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Transcriptional Targeting of Herpes Simplex Virus for Cell-Specific Replication

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Tissue- or cell-specific targeting of vectors is critical to the success of gene therapy. We describe a novel approach to virus-mediated gene therapy, where viral replication and associated cytotoxicity are limited to a specific cell type by the regulated expression of an essential immediate-early viral gene product. This is illustrated with a herpes simplex virus type 1 (HSV-1) vector (G92A) whose growth is restricted to albumin-expressing cells. G92A was constructed by inserting an albumin enhancer/promoter-ICP4 transgene into the thymidine kinase gene of mutant HSV-1 *d*120, deleted for both copies of the ICP4 gene. This vector also contains the *Escherichia coli lacZ* gene under control of the thymidine kinase promoter, a viral early promoter, to permit easy detection of infected cells containing replicating vector. In the adult, albumin is expressed uniquely in the liver and in hepatocellular carcinoma and is transcriptionally regulated. The plaquing efficiency of G92A is $>10^3$ times higher on human hepatoma cells than on non-albumin-expressing human cells. The growth kinetics of G92A in albumin-expressing cells is delayed compared with that of wild-type HSV-1, likely due to aberrant expression of ICP4 protein. Cells undergoing a productive infection expressed detectable levels of ICP4 protein, as well as the reporter gene product β-galactosidase. Confining a productive, cytotoxic viral infection to a specific cell type should be useful for tumor therapy and the ablation of specific cell types for the generation of animal models of disease.

Gene therapy strategies for cancer have used various delivery methods, including viral vectors, to transfer suicide genes (21, 48) or immune-modulator genes (64, 73) to neoplastic cells. We recently developed an alternative approach that uses the inherent cytotoxic capabilities of replication-competent herpes simplex virus type 1 (HSV-1) to destroy tumor cells in vivo and in the process replicate and spread throughout the tumor (29, 42). Our studies with malignant brain tumors used mutants of HSV-1 that were unable to replicate in nondividing cells and/or were attenuated for neurovirulence (29, 41, 42, 46, 47).

To expand the clinical potential of this viral oncolytic therapeutic approach to other tumors and to avoid toxicity to normal dividing cells (endothelial, fibroblast, epithelial, etc.), it is possible to exploit the transcriptional differences between normal and neoplastic cells. The targeting of gene therapy to the appropriate tumor cell type so that normal cells are not adversely affected has been achieved at the level of physical transfer, the replicative status of the cell, or the transcriptionally regulated expression of the transferred gene (45). Tissue-specific regulatory sequences have been used to drive expression of suicide genes following retrovirus- or adenovirus-mediated gene transfer (23, 28, 39, 69, 72, 75), direct injection of DNA (76), and adenovirus-polylysine-mediated transduction (14). Rather than target expression of a specific gene product for therapy, we have chosen to target the complete lytic growth cycle of the virus. For tumor therapy, the ability to synthesize new infectious viral particles that will spread to adjacent cells throughout the tumor should amplify the cytotoxic potential of the vector.

The feasibility of confining the host range of HSV-1 to a particular cell type was tested in the following model system that uses the mouse albumin enhancer/promoter sequence (26, 55) as the cell-type-specific regulatory region. Albumin is expressed uniquely in liver and is regulated at the level of transcription initiation (74). The albumin gene contains a liver-specific promoter of about 150 bp just upstream of the cap site (24). A region from 8.5 to 10.4 kb upstream of the albumin promoter functions as an enhancer that, in combination with the albumin promoter (300 bp), drives high-level expression specifically in the adult liver of transgenic mice (55), in infected hepatocytes in vivo after delivery of recombinant retroviruses through the portal vein and partial hepatectomy (22, 36), and in human hepatoma cells after infection with recombinant adenovirus (25) or recombinant retroviruses (28, 39). The albumin enhancer/promoter was used to drive expression of the HSV ICP4 gene.

HSV genes, during a lytic infection, are expressed temporally in three major classes: immediate-early (IE or α), early (E or β), and late (L or γ) (7, 27). Progression through this growth cycle is dependent on two essential IE proteins, ICP4 and ICP27 (10, 57, 62). The ICP4 (Vmw175, IE175, IE-3, α4) gene is located in the short inverted repeat regions of the HSV genome (Fig. 1A) and encodes a 175-kDa phosphorylated nuclear protein that is the main *trans* activator of HSV transcription (54, 56). Mutants lacking ICP4 fail to synthesize early or late viral polypeptides but continue to express the other IE genes (10, 12, 56, 57, 78). Binding of ICP4 protein to a degenerate consensus sequence present upstream of a number of HSV-1 genes (e.g., ICP4, ICP0, latency-associated transcript, ICP8, thymidine kinase [TK], gC, and gD genes) results in either repression of IE genes or activation of E and L genes (3, 11, 19, 38, 44, 60). The negative regulation of ICP4's own transcription occurs in a temporal fashion postinfection (p.i.)

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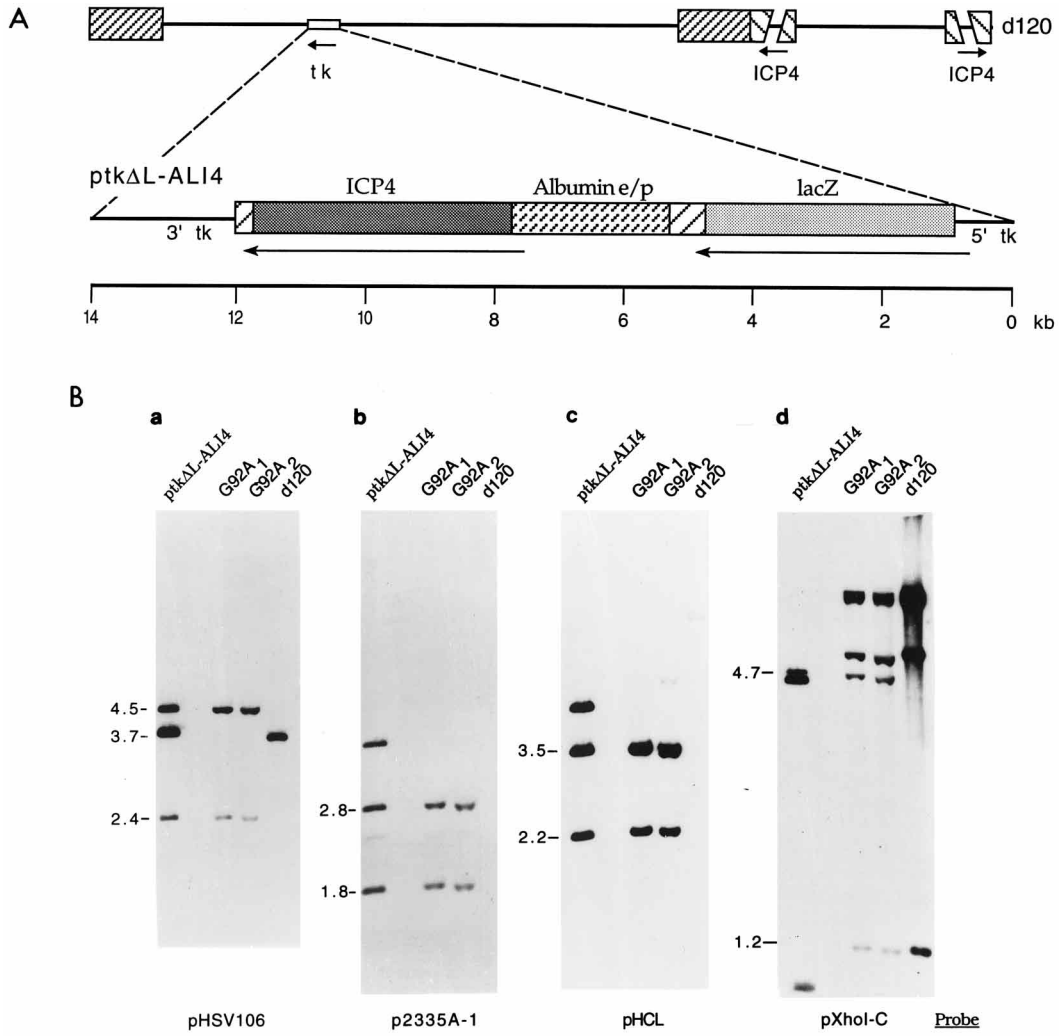

FIG. 1. DNA structure of G92A. (A) Schematic gene arrangement of G92A, illustrating the location of the diploid ICP4 gene in the short repeat regions and the TK gene in the long unique region as well as where the chimeric transgenes of pTKΔL-ALI4 (lower) were inserted into *d*120 (upper) via homologous recombination. E/p, enhancer/promoter; hatched box, polyadenylation sequence; arrows, direction of transcription. (B) Southern blot hybridization analysis of G92A DNA structure. (a) The presence of the transgene insertion into the TK coding sequence was confirmed by hybridization of *Bam*HI- and *Ssp*I-digested viral DNAs with labeled pHSV106 (including the TK gene). The two fragments (4.5 and 2.4 kb) derived from plasmid pTKΔL-ALI4 were present in G92A, whereas the native *Bam*HI Q fragment (3.6 kb) was present only in *d*120. (b) The presence of the albumin enhancer/promoter sequence was confirmed by hybridization of *Bam*HI- and *Ssp*I-digested viral DNAs with labeled p2335A-1 (containing the albumin enhancer/promoter). G92A contains the expected 2.8- and 1.8-kb fragments, not present in *d*120. (c) The presence of the *lacZ* and SV40 polyadenylation sequences were confirmed by hybridization of *Eco*RI-digested viral DNAs with labeled pHCL (containing *lacZ* and the SV40 polyadenylation site). G92A contains the expected 3.5- and 2.2-kb fragments, not present in *d*120. The faint band seen with *d*120 is due to hybridization with HSV ori and α sequences present on pHCL. (d) The presence of the correct ICP4 fragments was confirmed by hybridization of *Eco*RI-digested viral DNAs with labeled pXhoI-C (50) (containing the 9.5-kb HSV-1 *Xho*I C fragment, including the ICP4 gene). The *Xho*I-C probe should hybridize to HSV-1 KOS *Eco*RI fragments K, H, B/C, and E/J (50, 67). The 4.1-kb deletion of ICP4 in *d*120 and G92A is contained within the 1.2-kb terminal fragment (*Eco*RI-K) (10) and the high-molecular-weight submolar joint fragments (*Eco*RI-B/C). A 6-kb band is present in G92A and *d*120 (10). In addition, the 4.7-kb plasmid (pTKΔL-ALI4)-derived ICP4 fragment was seen only in G92A. The 4.6-kb band in pTKΔL-ALI4 is from the plasmid backbone.

(59). ICP4 also acts in concert with IE proteins ICP0 and ICP27 to regulate viral gene expression (13, 15, 51).

The second chimeric transgene, with the *Escherichia coli lacZ* gene under control of the HSV-1 TK promoter, was used to easily screen for recombinant viruses and identify cells containing replicating virus. TK is an E gene, and therefore *lacZ* expression should be dependent on synthesis of ICP4 protein (11, 16, 57, 65, 78). The chimeric transgenes (albumin enhancer/promoter-ICP4, TK promoter-*lacZ*) were inserted into the HSV-1 TK gene (UL23), resulting in a 0.5-kb deletion of the TK gene and inactivation of the UL24 gene (31). HSV-1 mutants lacking TK activity grow poorly in nondividing cells (32, 35) and have been shown to be effective in brain tumor viral therapy (4, 29, 33, 35, 42).

MATERIALS AND METHODS

Cells and viruses. Hep3B (with integrated hepatitis B virus [HBV] positive), HepG2 (HBV negative), HuH7 (HBV negative), and SW480 cells were grown in Dulbecco's modified Eagle's medium supplemented with 15% heat-inactivated fetal calf serum (IFCS) (HyClone). MCF7 cells were grown similarly except that the medium also contained 15 mg of endothelial cell growth supplement (Becton-Dickinson) per ml, and E5 cells were grown in medium supplemented with 10% newborn calf serum (HyClone). Detroit 551 cells were grown in Eagle's minimal essential medium supplemented with 10% newborn calf serum, 0.1% lactose hydrolysate, and 1 mM sodium pyruvate. HepG2, Hep3B, MCF7, Detroit

551, and SW480 cells were obtained from the American Type Culture Collection (Rockville, Md.), HuH7 cells were kindly supplied by J. Gerin (Georgetown University Medical Center, Washington, D.C.), and E5 cells were kindly supplied by N. DeLuca (University of Pittsburgh School of Medicine, Pittsburgh, Pa.). Viral stocks of wild-type HSV-1 strain KOS, obtained from D. Knipe (Harvard Medical School, Boston, Mass.), ICP4 deletion mutant *d*120 (parental strain KOS), obtained from N. DeLuca, and ICP6$^-$ recombinant *hr*R3 (parental strain KOS), obtained from S. Weller (University of Connecticut Health Center, Farmington), were generated from low-multiplicity infections.

Plasmid construction. PTKΔL-ALI4 was constructed as follows. The 0.5-kb *Bgl*II-*Kpn*I fragment from within the TK coding region in pHSV106 (43) (GIBCO/BRL Life Technologies, Gaithersburg, Md.) was replaced with a *Bgl*II-*Kpn*I polylinker from pSL301 (Invitrogen) to generate pTKΔ. This polylinker insertion also leads to a deletion in the overlapping gene UL24, of unknown function (31). The 4.3-kb blunt-ended *Hin*dIII-*Sal*I fragment from pHCL (34), containing *E. coli lacZ* and the simian virus 40 (SV40) polyadenylation site, was subcloned into the blunt-ended *Bgl*II site (+53 of TK) of pTKΔ to create pTKΔL. The 4.1-kb blunt-ended *Sal*I-*Mse*I fragment of pGH108 (60) (kindly provided by G. Hayward, Johns Hopkins School of Medicine, Baltimore, Md.), containing the ICP4 coding sequence, was subcloned into the blunt-ended *Bam*HI site of p2335A-1 (albumin sequences equivalent to those in construct NB [55]; kindly provided by R. Palmiter, University of Washington, Seattle) 22 bp downstream of the albumin cap site (55) to create pALI4. A 6.4-kb blunt-ended *Bst*XI-*Eco*RV fragment from pALI4 was subcloned into pTKΔL at the *Xba*I site (blunt ended) in the polylinker after the SV40 polyadenylation site to create pTKΔL-ALI4.

For Southern blot hybridization, viral DNA was isolated from viral stocks after a freeze-thaw/sonication regimen, DNase treatment, phenol-chloroform-isoamyl alcohol (1:1:0.04) extraction, and ethanol precipitation. DNA was digested with restriction endonucleases (New England Biolabs) as indicated, separated by agarose gel electrophoresis, and transferred to nylon membrane (Zeta-Probe; Bio-Rad) (70). The DNA was probed with plasmid DNA as indicated. Plasmid pXhoI-C was kindly provided by G. Hayward. Probes were labeled, and membranes were developed by using an ECL (enhanced chemiluminescence) random labeling kit (Amersham Corp., Arlington Heights, Ill.) as described by the manufacturer.

Generation of recombinant HSV-1. Linearized pTKΔL-ALI4 (*Sal*I site in plasmid backbone) and *d*120 DNA were cotransfected into E5 cells by using lipofectAMINE (GIBCO/BRL) as described by the manufacturer. Recombinant viruses, identified as plaques staining blue with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) agarose overlay (20), were plaque purified three times on E5 cells in the presence of ganciclovir (1 μg/ml). G92A virus stocks, for use in the experiments, were then prepared by low-multiplicity infection of HepG2 cells.

Single-step viral growth. Monolayer cultures of cells (Hep3B [8×10^4 cells/well] or MCF7 [4.3×10^5 cells/well]) in 12-well dishes (Falcon) were infected with HSV-1 strain KOS or G92A at a multiplicity of infection (MOI) of 1.5 in 0.5 ml of phosphate-buffered saline supplemented with 1% IFCS. The virus inoculum was removed after 60 min, and the cells were incubated in Dulbecco's modified Eagle's medium supplemented with 1% IFCS at 37.5°C in humidified 5% CO_2. At the times indicated, virus was harvested from the wells and titers were determined on E5 (ICP4$^+$) cells.

Virus titer. Monolayer cultures of cells in six-well dishes were infected with serial dilutions of virus as indicated. After removal of virus inoculum, the cells were incubated in Dulbecco's modified Eagle's medium supplemented with 1% IFCS and 0.1% Gammar (pooled human gamma globulin; Armour Pharmaceutical Co., Kankakee, Ill.) at 31.5, 34, or 37.5°C until plaques were visible. The cells were fixed with methanol (for Giemsa staining), 0.5% glutaraldehyde–2% formaldehyde (for X-Gal histochemistry [2]), or 4% paraformaldehyde (for ICP4 immunohistochemistry). For ICP4 immunohistochemistry, fixed cells were immunoreacted with mouse monoclonal antibody to HSV-1 ICP4 protein and purified from hybridoma cell line 58S (ATCC HB8183) (66), using an Immunopure (A/G) immunoglobulin G purification kit (Pierce, Rockford, Ill.) as described by the manufacturer. Immunoreactive material was detected with biotinylated anti-mouse immunoglobulin G (Vector Laboratories Inc., Burlingame, Calif.), a Vectastain ABC kit (Vector), and a DAB (diaminobenzidine) kit (Vector) as described by the manufacturers. Plaques were counted, and the average number of plaques was determined from ≥2 wells.

Western blotting. Monolayer cultures of HepG2 cells in six-well dishes were infected with *hr*R3 or G92A at an MOI of 0.5. After removal of the virus inoculum, the cells were incubated in Dulbecco's modified Eagle's medium supplemented with 1% IFCS at 37.5°C for the times indicated. To isolate protein, cells were washed with EDTA, scrapped off the dish in 0.5 ml of EDTA/well, pelleted, and resuspended in gel loading buffer (50 mM Tris [pH 6.8], 100 mM dithiothreitol, 2% sodium dodecyl sulfate [SDS], 0.1% bromphenol blue, 10% glycerol). Proteins (10 μg) were separated by electrophoresis on SDS–8% polyacrylamide gels by the method of Laemmli (40) and transferred to Immobilon-P membranes (Millipore) (6). ICP4 protein was detected by using an enhanced chemiluminescence detection kit (Renaissance; Dupont NEN) after immunoblotting with anti-ICP4 monoclonal antibody (1:200 dilution, purified from hybridoma cell line 58S) and horseradish peroxidase-conjugated goat anti-mouse antibody (Zymed). Protein determinations, from lysates of scraped cells, were performed by the method of Bradford (5), using the Bio-Rad protein assay kit according to the manufacturer's instructions and bovine gamma globulin as a standard.

RESULTS

Generation of albumin enhancer/promoter-regulated recombinant HSV-1 (G92A). We constructed an HSV-1 recombinant regulated by the albumin enhancer/promoter and termed G92A (Fig. 1A). G92A is derived from *d*120, an HSV-1 mutant with a deletion of the ICP4 gene (10, 12). *d*120 can grow only on ICP4-complementing cell lines such as E5 (10, 12). G92A was constructed by recombining sequences from plasmid pTKΔL-ALI4 into the TK locus of *d*120. Plasmid pTKΔL-ALI4 contains two chimeric transgenes (Fig. 1A) expressing ICP4 protein and β-galactosidase. The mouse albumin enhancer/promoter, containing upstream sequences from kb −10.5 to −8.5 fused to the proximal promoter sequences from bp −300 to +22 (55), was cloned upstream of the ICP4 coding sequence (at +178; ICP4 transcription begins at +301) and polyadenylation site. The *E. coli lacZ* coding sequence and SV40 polyadenylation site were inserted into the HSV-1 TK gene just downstream of the TK promoter so that β-galactosidase expression was regulated by the HSV-1 TK promoter (an early promoter). With *lacZ* upstream of the albumin enhancer/promoter, it is very unlikely that read-through transcription from the TK promoter could occur. The 3′ untranslated region of TK, downstream of the ICP4 transgene, overlaps the 5′ promoter region of gH (65), which should have no impact on the regulation of ICP4 expression. pTKΔL-ALI4 was cotransfected with *d*120 DNA into E5 (ICP4$^+$) cells, and the resulting recombinant viruses were thrice plaque purified on E5 cells. Recombinant plaques stained blue with X-Gal because of the presence of the *lacZ* gene, grew in the presence of ganciclovir because they were TK$^-$, and formed plaques on HepG2 cells, which express albumin.

Virus stocks were prepared on HepG2 cells to minimize the possibility of reversion of the ICP4 deletion by recombination with the complementing ICP4 gene in E5 cells. We prepared two independently isolated recombinant stocks, $G92A_1$ and $G92A_2$, which were identical by all criteria tested. The DNA structures of these recombinants (Fig. 1A) were confirmed by restriction endonuclease digestion and Southern blot analysis, using probes to ICP4 (pXhoI-C), *lacZ* (pHCL), albumin enhancer/promoter (p2335A-1), and HSV-1 TK (pHSV106) genes (Fig. 1B). The recombinant viruses maintain the ICP4 deletions of *d*120 (Fig. 1B, probe pXhoI-C). In addition, they contain the ICP4 and *lacZ* transgene insertions (Fig. 1B, probes pXhoI-C and pHCL) in the HSV-1 TK gene, resulting in two new fragments hybridizing to the TK gene and loss of the 3.6-kb fragment from *d*120 (Fig. 1B, probe pHSV106).

G92A replication in human albumin-expressing cells. The ability of G92A to replicate specifically in albumin-expressing cells was examined in a single-step growth experiment (Fig. 2). Human Hep3B hepatoma cells express albumin, whereas human MCF-7 breast adenocarcinoma cells do not (28). Infection of either Hep3B or MCF7 cells with wild-type, parental HSV-1 strain KOS resulted in similar viral replication kinetics and viral yields, indicating no cell-specific effects on HSV replication. However, no viral burst (10-fold lower than input virus) was detected with G92A-infected MCF7 cells (Fig. 2). A similar lack of viral burst was also seen with G92A-infected HeLa (human epitheloid carcinoma), CAKI-2 (human renal cell carcinoma), and PC3 (human prostate adenocarcinoma) cells (data not shown). G92A-infected Hep3B cells produced a significant viral burst, less than that obtained with KOS, and

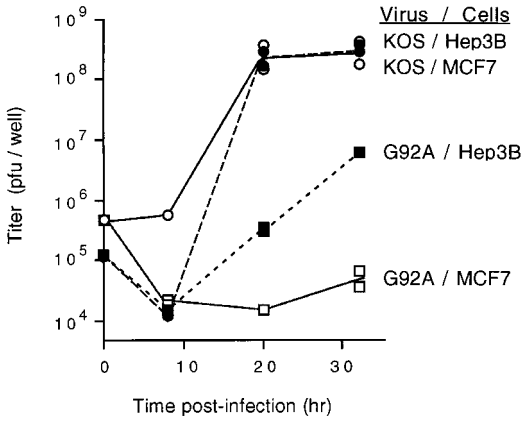

FIG. 2. Single-step growth curve for G92A and KOS on albumin-expressing Hep3B and albumin-nonexpressing MCF7 cells. Monolayers of Hep3B or MCF7 cells, in six-well dishes, were infected with KOS or G92A at an MOI of 1.5; at the times p.i. indicated, virus was harvested from the wells and titers were determined on E5 (ICP4$^+$) cells. The average number of PFU per well of infected Hep3B or MCF7 cells was determined. At some time points, duplicate wells of cells were infected (duplicate titer values).

growth of G92A was delayed and slower than that of KOS (Fig. 2).

Cell specificity of G92A growth on human cells in culture. The ability of G92A to form plaques on a number of different human cell lines was determined to examine both the replication and spread of the virus in culture. In addition to Hep3B, both HuH7 and HepG2, which are HBV-negative human hepatoma cell lines, express albumin (1, 9, 37, 49). Human SW480 colon adenocarcinoma cells and Detroit 551 diploid fibroblast cells do not express detectable albumin (28).

All cell types were similarly sensitive to wild-type HSV-1 infection, as illustrated by the ability of HSV-1 strain KOS to form plaques on all cells tested with similar efficiencies (Fig. 3 and 4C, E, and K). Plaque morphology varied depending on the cell type (Fig. 4). The parental virus, *d*120 (ICP4$^-$), formed plaques efficiently only on E5 (ICP4$^+$) cells (Fig. 3 and 4A). A few plaques formed on some other cell types at approximately 10^{-5} to 10^{-6} the frequency on E5 cells, possibly due to the presence of revertants of *d*120 in the virus stock (10, 12). In contrast to wild-type HSV-1, G92A formed plaques with high efficiency only on albumin-expressing hepatoma cells (Fig. 3 and 4D and F), with an efficiency similar to that seen on E5 cells (Fig. 3 and 4B). Both independently isolated G92A stocks behaved similarly (Fig. 3). The abilities of G92A or KOS to produce plaques were the same at the three incubation temperatures tested (31.5, 34, and 37.5°C).

Albumin-nonexpressing cells were highly refractory to G92A replication, and so there was a markedly decreased plaquing efficiency on Detroit 551, SW480, and MCF7 cells (Fig. 3 and 4L, N, and P). There was a greater than 10^3-fold difference between plaquing ability on albumin-expressing and -nonexpressing cells. We used very broad latitude in defining plaques so that all clusters of cells demonstrating cytopathic effect (CPE) were counted, regardless of their size or extent of CPE. In many cases, staining with X-Gal (Fig. 4L, N, and P) or antibody to ICP4 (Fig. 4J) was the only way to detect these plaques (for example, compare G92A plaques with KOS plaques on albumin-expressing [Fig. 4E and F] and -nonexpressing [Fig. 4L and K] cells). The time of incubation for the G92A-infected nonexpressing cells was usually 1 to 7 days longer than the corresponding time for wild-type KOS, whereas for the albumin-expressing cells, the incubation times were similar. It is possible that at the high titers of G92A plated on nonexpressing cells, cells infected with multiple viral particles were able to overcome the block to viral replication (63, 71). The MCF7 cells consistently yielded higher titers than the other albumin-nonexpressing cells, even with the parental ICP4 deletion mutant *d*120, and may contain factors able to complement the ICP4 deficiency at a low level.

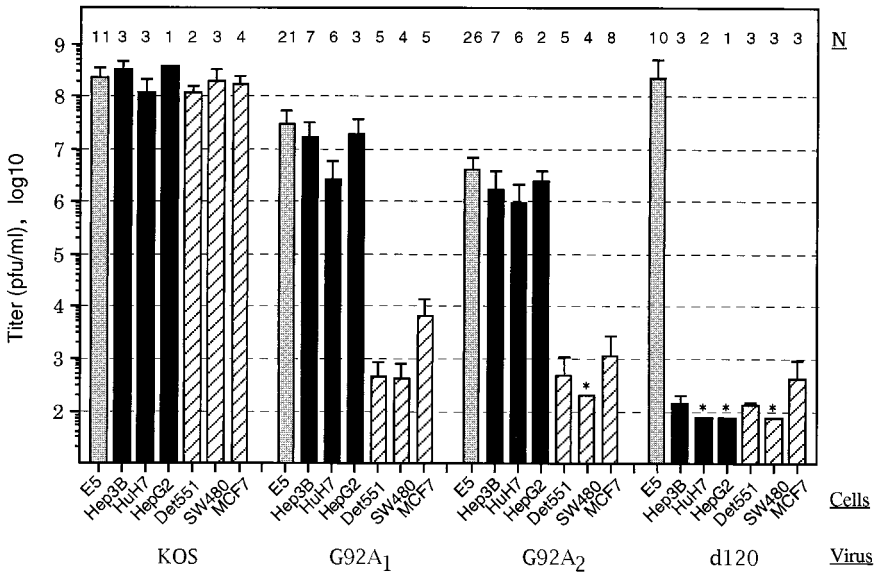

FIG. 3. Plaquing ability of G92A is restricted to albumin-expressing (Alb$^+$) human cell lines, as opposed to wild-type HSV-1 KOS, which has no cell specificity. Monolayers of E5 (ICP4$^+$), Hep3B (Alb$^+$), HuH7 (Alb$^+$), HepG2 (Alb$^+$), Detroit 551, SW480, and MCF7 cells grown in six-well dishes were infected with serial dilutions of HSV-1 strains KOS (wild type), G92A$_1$, G92A$_2$, and *d*120 (ICP4$^-$). No consistent significant difference in titer was seen at different incubation temperatures or between Giemsa- and X-Gal-stained plates. The number of PFU was calculated from the mean number of plaques formed ± standard deviation on albumin-expressing cells (solid bars) and -nonexpressing cells (hatched bars). *N* is the number of separate experiments in which titers were determined (each titer is the average of plaque counts obtained from ≥2 wells, usually of two different dilutions). *, at the lowest possible viral dilution (usually 10^{-2}), no plaques were detected.

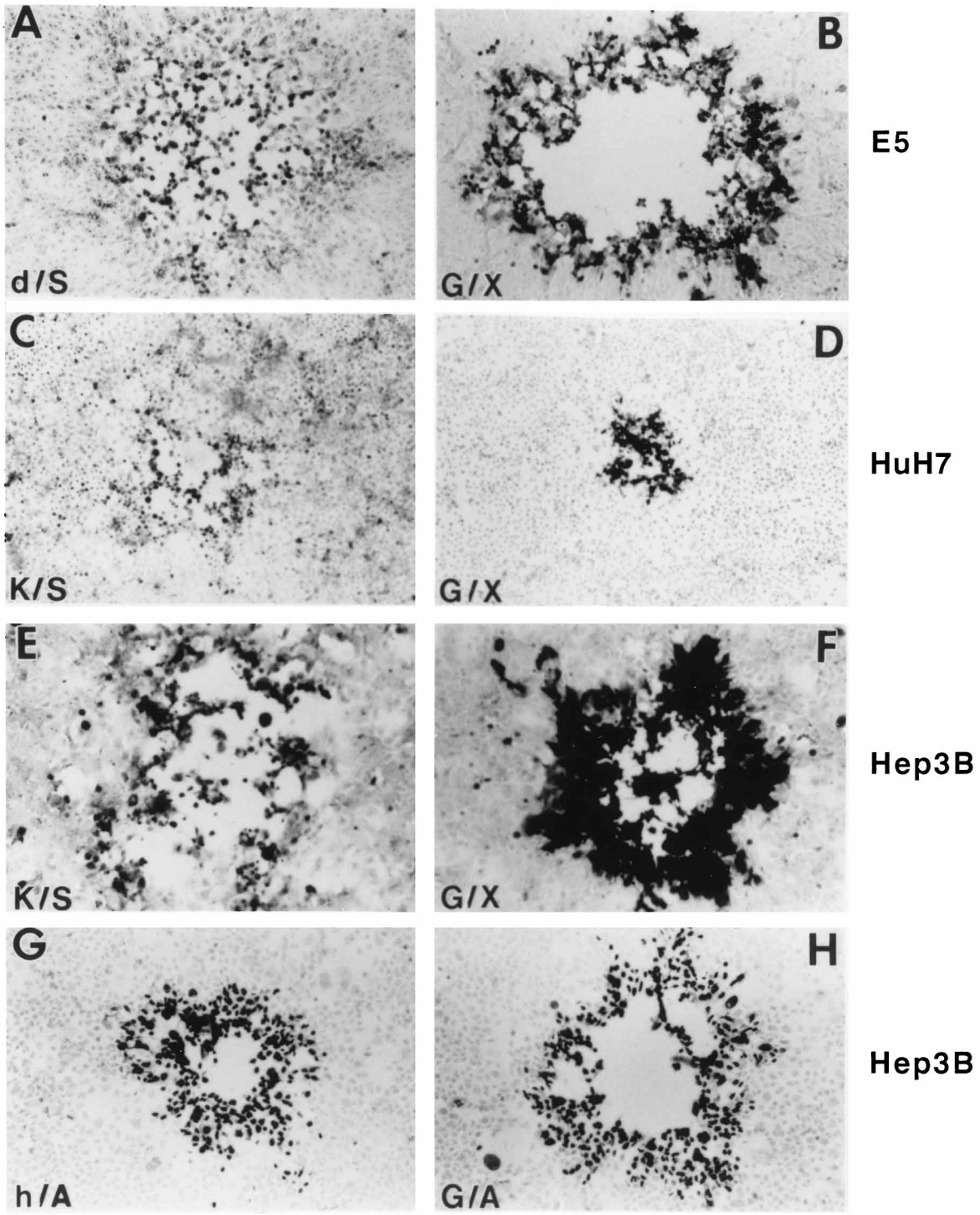

FIG. 4. HSV-1 plaque morphology on albumin-expressing (HuH7 [C and D] and Hep3B [E to H]) and -nonexpressing (MCF7 [I to L], SW480 [M and N], and Detroit 551 [O and P]) human cells and E5 cells (A and B). Cells were infected with HSV-1 strains KOS (wild type; **K**; C, E, and K), *hr*R3 (ICP6$^-$ *lacZ*$^+$; **h**; G, I, M, and O), *d*120 (ICP4$^-$; **d**; A), and G92A (**G**; B, D, F, H, J, L, N, and P). G92A infection of albumin-nonexpressing cells (J, L, N, and P) was at a dilution of 10^{-2} to 10^{-3}, compared to 10^{-5} to 10^{-6} for albumin-expressing (D, F, and H) and E5 (B) cells. Fixed cultures were stained with Giemsa (**S**; A, C, E, and K) or X-Gal (**X**; B, D, F, L, M, N, O, and P) or immunoreacted with mouse monoclonal antibody to HSV-1 ICP4 protein (**A**; G, H, I, and J). Some cultures were counterstained with neutral red. ICP4-immunoreactive cells and β-galactosidase-expressing cells appear darkly staining. Plaques are areas cleared of cells surrounded by cells exhibiting CPE. The bar represents 0.25 mm.

Transgene expression. Substantial β-galactosidase expression was detected in cells surrounding plaques of G92A-infected albumin-expressing cells (Fig. 4B, D, and F). Infection with HSV-1 *hr*R3 (ICP6$^-$ *lacZ*$^+$ [20]) produced ICP4$^+$ (Fig. 4G and I) and X-Gal$^+$ (Fig. 4M and O) plaques on all cell types tested.

ICP4 protein localized to the nucleus and was detected where plaques formed on G92A-infected albumin-expressing

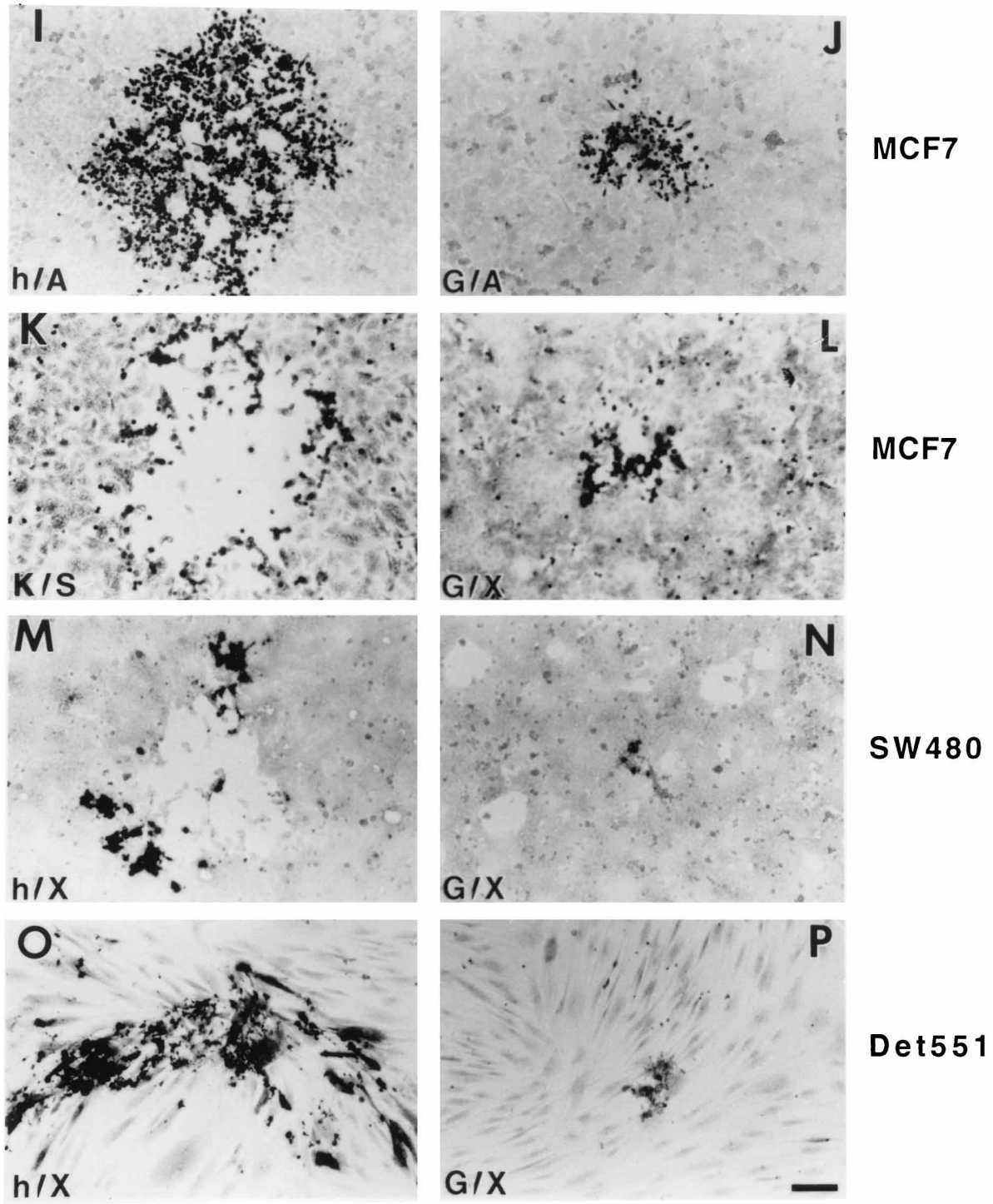

FIG. 4—*Continued.*

cells (Fig. 4H). Clusters of ICP4$^+$ cells were sometimes seen after infection of nonexpressing cells (at approximately 10^{-3} the efficiency seen on albumin-expressing cells) (Fig. 4J), likely due to non-albumin-specific expression. Low levels of reversion at the ICP4 loci should have produced wild-type-sized plaques. Synthesis of ICP4 protein in the various cell types was also determined by immunofluorescence (data not shown). At 48 h p.i. (prior to the formation of plaques), ICP4$^+$ cell clusters were detected only in G92A-infected hepatoma cultures (Hep3B and HepG2) or *hr*R3-infected MCF7 cells. ICP4 expression in E5 cells was detected only after infection with HSV-1 (data not shown). When ICP4 expression was examined, all plaques or clusters of cells exhibiting CPE were ICP4 immunoreactive.

The time course of ICP4 protein expression was determined by Western blotting of cell lysates from HepG2 cells infected

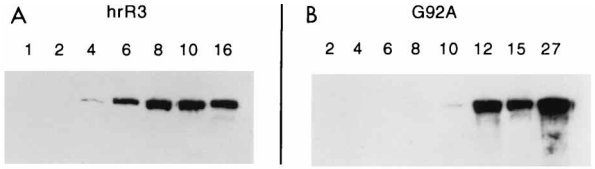

FIG. 5. Temporal expression of ICP4 protein. HepG2 cells were infected with *hr*R3 (A) or G92A (B), and cell lysates were isolated at the times (hours) p.i. indicated above the lanes. Western blot analysis was performed with anti-ICP4 antibody. ICP4 protein migrates with an apparent molecular weight of approximately 175.

with *hr*R3 or G92A, at an MOI of 0.5, after SDS-polyacrylamide gel electrophoresis (Fig. 5). ICP4 protein was detected as early as 4 h p.i. in *hr*R3-infected cells, with protein levels increasing until 10 to 12 h p.i. (Fig. 5A) and then decreasing from 15 to 27 h p.i. (data not shown). In contrast, in G92A-infected cells, ICP4 protein was not detectable until about 10 h p.i., and then levels remained high until at least 27 h p.i. (Fig. 5B). This very slow accumulation of ICP4 protein likely contributed to the delayed and slower kinetics of virus replication seen in Fig. 2.

DISCUSSION

Viral vectors whose replication is limited to specific cell types should be especially useful in tumor therapy. We have generated a recombinant HSV (G92A) whose replication is restricted to albumin-expressing cells in culture. This was accomplished by placing the IE ICP4 gene under control of the albumin enhancer/promoter and recombining this chimeric transgene into the HSV genome at the TK locus. G92A is able to efficiently replicate in three human hepatocellular carcinoma cell lines which express albumin and not in six other human cell lines, including five carcinoma cell lines, which do not express albumin. Much human hepatocellular carcinoma is associated with chronic HBV and hepatitis C virus infection (52). We have shown that G92A replicates similarly in both HBV-positive and HBV-negative hepatocellular carcinoma cell lines. A range of tumor suppressor gene alterations have been detected in different human hepatocellular carcinomas. In the cell lines that we used, these include Hep3B, lacking p53 and retinoblastoma protein; HuH7, with abnormal p53 expression; and HepG2, with normal p53 and retinoblastoma gene expression (58). Replication of G92A and HSV in general does not seem to be dependent on either of these tumor suppressor gene products.

It is fairly clear from the complex regulation of expression of ICP4 protein that the absolute levels of ICP4 protein in the infected cell at different times p.i. is important in the overall growth of the virus. It has been shown that the level of ICP4 protein affects the regulation of promoters with ICP4 binding sites, both positively and negatively (11, 60). In addition, the nuclear localization of ICP4 in infected or transfected cells is somewhat dependent on the MOI or amount of DNA transfected (80). In both albumin-expressing and -nonexpressing cells, ICP4 protein was mostly localized to the nucleus after infection with G92A. ICP4 protein negatively regulates its own promoter in the viral genome; however, the target sequence in the ICP4 promoter maps between positions −7 and +17, which are deleted from the ICP4 transgene in G92A (60).

Even in permissive, albumin-expressing hepatoma cells, G92A exhibited very delayed growth kinetics and synthesis of ICP4 protein compared to wild-type KOS or *hr*R3. ICP4 expression in G92A-infected cells is presumably regulated by cellular factors, in contrast to viral factors for KOS or *hr*R3. The viral growth difference observed is likely due to differences in the regulation of ICP4 protein expression and its consequent downstream effects on E and L gene expression. Some of the decreased growth could be due to the deletion in the UL24 gene, which has been shown to result in smaller burst sizes (31). Other HSV-1 mutants with decreased viral burst size (*hr*R3 and G207) have been found to be effective in inhibiting tumor growth in vivo (46, 47).

A strategy alternative to that described in this study is to replace the ICP4 promoter of the endogenous ICP4 gene with a novel regulatory sequence. The pseudorabies virus IE180 (homolog of HSV ICP4 [79]) promoter was replaced with the inducible promoter of the drosophila HSP70 gene (17) or the bovine cytokeratin IV promoter (17). A 2- to 3-log difference in pseudorabies virus titer was seen upon comparison of the plaquing abilities of the HSP70 promoter recombinant at inducing and noninducing temperatures or the cytokeratin IV promoter recombinant in a cytokeratin-expressing bovine epithelial cell line and in a swine kidney cell line (18). A drawback to this approach for HSV is illustrated by the studies of Smith et al. (68), who generated deletions in both copies of the ICP4 promoter region in HSV-2. A mutant deleted for the entire ICP4 promoter had undetectable amounts of ICP4 mRNA in the presence of cycloheximide yet was still viable in cells that do not express ICP4 (68). The replication of this mutant could be due to the presence of promoter sequences in ori_S that are regulated as early or late transcripts (blocked by cycloheximide treatment); alternatively, transcript levels below those detectable may have been sufficient for viral replication. Larger deletions that included ori_S were not viable (68). Igarashi et al. (30) were able to generate HSV-1 mutants deleted for both ori_S sequences if one of the ICP4 promoters was replaced with the SV40 promoter. These recombinants grew similarly to, though more slowly than, parental ori_S^+ HSV-1 (30), suggesting that the viral transcriptional program was not greatly altered by the SV40 promoter-ICP4 transgene present at the endogenous ICP4 location.

The second transgene inserted into G92A contains the *E. coli lacZ* gene regulated by an E HSV-1 promoter (TK). LacZ is expressed in those cells where the recombinant virus is replicating and can be easily detected by X-Gal histochemistry. Other coding sequences, such as for immune-modulatory proteins, could be placed under control of viral E and L promoters (in place of or in addition to the *lacZ* gene used here) such that they will be expressed only in those cells where virus is replicating. Thus, it may be possible to elicit an immune response specifically directed at tumor cells while minimizing the damage to normal tissue. HSV-1 is an especially useful vector for these purposes because of its large size (with many nonessential genes) and broad host range. Transgene insertions need not be in the TK locus. Other loci in the virus, for example, the ICP6 (ribonucleotide reductase) gene (47), may also be useful sites for recombination. $ICP6^-$ mutants retain sensitivity to acyclovir and ganciclovir, whereas TK^- mutants are resistant (8, 46).

These studies demonstrate the feasibility of transcriptionally targeting viral replication to specific cell types. Unlike most HSV-1 host range mutants, where viral growth is limited to specially isolated or constructed complementing cells (such as the E5 cells), the strategy described here targets normal or transformed cells and is applicable to in vivo models. In previous studies, promoter elements inserted into the HSV-1 genome were affected by the regulatory properties of the surrounding HSV-1 sequences (53, 61). One of the reasons that G92A is successfully regulated may be due to the lack of

normal IE gene expression. In the absence of ICP4 protein, the HSV-1 genome is relatively inactive transcriptionally, except for the other IE genes (10), and therefore recombinant sequences may be less prone to the normally strong positional effects of the viral genome. In addition, regulated expression of the ICP4 transgene in G92A does not have to be maintained for extended periods of time as is the case with standard gene delivery vectors, but just long enough to initiate and maintain viral replication.

G92A would likely be of limited application in humans due to the expression of albumin in both normal hepatocytes and hepatoma cells. However, G92A may be useful in experimental models of hepatocellular carcinoma. This is because the vector is TK^- and should grow poorly in nondividing adult hepatocytes (which express high levels of albumin). In a preliminary experiment, we found that inoculation of G92A (10^6 PFU) into the livers of young BALB/c mice was not lethal. HSV-1 TK^- mutants have been used as viral therapy for brain tumors (4, 29, 33, 35, 42), where glioma cells are dividing and the surrounding neurons and glia are nondividing. This growth differential is less pronounced in human hepatocellular carcinoma because of its association with chronic liver disease and hepatocyte regeneration (77). G92A might also prove useful for the generation of animal models of hepatocyte necrosis, a pathology associated with many nonmalignant diseases of the liver.

Other, more tumor-specific enhancers and/or promoters are the obvious regulatory regions to be attempted next. Examples include the tyrosinase promoter and melanoma cells (75), the secretory leukoprotease inhibitor promoter and carcinoma cells (14), the *erbB2* promoter and breast cancer cells (23), the α-fetoprotein promoter and hepatocellular carcinoma (28), and the carcinoembryonic antigen promoter and gastric cancer (72). This approach of generating cell-specific viral vectors should be applicable to many systems other than tumors, such as the ablation of specific cell types in animals at different stages in development or in adult life.

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