

## Identification of Sites in Adenovirus Hexon for Foreign Peptide Incorporation

Hongju Wu, Tie Han, Natalya Belousova, Victor Krasnykh,<sup>†</sup> Elena Kashentseva, Igor Dmitriev, Manjula Kataram, Parameshwar J. Mahasreshti, and David T. Curiel\*

*Division of Human Gene Therapy, Departments of Medicine, Pathology and Surgery, and the Gene Therapy Center, University of Alabama at Birmingham, Birmingham, Alabama*

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**Adenovirus type 5 (Ad5) is one of the most promising vectors for gene therapy applications. Genetic engineering of Ad5 capsid proteins has been employed to redirect vector tropism, to enhance infectivity, or to circumvent preexisting host immunity. As the most abundant capsid protein, hexon modification is particularly attractive. However, genetic modification of hexon often results in failure of rescuing viable viruses. Since hypervariable regions (HVRs) are nonconserved among hexons of different serotypes, we investigated whether the HVRs could be used for genetic modification of hexon by incorporating oligonucleotides encoding six histidine residues (His<sub>6</sub>) into different HVRs in the Ad5 genome. The modified viruses were successfully rescued, and the yields of viral production were similar to that of unmodified Ad5. A thermostability assay suggested the modified viruses were stable. The His<sub>6</sub> epitopes were expressed in all modified hexon proteins as assessed by Western blotting assay, although the intensity of the reactive bands varied. In addition, we examined the binding activity of anti-His tag antibody to the intact virions with the enzyme-linked immunosorbent assay and found the His<sub>6</sub> epitopes incorporated in HVR2 and HVR5 could bind to anti-His tag antibody. This suggested the His<sub>6</sub> epitopes in HVR2 and HVR5 were exposed on virion surfaces. Finally, we examined the infectivities of the modified Ad vectors. The His<sub>6</sub> epitopes did not affect the native infectivity of Ad5 vectors. In addition, the His<sub>6</sub> epitopes did not appear to mediate His<sub>6</sub>-dependent viral infection, as assessed in two His<sub>6</sub> artificial receptor systems. Our study provided valuable information for studies involving hexon modification.**

The adenovirus type 5 (Ad5)-based gene delivery system has shown great potential for gene therapy applications because Ad5 viruses have a minor pathological effect on humans, can efficiently infect a wide variety of cell types, and can be readily generated to high titers *in vitro* (3, 26, 42). However, several problems need to be solved before Ad5 vectors become effective in clinics. For example, the receptor for Ad5, coxsackievirus and adenovirus receptor (CAR), is expressed in many cells; thus, infection of Ad5 vectors is not specific to target cells (4, 35). On the other hand, some target tissue express a very low level of CAR and thus are difficult to infect (7, 18, 44). In addition, some patients have preexisting neutralizing antibodies against Ad5 resulting from natural adenovirus infection or previously administered Ad5 vectors (8, 17, 36, 38, 49, 50, 52). This preexisting immunity can prevent subsequent Ad5 infections.

One strategy to address these problems is capsid modification. The capsid of each Ad virion is composed of three major proteins: hexon, fiber, and penton base. Infection of cells by Ad5 is mediated by attachment of fiber protein to the cell surface receptors, CAR (4, 22, 34), followed by interaction of the penton base with  $\alpha_v$  integrins, which triggers the internal-

ization of the viruses (2, 27, 43). Based on this knowledge, fiber modification has been employed to redirect Ad5 infection by incorporating extra targeting motifs. For example, RGD and polylysine motifs have been incorporated into fiber, and the modified vectors demonstrated CAR-independent Ad5 infection (10, 19, 44, 45, 48). As the largest and most abundant capsid protein, hexon is found to be the major target for host neutralizing antibodies against Ad5 (14–16, 25, 31, 46, 47). Therefore, hexon modification may be used to circumvent the host's preexisting immunity. In this regard, gene therapy investigators have sought both nongenetic and genetic strategies (29).

The nongenetic strategy is to mask the antigens on hexon surface with protective polymers such as polyethylene glycol (PEG), polylactic glycolic acid (PLGA) microspheres, or lipids by *in vitro* chemical coupling methods (21, 23, 24, 28). These approaches require additional procedures, and their efficacy and safety remain to be tested. The genetic strategy is to modify the hexon gene so that the expressed hexon protein cannot be recognized by the preexisting neutralizing antibodies. For example, since neutralizing antibodies against adenoviruses have serotype specificity and are mostly targeted on the major capsid protein hexon, replacement of Ad5 hexon with hexons from different serotypes may accomplish this goal. Indeed, studies have shown that Ad5 vectors bearing hexons from Ad1, Ad2, Ad3, Ad6, and Ad12 can escape the host's preexisting immunity against Ad5 (16, 31, 47, 51). In addition, Crompton et al. have genetically incorporated an eight-amino-acid sequence from the major antigenic site in the VP1 capsid protein of poliovirus type 3 into the Ad2 hexon and have

\* Corresponding author. Mailing address: Division of Human Gene Therapy, Departments of Medicine, Pathology and Surgery, and the Gene Therapy Center, The University of Alabama at Birmingham, Birmingham, AL 35294. Phone: (205) 934-8627. Fax: (205) 975-7476. E-mail: david.curiel@ccc.uab.edu.

<sup>†</sup> Present address: Experimental Diagnostic Imaging, The University of Texas, M. D. Anderson Cancer Center, Houston, TX 77030.

discovered that this modification can alter the immune response to this virus (6). Hexon modification, however, often results in failure of rescuing viruses or poorly growing viruses (6, 16, 31, 47, 51), suggesting hexon modification may interfere with viral formation. Therefore, it will be very useful to know what regions of hexon can be modified without affecting viability of the Ad5 viruses.

The hexon monomer of Ad5 is composed of 952 amino acid residues. Electron microscopy and X-ray crystallography of human Ad2 and Ad5 hexon revealed that hexon has a dense pedestal base formed by two eight-stranded, antiparallel  $\beta$  barrels stabilized by an internal loop (L3). Three other loops, L1, L2, and L4, project away from the surface of the virion (1, 30, 32, 33, 39, 40). Analysis of the protein sequences of different hexons revealed that, in addition to the conserved regions, there are seven discrete hypervariable regions (HVRs), and these HVRs do not appear to be involved in binding any other viral proteins (5). In addition, previous studies have found that the HVRs of hexon contain the serotype-specific epitopes (5, 32). Therefore, genetic modification of these regions seems to be very promising in circumventing the host immunization response against Ad5. Furthermore, since HVRs are mostly located within the extruding domains of the hexon, genetic incorporation of foreign targeting motifs into these HVRs may allow CAR-independent viral infection. Since hexon has more copies than fiber in the capsid, hexon modification-mediated targeting may be more profound than fiber modification.

In this study, we tested whether foreign peptides can be incorporated into HVRs without affecting the normal function of Ad5 as a gene therapy vector. We genetically incorporated six-histidine ( $\text{His}_6$ ) epitopes into different HVRs of Ad5 hexon and characterized the viruses in detail. Our study provided valuable information for Ad5-based gene therapy applications involving hexon modification.

#### MATERIALS AND METHODS

**Antibodies.** Goat polyclonal antibody against Ad2 hexon AB1056, and mouse anti-His-Tag monoclonal antibody (MAB3112) were purchased from Chemicon International, Inc. Alkaline phosphatase (AP)-conjugated donkey anti-goat and goat anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

**Cells and cell culture.** The human embryonic kidney cell line 293, transformed with Ad5-E1 DNA, was purchased from Microbix (Toronto, Ontario, Canada). Human cervix carcinoma HeLa cells and human glioma U118MG cells were obtained from the American Type Culture Collection (ATCC). U118MG.Hiss Fv.Rec cells were obtained by stably transfecting U118MG cells with single-chain antibody against His tag (12). 293, U118MG, and U118MG.HissFv.Rec cells were cultured in Dulbecco's modified Eagle's medium-Ham's F-12 (DMEM/F-12) containing 10% fetal calf serum (FCS) and 2 mM L-glutamine. HeLa cells were grown in Eagle's minimum essential medium (E-MEM) containing 10% FCS and 2 mM L-glutamine. All of the cells were cultured at 37°C in a 5%  $\text{CO}_2$  atmosphere.

**Construction of adenoviral vectors.** The  $\text{His}_6$  epitope composed of six histidine residues and flanking Lys-Gly-Ser spacers were genetically incorporated into hexon HVRs at positions marked in Fig. 1. To accomplish this genetic modification, we first obtained hexon fragments containing sequences encoding the  $\text{His}_6$  epitope in different HVRs via three-step PCR. For instance, to obtain a  $\text{His}_6$  insertion in HVR2 (HVR2- $\text{His}_6$ ), using Ad5 hexon as template, we first amplified fragment 2L (left to the HVR2 insertion) with primers L (Dra III) and HVR2- $\text{His}_6$  (antisense), and fragment 2R (right to the HVR2 insertion) with primers HVR2- $\text{His}_6$  (sense) and R (SacI) (Table 1). After purification of fragments 2L and 2R, 25 to 50 ng of each fragment (equal molar ratio) was mixed and used as a template and primers for the second step of PCR, resulting in insertion of sequences encoding  $\text{His}_6$  and the spacers in HVR2. Next, primers L and R were

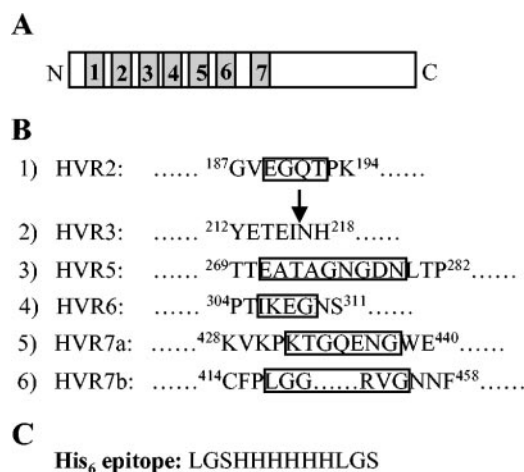


FIG. 1. Diagram of the  $\text{His}_6$  incorporation sites in Ad5 hexon. (A) Domain structure of hexon. HVRs (□) are numbered as 1 to 7. (B) Sites in the HVRs that were modified with  $\text{His}_6$  motif. The amino acid residues marked in the rectangles were replaced with the  $\text{His}_6$  motif. For HVR3, the arrow marks the position where the  $\text{His}_6$  motif was inserted. The numbers show the positions of the amino acid residues in the Ad5 hexon. (C) Amino acid residues of the  $\text{His}_6$  motif that was incorporated into HVRs of Ad5 hexon. LGS sequences flanking the six histidines were included as spacer.

added into the tubes, and a third step of PCR was used to amplify the HVR2- $\text{His}_6$  fragment. Other insertions were obtained in the same way with corresponding HVR- $\text{His}_6$  primers, which include HVR3- $\text{His}_6$  (antisense), HVR3- $\text{His}_6$  (sense), HVR5- $\text{His}_6$  (antisense), HVR5- $\text{His}_6$  (sense), HVR6- $\text{His}_6$  (antisense), HVR6- $\text{His}_6$  (sense), HVR7a- $\text{His}_6$  (antisense), HVR7a- $\text{His}_6$  (sense), HVR7b- $\text{His}_6$  (antisense), HVR7b- $\text{His}_6$  (sense) (Table 1).

The hexon fragments containing  $\text{His}_6$  epitope obtained above were purified and subcloned into Ad5 hexon shuttle vector H5/pH5S (described in reference 47) with DraIII and SacI. The resultant shuttle plasmids were named HVR2- $\text{His}_6$ /pH5S, HVR3- $\text{His}_6$ /pH5S, HVR5- $\text{His}_6$ /pH5S, HVR6- $\text{His}_6$ /pH5S, HVR7a- $\text{His}_6$ /pH5S, and HVR7b- $\text{His}_6$ /pH5S, respectively. To create Ad5 vector containing  $\text{His}_6$  epitopes in the HVRs of hexon, these plasmids were digested with EcoRI and PmeI, and the fragments containing the homologous recombination regions and the hexon genes were purified, then recombined with a SmaI-digested backbone Ad5 vector that lacks the hexon gene, pAd5/ $\Delta$ H5 (47) in *Escherichia coli* BJ5183. The resultant clones were designated pAd5/HVR2- $\text{His}_6$ , pAd5/HVR3- $\text{His}_6$ , pAd5/HVR5- $\text{His}_6$ , pAd5/HVR6- $\text{His}_6$ , pAd5/HVR7a- $\text{His}_6$ , and pAd5/HVR7b- $\text{His}_6$ , all of which contain the green fluorescence protein (GFP) gene and firefly luciferase (Luc) gene in the E1 region. The constructs were confirmed by restriction digestions and sequencing.

To rescue viruses, these modified plasmids were digested with PacI, and 2  $\mu$ g of each purified DNA was transfected into the Ad-E1-expressing 293 cells grown in 60-mm-diameter dishes with Superfect (QIAGEN). After plaques were formed, they were processed for large-scale proliferation in 293 cells, followed by purification by CsCl gradient centrifugation (47).

**SDS-PAGE and Western blotting.** A total of  $10^{10}$  virus particles (VPs) of each CsCl-purified virus were dissolved in Laemmli sample buffer without boiling and separated on 4 to 15% polyacrylamide gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The gels were either stained with Gelcode blue stain reagent (Pierce) according to the protocol from the manufacturer or transferred to nitrocellulose membrane (Bio-Rad). The membrane was processed to Western blotting with either anti-His-Tag monoclonal antibody or antihexon polyclonal antibody, essentially as described previously (47). In the Western blotting assay of the artificial receptor, cell extracts from both the transient and stable systems were boiled in Laemmli sample buffer, separated by SDS-PAGE on 4 to 15% polyacrylamide gradient gels, and stained with anti-hemagglutinin (HA) antibody.

**ELISA.** The enzyme-linked immunosorbent assay (ELISA) binding assay was performed essentially as described previously (48). In brief, different amounts of viruses ranging from  $4 \times 10^6$  to  $9 \times 10^9$  VPs were immobilized on wells of a 96-well plate (Nunc Maxisorp) by overnight incubation in (per well) 100  $\mu$ l of 100

TABLE 1. Primers used to incorporate the His<sub>6</sub> epitope into the HVRs of the Ad5 hexon

Name <sup>a</sup>	Sequence <sup>b</sup>
L (DraIII).....	CCTACGCACGACGTGACCACAG
R (SacI).....	CTAGGGAGCTCTGCAGAACCATG
HVR2-His <sub>6</sub> (as).....	TGAACCTAGGTGATGGTGTATGGTGTATGGGATCCGAGGACACCTATTTGAATACCCCTCCTTTG
HVR2-His <sub>6</sub> (s).....	CTCGGATCCCATCACCATCACCATCACCTAGGTTACCTAAATATGCCGATAAAACATTTTC
HVR3-His <sub>6</sub> (as).....	TGAACCTAGGTGATGGTGTATGGTGTATGGGATCCGAGTTCGTACCACTGAGATTCTCCTAT
HVR3-His <sub>6</sub> (s).....	CTCGGATCCCATCACCATCACCATCACCTAGGTTCAACTGAAATTAATCATGCAGCTGGG
HVR5-His <sub>6</sub> (as).....	TGAACCTAGGTGATGGTGTATGGTGTATGGGATCCGAGAGTAGTTGAGAAAAATTGCATTTC
HVR5-His <sub>6</sub> (s).....	CTCGGATCCCATCACCATCACCATCACCTAGGTTCACTTACTCCTAAAGTGGTATTGTAC
HVR6-His <sub>6</sub> (as).....	TGAACCTAGGTGATGGTGTATGGTGTATGGGATCCGAGAGTGGGCATGTAAGAAATATGAGTG
HVR6-His <sub>6</sub> (s).....	CTCGGATCCCATCACCATCACCATCACCTAGGTTCAAACTCACGAGAACTAATGGGCC
HVR7a-His <sub>6</sub> (as).....	TGAACCTAGGTGATGGTGTATGGTGTATGGGATCCGAGAGGTTTTACCTTGGTAAGAGTCTC
HVR7a-His <sub>6</sub> (s).....	CTCGGATCCCATCACCATCACCATCACCTAGGTTCACTTGGGAAAAAGATGCTACAGAATTTTC
HVR7b-His <sub>6</sub> (as).....	TGAACCTAGGTGATGGTGTATGGTGTATGGGATCCGAGTGGAAAGCAGTAATTTGGAAGTTC
HVR7b-His <sub>6</sub> (s).....	CTCGGATCCCATCACCATCACCATCACCTAGGTTCAATAATTTTGCCATGGAATCAATCTA

<sup>a</sup> as, antisense; s, sense.

<sup>b</sup> Underlined letters represent the sequences encoding the His<sub>6</sub> epitope.

mM carbonate buffer (pH 9.5) at 4°C. After extensive washes with 0.05% Tween 20 in Tris-buffered saline (TBS) and blocking with blocking solution (2% bovine serum albumin and 0.05% Tween 20 in TBS), the immobilized viruses were incubated with anti-His tag monoclonal antibody (Chemicon) for 2 h at room temperature, followed by AP-conjugated goat anti-mouse antibody incubation. Color reaction was performed with *p*-nitrophenyl phosphate (Sigma) as recommended by the manufacturer, and optical density at 405 nm (OD<sub>405</sub>) was obtained with a microplate reader (Molecular Devices).

**Gene transfer assay. (i) Gene transfer assay in HeLa cells, U118MG cells, and U118MG.HissFv.Rec cells.** Gene transfer efficacy of the viruses was evaluated by luciferase activity essentially as described previously (47). In brief, HeLa, U118MG, and U118MG.HissFv.Rec cells were plated in 24-well plates at a density of 10<sup>5</sup> cells per well the day before infection. Cells were infected at multiplicities of infection (MOI) of 1, 10, and 100 VPs/cell in triplicates. Twenty-four hours later, the cells were lysed in (per well) 250 μl of reporter lysis buffer (Promega), followed by one freeze/thaw cycle. Five microliters of each sample was used to measure the luciferase activity with a luciferase assay kit (Promega) and a luminometer (Berthold, Gaithersburg, Md.).

**(ii) Gene transfer assay in transient artificial system.** To establish a transient system expressing an artificial receptor (AR) for His-Tag, U118MG cells were infected with Ad5.MK.AR, which encodes anti-His-Tag single-chain antibody, the AR for His tag, in the E1 region under the control of a cytomegalovirus (CMV) promoter with an MOI of 300, and cultured for 3 days to allow the AR to express. A vector with E1 deleted, Ad5.E1dd, was used as a control. These cells were then infected with the His<sub>6</sub>-containing viruses at an MOI of 100, and their gene transfer efficacy was measured 24 h later as described above.

**Thermostability assay.** To test the thermostability of these viruses, viruses equivalent to an MOI of 100 were diluted in E-MEM containing 2% FCS and incubated at 45°C for different time intervals before infecting HeLa cells (11). Luciferase activity in infected cells was analyzed 24 h postinfection as described above.

## RESULTS

**Construction of hexon-modified viruses.** The HVRs of hexon are less conservative among different serotypes of adenoviruses and do not appear to interact with other viral proteins. Therefore, hexon modification may be made at these regions without affecting virus viability. To test this hypothesis, we explored the ability of the HVRs to accommodate foreign motifs. According to the X-ray crystallographic structure of adenovirus hexon, HVR1 and HVR4 contain internal β-sheet structure and are without a major ectodomain as predicted. In contrast, HVR2, HVR3, HVR5, HVR6, and HVR7 extrude from the capsid surface and contain flexible loop structures. We therefore focused on these HVRs and incorporated a His<sub>6</sub> epitope into each of them (Fig. 1). Two versions of HVR7

modification were created because HVR7 contains a large loop. Thus, we replaced a small fragment (HVR7a) and a large fragment (HVR7b) in HVR7. The His<sub>6</sub> epitope was chosen as a marker because antibody against His<sub>6</sub> is commercially available, which facilitates detection of the genetic incorporation and its effect. The amino acid residues marked in the box were replaced with the His<sub>6</sub> motif LGSHHHHHLLGS (Fig. 1), in which the LGS sequences acted as a spacer to present the six histidines (41). Genetic incorporation of the His<sub>6</sub> motif into Ad5 hexon was achieved by bacterium-based homologous recombination. The resultant HVR-His<sub>6</sub>-containing vectors encoded luciferase and GFP double reporters and were replication deficient. In the study, all of the viruses but Ad5/HVR7b-His<sub>6</sub> were successfully rescued in 293 cells stably expressing Ad5-E1 proteins. The inability to rescue Ad5/HVR7b-His<sub>6</sub> suggested that replacement of a large fragment of HVR7 interfered with viral formation. This will be discussed in detail later. Nonetheless, incorporation of His<sub>6</sub> in other HVRs resulted in similar virus yields to unmodified viruses, which ranged from 3 × 10<sup>12</sup> to 5 × 10<sup>12</sup> VPs in a culture of 3 × 10<sup>8</sup> cells, suggesting that the hexon modifications did not affect virus formation and the HVRs were able to accommodate foreign peptides.

**Expression and presentation of His<sub>6</sub> epitopes in hexons of modified viruses.** Next we examined whether the incorporated His<sub>6</sub> epitopes were indeed expressed in hexon proteins and how they were presented. In this regard, we performed SDS-PAGE and Western blotting analysis after lysis of purified virions in Laemmli sample buffer. Since several antihexon antibodies that we tested before do not recognize denatured (boiled) hexon protein (47), the viral samples for Western blotting assays were not boiled. In the experiments, 10<sup>10</sup> VPs per well were lysed and subjected to SDS-PAGE. The gels were either stained with Gelcode blue stain reagent (Pierce) or transferred to nitrocellulose membrane for Western blotting. Gelcode blue staining demonstrated that the modified viruses contained proper viral proteins, as compared to the unmodified Ad5, and the quantitation of the viruses is consistent (Fig. 2A and B). Western blotting assays with antihexon and anti-His tag antibody suggested the His<sub>6</sub> epitopes were indeed expressed in hexons of modified viruses but not in unmodified

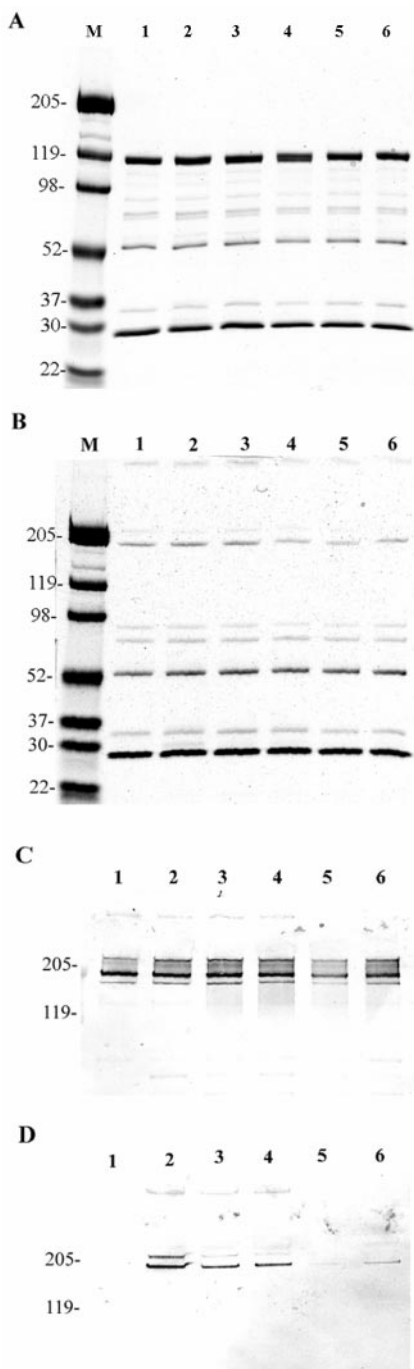


FIG. 2. SDS-PAGE and Western blotting confirmed the HVR-His<sub>6</sub>-containing viruses. In the assays, 10<sup>10</sup> VPs of purified viruses were lysed in Laemmli sample buffer, followed by boiling for 5 min (A) or without boiling (B to D), and separated on 4 to 15% polyacrylamide gradient SDS-PAGE gels. The proteins were either stained with Gelcode blue stain reagent (Pierce) (A and B) or transferred to nitrocellulose membrane and then stained with anti-hexon polyclonal antibody AB1056 (C) or anti-His tag monoclonal antibody (D). Lane M, molecular weight marker; lane 1, Ad5 (unmodified); lane 2, Ad5/HVR2-His<sub>6</sub>; lane 3, Ad5/HVR3-His<sub>6</sub>; lane 4, Ad5/HVR5-His<sub>6</sub>; lane 5, Ad5/HVR6-His<sub>6</sub>; lane 6, Ad5/HVR7a-His<sub>6</sub>. The weak anti-His tag staining of hexons of Ad5/HVR6-His<sub>6</sub> and Ad5/HVR7a-His<sub>6</sub> suggested that the His<sub>6</sub> epitopes incorporated in HVR6 and HVR7a were not well accessible to the anti-His antibody at the protein level.

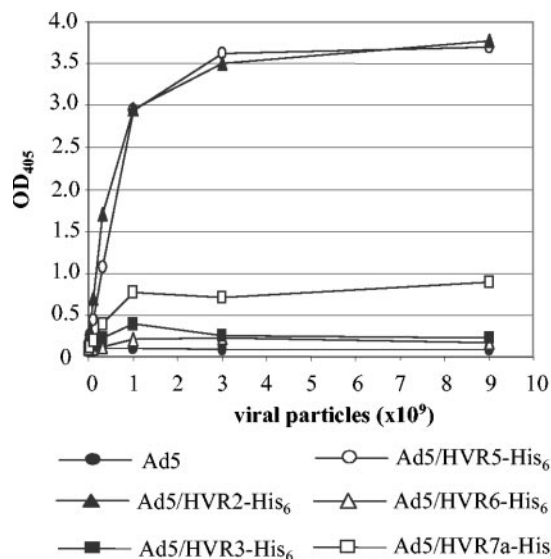


FIG. 3. Results of an ELISA binding assay suggesting that the His<sub>6</sub> epitopes incorporated in different HVRs exhibited differential accessibility to anti-His tag antibody in the context of intact virion. In the assay, various amounts of purified viruses were immobilized on the wells of 96-well ELISA plates and incubated with anti-His tag antibody. The binding was detected with AP-conjugated secondary antibody. The results suggested that the His<sub>6</sub> epitopes incorporated into HVR2 and HVR5 were accessible to anti-His tag antibody at the virion level, indicating they were exposed on the virion surface.

Ad5 hexon (Fig. 2C and D). However, His<sub>6</sub> epitopes incorporated in different HVRs exhibited differential antibody accessibility in the hexon protein, as reflected by the intensity of the bands in the anti-His tag Western blotting assay. Hexons of Ad5/HVR2-His<sub>6</sub>, Ad5/HVR3-His<sub>6</sub>, and Ad5/HVR5-His<sub>6</sub> appeared to be readily accessible to anti-His tag antibody, while hexons of Ad5/HVR6-His<sub>6</sub> and Ad5/HVR7a-His<sub>6</sub> were much less accessible. These data indicated that the His<sub>6</sub> epitopes inserted in HVR2, HVR3, and HVR5 were better presented on the surface of hexon protein than in HVR6 and HVR7.

**Presentation of His<sub>6</sub> epitope on virion surface.** Exposure of an epitope on the surface of a protein does not necessarily mean it is exposed on an intact virion. Therefore, we next examined whether the His<sub>6</sub> epitopes were presented on the surface of virions using an ELISA. In the assay, different amount of purified viruses were immobilized on the wells of ELISA plates and incubated with anti-His tag antibody. The binding of anti-His antibody to the intact virions was detected with AP-conjugated secondary antibody, followed by AP color reaction. The results showed that Ad5/HVR2-His<sub>6</sub> and Ad5/HVR5-His<sub>6</sub> bound anti-His tag antibody most strongly and Ad5/HVR7-His<sub>6</sub> showed weak binding, while Ad5/HVR3-His<sub>6</sub> and Ad5/HVR6-His<sub>6</sub> showed minimal binding (similar to unmodified Ad5) (Fig. 3). This suggested that the His<sub>6</sub> epitopes were exposed on the virion surfaces when incorporated in HVR2 and HVR5, partially exposed in HVR7, while not exposed in HVR3 and HVR6. As noted, the His<sub>6</sub> epitope in HVR3 was readily accessible to anti-His tag antibody at the protein level but not in the context of intact virion. The discrepancy may be due to the complicated surroundings of the epitopes in the virions. In particular, hexon interacts with many

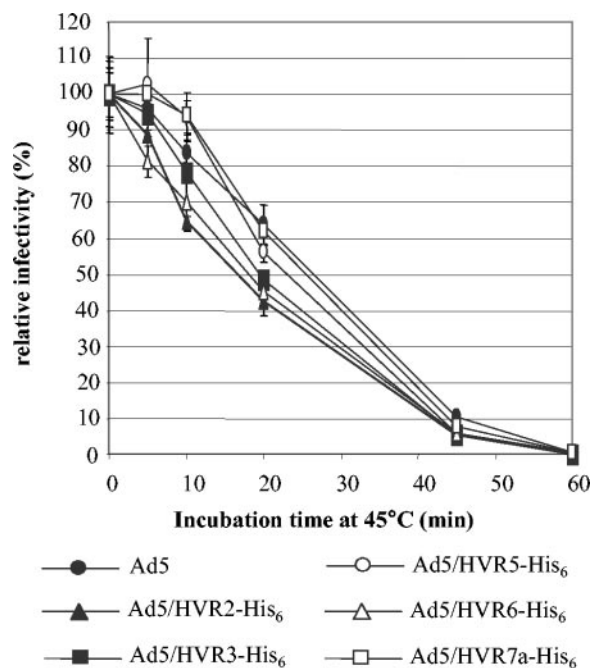


FIG. 4. Thermostability of HVR-His<sub>6</sub>-containing viruses. The viruses equivalent to an MOI of 100 were diluted in infection media (E-MEM plus 2% FCS) and incubated at 45°C for different time intervals before infecting HeLa cells in triplicates. The cell lysates were processed for luciferase activity assay. The relative light units (RLU) showing the activity of luciferase were detected with a luciferase assay kit (Promega) and a luminometer. The relative infectivity was obtained by changing the RLU readings of the heat-treated viruses to percentages of the readings of corresponding untreated viruses.

other proteins in a virion, which include polypeptides VI, VIII, and IX (13, 37). These interactions can affect the exposure of the His<sub>6</sub> epitopes by affecting the conformation of hexon or by simply covering the His<sub>6</sub> epitopes.

**Thermostability of the modified viruses.** One problem often associated with capsid modification of adenoviruses is instability of the modified viruses. This is especially evident with hexon modification, since hexon constitutes the major part of adenovirus capsid (16, 31, 47, 51). We therefore tested whether incorporation of His<sub>6</sub> epitopes in HVRs could affect the structural integrity of the virions by comparing their thermostability with that of unmodified Ad5. The viruses were incubated at 45°C in infection media (E-MEM containing 2% FCS) for different time intervals before infecting HeLa cells, and their relative gene transfer efficiencies were obtained by comparing to that of unheated viruses with luciferase reporter. We found that all of the modified viruses showed similar stability to the unmodified viruses (Fig. 4). Although Ad5/HVR2-His<sub>6</sub> and Ad5/HVR6-His<sub>6</sub> appeared to be less stable than unmodified and other modified viruses, the differences were not statistically significant. This suggested that incorporation of His<sub>6</sub> epitopes in HVRs of hexon did not significantly affect the stability of Ad5, consistent with our results on virus production showing the modified viruses had similar yields to unmodified Ad5.

**Gene transfer efficacies of the viruses in HeLa cells.** Since capsid proteins play critical roles in Ad5 infection, insertion of

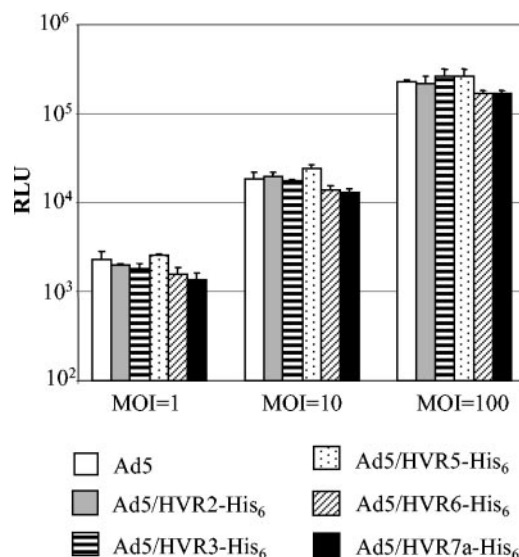


FIG. 5. Gene transfer efficacies of the HVR-His<sub>6</sub>-containing viruses in HeLa cells. In the assay, HeLa cells were infected with these viruses at three different MOI (MOI of 1, 10, and 100) in triplicate, and the cell lysates were processed for the luciferase activity assay as described in the legend to Fig. 4.

foreign peptide into hexon may interfere with the native infectivity of Ad5. We thus investigated whether incorporation of His<sub>6</sub> epitopes in HVRs affected gene transfer efficacies of the viruses in HeLa cells. HeLa cells were chosen because they express a high level of CAR, the native Ad5 receptor, and can be effectively infected by unmodified Ad5 vector. Viruses equivalent to MOI of 1, 10, and 100 were used to infect the HeLa cells, and the gene transfer efficacies were assessed with the reporter firefly luciferase. All of the modified viruses exhibited similar luciferase activity compared to unmodified Ad5 (Fig. 5). Ad5/HVR6-His<sub>6</sub> and Ad5/HVR7a-His<sub>6</sub> appeared to have less efficiency than unmodified and other modified viruses, but the differences were not statistically significant (Fig. 5). These data suggested that His<sub>6</sub> incorporation in HVRs of hexon did not interfere with native infectivity of Ad5.

**Gene transfer efficacies of the viruses in His<sub>6</sub> AR systems.** We further investigated whether the His<sub>6</sub> epitope incorporated in HVRs of Ad5 hexon could mediate independent viral infection. Two AR systems were employed in this regard. In the first one, we established a transient AR system by infecting CAR-negative U118MG cells with Ad5.MK.AR, an Ad5 vector encoding anti-His tag single-chain antibody (the AR for His<sub>6</sub> epitopes) in the E1 region (12; P. J. Mahasreshti et al., unpublished data). The AR was driven by CMV promoter and was composed of an immunoglobulin  $\kappa$ -chain signal peptide, an HA epitope, the single-chain antibody against His<sub>6</sub> epitope, and the transmembrane domain of platelet-derived growth factor (PDGF) receptor (12). As demonstrated previously (12), the Ig  $\kappa$ -chain leader directs the AR to the cell surface, the PDGF receptor transmembrane domain anchors the AR in the plasma membrane, and the HA epitope facilitates AR detection. As shown in Fig. 6A, the AR was expressed in the U118MG cells preinfected with Ad5.MK.AR, but not in uninfected cells or the cells preinfected with a negative control,

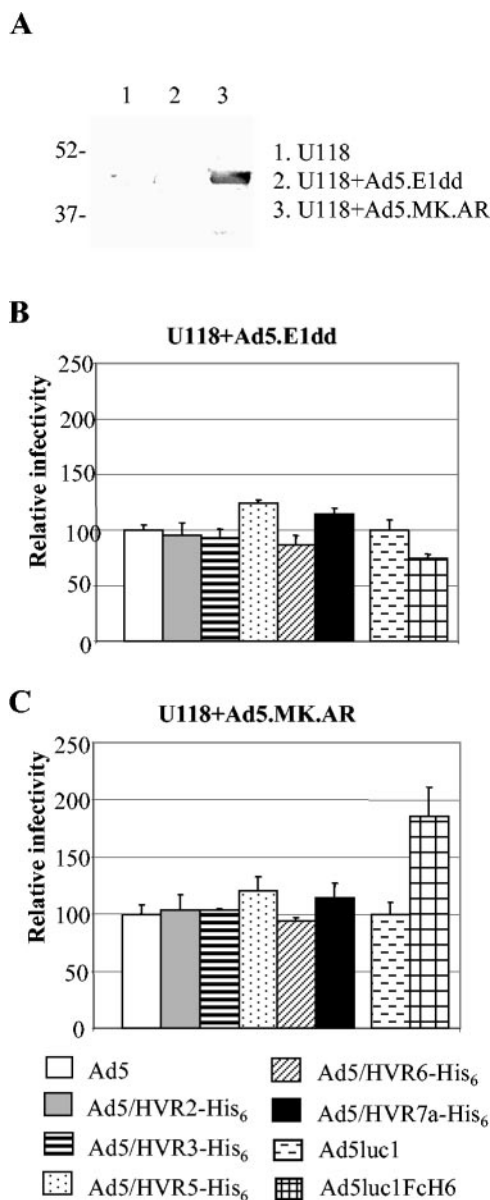


FIG. 6. Gene transfer efficacies of the modified viruses in a transient artificial receptor system. (A) Western blotting assay showing AR expression in Ad5.MK.AR-infected U118MG cells, but not in uninfected cells or Ad5.E1dd-infected cells. (B and C) Gene transfer efficacies of the modified viruses in the transient AR system. In the assay, U118MG cells were infected with Ad5.E1dd (B) or Ad5.MK.AR (C) at an MOI of 300. Three days later, the cells were infected with the His<sub>6</sub>-containing viruses at an MOI of 100, and their gene transfer efficacies were measured 24 h later as described in the legend to Fig. 4. A fiber-modified virus, Ad5luc1FcH6, which contains the His<sub>6</sub> motif at the C-terminal end of fiber, and its unmodified control, Ad5luc1, were used as positive controls. The relative infectivity was obtained by changing the RLU readings of the modified viruses to a percentage of the readings of unmodified viruses. The results suggested that the His<sub>6</sub> epitopes incorporated in HVRs did not confer the viruses' His<sub>6</sub>-mediated gene transfer ability.

Ad5.E1dd, an E1-deleted Ad5 vector. To test the gene transfer efficacies of the hexon-modified viruses in this AR system, the U118MG cells were preinfected with either Ad5.MK.AR or Ad5.E1dd and cultured at 37°C in 5% CO<sub>2</sub> for 3 days to allow

expression of the AR. Then the preinfected U118MG cells were infected with the HVR-His<sub>6</sub>-containing viruses at an MOI of 100, and their gene transfer efficacies were examined with the luciferase assay. A fiber-modified vector containing His<sub>6</sub> motif in fiber, Ad5luc1FcH6, has been shown to be able to infect cells via the His<sub>6</sub> AR expressed on the targeting cells (12) and thus was used as a positive control. Compared to the control vector Ad5luc1, Ad5luc1FcH6 showed enhanced gene transfer efficacy in the transient AR system, but not in the control cells, indicating the transient AR system was functional (Fig. 6B and C). In contrast, all of the hexon-modified viruses showed similar infectivity compared to unmodified Ad5 in U118MG preinfected with both Ad5.MK.AR and Ad5.E1dd (Fig. 6B and C). These data suggested that His<sub>6</sub> epitope incorporated in the hexon HVRs did not mediate independent viral infection in the transient system.

To confirm this conclusion, we employed a second AR system, the U118MG.HissFv.rec cells—cell line that stably expressed the AR for His tag on cell surface (12). Expression of the AR was confirmed by Western blotting assay with anti-HA antibody (Fig. 7A). We next infected the stable AR cells and the control U118MG cells with the hexon-modified viruses and compared their infectivity with that of unmodified Ad5 vector in both cell lines. Similarly, Ad5luc1 and Ad5luc1FcH6 were used as a positive control in this system. Consistent with studies in the transient system, hexon-modified viruses did not show enhanced gene transfer efficacy in the stable system, while the positive control did (Fig. 7B and C). Taken together, these data demonstrated that His<sub>6</sub> epitopes incorporated in HVRs of hexon did not mediate cell infection.

### DISCUSSION

In this study, we identified several HVRs in Ad5 hexon as proper sites for foreign peptide incorporation, using the His<sub>6</sub> motif as a marker. We found that the His<sub>6</sub> motif could be genetically inserted into HVR2, HVR3, HVR5, HVR6, and HVR7 of the Ad5 hexon without affecting virus formation, virion stability, and CAR-mediated Ad5 native infectivity. However, different HVRs appeared to present the His<sub>6</sub> epitopes differentially. The His<sub>6</sub> epitopes incorporated in HVR2, HVR3, and HVR5 appeared to be exposed on the protein surface because they were readily detected in Western blotting assays with anti-His tag antibody. Nonetheless, in the context of intact virion, only the His<sub>6</sub> epitopes inserted in HVR2 and HVR5 appeared to be exposed, as assessed by ELISA, suggesting protein interactions in the intact virion have some effect on epitope exposure. Furthermore, we found that in all of the modified adenovirus vectors, the His<sub>6</sub> epitopes did not mediate CAR-independent cell infection in the presence of fiber.

This study demonstrated that HVRs of hexon can accommodate insertions of foreign peptides. The definition of HVRs used in the study is based on analysis performed by Crawford-Miksza and Schnurr (5). While we were preparing the manuscript for this report, another study performed by Rux et al. was published, in which the HVRs of hexon were redefined (33). Using high-resolution X-ray crystallographic, molecular modeling, and sequence-based methods, Rux and his colleagues identified nine HVRs. In general, the positions of the

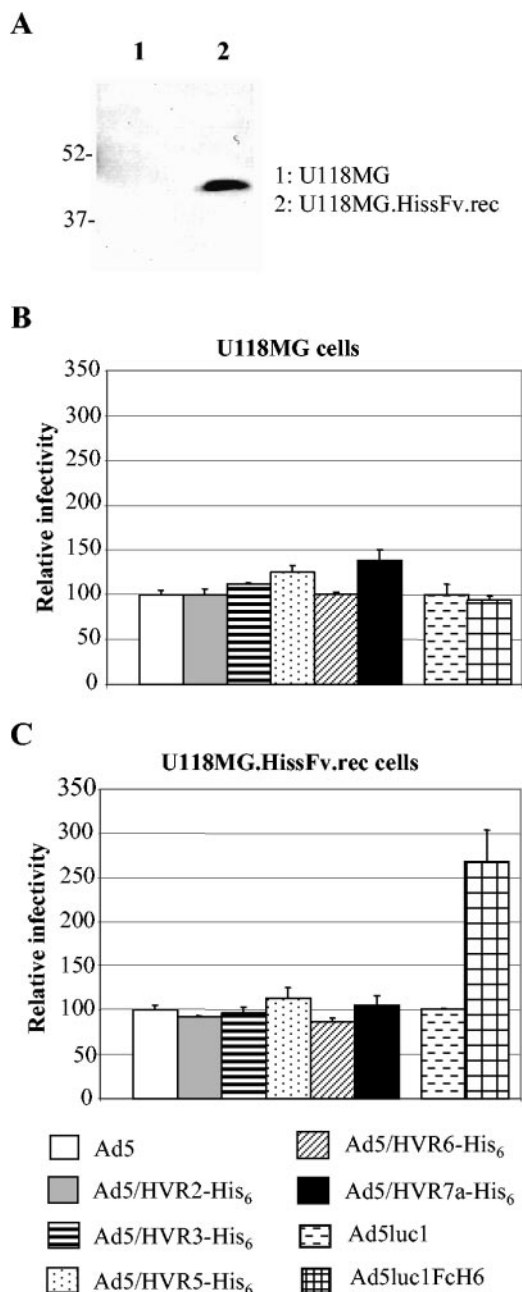


FIG. 7. Gene transfer efficacies of the hexon-modified viruses in a stable AR system. (A) Expression of the AR in the stable U118MG.HissFv.Rec cells. Cell extracts from U118MG cells or U118MG.HissFv.Rec cells were subjected to Western blotting analysis with anti-HA antibody which recognizes the HA epitope fused to the AR. (B and C) Gene transfer efficacies of the modified viruses in the stable AR system. In this assay, U118MG cells (B) and U118MG.HissFv.Rec cells (C) were infected with the modified viruses at an MOI of 100. Ad5luc1 and Ad5luc1FcH6 were used as positive controls. The cell lysates were subjected to luciferase assay, and the relative infectivity was obtained as described in the legend to Fig. 6. The data confirmed that the His<sub>6</sub> epitopes incorporated in HVRs did not confer the viruses' His<sub>6</sub>-mediated gene transfer ability.

newly defined HVRs are in agreement with what Crawford-Miksza and Schnurr have defined. The exceptions are HVR3 and HVR7. The authors assigned HVR3 to a different region, and HVR7 was defined more precisely as HVR7, HVR8, and

HVR9. According to the new definition, the previously defined HVR3 is in a well-conserved region. We successfully rescued Ad5.HVR3-His<sub>6</sub>, suggesting some regions outside HVRs may also accommodate certain modifications. As noted, we were able to rescue Ad5.HVR7a-His<sub>6</sub> viruses, but not Ad5.HVR7b-His<sub>6</sub>. This may be explained with the newly identified HVR7, HVR8, and HVR9. According to the sequence information, our HVR7a-His<sub>6</sub> modification is in HVR8, whereas the HVR7b-His<sub>6</sub> modification involved sequences ranging from part of HVR7 to part of HVR9. These results indicate that HVR8 is able to accommodate modifications. However, replacement of sequences ranging from HVR7 to HVR9 interferes with virion assembly since it involves the conserved sequences in this region. Our results are in agreement with an early study, in which 8-amino-acid sequences from the poliovirus type 3 VP1 capsid protein were used to replace residues 442 to 449 of the Ad2 hexon, resulting in viruses that grow poorly (6). The position is in HVR7 according to Crawford-Miksza and Schnurr's definition. However, the insertion extends between HVR7 and HVR8, which is a largely conserved region in the redefinition. Therefore, the redefinition of HVR7-HVR9 appears to explain the results better and thus may be more accurate.

Although His<sub>6</sub> epitopes inserted into HVR2 and HVR5 are exposed on the surface of the Ad5 virion, they do not appear to mediate viral infection of cells. This was demonstrated in both transient and stable AR systems, suggesting peptides incorporated into hexon are not able to mediate cell targeting. However, a previous study has shown that RGD peptide incorporated into HVR5 is able to mediate CAR-independent cell infection (41). The controversy may arise from the nature and length of the incorporated peptides. Longer and well-protruding ligands have better accessibility to their receptors. In addition, the ligand-receptor binding affinity can also have an effect on the ligand-mediated cell infection. It is possible that the AR for the His<sub>6</sub> epitope, the single-chain antibody against His tag, has weaker binding affinity to His<sub>6</sub> peptide inserted in the hexon than anti-His tag antibody; thus, even though it is readily exposed on the virion surface, it cannot mediate cell infection. Furthermore, the presence of fiber, the major cell targeting protein of Ad5, may spatially block access of the incorporated ligands to the receptors expressed on the cell surface. In this regard, our future study involving employment of fiberless Ad will help clarify whether the epitopes incorporated in hexon can mediate additional cell targeting.

Since hexon is the major target for the neutralizing antibodies against adenovirus vectors, previous studies involving hexon modifications mainly focused on circumventing the host neutralization response to Ad vectors (16, 31, 47, 51). A hexon switch strategy has been employed in this regard. In particular, the hexons of Ad5 vectors were replaced with hexons from different serotypes, including Ad1, Ad2, Ad3, Ad6, and Ad12. These hexon switch modifications demonstrated their ability to escape the preexisting neutralizing antibodies when administered sequentially (16, 31, 47, 51). However, a major problem associated with this strategy is the inability to rescue viable viruses with most alternate hexons. In this regard, hexons from more than 20 adenovirus serotypes have been investigated, but only the aforementioned five hexons were able to form viable viruses after replacement of the Ad5 hexon (16, 31, 47, 51). In

addition, the yields of the hexon-chimeric Ad5 vectors are lower than that of native Ad5 vector, and they are more susceptible to heat inactivation, suggesting the foreign hexons do not encapsidate the Ad5 genome as efficiently as the native Ad5 hexon. These properties of the hexon-chimeric Ad5 vectors may result from the incompatibility of the hexons with other Ad5 capsid proteins that are involved in virion assembly (9, 37). Since HVRs of hexons contain the serotype-specific epitopes and they do not appear to be involved in binding any other viral proteins, it is speculated that switching only the HVR regions of hexon may allow better rescue of hexon chimeras while retaining their ability to circumvent the host neutralizing response. Our current study suggest that the HVRs of the Ad5 hexon can accommodate modifications without affecting viral formation and stability, thus providing support for the possibility of switching HVRs to escape host neutralization response. An alternative strategy in this regard is to genetically incorporate some ligands to mask the antigenic epitopes of HVRs in a similar way to the nongenetic strategy to overcome host immunity.

In addition to potential utility of hexon modifications in cell targeting and in escaping host neutralization response, hexon modification may be used for other purposes, such as in vivo imaging of Ad virions. Some imaging reagents such as GFP have been incorporated in Ad5 capsid protein pIX, which allowed live imaging of the Ad5 virion (20). Hexon is the most abundant capsid protein, and each virion contains 240 copies of hexon homotrimer. Therefore, incorporation of imaging reagents in hexon may provide strong signals for live and in vivo imaging purposes. An unsolved problem, however, is the size limit for insertions in hexon. This will be investigated in our future studies.

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