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A Hybrid Herpesvirus Infectious Vector Based on Epstein-Barr Virus and Herpes Simplex Virus Type 1 for Gene Transfer into Human Cells In Vitro and In Vivo

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We have developed a miniviral vector, pH300, based on the human herpesviruses 1 and 4, herpes simplex virus type 1 (HSV-1), and Epstein-Barr virus (EBV), carrying EBV sequences for plasmid episomal maintenance and HSV-1 sequences for amplification and packaging in multimeric form into HSV-1 capsids in the presence of a helper virus and helper cell line. A reporter gene, the bacterial *lacZ* gene, which expressed β-galactosidase, was inserted into the multiple cloning site of pH300 to make pH300-lac. The packaged pH300-lac DNA was very efficient in infecting human cells in tissue culture. The pH300-lac miniviral stock was used to infect in vitro various human cell types derived from breast cancer, lung cancer, and liver cancer. Up to 95% of cells were infected and expressed β-galactosidase activity after exposure to viral stock at a multiplicity of infection of 3. There was essentially no apparent cytotoxicity after infection of cultured cells in vitro. To test in vivo gene delivery, human liver tumor cells preimplanted subcutaneously in nude mice and injected *in situ* with pH300-lac showed high efficiency of ectopic gene expression. The pH300 miniviral vector is a simple and effective gene transfer system which shows potential for gene therapy of cancer and inherited diseases.

Gene therapy has been considered a revolutionary method for treating genetic diseases, cancer, and infectious diseases, such as AIDS. The method of transfer of foreign genetic material into cultured human cells and/or the human body will play a fundamental role in the gene therapy practice. As current vectors, such as retroviruses, adenoviruses, and vaccinia viruses, are tested in clinical trials, their disadvantages and limitations are becoming apparent (1, 12, 17, 19, 33). Manipulation of an entire virus as a gene transfer vector generally involves recombination of the gene of interest into the viral genome. Such recombinant viruses are rather difficult to handle, and their capacity for insertion of exogenous DNA is relatively limited, from 7.5 kb (adenovirus) to 30 kb (herpes simplex virus type 1 [HSV-1]). Although HSV-1 has a relatively larger insert capacity, current vectors appear cytotoxic as a result of residual viral proteins produced by the virus (11). Helper virus-dependent miniviral vectors whose capacity for insertion is theoretically as large as the size of the original viral genome are being developed (31, 33). These vectors carry *cis*-acting viral elements required for replication and packaging into infectious virions. Since these vectors are defective for viral production, they are dependent on a helper virus to provide the missing viral proteins in trans. A miniviral vector combines the advantages of cloning the transgene in bacteria and virus-mediated high efficiency of gene transfer. The theoretical capacity for large insertion into such a vector offers the possibility to carry large DNA fragments including regulatory genomic elements. Importantly, a miniviral vector can be designed for the desired mode of action by assembling several elements from different viruses, therefore creating a hybrid miniviral vector system. Finally, a helper virus-free packaging system similar to that of other viral vectors (5, 18, 23) could be developed for an HSV-1 amplicon (7a).

The linear double-stranded DNA genome of HSV-1 is 152 kb in length and encodes at least 72 unique proteins (16). The viral genome contains three origins of replication, one within the unique long segment (oriL) and two within the repeats flanking the unique short segment (oriS) (25, 27). An HSV-1 vector has a number of advantages as a gene delivery system. These include a wide host range, the ability to infect nonreplicating cells like neurons in which the vectors can be maintained indefinitely in a latent state (26), and the ability to prepare high-titer viral stocks. By using a plasmid containing an HSV lytic origin of replication and HSV terminal packaging signal sequences, Frenkel et al. (15, 24, 25, 32) demonstrated that such an amplicon was amplified and packaged into infectious HSV virions in the presence of a wild-type helper virus. The virions contained multimeric forms of the original monomeric vector conforming to a rolling-circle replication mode. Since the wild-type helper virus invariably caused cell death due to lytic replication in infected cells, two replication-defective HSV systems have been developed as helper viruses. In a helper virus temperature-sensitive system, the virion stocks were produced at the permissive temperature (31°C). Infection of cells at 37°C allowed miniviral vector delivery to the target cell, whereas the temperature-sensitive mutant helper virus was incapable of entering the lytic cycle and thereby cell death was prevented (8, 9, 28). In another system, an essential immediate-early gene 3 (IE3), encoding the ICP4 protein necessary for early and late viral gene expression and virus replication, had been deleted from the helper viral genome. The miniviral vector DNA was transfected into a helper cell line which expressed a functional IE3 gene for complementation and virion propagation was induced by infecting cells with the IE3 deletion mutant replication-incompetent helper virus (6, 9).

Epstein-Barr virus (EBV) is another member of the human

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herpesvirus family. EBV carries a unique latent replication origin (oriP) which allows viral self-replication in human cells without entering the lytic cycle (14, 21, 36). oriP-based vectors allow episomal replication and maintenance in cells expressing the EBV nuclear antigen EBNA-1, the only virus-encoded transactivator of oriP (37). EBV-based miniviral vectors for delivering large genes to human cells which can be stably maintained in an episomal form in these cells have been developed (3, 29–31). In this study, we present a novel EBV/HSV-based miniviral vector, pH300, allowing high-efficiency *lacZ* gene transfer into various human cells in vitro and in vivo. The vector contains the HSV lytic replication origin, oriS, and an HSV packaging sequence, a, which allow vector replication and packaging in the presence of the IE3 gene-deleted helper virus in an IE3 gene-expressing helper E5 cell line. The latent replication origin, oriP, and the transactivator, EBNA-1, from EBV allow vector episomal maintenance in the E5 cells so that viral stocks of high titers can be made. Effective β-galactosidase expression in infected human cells, particularly in various tumor-derived cell lines, is demonstrated. The efficiency of infection can be as high as 95 to 99% in cultured human fibroblast cells and epithelial cells without apparent cytotoxicity. As an in vivo model for gene transfer, packaged pH300-lac virions were injected into preimplanted human liver tumors in nude mice. Histological 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining showed *lacZ* gene expression in many areas of the injected tumor. The pH300 miniviral vector system should be a useful and efficient DNA delivery system for gene expression studies and gene therapy experiments.

MATERIALS AND METHODS

Construction of pH300 and pH300-lac. pH300 is a 15.6-kb plasmid which was constructed from a combination of several plasmids. The HSV replication origin, oriS, is a *Bam*HI fragment of pT011, kindly provided by N. Stow, Medical Research Council, Glasgow, United Kingdom). The HSV packaging sequence, a, is an *Eco*RI-*Hin*dIII fragment of pT011. The EBNA-1 gene and oriP of EBV, and the Hyg^r gene, which codes for hygromycin B phosphotransferase, are from a BamHI fragment of p500 (34). The backbone of the vector is pCDMV2A (kindly provided by T. Tsukada, Kyoto University, Kyoto, Japan), which includes a cytomegalovirus (CMV) promoter and simian virus 40 (SV40) poly(A) site, elements required for bacteria growth, as an expression cassette consisting of a *Sca*I-*Nru*I fragment from pCDM8 (INVITROGEN); a multiple cloning site and a 1.1-kb *Eco*RI-*Pst*I stuffer fragment are derived from pCH110 (LKB, Pharmacia). pH300-lac was constructed by inserting a *lacZ* gene into the *Hin*dIII and *Not*I sites of the multiple cloning site of pH300. The *lacZ* gene was a *Hin*dIII-*Not*I fragment from pCDMV3-lac kindly provided by H. Takebe, Kyoto University.

Cells and viruses. All cells were from the American Type Culture Collection except where indicated. Cells were grown and maintained in Eagle's minimal essential medium (E-MEM; Gibco BRL) (or in RPMI 1640 where indicated) containing 10% fetal bovine serum (HyClone), glutamine, and penicillin-streptomycin and incubated at 37°C in a humidified 5% CO_2 incubator. E5 is a helper cell line derived from African green monkey cells (Vero) and stably transfected with an IE3 gene of HSV (6) (kindly provided by S. Bachenheimer, University of North Carolina at Chapel Hill [UNC-CH]). The cells were maintained in 400 μg of G-418 (Geneticin; GibcoBRL) per ml. NHF cells are fibroblasts derived from normal skin (kindly provided by W. Kaufmann, UNC-CH). VA13 cells (American Type Culture Collection) were established from human WI38 cells by SV40 transformation. XP4BE cells are SV40-transformed skin fibroblasts derived from a patient with xeroderma pigmentosum, group variant (an inherited DNA repair defect). JML cells were established from tumor cells of a patient with Li-Fraumeni syndrome. GM6914A cells are SV40-transformed skin fibroblasts derived from a patient with Fanconi's anemia. T98G cells are fibroblast-like human glioma cells. SKBR-3, MCF-7, T47D, and DT-20 are all human breast carcinoma cell lines. T47D and SKBR-3 cells were grown and maintained in RPMI 1640. RD cells are spindle-shaped human rhabdomyosarcoma cells (kindly provided by B. Weissman, UNC-CH). SW1271, A498, and HepG2 are human lung, kidney, and liver carcinoma cell lines, respectively. The HSV-1 strain 17$^+$ IE3 deletion mutant D30EBA (20) was kindly provided by P. Johnson, University of California, San Diego. The virus was grown and titers were determined in E5 cells.

Transfection and selection for hygromycin resistance. Transfection of pH300-lac into E5 cells was carried out with Lipofectin as recommended by the manufacturer (GIBCO BRL/Life Technologies, Gaithersburg, Md.). Optimal results were obtained by using 2 μg of plasmid DNA and 10 μg of Lipofectin, each diluted in 1 ml of Opti-MEM (GIBCO BRL/Life Technologies); the components were mixed well and incubated at room temperature for 15 min. The liposome-DNA complex was added to 5×10^5 actively growing E5 cells (washed previously with Opti-MEM) in 2 ml of Opti-MEM in a six-well plate (Falcon, Lincoln Park, N.J.). The Opti-MEM was replaced with complete medium after the cells were incubated for 12 to 15 h at 37°C in a humidified 5% CO_2 incubator. Two days after transfection, the cells were trypsinized and seeded into 10-cm-diameter dishes at 10^6 per dish. The medium was replaced the following day by fresh medium containing 200 μg of hygromycin B (ICN Biomedical, Inc., Aurora, Ohio) per ml to select stable cell transformants.

Packaging and preparation of virion stocks. Hygromycin-resistant colonies carrying the episomal pH300-lac were trypsinized, and 5×10^5 cells were plated onto a 35-mm-diameter dish. At cell confluency, helper virus in 0.5 ml of Opti-MEM was added to the dish at an MOI (multiplicity of infection) of 1. The viruses were allowed to adsorb to the cells for 3 h at 37°C in a humidified 5% CO_2 incubator. Viral solutions were then aspirated, and 3 ml of E-MEM with 10% of fetal bovine serum was added to the cells, which were kept for 3 days at 37°C in a humidified 5% CO_2 incubator. The medium was collected and centrifuged at $1{,}000 \times g$ for 10 min, and the supernatant was used for virus titration and infection. For production of helper virus, helper virus was used at an MOI of only 0.1.

Titration. (i) Helper virus. Viral stocks were diluted in 100 μl of Opti-MEM, and 10^{-3} to 10^{-8} dilutions were used to infect confluent monolayers of E5 cells grown in 24-well plates. The viruses were allowed to adsorb to the cells for 3 h at 37°C in a humidified 5% CO_2 incubator. The virus solutions were aspirated and overlaid with 1 ml of E-MEM containing 5% fetal bovine serum and 1% low-melting-point agarose which was previously equilibrated to 42°C. The low-melting-point agarose medium was allowed to solidify at room temperature and then placed at 37°C in a humidified 5% CO_2 incubator for 3 days. Plaques were then visualized by staining with 0.5 ml of 0.6% crystal violet in 50% ethanol for 5 min. The plates were dried, and plaques were counted. Titers of helper virus were expressed as PFU per milliliter.

(ii) Minivirus. pH300-lac miniviral vector stocks were titered by infection of T98G cells as described above. Assuming that one X-Gal-positive T98G cell represented one infectious pH300-lac virion, the titers were expressed as blue cell-forming units per milliliter.

In vitro and In vivo infection with pH300-lac. For in vitro infection, cultured human cells from different sources, including a number of tumor cell lines (see Table 2), were trypsinized, counted, and seeded at appropriate cell densities. When cells reached confluency, they were infected by defective viral pH300-lac at an MOI of 3 for 3 h. For in vivo studies, female nude mice (Harlan Laboratories) were injected subcutaneously at two symmetrically located sites with 10^7 HepG2 cells suspended in 0.1 ml of phosphate-buffered saline (PBS). When visible tumors approximately 0.5 cm^3 in size developed 1 month later, miniviral pH300-lac was administered to the tumor at one site by a multidirection injection in situ with 0.2 ml of viral solution, approximately 2×10^6 PFU. As a control, the tumor on the other side of the mouse was injected with 0.2 ml of PBS. The β-galactosidase activity was measured 24 h later for both in vitro and in vivo infection as described below.

Assays for β-galactosidase activity. Two assays were used to detect β-galactosidase activity in pH300-lac infected cells. For visualization of virions carrying the *lacZ* gene, infected cells were rinsed with PBS on day 2 following infection. The cells were then fixed for 5 min at room temperature in 2% formaldehyde–0.3% glutaraldehyde in PBS and stained by incubation in a chromophore solution containing 0.1% X-Gal (Promega), 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 5 mM $K_3Fe(CN)_6$, and 2 mM $MgCl_2$ in PBS. Incubation continued at 37°C until color developed (usually within 30 to 60 min). For quantitative measurement of β-galactosidase activity, all cells were trypsinized after 48 h of infection and washed once in PBS solution. Then 10^6 cells were resuspended in an *o*-nitrophenyl-β-D-galactopyranoside (ONPG) lysis solution (0.45 mM ONPG and 0.5% Nonidet P-40 in Hanks balanced salt solution) and incubated at 37°C in a 5% CO_2 humidified incubator, and the optical density at 420 nm was read 1 h later. Expression of β-galactosidase activity in tumor tissue was detected as described previously (4). Briefly, freshly isolated tissue was flash-frozen in isopentane and cooled in liquid nitrogen for the preparation of cryosections. These cryosections (~8 to 10 mm) were fixed briefly with glutaraldehyde and histochemically stained as described for cell monolayers but incubated for 4 to 16 h.

UV irradiation of virus. Stocks of packaged pH300-lac were irradiated with UVC light (254 nm) at various dosages. The effect of UV irradiation on *lacZ* gene expression of pH300-lac, i.e., the β-galactosidase activity, was determined by infection of T98G cells at an MOI of 3. X-Gal staining and measurement of ONPG expression levels were both carried out 24 h later.

Slot blot and DNA hybridization. DNA from packaged pH300-lac and helper virus (D30EBA) was prepared and transferred to a nylon membrane (Magnagraph Nylon; MSI) for slot blot hybridization analysis (2). One million copies of pH300-lac and pGH83, the plasmid carrying the HSV IE110 promoter (kindly provided by S. Bachenheimer), were also loaded as copy number standards. Membranes were hybridized with pH300-lac miniviral vector-specific (hygromycin, *Bgl*II-*Eco*RI fragment, 1,068 bp) and HSV helper virus-specific (IE110 promoter, *Hin*dIII-*Bam*HI fragment, 950 bp) probes. The radioactive signals were exposed and analyzed on a PhosphorImager (Molecular Dynamics). The data were corrected by the fact that the packaged multimeric pH300-lac virions

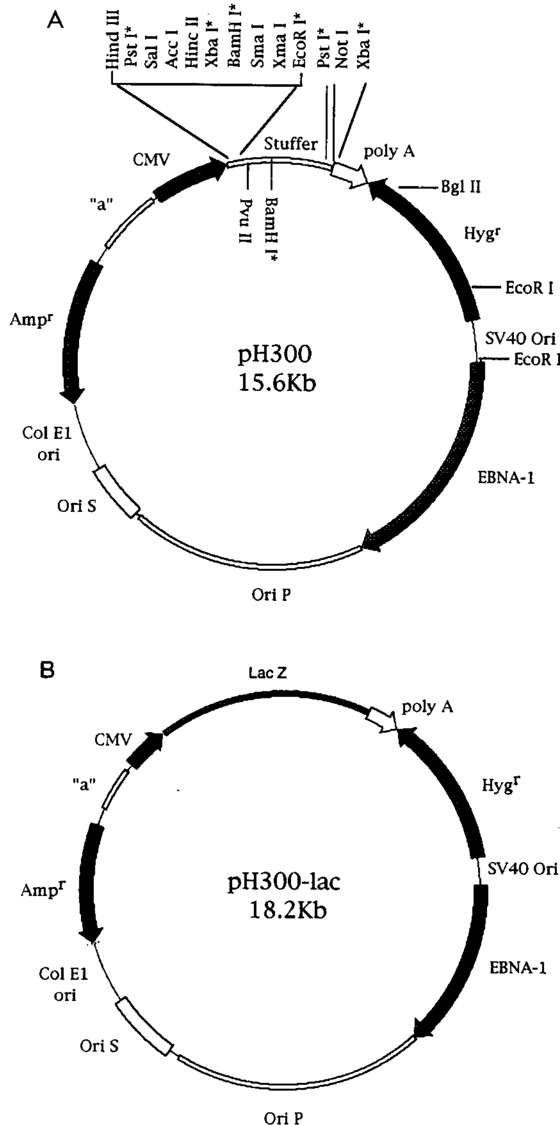

FIG. 1. Structures of pH300 and pH300-lac mini-EBV/HSV-1 vectors. pH300 and pH300-lac have the same structure. pH300 has a multiple cloning site and a stuffer fragment for multipurpose insertion and cloning. pH300-lac contains a constitutively expressed *lacZ* gene inserted at the *Hin*dIII and *Not*I sites of pH300. Ori S, HSV-1 origin of DNA replication. "a," HSV packaging sequence; Ori P, EBV origin of DNA replication; SV40 Ori, SV40 origin of DNA replication; CMV, CMV immediate-early promoter; poly A, SV40 early region poly(A) sequence for termination of transcription; Amp^r, ampicillin resistance gene; Col E1 ori, pBR322 replication origin.

contained about eight copies of pH300-lac plasmid whereas the helper virus contained only two copies of the HSV IE110 promoter.

RESULTS

Strategy for the generation of the hybrid EBV/HSV-1 pH300 and pH300-lac miniviral vectors. The 15.6-kb mini-HSV-1 vector, pH300, is schematically shown in Fig. 1A. pH300 contains the HSV-1 sequences oriS (HSV-1 origin of DNA replication) and a (viral packaging). oriP and EBNA-1 of EBV allow episomal maintenance of pH300 in human cells under hygromycin B selection. The SV40 origin has a bidirectional promoter function, with the early and late promoters driving the Hyg^r and EBNA-1 genes, respectively. The expression cassette consists of a CMV immediate-early promoter and the SV40 early region poly(A) sequence for termination of transcription to drive transgene expression. In addition, a multiple cloning site and a stuffer fragment are included for easy subcloning. The miniviral reporter vector pH300-lac (Fig. 1B) is essentially the same as pH300 except for the insertion of the *lacZ* gene between the *Hin*dIII and *Not*I sites of pH300.

TABLE 1. Efficiency of packaging pH300-lac DNA into HSV-1 particles

Viral stock[a]	pH300-lac titer[b]	D30EBA titer[c]	Ratio[d]
1	3.0×10^6	2.0×10^5	15
2	2.3×10^6	2.0×10^6	1.15
3	2.56×10^6	1.0×10^7	0.256
4	2.56×10^6	8.0×10^6	0.32
5	1.3×10^6	3.0×10^6	0.433
6	4.3×10^6	2.2×10^6	1.95
7	4.1×10^6	1.3×10^7	0.35
8	4.0×10^4	1.0×10^6	0.04
9		2×10^6	
10		4×10^6	
11		3.5×10^6	

[a] Viral stocks consist of a mixed population of pH300-lac and helper virus D30EBA (1 to 8) or helper virus only (9 to 11). Viral stocks 1 to 7 were produced after pH300-lac transfection into E5 cells and selection with hygromycin; viral stock 8 was produced immediately after transfection without hygromycin selection; viral stocks 9 to 11 were produced from original E5 cells.
[b] Titers of pH300-lac miniviral vector stocks were determined by infection of T98G cells as blue cell-forming units per milliliter.
[c] D30EBA (helper virus) titers were determined in E5 cells as PFU per milliliter.
[d] Ratios were obtained by dividing pH300-lac titer by D30EBA titer.

Production of packaged pH300-lac HSV-1 miniviral virions. pH300-lac was transfected into cultured E5 cells and subjected to selection with hygromycin B. After 2 to 3 weeks, stable resistant colonies were replated and infected with the helper virus D30EBA. The virion stocks consisted of a mixed population of miniviral pH300-lac and helper D30EBA. Titers of the produced stocks and ratios of pH300-lac to D30EBA are presented in Table 1. The packaging efficiency of pH300-lac was very reproducible in different preparations, ranging between 2.5×10^6 and 5×10^6 blue cell-forming units/ml. The production of helper virus, however, varied between different stocks, resulting in ratios of packaged pH300-lac to D30EBA ranging from 15 to 0.256. When a pH300-lac viral stock was prepared after transient transfection of pH300-lac into E5 cells without hygromycin selection and after infection with helper virus, less pH300-lac DNA was packaged and the ratio of pH300-lac to helper virus was only 0.04. Such a low ratio is similar to those observed by others by others with HSV amplicon vectors (13). To confirm the packaging of pH300-lac, slot blot hybridization was performed (Fig. 2). This analysis showed that the ratios of pH300-lac to D30EBA viral particles were in the range of 1.98 to 0.165.

Effective gene delivery by infectious pH300-lac into various human cell types. Cultured normal and genetically defective human cells as well as a number of tumor cells from various tissues (Table 2) were infected with miniviral pH300-lac, and β-galactosidase activity was evaluated by X-Gal staining. As illustrated in Fig. 3, NHF cells, normal primary human fibroblasts which are resistant to standard transfection methods, were very efficiently infected (Fig. 3A), as was JML, a tumor cell line derived from a patient with Li-Fraumeni syndrome (Fig. 3B). XP4BE and GM6914A, SV40-transformed human fibroblast lines derived from patients with the DNA repair defect xeroderma pigmentosum variant and Fanconi's anemia,

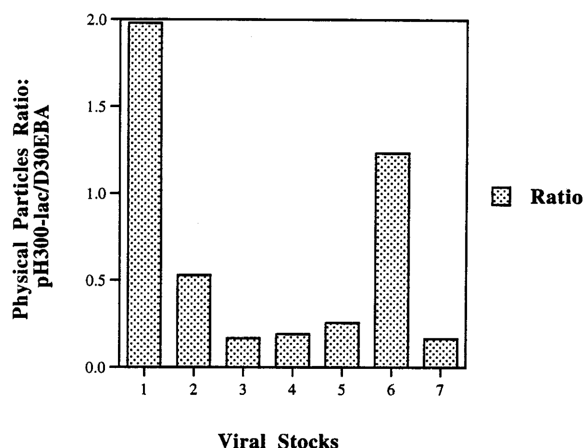

FIG. 2. Slot blot DNA hybridization analysis of the ratio of pH300-lac and helper virus (D30EBA) particles in viral stocks. The pH300-lac-specific probe was a 1.07-kb *Bgl*II-*Eco*RI Hyg^r fragment. The HSV helper-specific probe was the 0.95-kb IE110 promoter *Hin*dIII-*Bam*HI fragment. The radioactive signals were exposed and analyzed on a PhosphorImager (Molecular Dynamics). The ratios were corrected for copy numbers of the probes Hyg^r and IE110 promoter in the packaged multimeric pH300-lac and monomeric HSV-1, respectively.

respectively, were also infected efficiently (Fig. 3C and D). In contrast, maximum transfection efficiencies of 10 to 20% were obtained by lipofection in these cell lines (data not shown). The highest efficiency was obtained with T98G glioblastoma cells (Fig. 3E and F). Figure 4 illustrates the infectivity of pH300-lac on tumor cells derived from different tissues. Tumor cells from breast (T47D, SKBR-3, MCF-7, and DT-20), lung (SW1271), and liver (HepG2) carcinomas were efficiently infected at an MOI of 3 (Fig. 4), while cells from muscle (RD) and kidney (A498) tumors were less efficiently infected (not shown). The results are summarized in Table 2. Most of the cells were efficiently infected in vitro by pH300-lac at an MOI of 3. Therefore, the pH300 miniviral vector can deliver and express transgenes such as *lacZ* with high efficiency in various human cell types.

To demonstrate de novo expression from the mini-EBV/HSV-1 virus, pH300-lac virions were irradiated with various doses of UVC (254 nm) and then used to infect T98G cells. X-Gal staining 24 h after infection showed that the number of X-Gal-positive cells was decreased in response to the UVC doses (data not shown). ONPG measurements taken 1 week after infection showed decreasing *lacZ* expression with increasing UVC doses (Fig. 5). The dose of UVC irradiation needed to reduce *lacZ* expression to 37% (1 lethal hit) was approximately 160 J/M^2. About 1 to 2% X-Gal staining in T98G cells was observed at 4 weeks following infection at an MOI of 3. ONPG measurements of pH300-lac-transduced T98G cells at this MOI showed a peak of *lacZ* gene expression 3 days postinfection, with a continuous decline of ONPG levels thereafter. ONPG levels above that of uninfected controls were obtained up to 5 weeks following infection (data not shown). In these experiments, the transduced cells were cultured in the absence of selection and split at a ratio of 1:4 when confluent. The packaged pH300-lacZ expressed the *lacZ* gene after infection of target cells. Trypan blue staining did not reveal any apparent cytotoxicity in infected cells, even at an MOI of 3. Hence, such infected cells could be easily grown in large quantities for further analysis.

Functional gene delivery in vivo by pH300-lacZ virus into human tumor cells. Infection and expression of pH300-lac in vivo were evaluated in animals (female nude mice from Harlan Laboratories) by in situ injection of pH300-lac virions into HepG2 tumors that were preimplanted by subcutaneous injection (Fig. 6). In vivo infection and expression of pH300-lac was demonstrated by X-Gal staining 24 h after injection of the virus into the tumor tissue. Cells prepared from freshly isolated tumor tissue stained blue, indicating that the *lacZ* gene was delivered to and expressed β-galactosidase in these tumor cells. Among the randomly prepared cryosections, 25% fully expressed *lacZ*, with all areas showing blue staining (Fig. 6A and B); 38% exhibited partial expression, with most areas showing blue staining (Fig. 6C and D); 15% exhibited less expression (Fig. 6E and F), and about 22% of the cryosections showed only scattered blue staining. In contrast, in cryosections prepared from mock-injected control tissue on the same animal, blue staining was not detectable (Fig. 6G and H).

DISCUSSION

An infectious hybrid EBV/HSV-based miniviral vector, pH300, was constructed and used to deliver a reporter gene, *lacZ*, into human cells both in vitro and in vivo. pH300 is capable of high efficient gene transfer and expression in a variety of human cells, both in vitro and in vivo. It also carries

TABLE 2. Infection and expression of pH300-lac in human cells

Cell line	Tissue source	Morphology	Pathology	% X-Gal-positive cells[a]
NHF	Skin	Fibroblast	Normal	90–95
VA13	Lung	Epithelial-like	SV40 transformed	≥95
JML	Skin	Fibroblast	Li-Fraumeni syndrome	90–95
XP4BE	Skin	Fibroblast	DNA repair defect	≥95
GM6914A	Skin	Fibroblast	Fanconi's anemia	80–90
T98G	Nerve	Fibroblast	Glioblastoma	≥95
T47D	Breast	Epithelial	Carcinoma	≥95
SKBR-3	Breast	Epithelial	Carcinoma	≥95
MCF-7	Breast	Epithelial	Carcinoma	80–90
DT-20	Breast	Epithelial	Carcinoma	≥95
RD	Embryonal	Spindle-like	Rhabdomyosarcoma	50–55
SW1271	Lung	Epithelial	Carcinoma	80–90
A498	Kidney	Epithelial	Carcinoma	50–60
HepG2	Liver	Epithelial	Carcinoma	≥95

[a] Cells were infected with pH300-lac at an MOI of 3, and X-Gal staining was carried out 24 h later. Fractions of X-Gal-positive cells were evaluated in at least two independent experiments.

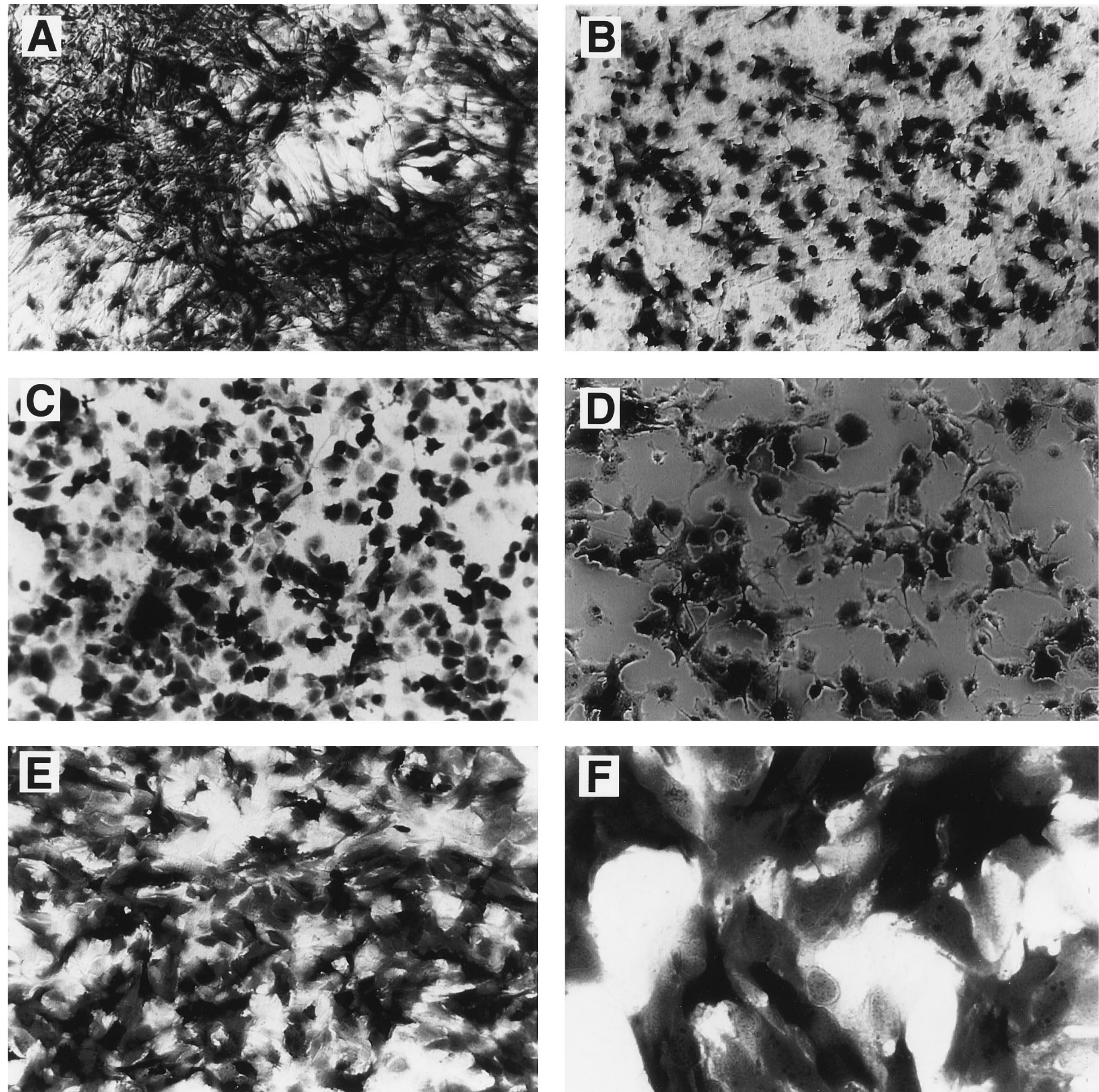

FIG. 3. X-Gal staining of human cells infected in vitro with pH300-lac virions. Cells were infected at an MOI of 3 for 3 h and stained with X-Gal 24 h later. (A) NHF (normal fibroblasts), magnification, ×60; (B) JML (Li-Fraumeni syndrome), magnification, ×60; (C) XP4BE (xeroderma pigmentosum variant), magnification, ×63; (D) GM6914A (Fanconi's anemia), magnification ×95; (E and F) T98G (glioblastoma), magnifications, ×95 and ×304, respectively.

a bacterial plasmid backbone for easy manipulation and amplification in prokaryotic cells.

Latent episomes as a strategy for efficient packaging. One notable characteristic of pH300 is that it contains the latent EBV replication origin, oriP, and its transactivator, EBNA-1. These two elements make the pH300 vector capable of self-replication and mitotic segregation in an episomal form in dividing human cells (22, 29, 35, 36). Episomal maintenance of the vector is an essential step for effective vector replication and packaging into HSV capsids in the presence of HSV-1 helper virus. In addition, the Hyg^r gene ensures that every helper cell contains the miniviral vector and that high titers of viral stock can be made. Under hygromycin selection, helper cells maintained episomal pH300-lac very stably, and pH300-lac virions could be produced from the helper cells for at least 6 months in culture (unpublished observations). By contrast, the HSV-1 amplicon could not be episomally maintained in helper cells for extended periods of time, and viral stocks had to be produced immediately after transfection (8, 10, 15, 24, 25, 28, 32). Because of low transfection efficiency, only a fraction of cells in the transiently transfected population contained the amplicon. Since the ratio of amplicon to helper virus was very low in the initial passage, higher ratios of viral stocks could be obtained only by successive propagation at a higher MOI for at

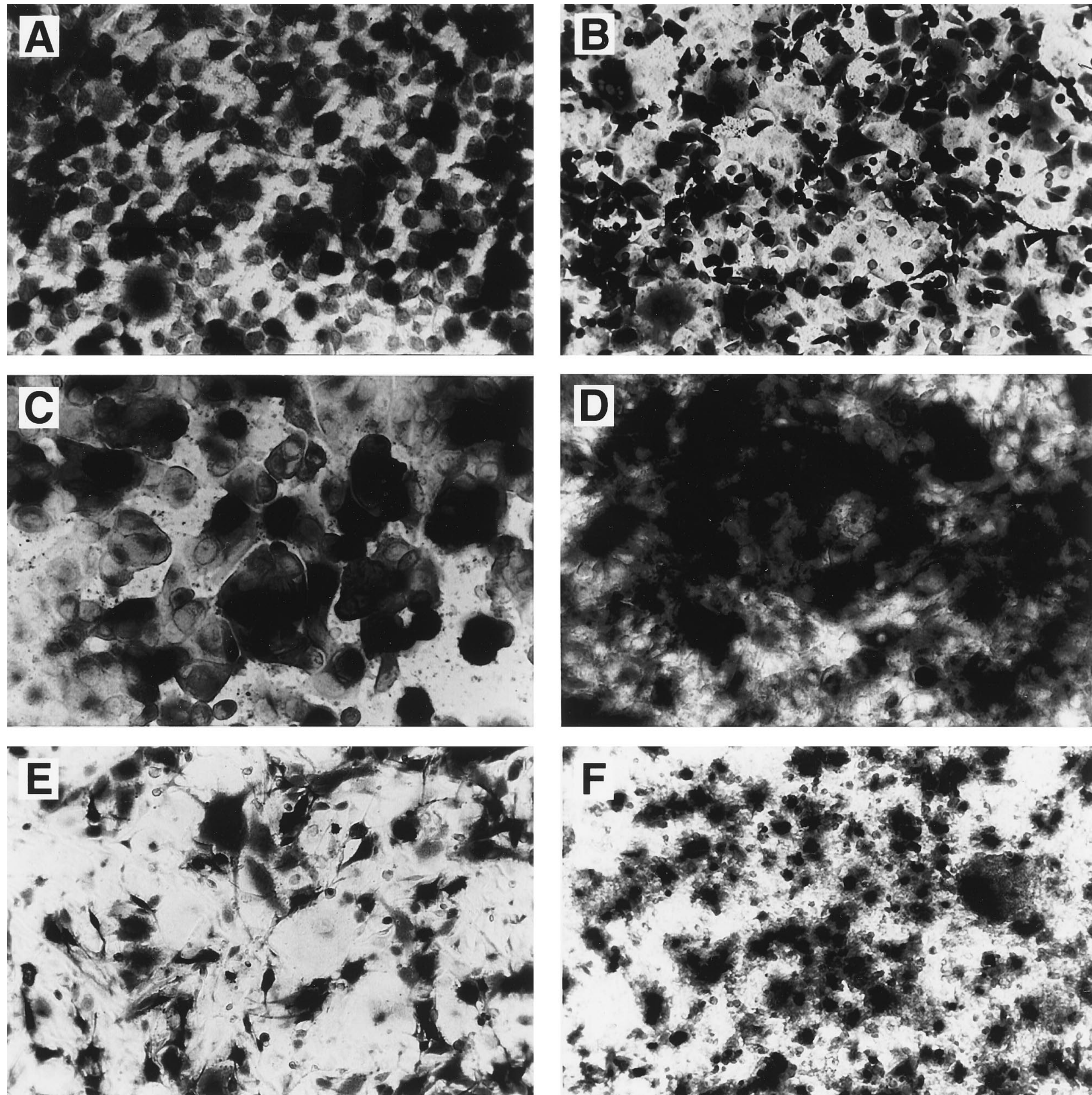

FIG. 4. X-Gal staining of human tumor cell lines infected with mini pH300-lac virions. Cells were infected at an MOI of 3 for 3 h and stained with X-Gal 24 h later. (A) T47D (breast cancer), magnification, ×60; (B) SKBR-3 (breast cancer), magnification, ×60; (C) MCF-7 (breast cancer), magnification, ×190; (D) DT-20 (breast cancer), magnification, ×190; (E) SW1271 (lung cancer), magnification, ×60; (F) HepG2 (liver cancer), magnification, ×60.

least five to eight passages (8, 13, 24). However, what limits the use of this strategy is the apparent difficulty of reproducing such a high ratio of amplicon to helper virus (7). In accordance with these results, the ratio of miniviral pH300-lac vector to helper virus after a single passage of viral stock propagated in helper cells without hygromycin selection was only about 4% (Table 1). In contrast, slot blot hybridization analysis showed that the pH300-lac/D30EBA ratios were in the range of 1.98 to 0.165 (Fig. 2), while biological titration indicated ratios of packaged pH300-lac to D30EBA varying between 15 and 0.256 (Table 1). A lower ratio of pH300-lac to helper by biological titration than by molecular hybridization analysis may be due to the fact that the lysis plaque assay requires multiple rounds of viral cycles in E5 cells, while the *lacZ*-based assay relies on a single infection cycle. Titers of pH300-lac virions, however, are reproducible because expression of the *lacZ* gene is more direct than viral titration based on lysis plaques.

Hybrid mini-EBV/HSV-1 for reduced helper interference. The pH300-lac is defective for viral production for both EBV and HSV-1 and is thus dependent on a helper virus to provide all needed viral proteins in *trans*. The helper HSV-1 (D30EBA) used for pH300-lac production is also replication defective and incapable of lytic infection as a result of a deletion in both copies of the IE 3 gene. Viral functions of IE3 are

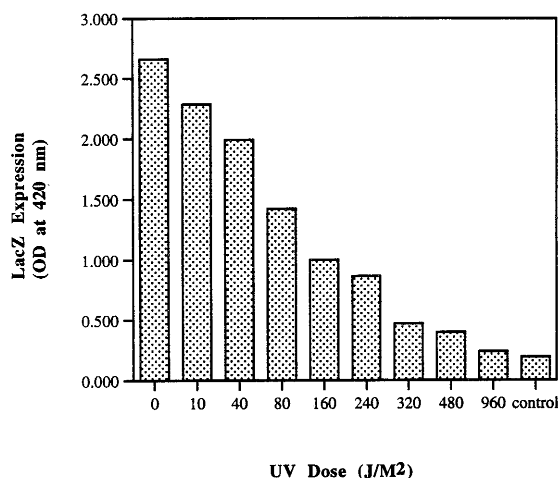

FIG. 5. Effect of UVC on infection and expression of pH300-lac in cultured human glioblastoma T98G cells. The viral stock was irradiated with various doses of UVC as indicated and then used to infect T98G cells. The β-galactosidase activity of miniviral pH300-lac was determined 1 week later by the measurement of optical density (OD) at 420 nm. Each bar represents the mean value of three independent determinations of ONPG.

essential for early gene expression, viral DNA replication, late gene expression, and thus the production of progeny virus (6). Consequently, the helper virus can be replicated and propagated only through the lytic cycle in helper cells such as E5 expressing the IE3 gene through complementation. Human cells do not contain a homologous IE3 gene and are therefore not permissive for HSV helper virus replication. However, the regeneration of wild-type HSV-1 through recombination between the helper viral genome and the chromosomally integrated complementing viral genes during in vitro passage has been reported (11). In contrast, we have not been able to detect any rescued wild-type virus in our experiments (data not shown). Such observation may be due to a combination of the low frequency of such recombination events (10^{-6} to 10^{-7} in reference 11) and the usage of a single short-term infection cycles at low MOI of helper for making viral stocks (instead of multiple repetitive passages at high MOI of helper). As additional safety precautions, several strategies can be envisioned to reduce the probability of recombination in vitro or in vivo, such as the engineering of a cell line expressing multiple viral genes codeleted from a helper virus (10a) or the development of complementing viral subgenomes to passage a fragmented helper virus (7a). Our observations indicate that there were no apparent cytopathic effects after infection of human cells with pH300-lac virions in vitro. In a recent study, Johnson et al. (11) detected cytotoxicity in cells infected with a replication-defective mutant (IE3 deletion) of HSV-1, CgalD3. In confirmation, the helper virus only (D30EBA) caused cell death 3 days after infection (unpublished observations). The miniviral system pH300 also carries helper virus in viral stocks. Specifically, the pH300-lac viral stocks consisted of an average vector/helper ratio of 0.74 (Table 1, viral stocks 2 to 7), with the noticeable exception of a ratio of 15.0 for viral stock 1. In contrast, a ratio of 0.04 was observed when the traditional method of transient transfection of the amplicon was used (viral stock 8). Hence, the presence of helper in the pH300 system was reduced by ca. 18-fold (0.74/0.04). Such a lower amount of helper virus should help reduce the cytopathic effects observed after infection with HSV-1 recombinants or previous HSV amplicons. In addition, the lower amount of helper virus could also diminish potential immune reactions to viral components in vivo. However, the usage of helper HSV to generate pH300 viral stocks still presents the disadvantage of some helper contamination, which could preserve some virus-induced *in vivo* cytotoxicity. Such a potential cytotoxicity of HSV is a major concern, which could be reduced if not completely eliminated through the development of helper virus-free packaging systems for HSV vectors.

Multimeric packaging for efficient gene expression. The EBV/HSV-1 hybrid vector pH300 is capable of infecting a variety of types of human cells, both in vitro and in vivo (Fig. 3 to 5). The β-galactosidase activity detected after infection of pH300-lac may not necessarily derive from de novo expression of the transduced *lacZ* gene, since the β-galactosidase protein may be encapsidated into helper and/or defective virions during the packaging process. To exclude such a possibility, we examined the effect of UVC irradiation on β-galactosidase activity after infection. As shown in Fig. 6, β-galactosidase activity of miniviral pH300-lac was inhibited by UV irradiation in proportion to the dose (Fig. 6), demonstrating that β-galactosidase activity of the *lacZ* gene was indeed conferred by the transduced pH300-lac DNA and due to de novo expression. Because the pH300-lac DNA is packaged into HSV particles, its tropism of infection is probably as wide as that of the wild-type HSV-1. We have also successfully infected primary mouse liver cells with pH300-lac and achieved about 95% X-Gal-stained cells (34a). Such a result indicates a host range wider than human cells. However, episomal replication of the vector is not expected to occur in rodent cells since the oriP/EBNA-1 replication system from EBV is limited essentially to human and primate cells. For certain types of human cells such as RD and A498, derived from muscle and kidney, respectively, infection efficiency was somewhat reduced. This could be due to a lower number of HSV-1 receptors on the membranes of those cells. Alternatively, such cell types may not sustain efficient expression from the CMV immediate-early promoter. Our data indicated that *lacZ* gene expression generally lasted for approximately 2 weeks in the majority of human cell lines tested. In T98G cells, β-galactosidase activity could be detected for up to 5 weeks after in vitro infection (data not shown). Such a decrease of β-galactosidase activity as a function of time in infected cells might also be due to promoter inactivation or, alternatively, to vector loss in the actively growing cells. The very intense staining of cells infected both in vitro and in vivo indicates that the CMV promoter was very active. Because of the smaller size of the pH300-lac and its lytic replication via a rolling circle, a mini-HSV-1 vector is packaged as linear multimeric concatemers consisting of identical head-to-tail repeat units (24). Hence, the number of multimers in a virion is dictated by the size ratio of the monomeric vector to the overall 152 kb of HSV-1 DNA (33). Such a multimeric structure may also contribute to high expression levels per cell. By using pulsed-field gel electrophoresis and Southern blot methods, Sun and Vos (31) measured a packaging size range of 150 to 200 kb for a multimeric mini-EBV, another member of the human herpesvirus family. Hence, the pH300-lac vector is expected to be packaged as 8-mers into HSV-1 viral particles. The maximum size of insert DNA that can be packaged into mini-HSV-1 viral capsids is not known. Theoretically, insertion of nonviral sequences into mini-HSV-1 as a monomeric vector may reach the total size of the viral genome. In the case of pH300, the theoretical maximum insertion size of foreign DNA might be as large as 130 to 140 kb.

Effective in vivo delivery in tumors as a potential tool for gene therapy. Successful infection of tumor cells in vivo (Fig. 5) by in situ injection of pH300-lac virions indicates that the miniviral pH300 has potential use for human tumor treatment

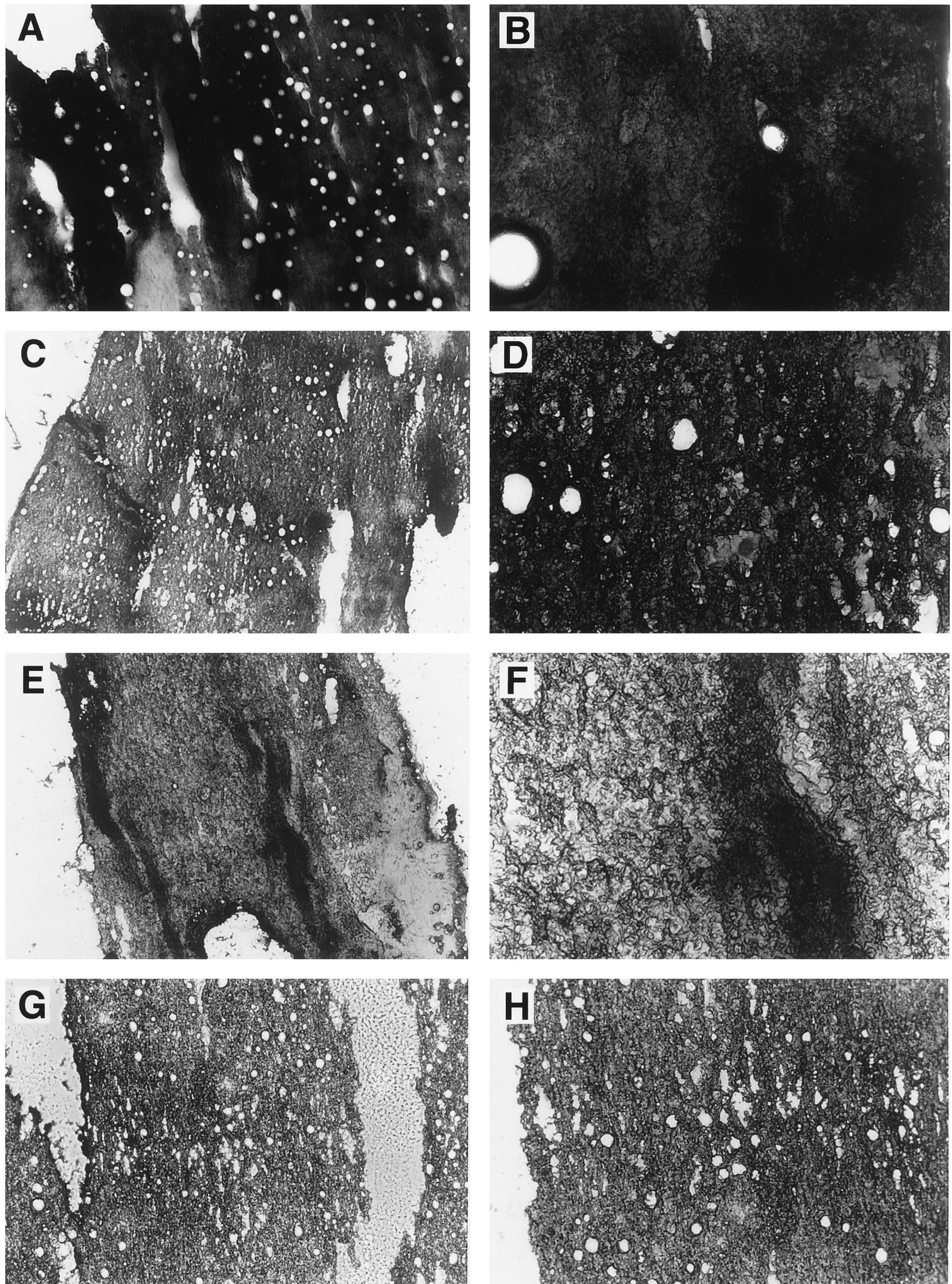

FIG. 6. Histological detection of β-galactosidase activity in nude mouse tumors 24 h after pH300-lac injection into human tumor cells grown in nude mice. (A and B) Area with high *lacZ* expression; (C and D) area with medium *lacZ* expression; (E and F) area with less *lacZ* expression; (G and H) control area. Magnifications: A, C, E, and G, ×19; B, D, F, and H, ×190.

in vivo. The observed strong and transient expression of an inserted gene in pH300 may be sufficient in killing tumor cells. Alternatively, the mini-HSV-1 vector could be used in the central nervous system for treating acquired and hereditary diseases due to the neurotropism of HSV-1. Geller and Breakefield (8) explored the feasibility of the mini-HSV-1 amplicon to transfer expressed genes into nervous cells. A follow-up study by Kaplitt et al. (13) also using the *lacZ* gene on an HSV-1 amplicon confirmed mini-HSV-1 as a promising viral vector for gene transfer and expression in the central nervous system of the adult rat brain in vivo. Preexisting or future HSV infection could theoretically induce vector reactivation and its spread to other parts of the human body. This issue is a primary concern for future gene therapy protocols based on any infectious vector derived from endemic human viruses. Such potential pathogenesis induced by rescued infectious HSV vector spreading at new sites would not be expected to be as severe as that of wild-type virus infection, since all herpesvirus genes have been deleted from the vector. Nonetheless, strategies such as controlled tissue-specific gene expression and inclusion of suicide genes for viral destruction will have to be considered as safety barriers. In summary, the wide tropism of infection, the simplicity of handling, the potentially large capacity of DNA insertion, the undetectable cytotoxicity in vitro, and the high efficiency of infection and expression in vivo may render the EBV/HSV-1 hybrid vector pH300 a potentially excellent gene transfer system for future gene therapy applications.

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