lymph nodes, skeletal muscle [thigh], and myocardia) were taken aseptically and divided into two fragments, which were weighed. One was used for viral isolation, and the other was used for monitoring luciferase activity. Fragments for viral isolation were ground in 1 ml of MEM with an Ultra-turrax grinder (1KA Labortechnik; Janke & Kunkel). Blood samples were collected just before sacrifice by retro-orbital puncture, and cells were then pelleted. Erythrocytes were lysed in blood cell lysis buffer (0.32 M sucrose, 10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1% Triton X-100). Nasal washes were obtained by inserting and twirling an Eppendorf yellow tip in each nostril and pipetting several times with MEM (approximately 100 μl). Nasal swabs were sampled and titrated every day for each group of animals maintained for 7 days. Fecal swabs were collected every day from inoculation to sacrifice and were obtained in the same way as nasal swabs were by pipetting in the rectum. Urine samples were collected by inserting a syringe directly into the bladder just after sacrifice. Organ extracts were plated on Vero cells, and viral isolation was then performed in four passages. The first inoculum was 200 μl of organ extract in MEM adjusted to 200 mg/ml (for lymph nodes, 1 mg/ml). For each passage, virus was released from cells by three cycles of freezing and thawing. For nasal swabs, fecal swabs, urine samples, and blood cells, extracts were plated on Vero cells. These cells were lysed 7 days later (with luciferase assay lysis buffer) for luciferase activity assays.

β-Galactosidase cytochemistry. Cell extracts from Vero cells showing a cytopathic effect (CPE) were made by three cycles of freezing and thawing and then used to inoculate Vero cells again. When the CPE developed or 4 to 5 days later if no CPE appeared, cells were rinsed with phosphate-buffered saline (PBS; 150 mM NaCl, 15 mM sodium phosphate [pH 7.3]) and fixed for 5 min in 0.37% formaldehyde–0.2% glutaraldehyde in PBS. Cells were then washed twice with PBS and incubated with the substrate (0.4 mg of X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside] per ml, 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS) for 16 h at 37°C. The X-Gal stock solution was made in dimethyl sulfoxide.

Luciferase assay. For luciferase assays, organs were ground in a liquid nitrogen grind mill and then tested by using the Promega luciferase assay system. Briefly, the organ powder was lysed in 1 ml of lysis buffer (25 mM Tris-phosphate [pH 7.8], 2 mM dithiothreitol 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, 1% Triton X-100), debris was removed by centrifugation, 20 μl of extract was mixed with 100 μl of luciferase assay reagent [20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP (pH 7.8)], and then chemiluminescence was measured in a scintillation counter for 30 s.

TABLE 1. In vitro detection of Ad-luc in phenotypically mixed virus populations[a]

wt Ad5 MOI	Amt of luciferase (pg/10^5 cells) at an Ad-luc MOI of:			
	0.001	0.01	0.1	1
1	0	<1	3.4	220
0.1	0	0	0	2.25
0.01	0	0	0	0
0.001	0	0	0	0

[a] Vero cells (10^5) were inoculated simultaneously with a mixture of Ad-luc and wt Ad5 at different MOI. Five days postinoculation, cells were assayed for firefly luciferase expression.

RESULTS

Generation of recombinant Ad-β-gal-nls and Ad-luc. A recombinant adenovirus expressing the *E. coli* β-galactosidase gene with a nuclear localization signal under the control of the Ad2 major late promoter was generated by homologous recombination. pMLP-β-gal was generated first by cloning the *Hin*dIII-*Bam*HI fragment from pCH-nls which contains the nuclear localization signal-β-galactosidase open reading frame into pMLP10CAT at cohesive ends. The *Bgl*II-*Nru*I fragment (2,984 bp) of Ad5 (nucleotides 3328 to 6306) was then cloned into pMLP-β-gal (*Bam*HI-*Nru*I sites) by using *Bam*HI-*Bgl*II ligation compatibility. pMLPA5-β-gal was thus generated. In this plasmid, the nuclear localization signal (bp 1237 to 1333) is localized just before the *E. coli lacZ* gene. Plasmid pMLPA5-β-gal was linearized by cleavage at the unique *Xmn*I site and cotransfected with Ad-gp50 DNA cleaved by *Cla*I (to remove the first 914 bp and the gp50 gene) in 293 cells. Recombinant Ad-β-gal-nls was isolated from one of the plaques, identified by restriction analysis, and screened for β-galactosidase activity.

Recombinant Ad-luc was generated by homologous recombination as was Ad-β-gal-nls, except that the *Bgl*II-*Xho*I fragment (nucleotides 3322 to 5778 on the Ad5 genome) which had been cloned between the *Bam*HI and *Sal*I sites of pMLP10CAT at cohesive ends by using the ligation compatibility of these sites was used, generating pMLP10CAT-A5 (which will be described in detail elsewhere). The firefly luciferase gene was extracted from pRSVL by *Hin*dIII-*Sma*I digestion and cloned into the *Hin*dIII-*Hpa*I sites of pMLP10CAT-A5, generating pMLPA5-luc.

In vitro *trans* complementation assays. To verify that wt Ad5 can provide *trans* complementation to E1A/E1B-deleted recombinant adenoviruses, non-*trans*-complementing Vero cells were used. We first tested the sensitivities of this cell line (Tables 1 and 2). Vero cells were infected with a mixture of Ad-β-gal-nls or Ad-luc and wt Ad5 at various multiplicities of infection (MOI). At an MOI of 1 PFU per cell or less, no foreign gene activity was evident in the absence of wt virus as a helper virus. β-Galactosidase proved to be the most sensitive marker and was evident when cells were infected at an MOI of 0.01 in the presence of 1 PFU of helper virus per cell. At this concentration of helper virus, a CPE developed rapidly. At lower concentrations of helper virus, the threshold of Ad-β-gal-nls detection was higher. Surprisingly, A549 cells tested in

TABLE 2. In vitro detection of Ad-β-gal-nls in phenotypically mixed virus populations[a]

wt Ad5 MOI	No. of blue nuclei/10^5 cells at an Ad-β-gal-nls MOI of:			
	0.001	0.01	0.1	1
1	0	2	550	950
0.1	0	0	1	100
0.01	0	0	0	5
0.001	0	0	0	0

[a] Vero cells (10^5) were inoculated simultaneously with a mixture of Ad-β-gal-nls and wt Ad5 at different MOI. Five days postinoculation, cells were assayed for β-galactosidase expression (number of blue nuclei).

TABLE 3. In vitro phenotypic complementation[a]

Passage	Luciferase (pg/10^7 cells)	β-Galactosidase (blue nuclei/10^7 cells)
1	44.01	3,025
2	474.99	9,112
3	462.82	11,345
4	3,711.13	20,416
5	7,892.36	26,465
6	3,920.93	24,689
7	3,304.33	25,090
8	687.14	20,359
9	534.52	19,667
10	372.06	19,079

[a] Vero cells inoculated with 200 PFU of Ad-β-gal-nls per cell and 200 PFU of Ad-luc per cell were passaged three times. No infectious virus was recovered in 293 cells at the third passage. Cells from the third passage were inoculated with 10 PFU of wt Ad5 per cell or no virus as a control (Fig. 1). Results of β-galactosidase and luciferase assays on these cells are presented in row 1 (passage 1). To verify the stability of expression and complementation, all extracts were then prepared, inoculated onto Vero cells (10 $TCID_{50}$ per cell), and assayed for β-galactosidase and luciferase expression. This was repeated for nine other passages.

a parallel experiment proved to be less sensitive than Vero cells for the detection of small amounts of enzymes encoded by Ad-β-gal-nls and Ad-luc DNAs (data not shown). To study *trans* complementation, Vero cells were inoculated at the same time with 200 PFU of Ad-β-gal-nls per cell and 200 PFU of Ad-luc per cell and passaged three times until no virus was recovered after inoculation of 293 cells (Fig. 1). The cells from the third passage were either inoculated with wt virus (10 PFU per cell) or not inoculated (as control cells). Cells were harvested 3 days later (after CPE), and three cycles of freezing and thawing were performed. Vero cells were infected with each extract at an MOI of 10 50% tissue culture infective doses ($TCID_{50}$) per cell and tested for β-galactosidase and luciferase activities. The results are summarized in Table 3. While control cells showed no cells with blue nuclei for 10^7 cells, tested cells showed 3,025 blue nuclei for the same number of cells. Luciferase assays showed no activities for control cells compared

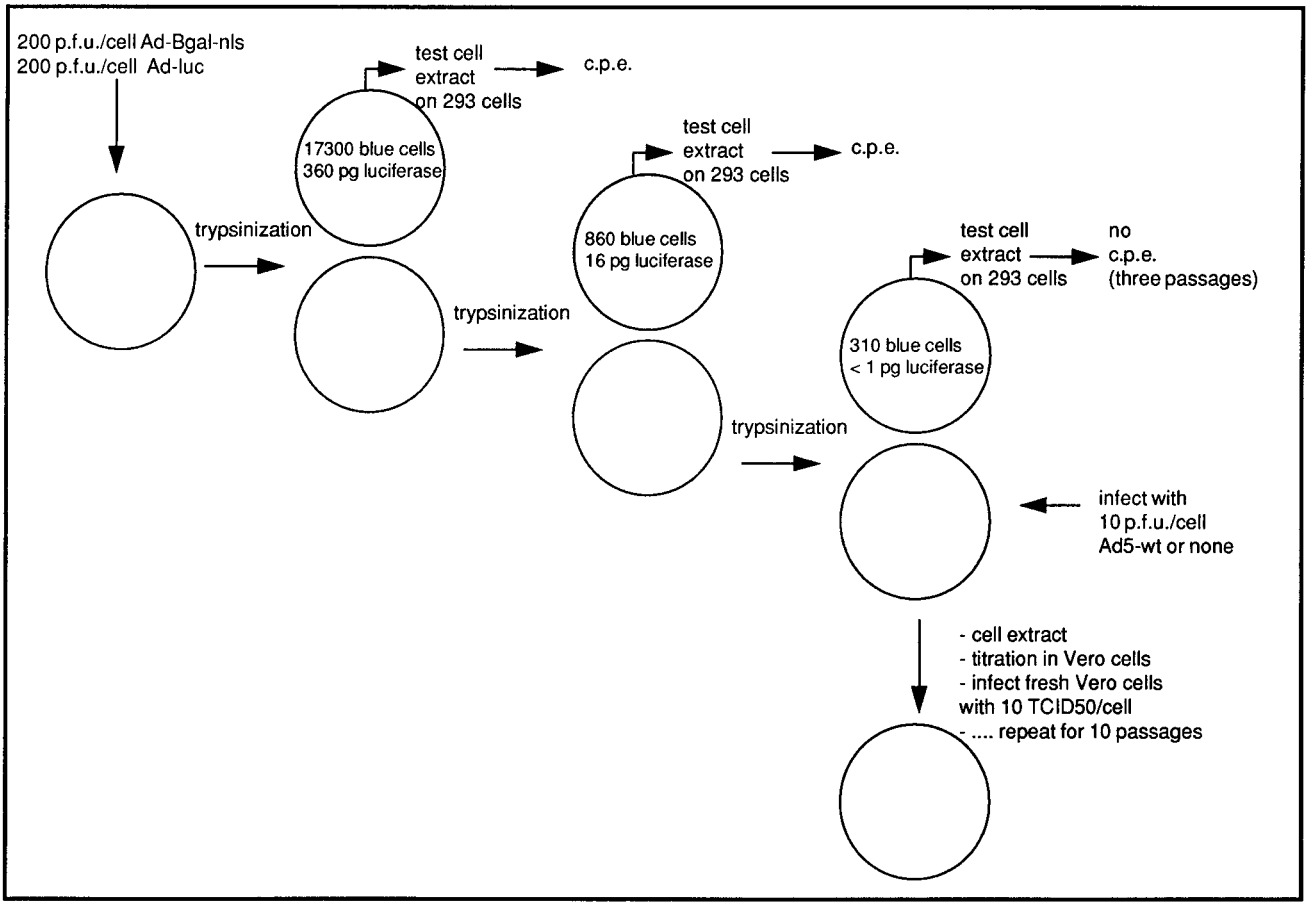

FIG. 1. Protocol used to study in vitro *trans* complementation. The protocol used to eliminate infectious replication-defective virus, together with the results of monitoring the two marker genes for the first three passages, is depicted. The steps after the third passage in the in vitro *trans* complementation assay are summarized in Table 3.

with 44 pg for tested cells. These results show that latent DNA from replication-defective viruses can be efficiently encapsidated upon superinfection of cells by wt virus. This virus population was then passaged 10 times in Vero cells to confirm that the mixed population was maintained at a stable state through passage. To do so, cells were inoculated with 10 $TCID_{50}$/cell of titrated extracts at each passage. *trans* complementation appears to be maintained through 10 passages (Table 3).

In vivo *trans* complementation assays. Twenty-four cotton rats were inoculated and divided into eight lots, each consisting of three animals. On day 0, all animals received a mixture of Ad-β-gal-nls and Ad-luc (10^9 PFU of each purified recombinant [under 100 μl] per animal), one half by the intramuscular route and the other half by the intravenous route. The animals tested for *trans* complementation were inoculated intranasally with 10^8 PFU of Ad5 (50 μl per nostril), while controls did not receive Ad5. wt Ad5 inoculations occurred 24 h after recombinant virus inoculations. Animals were sacrificed on day 3 or day 7, and the luciferase activities in the following organs were checked: livers, lungs, spleens, kidneys, brains, ilea, popliteal nodes, inguinal nodes, myocardia, thigh muscles, and blood cells. Adenovirus isolation in each of these organs was also undertaken, and β-galactosidase activities in infected cells were verified. To study the possibility of virus excretion, we also assayed nasal swabs, urine samples, and fecal swabs. The results of viral isolation and luciferase assays are summarized in Tables 4 and 5. No clear differences between groups of cotton rats (either infected by wt Ad5 or not) were evident. No β-galactosidase activity was evident in any viral population isolated from organ extracts. No infectious virus or luciferase activity was found in fecal swabs either. The results of viral isolation from nasal swabs are presented in Fig. 2. Virus titers, which reached their peaks on the second day after inoculation, were maintained until day 3 and then decreased, reaching titers of from $10^{1.5}$ to $10^{3.2}$ PFU/ml of extract. No β-galactosidase activities were evident in cells infected with these samples.

TABLE 4. In vivo phenotypic complementation 3 days after inoculation with E1A/E1B-deleted virus[a]

Inoculation route	Group	Viral isolation result[b]/amt of luciferase (pg/mg of organ)[c]													
		Liver	Lung	Spleen	Kidney	Brain	Ileum	Popliteal node	Inguinal node	Heart	Thigh muscle	Nasal swab	Leukocytes	Urine	Fecal swab
Intravenous	Tested	−/10.43, +/0.27, +/0.17	4.7/0.95, 4.9/0, 5.3/0.11	2/0.12, 2.1/0, +/0	+/0.15, +/0, −/0	1.7/0, +/0, 2/0	1.5/0, +/0, */0	−/0, +/0, +/0	−/0, +/0, +/0	*/0.18, −/0, −/0	−/0.21, */0, −/0	4.8/0, 5.9/0, 4.5/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0
	Control	−/0.28, −/8.91, −/0.19	−/0.18, −/0.37, −/0.15	−/0, −/0.10, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0
Intramuscular	Tested	+/0, +/0.14, −/0	3.9/0, 5.6/0, 4.2/0	−/0, +/0, −/0	−/0, */0, +/0	+/0, +/0, 1.9/0	*/0, +/0, +/0	−/0, +/0, −/0	+/0, −/0, +/0	−/0, −/0, */0	−/0, −/0, −/0	5.3/0, 4.6/0, 5/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0
	Control	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0

[a] Tested animals ($n = 3$) received recombinant viruses Ad-β-gal-nls and Ad-luc (10^9 PFU of each) either intramuscularly or intravenously and wt Ad5 (10^8 PFU) 24 h later intranasally (tested). Controls ($n = 3$) received the same inoculations except wt Ad5. All animals were sacrificed 3 days after inoculation with recombinants.

[b] If viral isolation was positive at the first passage, the titer is given as the $\log_{10}$ per gram of organ. +, positive at second passage; *, positive at third passage; −, negative isolations (the fourth passage never gave a positive result). For nasal swabs, the titer is given as $\log_{10}$ per ml.

[c] When the results of counting gave levels in the background, they were considered negative and are indicated as 0 pg of luciferase for that extract.

DISCUSSION

By passaging Vero cells infected with replication-defective adenoviruses until no virus was isolated in 293 cells, we have mimicked the state of DNA in the target cells of animals inoculated with these kinds of vectors. We deduce from our results that phenotypic complementation of the E1A and E1B genes occurred after the infection of cells with wt Ad5 and that extrachromosomal DNA can replicate and be encapsidated. This phenotypic complementation was maintained through several passages at high MOI. Although it has not been verified, it is obvious that this complementation occurred in *trans*, because it is unlikely that a homologous recombination preserving the marker gene under the control of the promoter would have occurred. In fact, in our hands, no E1A/E1B$^+$ virus harboring a functional foreign gene ever appeared in 293 cells, despite production of a lot of different recombinant viruses, which seems to be good evidence of the rarity of such an event even at a very high MOI. Therefore, phenotypic complementation seems to occur in *trans* and to require a high MOI of wt virus so that even small quantities of E1A/E1B-deleted DNA can be encapsidated as infectious virions.

The theory and results that we obtained in vitro might have indicated that high risks were to be expected in vivo. Cotton rats were used as a species permissive of human Ad5 to study phenotypic complementation in vivo. The intravenous route was chosen to maximize risks, providing wide and rapid dissemination of the virus. The intramuscular route was also

TABLE 5. In vivo phenotypic complementation 7 days after inoculation with E1A/E1B-deleted virus[a]

Inoculation route	Group	Viral isolation result[b]/amt of luciferase (pg/mg of organ)[c]												
		Liver	Lung	Spleen	Kidney	Brain	Ileum	Popliteal node	Inguinal node	Heart	Thigh muscle	Nasal swab	Leukocytes	Urine	Fecal swab
Intravenous	Tested	−/0.12, −/1.07, −/0.13	1.5/0.15, +/0.34, +/0.11	−/0, −/0, −/0.10	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0.12, −/0.17, −/0.10	−/0.10, −/0.20, −/0	3.2/0, 1.7/0, 3.4/0	−/0, −/0, −0	−/0, −/0, −/0	−/0, −/0, −/0
	Control	−/0.21, −/0.15, −/0.70	−/0.16, −/0.12, −/0.25	−/0, −/0.10, −/0	−/0.11, −/0.12, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0.13	−/0.13, −/0.12, −/0.13	−/0.10, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −0	−/0, −/0, −/0	−/0, −/0, −/0
Intramuscular	Tested	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0.11	−/0.11, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	2.2/0, 1.5/0, 2.1/0	−/0, −/0, −0	−/0, −/0, −/0	−/0, −/0, −/0
	Control	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −/0	−/0, −/0, −0	−/0, −/0, −/0	−/0, −/0, −/0

[a] The conditions for inoculations were the same as those noted in Table 4, footnote *a*, except that all animals were sacrificed 7 days after inoculation with recombinants.
[b] See Table 4, footnote *b*.
[c] See Table 4, footnote *c*.

FIG. 2. Viral isolations on nasal swabs. The route of inoculation with E1A/E1B-deleted virus is indicated (either intramuscular [IM] or intravenous [IV]). All animals were inoculated intranasally with wt virus. Datum points of daily kinetics are decimal logarithms of titers (in PFU/ml). Nasal swabs were sampled every day, and each sample was titrated with Vero cells.

tested as it is the route of choice for certain trials of gene therapy; the cotton rat has proved to be a permissive host by this route of infection (18). To document the biosecurity of replication-defective adenovirus vectors, we decided to inoculate the cotton rats with wt virus just after (24 h) inoculation with the recombinant vector. The risks which may result from prior infection by the wt virus can be precluded by two precautions. First, patients enrolled for gene therapy should be controlled by testing for anti-adenovirus antibodies (to avoid the risk of prior infection). Second, patients should be quarantined before gene therapy to preclude the risks of recent infection before inoculation with the recombinant. On the contrary, wt virus infection after the administration of recombinant virus is a major concern for two reasons. First, the recipient of adenovirus therapy can be infected a short time after treatment via the staff in charge of medical care, in spite of the usual precautions which are taken. This point was addressed in our work by inoculation with wt virus 24 h after inoculation with the recombinant. Second, as recombinant vectors persist a long time (24) in treated patients (as extrachromosomal

DNA), it may be very difficult to isolate them for a sufficient period. This is never done in current protocols of adenovirus-mediated gene therapy. Nevertheless, in this case, a vigorous immune response against Ad5 should develop and the multiplication of the wt virus should be greatly reduced. Thus, it appears that inoculation with the wt virus a short time after inoculation with defective virus represents a worst-case scenario.

To maximize the probability of complementation, inoculations with wt Ad5 were administered 24 h after inoculations with recombinant vectors by the nasal route, a natural route of infection. The Vero cells used for viral isolation were shown to be sensitive enough to detect at least 0.01 PFU of Ad-β-gal-nls per cell in the presence of 1 PFU of wt Ad5 per cell. These cells yielded the same pattern of dissemination in cotton rats as observed for viral isolation on 293 cells (18). The following criteria ensured that no complementation occurred: direct assessments of luciferase activities in organ extracts and indirect viral isolation and β-galactosidase assays of cells inoculated with these extracts. However, as the marker genes used by us are under the control of the major late promoter of Ad2, their expression depends on the tissue-specific preference of this promoter. It was previously demonstrated (6) that the major late promoter permits gene expression (luciferase) in various organs (dermis, epidermis, skeletal muscle, liver, and pancreas), with a notable preference for skeletal muscle. Our results confirm those of Cheng et al. (6); for the cotton rat, which showed stronger luciferase activity, the results for thigh muscle and myocardium were positive (stronger than those for other organs, except livers and lungs, which are among the best blood-irrigated organs). In the two animals with positive responses in luciferase assays, coinfected and control, the route was intravenous. Stronger responses were obtained in the lungs and liver, implying that even if the major late promoter is not the best promoter in these organs, it is sufficient to study in vivo phenotypic complementation. No marker gene was expressed in the excretion products studied, especially those from nasal swabs, from which high titers of infectious wt virus were isolated. This result is particularly important because of the very high titers of wt Ad5 which were isolated from these excretions (Fig. 2). Vero cells infected with swabs collected at the peak of excretion were clearly infected at an MOI of more than 10 $TCID_{50}$/cell, which would have permitted the identification of small amounts of contaminating Ad-β-gal-nls (Table 3). Even under the worst conditions, no β-galactosidase activity was evident, while the marker gene from the defective virus was expressed in lungs (Tables 4 and 5), organs in which Ad5 replicates extensively (20).

Therefore, although complementation is evident in vitro, it was not observed in vivo. In fact, complementation might only occur if the same cell is infected by both E1A/E1B-deleted virus and the wt. This occurs quite easily on a culture plate (in vitro) with high MOI (so that it becomes visible). In vivo, the number of cells which can be coinfected by both viruses is dramatically reduced, because both viruses are not inoculated in situ and because they are diluted in the bloodstream and put in possible contact with a far greater number of cells than they are in vitro. Moreover, after penetration of a cell by the E1A/E1B-deleted virus, replication of the wt 24 (or more) h later is more difficult because of the production of cytokines, such as alpha interferon, following the first infection. Finally, under field conditions, the probability of infection of recipients of adenovirus-mediated gene therapy with wt virus just after E1A/E1B-deleted virus inoculation is low; some days later, an immune response could develop and dramatically reduce the efficiency of cell transduction (15).

From our results, we conclude that even under the worst conditions (wt Ad5 inoculated at a dose of 10^8 PFU intranasally 24 h after intravenous inoculation with the defective virus), phenotypic complementation would not be evident. As we have used only a few animals per assay, this does not mean that such an event is impossible, but only that its frequency is very low. The risk of a recombination between wt virus and an E1/E3-deleted vector generating an E3-deleted replication-competent virus remains. This virus may be more pathogenic than the wt because the E3 region will be deleted (9). However, from our results, if elementary precautions are taken before and during gene therapy, the conditions required for such an occurrence should not exist. Our results may be of general interest in assessing the risks involved in using viruses deleted of essential genes as vectors.

ACKNOWLEDGMENTS

wt Ad5 was kindly provided by T. Ragot (Laboratoire de Génétique des Virus Oncogènes, CNRS UA 1301, IGR, Villejuif, France). We thank Lorna Hovarth and Sophia C. Tenold for revision of the English manuscript.

REFERENCES

1. **Alam, J., and J. L. Cook.** 1990. Reporter genes: application to the study of mammalian gene transcription. Anal. Biochem. **188:**245–254.
2. **Ali, M., N. R. Lemoine, and C. J. A. Ring.** 1994. The use of DNA viruses as vectors for gene therapy. Gene Ther. **1:**367–384.
3. **Ballay, A., M. Levrero, P. Tiollais, and M. Perricaudet.** 1987. A hepatitis B adenovirus recombinant as potential live vaccine. UCLA Symp. Mol. Cell. Biol. **70:**481–493.
4. **Bonnerot, C., D. Rocancourt, P. Briand, G. Grimber, and J. F. Nicolas.** 1987. A beta-galactosidase hybrid protein targeted to nuclei as a marker for developmental studies. Proc. Natl. Acad. Sci. USA **84:**6795–6799.
5. **Caillaud, C., S. Akli, E. Vigne, A. Koulakoff, M. Perricaudet, L. Poenaru, A. Kahn, and Y. Berwald-Netter.** 1993. Adenoviral vector as a gene delivery system into cultured rat neuronal and glial cells. Eur. J. Neurosci. **5:**1287–1291.
6. **Cheng, L., P. R. Ziegelhoffer, and N.-S. Yang.** 1993. *In vivo* promoter activity and transgene expression in mammalian somatic tissues evaluated by using particle bombardment. Proc. Natl. Acad. Sci. USA **90:**4455–4459.
7. **de Wet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7:**725–737.
8. **Eloit, M., P. Gilardi-Hebestreit, B. Toma, and M. Perricaudet.** 1990. Construction of a defective adenovirus vector expressing the pseudorabies virus glycoprotein gp50 and its use as a live vaccine. J. Gen. Virol. **71:**2425–2431.
9. **Ginsberg, H. S., U. Lundholm-Beauchamp, R. L. Horswood, B. Pernis, W. S. M. Wold, R. M. Chanock, and G. A. Prince.** 1989. Role of early region 3 (E3) in pathogenesis of adenovirus disease. Proc. Natl. Acad. Sci. USA **86:**3823–3827.
10. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36:**59–72.
11. **Graham, F. L., and A. J. Van Der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
12. **Guzman, R. J., P. Lemarchand, R. G. Crystal, S. E. Epstein, and T. Finkel.** 1993. Efficient gene transfer into myocardium by direct injection of adenovirus vectors. Circ. Res. **73:**1202–1207.
13. **Haddada, H., M. Lopez, C. Martinache, T. Ragot, M. A. Abina, and M. Perricaudet.** 1993. Efficient adenovirus-mediated gene transfer into human blood monocyte-derived macrophages. Biochem. Biophys. Res. Commun. **195:**1174–1183.
14. **Jaffe, H. A., C. Danel, G. Longenecker, M. Metzger, Y. Setoguchi, M. A. Rosenfeld, T. W. Gant, S. S. Thorgeirsson, L. D. Stratford-Perricaudet, and M. Perricaudet.** 1992. Adenovirus-mediated *in vivo* gene transfer and expression in normal rat liver. Nat. Genet. **1:**372–378.
15. **Kass-Eissler, A., E. Falck-Pedersen, D. H. Elfenbein, M. Alvira, P. M. Buttrick, and L. A. Leinwand.** 1994. The impact of developmental stage, route of administration and the immune system on adenovirus-mediated gene transfer. Gene Ther. **1:**395–402.
16. **Le Gal La Salle, G., J. J. Robert, S. Berrard, V. Ridoux, L. D. Stratford-Perricaudet, M. Perricaudet, and J. Mallet.** 1993. An adenovirus vector for gene transfer into neurons and glia in the brain. Science **259:**988–990.
17. **Mittal, S. K., M. R. McDermott, D. C. Johnson, L. Prevec, and F. L. Graham.** 1993. Monitoring foreign gene expression by a human adenovirus-based vector using the firefly luciferase gene as a reporter. Virus Res. **28:**67–90.

18. **Oualikene, W., P. Gonin, and M. Eloit.** 1994. Short and long term dissemination of deletion mutants of adenovirus in permissive (cotton-rat) and non-permissive (mouse) species. J. Gen. Virol. **75:**2765–2768.
19. **Pacini, D. L., E. J. Dubovi, and W. A. Clyde.** 1974. A new animal model for human respiratory tract disease due to adenovirus. J. Infect. Dis. **150:**92–97.
20. **Prince, G. A., D. D. Porter, A. B. Jenson, R. L. Horswood, R. M. Chanock, and H. S. Ginsberg.** 1993. Pathogenesis of adenovirus type 5 pneumonia in cotton rats (*Sigmodon hispidus*). J. Virol. **67:**101–111.
21. **Ragot, T., N. Vincent, P. Chafey, E. Vigne, H. Gilgenkrantz, D. Couton, J. Cartaud, P. Briand, J. C. Kaplan, and M. Perricaudet.** 1993. Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of mdx mice. Nature (London) **361:**647–650.
22. **Rosenfeld, M. A., K. Yoshimura, B. C. Trapnell, K. Yoneyama, E. R. Rosenthal, W. Dalemans, M. Fukayama, J. Bargon, L. E. Stier, L. Stratford-Perricaudet, M. Perricaudet, W. B. Guggino, A. Pavirani, J.-P. Lecocq, and R. Crystal.** 1992. *In vivo* transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. Cell **68:**143–155.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 16.8. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Stratford-Perricaudet, L. D., M. Levrero, J. F. Chasse, M. Perricaudet, and P. Briand.** 1990. Evaluation of the transfer and expression in mice of an enzyme-encoding gene using a human adenovirus vector. Hum. Gene Ther. **1:**241–256.
25. **Van Trung, N., M. Morange, and O. Bensaude.** 1988. Firefly luciferase luminescence assays using scintillation counters for quantitation in transfected mammalian cells. Anal. Biochem. **171:**404–408.