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Plasmid DNA Sequences Present in Conventional Herpes Simplex Virus Amplicon Vectors Cause Rapid Transgene Silencing by Forming Inactive Chromatin

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The herpes simplex virus (HSV)-based amplicon vector, a bacterial-viral-mammalian cell shuttle system, holds promise as a versatile gene delivery vehicle because of its large transgene capacity. However, amplicon-mediated transgene expression is often transient. We hypothesized that the presence of prokaryotic DNA sequences within the packaged vector genome can trigger transcriptional silencing of the entire vector sequence. To test this, we constructed a novel amplicon vector devoid of bacterial sequences (minicircle [MC] amplicon). Although the same dose of the minicircle amplicon vector in normal human fibroblasts resulted in an expression of luciferase approximately 20 times higher than that caused by the conventional amplicon vector, no significant difference was observed in copy numbers of luciferase DNA between MC amplicon- and control-transduced cells. Quantitative analyses of levels of luciferase mRNA revealed that differential expression of luciferase was controlled at the transcriptional level. Chromatin immunoprecipitation PCR analyses of several regions of vector genomes revealed that the bacterial sequences found in the conventional amplicon DNA were associated with an inactive form of chromatin immediately after infection. The presence of bacterial sequences also affected the remaining vector sequences in the conventional amplicon vector. Finally, nude mice injected with the MC amplicon exhibited higher and more sustained expression of luciferase than those injected with the conventional amplicon, confirming the usefulness of the MC amplicon devoid of bacterial sequences. Although additional improvements are absolutely required, these findings are a significant first step toward developing a novel HSV amplicon vector that can achieve enhanced long-term transgene expression.

Most popular gene therapy vectors, including those based on retroviruses, lentiviruses, and adeno-associated viruses, can deliver no more than 8 to 10 kb of exogenous DNA. Their ability to integrate vector sequences into host chromosomes enables therapeutic genes to remain permanently in transduced cells. However, such integrating vectors still suffer from transcriptional silencing (19), host immune responses (35, 38), and potential tumorigenesis (9). An alternative to vector integration is extrachromosomal maintenance of gene therapy vectors, in cases such as adenovirus- and herpes simplex virus (HSV)-based vectors. The HSV-based amplicon vector is a bacterial-viral-mammalian cell shuttle system that was originally developed by Spaete and Frenkel (29). When transfected into HSV-permissive cells and supplied with full HSV helper function, virtually any plasmid DNA with two noncoding sequences of HSV (an origin of DNA replication [*ori*] and a DNA cleavage/packaging signal [*pac*]) can be replicated and packaged into infectious HSV virions as a 150-kb linear vector DNA genome comprising "head-to-tail" concatenated structures of the original plasmid (27). Therefore, each vector particle generated from a 10-kb amplicon vector plasmid contains 15 copies of the seed plasmid sequence. This vector system holds considerable promise as a versatile gene delivery vehicle because of its unique features, including (i) a large transgene capacity of up to 150 kb (12, 33, 34), (ii) the ability to transduce a wide variety of cell types across a broad range of species, (iii) the ease and flexibility of vector construction, and (iv) limited cytotoxicity and immunogenicity because of the lack of viral coding sequences and availability of helper virus-free packaging systems (7, 22). However, the vector system needs to be further characterized and improved with regard to its stability of transgene expression. Recent efforts to improve the vector system have focused on generating hybrid amplicon vectors by incorporating DNA elements from other viruses, such as adeno-associated viruses (10, 37), Epstein-Barr virus (12, 26, 33, 34, 36), or retroviruses (24, 26). Although significant improvements in the stability of transgene expression have been made with these hybrid vectors, little study has been made of the molecular mechanism of the rapid transcriptional silencing of conventional amplicon vectors both in vitro and in vivo.

When plasmid DNA is delivered nonvirally to liver, muscle, or lung in vivo, transgene expression often lasts only for a short period of time, even though vector DNA is not lost (5, 8, 16, 23). The immunogenic CpG dinucleotides in the bacterial backbone of plasmid and the interaction between the DNA elements of the vector and a variety of cytokines have been suggested to play key roles in episomal gene silencing (4, 20). Recently, Chen and colleagues developed a novel method to prepare a minicircle (MC) DNA vector devoid of bacterial sequences from a plasmid vector through site-specific intramolecular recombination using the integrase from *Streptomyces*

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temperate phage φC31 and demonstrated MC-mediated long-term transgene expression in mouse liver (3).

In this study, we adopted the φC31 integrase-mediated recombination method to generate amplicon vectors devoid of bacterial sequences (MC amplicons). A firefly luciferase (Luc)-expressing MC amplicon was thus constructed and evaluated for expression of Luc in cultured normal human cells; a Luc-expressing, conventional HSV amplicon vector with the pBR322-derived plasmid backbone was used as a control. The MC amplicon expressed significantly higher levels of Luc activity than the control vector immediately after infection and maintained the high levels. We next investigated the molecular mechanisms underlying this phenomenon by examining the amounts of remaining vector DNA and transcriptional levels of luciferase gene (*luc*) in the infected cells. The results revealed that Luc activity in the transduced cells was controlled entirely at the transcriptional, not the translational, level. Chromatin immunoprecipitation (ChIP) PCR analyses of the introduced vector DNA suggested that the presence of bacterial sequences in the packaged vector genome triggers an association with inactive forms of chromatin immediately after infection. These findings thus provide a critical foundation for further investigation of the molecular mechanisms responsible for the transient nature of HSV amplicon-mediated gene expression.

MATERIALS AND METHODS

Plasmid construction and MC preparation. The HSV amplicon vector plasmid pHGCag was constructed by replacing the CMV promoter of pHGCX (21) with the CAG promoter obtained from pCAGGSneo (17) (a gift from J. Miyazaki, Osaka University, Osaka, Japan). The *luc*-expressing conventional amplicon plasmid pHGCag-Luc was constructed by inserting a 2-kb HindIII-BamHI fragment of pGL3-basic (Promega, Madison, WI) between unique HindIII and BamHI sites of the pHGCag vector plasmid. The MC-producing plasmid pBAD.φC31.RHB (3) was kindly provided by M. A. Kay (Stanford University, Stanford, CA). The 7.6-kb SalI fragment of the pHGCag-Luc was inserted between the two XhoI sites of the pBAD.φC31.RHB, replacing the hAAT expression cassette, and the resulting construct was designated pBAD.φC31.HGCag-Luc. MC amplicon DNA was prepared as described previously with minor modifications (3). Briefly, MC-producing pBAD.φC31 .HGCag-Luc was transformed into DH10B *Escherichia coli* (Invitrogen, Carlsbad, CA) and grown in LB broth containing 50 μg/ml ampicillin at 32°C. Overnight culture was diluted fivefold with LB medium with ampicillin and 1% L-(+)-arabinose (Sigma, Saint Louis, MO) for the induced expression of φC31 integrase. After a 2-hour culturing at 32°C, extrachromosomal DNA was collected using the QIAGEN plasmid maxi kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. The prepared DNA sample, a mixture of unresolved pBAD.φC31.HGCag-Luc, MC-HGCag-Luc, and excised φC31 backbone (Fig. 1), was digested with restriction endonucleases KpnI and NotI (New England Biolabs, Ipswich, MA). Supercoiled MC-HGCag-Luc DNA was then purified from the digested sample through CsCl density gradient ultracentrifugation followed by ethanol precipitation.

Cell culture. MRC9 (normal human fibroblasts purchased from ATCC, Manassas, VA) and Vero 2-2 (28) (a gift from R. M. Sandri-Goldin, University of California, Irvine, CA) cells were cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). G16-9 (Gli36 human glioma cells stably transduced with a retrovirus vector expressing the HSV VP-16 protein to enhance expression of reporter gene from the IE4/5 promoter) titration cells were maintained and propagated in DMEM supplemented with 10% FBS, penicillin, streptomycin, and hygromycin B (200 μg/ml). All cells were cultured at 37°C in an atmosphere containing 5% carbon dioxide.

Preparation of HSV amplicon vectors. HSV amplicons were packaged using the improved helper virus-free packaging system as described previously (21, 22). Briefly, 2-2 cells (plated at ~90% confluence in 6-cm culture dishes) were transfected with 0.6 μg amplicon DNA, 0.2 μg pEBH-ICP27 DNA, and 2 μg fHSVΔpacΔ27-0+ DNA using Lipofectamine Plus (Invitrogen) following the manufacturer's protocol. Three days later, the transfected cells and medium were harvested. The crude vector stocks were concentrated by ultracentrifugation at 75,000 × *g* for 3 h at 4°C over a 25% (wt/vol) sucrose cushion. The pellet was then resuspended in Hanks' balanced salt solution (HBSS) and stored at −80°C until use. The titer (green fluorescent protein [GFP] transducing units [TU]/ml) of each vector stock was determined on G16-9 cells.

Luciferase activity assay in vitro. MRC9 cells were seeded at 100% confluence in 24-well plates (1×10^5 cells/well). After 24 h, the cells were infected with 1×10^6 TU of HSV amplicon vectors in 250 μl DMEM supplemented with 2% FBS. After 24 h of incubation with the vectors, the cells were washed with HBSS and fed with 1 ml of fresh DMEM supplemented with 2% FBS. At various time points (1, 2, 3, and 6 days after infection), each well of cells was washed three times with phosphate-buffered saline (PBS), and 500 μl of passive lysis buffer (Promega) was added. After a freeze-thaw cycle, the cell extracts were transferred into tubes and centrifuged at 7,000 × *g* for 3 min at 4°C. The resulting supernatant was measured for Luc activity using the firefly luciferase assay reagent (Promega) and an Auto Lumat LB953 luminometer (Berthold Technologies, Oak Ridge, TN).

Vector genome copy number analysis. MRC9 cells were seeded in 24-well plates and infected with HSV amplicon vectors as described above. At each time point (1, 2, 3, and 6 days after infection), each well was washed once with HBSS and trypsinized, and the contents were transferred into a sterile 1.5-ml tube. The collected cells were washed once with PBS and resuspended in 200 μl PBS. Whole cellular DNA was then extracted using the QIAamp DNA blood mini kit (QIAGEN) and subjected to quantitative PCR analysis (10 min at 95°C and then 40 cycles of 15 s at 95°C and 1 min at 60°C) using a model 7500 real-time PCR system (Applied Biosystems, Foster, CA). A set of PCR primers and a VIC-TAMRA-labeled TaqMan probe specific to the *luc* gene (5′-CCTATGATTAT GTCCGGTTATGTAAACA-3′, 5′-TGTAGCCATCCATCCTTGTCAA-3′, and 5′-VIC-TCCGGAAGCGACCAACGCCTT-TAMRA-3′) were used to quantitate the *luc*-containing vector genomes. The pGL3-basic plasmid DNA was used as a control standard.

Quantitative RT-PCR analysis. MRC9 cells were seeded at 100% confluence in 12-well plates (2×10^5 cells/well) and infected with HSV amplicon vectors as described above. With TRIzol reagent (Invitrogen), total mRNA was isolated from the infected cells at various time points, and first-strand cDNA was synthesized using the SuperScript first-strand cDNA synthesis system (Invitrogen). Quantitative reverse transcription (RT)-PCR amplification was performed using a model 7500 real-time PCR system with *luc*-specific probe and primers (listed above) and ready-made human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)-specific probe and primers (Hs99999905-m1; Applied Biosystems) as an internal control.

ChIP assay. MRC9 cells were seeded at 100% confluence in 10-cm dishes (2×10^6 cells/well) and infected with HSV amplicon vectors (2×10^7 TU/well). The ChIP assay was performed using the ChIP assay kit (Upstate, Charlottesville, VA) with anti-K9-dimethylated H3 and anti-K4-dimethylated H3 antibodies (Upstate) as recommended by the manufacturer. Briefly, chromatin was cross-linked with 1% formaldehyde for 10 min at room temperature. After quenching with 125 mM glycine, the cells were washed and resuspended in sodium dodecyl sulfate lysis buffer and then sonicated. The soluble chromatin in the supernatant was diluted in ChIP dilution buffer and precleared by incubating with protein A-agarose–salmon sperm DNA slurry. Antibodies were added to the precleared supernatant and immunoprecipitated overnight at 4°C. After washing, antibody-bound histone-DNA complex was eluted from the agarose beads, and histone-DNA cross-links were reversed by heating to 65°C for 4 h. The immunoprecipitated DNA from each sample was subjected to quantitative PCR analyses using a model 7500 real-time PCR system and SYBR green PCR master mix (Applied Biosystems). PCR amplification was performed in the following conditions: 15 min at 95°C, 40 cycles of 1 min at 94°C, 55 s at 55°C, and 1 min at 72°C, and then 10 min at 72°C. The following seven pairs of primers were used for the assay: (i) IE4/5-EGFP, 5′-AGGAACGTCCTCGTCGATA-3′ and 5′-CGCTGAACTTGT GGCCGTTTA-3′; (ii) *EGFP*, 5′-TATCATGGCCGACAAGCAGA-3′ and 5′-CGTCCATGCCGAGAGTGA-3′; (iii) CAG promoter, 5′-GCATGGACGAGC TGTACAAG-3′ and 5′-CTATTGGCGTTACTATGGGAACAT-3′; (iv) *Luc*-1, 5′-ACATCACTTACGCTGAGTACTTCG-3′ and 5′-CAACCCCTTTTTGGA AACGA-3′; (v) *Luc*-2, 5′-GGAATCCATCTTGCTCCAAC-3′ and 5′-TCTCTCTGATTTTTCTTGCGTC-3′; (vi) *Luc-pac*, 5′-CATATCAGCAAAA GTGATACGGGT-3′ and 5′-GGGCATCTCTACCTCAGTGC-3′; and (vii) ampicillin resistance (*amp*) gene, 5′-CCCCGAAGAACGTTTTCC-3′ and 5′-GCGAGT TACATGATCCCCCA-3′. The amplicon plasmid pHGCag-Luc was used as control standard.

Immunoblot assay for phosphorylation of eukaryotic initiation factor 2α (eIF-2α). MRC9 cells were seeded at 100% confluence in six-well plates (3×10^5 cells/well). The following day, the cells were infected with the HSV amplicon

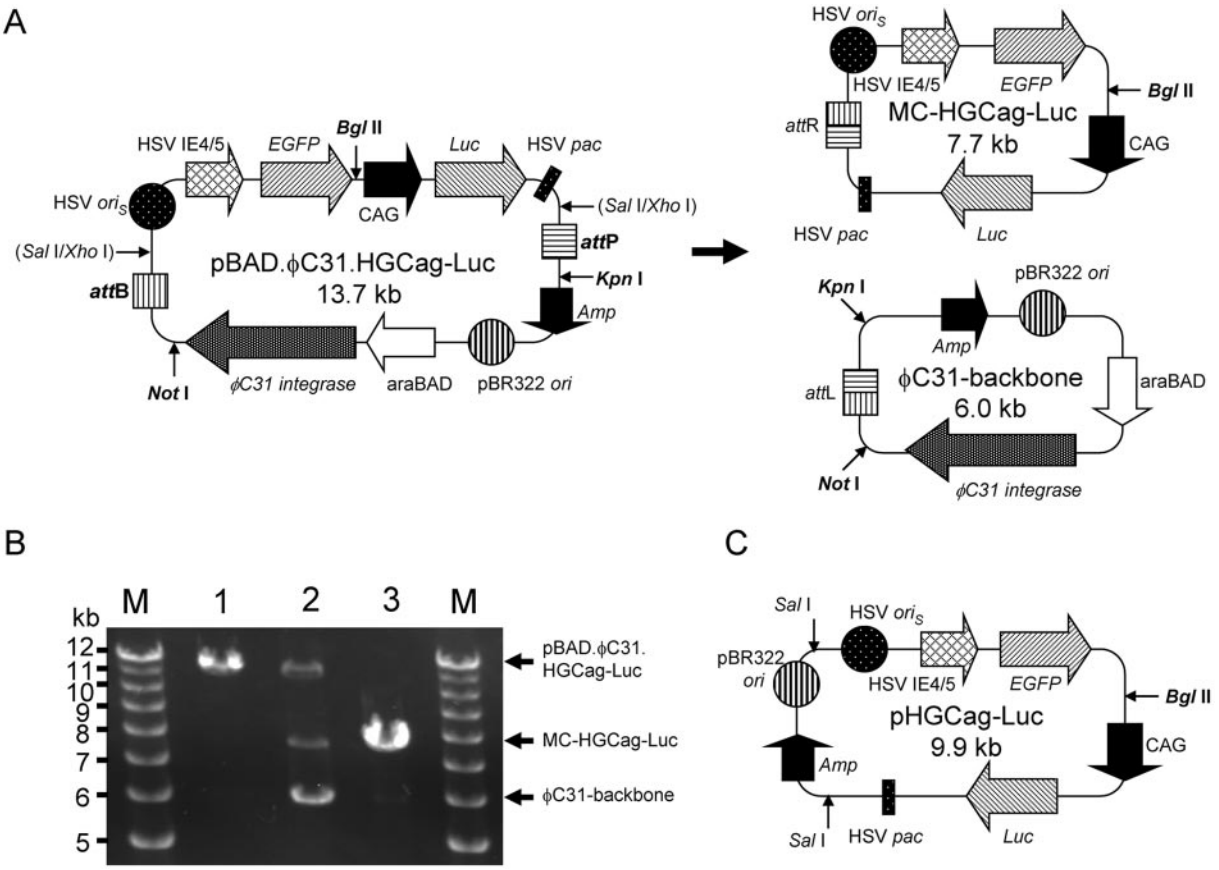

FIG. 1. Production of the MC amplicon vector using φC31 integrase-mediated recombination in *Escherichia coli*. (A) Flow chart of φC31 integrase-mediated intramolecular recombination of pBAD.φC31.HGCag-Luc. The resulting products are shown and designated as MC-HGCag-Luc and φC31-backbone. MC-HGCag-Luc DNA was purified from contaminated pBAD.φC31.HGCag-Luc and φC31-backbone by digesting with KpnI and NotI followed by CsCl gradient ultracentrifugation. (B) Electrophoretic analysis of the MC amplicon, its precursor, and intermediates. One hundred nanograms of each DNA sample was digested with BglII and NotI and electrophoresed on a 0.8% agarose gel. Lane 1, pBAD.φC31.HGCag-Luc before induction; lane 2, DNA sample obtained after a 2-hour induction of φC31 integrase expression (mixture of pBAD.φC31.HGCag-Luc, MC-HGCag-Luc, and φC31-backbone); lane 3, purified MC-HGCag-Luc; and lane M, 1-kb-plus DNA ladder (Invitrogen). (C) Map of pHGCag-Luc amplicon vector plasmid used for a control in the study.

vectors (6×10^6 TU/well) or were mock infected. At each time point (1, 2, 3, and 6 days after infection), each well of cells was harvested and collected into a sterile 1.5-ml tube. The pelleted cells were resuspended in 150 μl protein-loading buffer and boiled for 5 min. The protein samples were electrophoresed on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA), and incubated with either a mouse monoclonal anti-eIF-2α antibody or a rabbit polyclonal antibody against the Ser51-phosphorylated form of eIF-2α (Cell Signaling Technology, Beverly, MA). Proteins were detected using the ECL Plus kit (Amersham Pharmacia Biotech, Piscataway, NJ) as recommended by the manufacturer.

In vivo bioluminescence imaging studies. Nude (*nu/nu*) mice, purchased from Charles River Laboratories (Wilmington, MA), were injected with 1×10^7 TU of HSV amplicon vectors in 200 μl HBSS via the tail vein (day 0). At various time points, mice injected with Luc-expressing HSV amplicon vectors were imaged for in vivo Luc activity using a NightOWL LB 981 bioluminescence imaging system (Berthold Technologies). Mice were injected intraperitoneally with 500 μl D-luciferin sodium solution (100 mg/kg body weight; Biotium Inc., Hayward, CA), and images were acquired 5 min after administration of substrate. A light image of the animal was also taken in the dark chamber using dim illumination. The intensity and spatial distribution of Luc activity within the mice were then measured by recording photon counts for 5 min using the cooled charge-coupled device camera with no illumination. Following data acquisition, postprocessing and visualization were performed using the CMIR-Image program (32). Regions of interest were defined using an automatic intensity contour procedure to identify bioluminescent signals with intensities significantly greater than the background. The sum of the photon counts in these regions was then calculated.

RESULTS

Construction and production of MC amplicon. Plasmid pBAD.φC31.HGCag-Luc (Fig. 1A, left, and Fig. 1B, lane 1), which serves as a precursor of MC-HGCag-Luc (Fig. 1A, middle), was constructed as described in Materials and Methods. The *φC31 integrase* gene in the pBAD.φC31.HGCag-Luc was designed to be expressed in an L-arabinose-inducible manner; expression of the integrase facilitates intramolecular recombination between the *attB* and *attP* sites of the plasmid, resulting in generation of MC-HGCag-Luc and φC31 plasmid backbone (Fig. 1A, right). After bacteria carrying the precursor plasmid were grown for 2 h at 32°C in the presence of 1% L-arabinose induction, extrachromosomal DNA was purified using the QIAGEN plasmid maxi kit (Fig. 1B, lane 2). After linearizing the bacterial backbone-containing plasmids (pBAD.φC31 .HGCag-Luc and φC31-backbone) by digestion with KpnI and NotI, MC amplicon vector plasmid (MC-HGCag-Luc) was successfully purified by standard CsCl-gradient ultracentrifugation (Fig. 1B, lane 3). The isolated MC-HGCag-Luc DNA was then packaged into infectious HSV virions using the improved

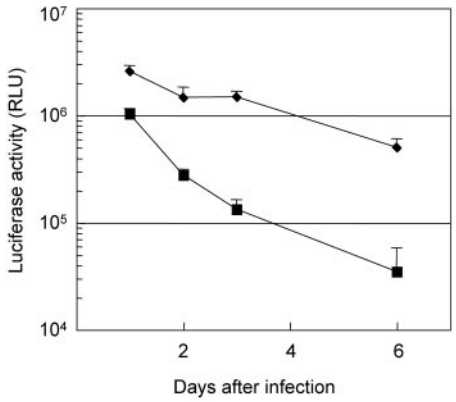

FIG. 2. Time course of Luc activity in MRC9 human fibroblasts infected with either MC (◆) or conventional HGCag (■) amplicon vectors in culture. Luc activity is presented as mean ± standard deviation (SD) ($n = 4$).

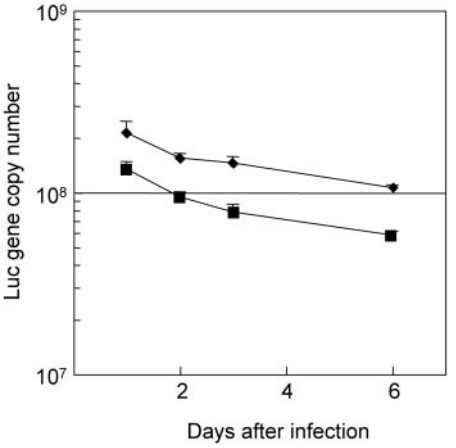

FIG. 3. Time course of vector genome copy numbers in MRC9 human fibroblasts infected with either MC (◆) or conventional HGCag (■) amplicon vectors in culture. *luc* gene copy numbers were determined by real-time PCR analysis. Data are presented as mean ± SD ($n = 4$).

HSV amplicon helper virus-free packaging system (21, 22). The titers obtained with the MC amplicon plasmid DNA were similar to those obtained with conventional amplicon vector plasmid. In this study, a pBR322 backbone-containing conventional amplicon plasmid pHGCag-Luc (Fig. 1C) was used as a control. MC-HGCag-Luc and pHGCag-Luc share all expression cassettes and DNA elements for the HSV amplicon.

Evaluation of luciferase expression in vitro. We first evaluated time course expression of the *luc* gene product in nondividing normal human fibroblast cells. As described in Materials and Methods, 1×10^5 MRC9 cells were infected with 1×10^6 TU of either MC-HGCag-Luc (MC) or HGCag-Luc (HGCag) amplicon vectors. At various time points after infection, the infected cells were harvested and measured for Luc activity (Fig. 2). At day 1, Luc expression in cells infected with the MC amplicon was almost double that of cells infected with the conventional HGCag amplicon. Luc activity in HGCag-infected cells started to decline by day 2 and was only about 1/30 its original level by day 6, whereas that in cells infected with the MC amplicon decreased rather gradually to about 1/5 the original level by day 6 (Fig. 2). The same experiment was repeated three times; additional experiments employing different vector doses (1×10^5, 5×10^5, and 2×10^6 TU/well) were also performed and produced very similar results (data not shown).

Quantitation of vector genome copy numbers in infected cells. To determine the mechanisms underlying differential Luc activity in cells infected with the MC amplicon or HGCag amplicon, vector genome copy numbers were determined using the primers and probe specific to the *luc* gene (Fig. 3). Whole cellular DNA was isolated from the infected MRC9 cells at different time points. Quantitative PCR amplification was performed with these DNA samples. The copy number of the *luc* gene from each sample was determined using a standard curve generated with samples containing known DNA amounts of a *luc*-containing plasmid. From day 1 through day 6, the copy number of the *luc* gene of the cells infected with the MC amplicon was approximately 1.5 times that of those infected with the HGCag amplicon, probably as a result of the size difference of their monomer DNA (7.7 versus 9.9 kb). Vector genome copy numbers of HSV amplicon vectors were shown to be rather stable with or without prokaryotic plasmid DNA sequences in the nondividing cultured cells. In both cases, no more than 50% of the originally introduced vector DNA was lost during the 5 days of culturing. These data indicated that the decline of Luc expression in the infected cells was not caused by loss of vector DNA.

Quantitation of luciferase mRNA in infected cells. We next investigated the transcriptional levels of the *luc* transgene by measuring *luc* mRNA in the infected cells. Total RNA was isolated from the cells infected with either of the amplicon vectors and converted to first-strand cDNA. Quantitative RT-PCR was performed with these cDNA samples using a probe and primers specific to *luc*. *GAPDH* mRNA was also quantitated as an internal control. The amount of *luc* mRNA in each sample was calculated relative to the endogenous *GAPDH* mRNA and graphed in Fig. 4. At day 1, the MC amplicon expressed 1.2 times the *luc* mRNA expressed by the conventional HGCag amplicon. The transcriptional level of *luc* transgene in the MC amplicon vector was maintained for up to at

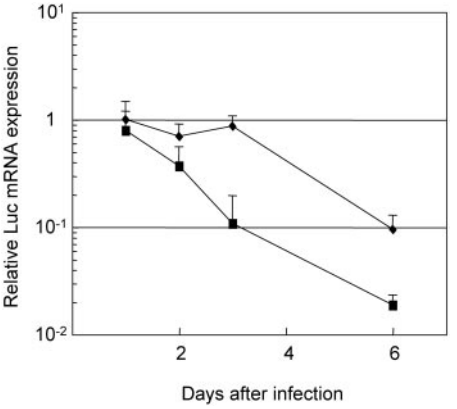

FIG. 4. Time course expression of *luc* mRNA in MRC9 human fibroblasts infected with either MC (◆) or conventional HGCag (■) amplicon vectors in culture. Each value was calculated relative to that of the MC amplicon at day 1. Data are presented as mean ± SD ($n = 3$).

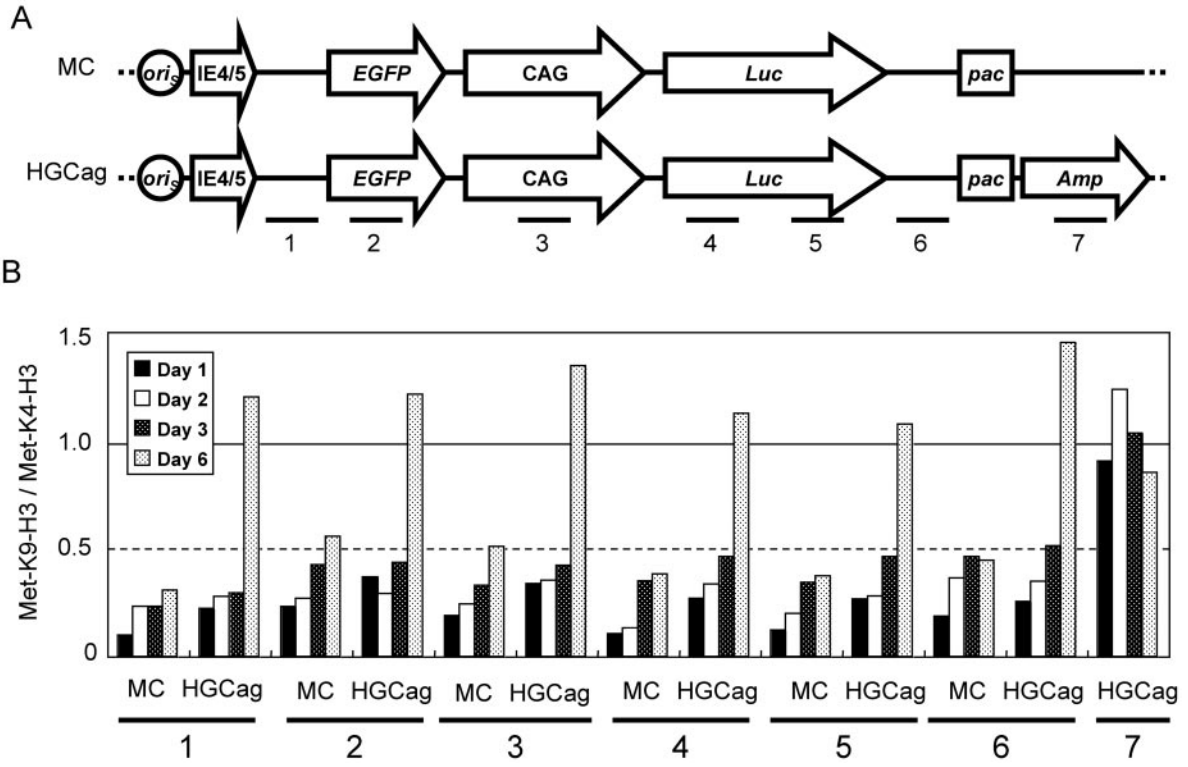

FIG. 5. Time course modifications in chromatin of various regions of vector genomes in MRC9 human fibroblasts infected with either MC or conventional HGCag amplicon vectors. (A) Schematic structures of MC and HGCag amplicon vector genomes and approximate locations of the seven primer pairs examined. (B) Ratio of DNA associated with Met-K9-H3 to that with Met-K4-H3 was calculated for each of the seven genetic regions shown in panel A. The higher the ratio, the more inactive the transcription in the region.

least 3 days and declined to approximately 1/10 of the original at day 6, whereas that in the HGCag rapidly declined to approximately 1/40 at day 6. The time course of expression of *luc* mRNA was strikingly similar to that of Luc activity in the amplicon-transduced cells, indicating that Luc activity was regulated primarily at the transcriptional level. The presence of bacterial sequences in the conventional amplicon vector, therefore, seems to affect transcriptional activity of the vector genome.

ChIP PCR analysis of vector genomes in mammalian cells. To determine if bacterial sequences affect the chromatin status of the vector genomes in the infected cells, ChIP PCR analysis was performed. DNA samples were isolated from MRC9 human fibroblasts infected with either MC or HGCag amplicon vectors and immunoprecipitated with anti-methylated lysine (K) 9 histone H3 (Met-K9-H3) or anti-methylated K4 histone H3 (Met-K4-H3) antibodies. These modified histone molecules (Met-K9-H3 and Met-K4-H3) have been reported to localize selectively in the heterochromatin and euchromatin domains, respectively (18). After immunoprecipitation with each antibody, the presence of enhanced GFP (EGFP) coding, CAG promoter, and Luc coding sequences were evaluated by quantitative PCR using the specific primers. Quantitative PCR of the *amp* gene was also performed with the samples using the conventional HGCag amplicon (Fig. 5A). All the amplified products were electrophoresed and confirmed to have no nonspecific PCR products, such as primer dimers (data not shown). The ratio of Met-K9-H3-associated DNA to Met-K4-H3-associated DNA was calculated with each primer set at each time point and was plotted (Fig. 5B). All the examined DNA segments of HGCag amplicon DNA were shown to be associated preferentially with transcriptionally inactive Met-K9-H3 compared to the corresponding DNA segments of the MC amplicon. The *amp* gene present in the HGCag amplicon demonstrated a significantly higher ratio of Met-K9-H3 to Met-K4-H3 than other DNA segments even at day 1, and the ratio persisted. The ratios of Met-K9-H3 to Met-K4-H3 of the other regions of the HGCag amplicon genome increased over time and reached levels similar to that of the *amp* gene by day 6. On the other hand, all regions of the MC amplicon genome examined were shown to be maintained at lower ratios of Met-K9-H3 to Met-K4-H3, although the ratios gradually increased. These results indicate that prokaryotic sequences in conventional HSV amplicon vectors can be sensed by unknown molecular mechanisms and associated with inactive forms of histone molecules immediately after infection. The inactive form of chromatin, originating at the bacterial sequences, seems to spread through the vector genome rather rapidly in mammalian cells. The same experiment was repeated two more times with similar results (data not shown). Similar results were also obtained with nondividing normal human astrocyte culture (data not shown), precluding the possibility that this phenomenon is fibroblast specific.

Immunoblotting assay for eIF-2α phosphorylation. In virally infected cells, eIF-2α has been reported to be phosphorylated by a double-stranded RNA-dependent protein kinase

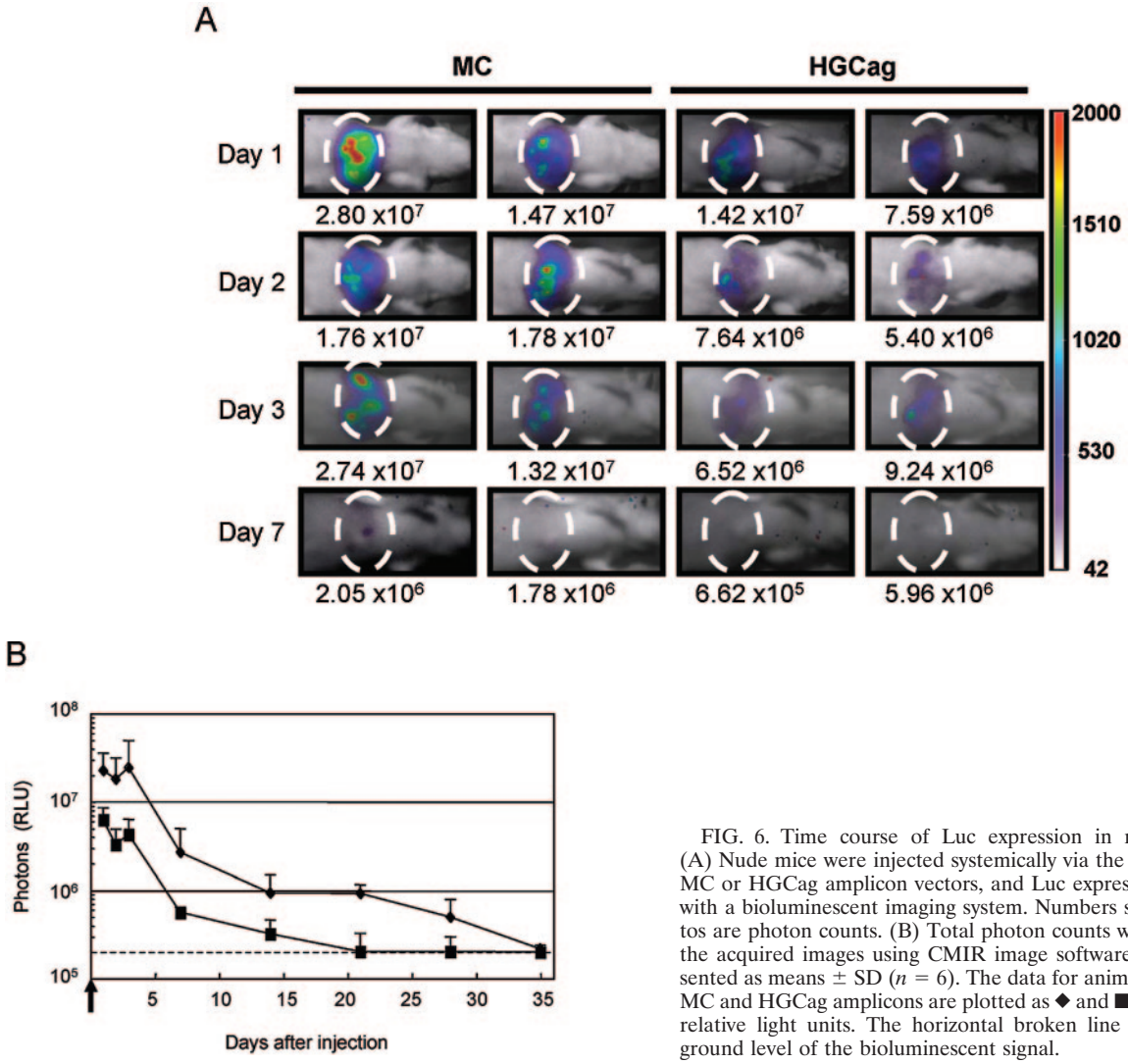

FIG. 6. Time course of Luc expression in nude mice in vivo. (A) Nude mice were injected systemically via the tail vein with either MC or HGCag amplicon vectors, and Luc expression was monitored with a bioluminescent imaging system. Numbers shown beneath photos are photon counts. (B) Total photon counts were calculated from the acquired images using CMIR image software, and data are presented as means ± SD ($n = 6$). The data for animals injected with the MC and HGCag amplicons are plotted as ◆ and ■, respectively. RLU, relative light units. The horizontal broken line indicates the background level of the bioluminescent signal.

(PKR), shutting off host translation (6). Infection of cells with replication-conditional HSV vectors has also been demonstrated to activate PKR, resulting in increased phosphorylation of eIF-2α (13). To verify whether infection with the HSV amplicon also triggers PKR activation, we evaluated phosphorylation of eIF-2α in the amplicon-transduced MRC9 cells (>70% of the cells were GFP positive) or mock-infected cells at several time points. There was no significant increase in the amount of phosphorylated eIF-2α in the cells infected with either MC or HGCag amplicon vectors compared to mock-infected cells, precluding the possibility of suppression of PKR-mediated translation (data not shown).

Evaluation of luciferase expression in vivo. Finally, we evaluated MC and HGCag amplicon vectors for their expression of Luc in vivo. An injection of 1×10^7 TU of each amplicon vector was made into the tail veins of nude mice (six animals per group). Expression of Luc was measured by a NightOWL LB 981 bioluminescence imaging system (Berthold Technologies) at several time points (Fig. 6A). Luc activity detected from only the liver suggested that the majority of intravenously administered vector particles were taken up by the liver. Total Luc expression from each animal was evaluated by calculating the sum of the photon counts from the liver. Figure 6B shows that mice injected with the MC amplicon demonstrated higher and more sustained Luc activity than those injected with the conventional HGCag amplicon. Luc expression with the conventional amplicon declined to an undetectable level within 21 days after injection, whereas that with the MC amplicon was detectable up to at least day 28. These results indicate that the MC amplicon devoid of bacterial sequences can mediate enhanced and sustained transgene expression not only in vitro, but also in vivo.

DISCUSSION

HSV-based amplicon vector is a versatile plasmid-based infectious gene delivery system that can carry a transgene cassette as large as 150 kb (27, 34). Recent improvement of the bacterial artificial chromosome-based helper virus-free packaging system has allowed complete elimination of replication-competent helper virus in HSV amplicon vector stocks (22). Although we anticipated this would solve most of the short-

comings of the vector, such as transient transgene expression, cytotoxicity, and immunogenicity, it has become evident that there are still problems remaining with the HSV amplicon vector itself. For example, we often experience rapid transgene silencing of HSV amplicon vectors, even in nondividing tissue culture cells.

In this study, we constructed an HSV amplicon vector devoid of plasmid backbone and compared it with a control vector with plasmid backbone to examine whether the presence of bacterial sequences (plasmid backbone) in the vector genome contributes to rapid transgene silencing. Bacterial sequence-containing transgene cassettes and nonviral plasmid-based expression vectors were reported to undergo gene silencing. Recently, Chen and colleagues elegantly developed a method to generate MC DNA vectors devoid of bacterial sequences using a phage ϕC31 integrase-mediated intramolecular recombination technology (3). They further demonstrated that MC DNA vectors are capable of persistently high transgene expression in vivo and that bacterial DNA elements covalently linked to expression cassettes are responsible for the silencing of transgene expression mediated by plasmid-based expression vectors (3, 4). We thus adopted the ϕC31 integrase-mediated method to generate an HSV amplicon vector free of bacterial sequences (MC amplicon).

The MC amplicon vector carrying a firefly luciferase (*luc*) transgene under the control of the CAG promoter (17) expressed persistently higher levels of Luc activity than the conventional HGCag amplicon vector carrying the identical transgene cassettes in normal human fibroblasts and astrocytes in culture. When we determined the quantities of retained vector DNA in the infected cells, no significant difference was observed between the MC amplicon and the HGCag amplicon, precluding differential DNA retention efficiencies of these vectors. Quantitative analyses of *luc* mRNA revealed that the differential Luc activities in the cells infected with these two vectors were regulated by the transcriptional levels of the *luc* gene. ChIP PCR was performed to investigate molecular mechanisms responsible for the transcriptional silencing of the HGCag amplicon bearing bacterial sequences. Euchromatic and heterochromatic chromosomal domains are known to have distinctive site-specific histone H3 methylation patterns. Met-K9-H3 is strictly localized at transcriptionally silent heterochromatic regions, whereas Met-K4-H3 is specific to transcriptionally active euchromatic regions (39). The regions having a higher ratio of Met-K9-H3 to Met-K4-H3 are considered transcriptionally silent or heterochromatic. The *amp* gene, a part of the prokaryotic sequences of the HGCag amplicon, showed quite a high ratio of Met-K9-H3 to Met-K4-H3 immediately after infection that persisted, while mammalian expression DNA elements, such as the CAG promoter, EGFP, and Luc coding sequences, showed lower ratios of Met-K9-H3 to Met-K4-H3 at day 1 that increased gradually to that of the *amp* gene in the HGCag amplicon genome. These data indicate that the bacterial sequences are rapidly sensed by unknown host defense mechanisms and transcriptionally silenced by chromatin modifications and that the inactive chromatin gradually spreads along the surrounding vector sequences to completely abolish transgene expression. The ratios of Met-K9-H3 to Met-K4-H3 of DNA elements in the MC amplicon genome also increased slightly with time, suggesting a possible intrinsic silencing mechanism in the HSV amplicon system in addition to the bacterial sequences.

Heterochromatic modifications in histone are often linked to DNA methylation (2). The frequency of CpG dinucleotides is much higher in bacterial DNA than in vertebrate DNA (31). In bacterial DNA, CpG sequences are usually unmethylated, whereas in mammalian DNA, about 75% of the CpGs are methylated to 5′-methylcytosine (1). CpG dinucleotides in episomal vector DNA could undergo de novo methylation in mammalian cells, and this could be a potential mechanism for episomal transgene silencing (11). Methylated CpG dinucleotides could become targets for a group of cellular proteins, including CPM1 and CPM2, that are known to bind to and condense DNA, causing transcriptional silencing of the regions (2). Moreover, Chen and associates pointed out that the high frequencies of unmethylated CpG dinucleotide present in all bacterial DNA elements may be responsible for the sequence-independent inhibitory effects of the prokaryotic sequences (4).

Our study of gene transfer in vivo revealed that the MC amplicon was capable of higher and more sustained transgene expression than the conventional HGCag amplicon in nude mice, suggesting that bacterial DNA elements in the vector genome also cause transcriptional silencing in vivo. However, the Luc activity transduced with MC amplicon vector also declines to a background level within a month, indicating the presence of additional factors (e.g., host innate immune responses and vector genome structures) causing transgene silencing of HSV amplicon vectors. It is well known that unmethylated CpG motifs in the genomes of bacteria, insects, and some viruses can directly activate B cells and antigen-presenting cells in vitro and in vivo, producing effector molecules that induce a potent adaptive Th1-type response (14, 31). Moreover, innate defense mechanisms are triggered by host reactions mediated by Toll-like receptors (TLR) to pathogen-associated molecular patterns that distinguish invading pathogens, such as infectious entities, from the host itself (25). Recently, unmethylated CpG motifs have been shown to be recognized by TLR-9 and to trigger innate immune responses (30), and the HSV genome has been reported to be undermethylated and immunostimulatory both in vitro and in vivo (15). It would be very interesting to investigate the methylation status of vector genomes in packaged HSV virions and in infected mammalian cells. Further investigation including careful assessment of host innate responses against HSV amplicon vectors would be necessary to uncover the molecular mechanisms of transgene silencing of HSV amplicon vectors. These studies will provide a critical basis for future improvement of the vector system through the development of a truly versatile gene delivery vector.

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