

Promoter Sequences of Varicella-Zoster Virus Glycoprotein I Targeted by Cellular Transactivating Factors Sp1 and USF Determine Virulence in Skin and T Cells in SCIDhu Mice In Vivo

Hideki Ito,^{1,2} Marvin H. Sommer,^{1,2} Leigh Zerboni,^{1,2} Hongying He,³ Dwayne Boucaud,³ John Hay,³ William Ruyechan,³ and Ann M. Arvin^{1,2*}

*Departments of Pediatrics¹ and Microbiology and Immunology,² Stanford University, Stanford, California, and
Department of Microbiology, State University of New York at Buffalo, Buffalo, New York³*

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Varicella-zoster virus (VZV) glycoprotein I is dispensable in cell culture but necessary for infection of human skin and T cells in SCIDhu mice in vivo. The gI promoter contains an activating upstream sequence that binds the cellular transactivators specificity factor 1 (Sp1) and upstream stimulatory factor (USF) and an open reading frame 29 (ORF29)-responsive element (29RE), which mediates enhancement by ORF29 DNA binding protein of immediate-early 62 (IE62)-induced transcription. Recombinants, rOKAgI-Sp1 and rOKAgI-USF, with two base pair substitutions in Sp1 or USF sites, replicated like rOKA in vitro, but infectivity of rOKAgI-Sp1 was significantly impaired in skin and T cells in vivo. A double mutant, rOKAgI-Sp1/USF, did not replicate in skin but yielded low titers of infectious virus in T cells. The repaired protein, rOKAgI:rep-Sp1/USF, was as infectious as rOKA. Thus, disrupting gI promoter sites for cellular transactivators altered VZV virulence in vivo, with variable consequences related to the cellular factor and the host cell type. Mutations in the 29RE of the gI promoter were made by substituting each of four 10-bp blocks in this region with a 10-bp sequence, GATAACTACA, that was predicted to interfere with enhancer effects of the ORF29 protein. One of these mutants, which was designated rOKAgI-29RE-3, had diminished replication in skin and T cells, indicating that ORF29 protein-mediated enhancement of gI expression contributes to VZV virulence. Mutations within promoters of viral genes that are nonessential in vitro should allow construction of recombinant herpesviruses that have altered virulence in specific host cells in vivo and may be useful for designing herpesviral gene therapy vectors and attenuated viral vaccines.

Varicella-zoster virus (VZV) is a human herpesvirus that causes varicella, establishes latency in sensory ganglia, and may reactivate, resulting in herpes zoster (3, 32). The VZV genome encodes glycoproteins gB, gC, gE, gH, gI, gK, and gL and putative glycoproteins gM and gN (9, 13, 32). VZV glycoproteins, like their herpes simplex virus (HSV) homologues, are presumed to be involved in virion attachment, entry, envelopment, cell-cell spread, and egress (31, 36). VZV gE and gI form heterodimers; gI enhances gE endocytosis from plasma membranes and facilitates its trafficking to the *trans*-Golgi network (1, 6, 8, 16, 19, 24, 25, 39, 40, 41, 43). VZV gE resembles HSV gD in being essential, whereas HSV gE is dispensable (19). Full and partial gI deletion mutants are infectious in vitro, although syncytium formation and virus yields are diminished (16), and few virions reach post-Golgi structures, indicating that gI is important for assembly and egress in cell culture (40).

During primary infection, VZV causes a lymphocytic cell-associated viremia and vesicular skin lesions (3). SCID mice with human xenografts are useful for defining genetic determinants of VZV virulence, because VZV infects CD4, CD8, and CD4-CD8 T cells in thymus-liver implants and dermal and epidermal cells in skin implants (21–23, 34, 35). Although the gI deletion mutant rOKAΔgI replicates in vitro, gI is essential

for infection in vivo (20). These characteristics suggested that VZV recombinants with gI promoter mutations could be used to investigate how viral promoter sequences influence virulence in differentiated T cells and skin in SCIDhu mice in vivo.

VZV gI promoter nucleotides were selected for mutation in the viral genome based upon mapping the residues that mediate effects of cellular and viral regulatory proteins in reporter construct assays (10). Like other VZV promoters, the putative gI promoter is inactive in the absence of the VZV immediate-early protein 62 (IE62) (5, 13, 26, 27). Optimal gI promoter function in reporter constructs requires an activating upstream sequence (AUS), from –54 to –35, which contains motifs characteristic of binding sites for the cellular transcription factors, specificity protein 1 (Sp1), upstream stimulatory factor (USF), and activator protein 1 (AP-1) (2, 10, 14, 15, 37). Sp1 is a ubiquitous mammalian cell protein that binds to GC box and GT/CA box motifs (14, 37), and USF is a member of the bHLHZ family of DNA-binding transcription factors (14, 15, 28, 37). Sp1 and USF bind to the AUS, while IE62 does not, but disrupting the putative Sp1 and USF binding sites blocks IE62-induced transcription in reporter construct assays (10). In contrast, AP-1 does not bind to the AUS, and substitutions in the putative AP-1 site do not alter transcription. VZV open reading frame 29 (ORF29) protein is a single-stranded DNA binding protein, homologous to HSV ICP8 (13). The gI promoter contains an ORF29-responsive element (29RE), from –220 to –180, that enhances IE62-dependent transcription in reporter assays (10).

* Corresponding author. Mailing address: 300 Pasteur Dr., Rm. G312, Stanford University School of Medicine, Stanford, CA 94305-5208. Phone: (650) 725-6574. Fax: (650) 725-8040. E-mail: aarvin@stanford.edu.

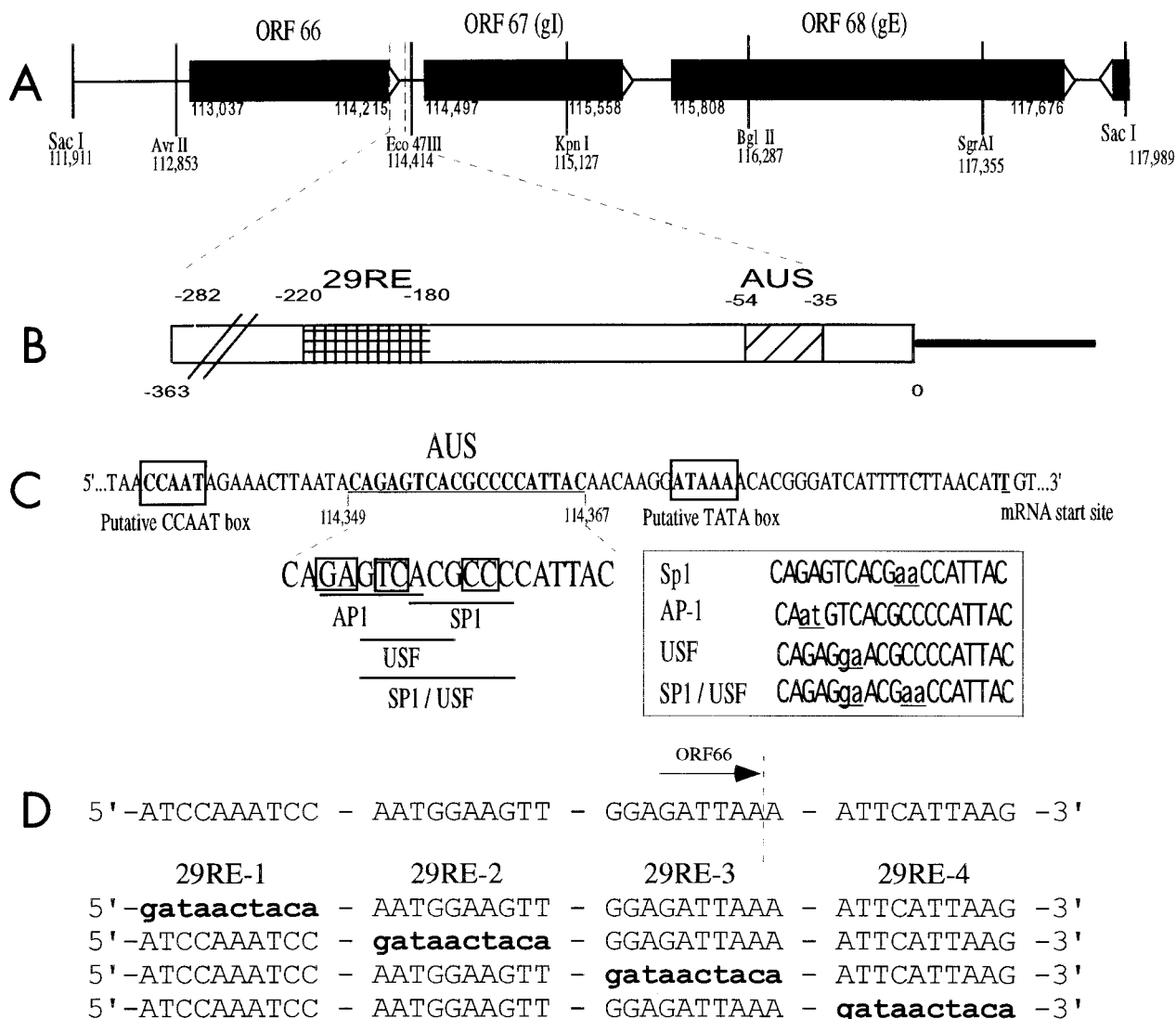


FIG. 1. Construction of VZV cosmids with gI promoter mutations. The gI coding region (ORF67) and its upstream sequences are located in the unique short region of the VZV genome. A *Sac*I fragment containing ORFs 66, 67, and 68 (A) was subcloned from cosmid pvSpe21 to construct gI promoter mutations, and the mutated segment was reinserted into the cosmid. (B) AUS and 29RE of the gI promoter. (C) Positions of Sp1, AP-1, and USF binding sites within the 19-bp AUS region (underlined). Mutations introduced into Sp1, AP-1, USF, and Sp1/USF sites are indicated by underlined, lowercase letters. CCAAT and ATAAA are putative CCAAT and TATA boxes; the underlined T is the putative mRNA start site. (D) 29RE region. Letters in boldface, lowercase type indicate the 10-bp sequences that were replaced with GATAACTACA in each 29RE subcomponent.

In the present experiments, VZV cosmids were used to construct viral recombinants carrying mutations within the AUS or 29RE regions of the gI promoter. VZV recombinants with gI promoter mutations were evaluated for effects on replication in cell culture and in human skin, and T cells in the SCID mouse model of VZV pathogenesis were evaluated *in vivo*.

MATERIALS AND METHODS

VZV recombinants. VZV recombinants were made from overlapping fragments of vaccine Oka virus DNA ligated into SuperCos1 cosmid vectors (Stratagene, La Jolla, Calif.) (12, 16, 35). The locations of ORFs 66, 67 (gI), and 68 (gE) in the cosmid, pvSpe21, are shown in Fig. 1A (16). The cosmid pvSpe21 was digested with *Sac*I, to make pSac6A (Fig. 1A), which contains ORF66, ORF67, and ORF68. The gI promoter regions, designated 29RE and AUS, that were the

targets of mutational analysis in these experiments are shown in Fig. 1B. The nucleotide sequence of the AUS region is depicted in Fig. 1C, including the locations of the putative CCAAT and TATA boxes. The region of the gI promoter that includes the sequences which constitute the targeted AP-1, Sp1, and USF sites are indicated below the complete AUS sequence in Fig. 1C (underlined). Eight sets of PCR primers were designed to introduce two nucleotide substitutions into the AUS (Fig. 1B and C). The Sp1, AP-1, USF, and Sp1/USF mutations that were introduced into the AUS are shown in the box (Fig. 1C).

The sequences of the PCR primers that were used to make these substitutions are shown in Table 1. Primers 2 and 4 anneal upstream and downstream, respectively, of the indicated *Kpn*I sites. Primer 1 anneals over the AUS region and changes the indicated nucleotides (underlined), in order to create Sp1, AP-1, USF, and Sp1/USF mutated sites. Primer 3 anneals adjacent to the end of primer 1. Using pSac6A as a template, PCRs were carried out using *Pfu* DNA polymerase (Stratagene, Inc.) with primers 1 and 2 as one primer set and primers 3 and 4 as the second primer set. The PCR products were gel isolated, digested with *Kpn*I, and reisolated; pSac6A was digested with *Kpn*I, and a 5.7-kb fragment was

TABLE 1. Primers used for mutagenesis of the AUS region of the gI promoter

Primer no.	Primer name	Annealing site	Primer sequence ^a
1	Mutagenic Sp1	114352	5'... GTTGTAAATGGTTCGTGACTCTG... 3'
	Mutagenic AP-1	114344	5'... GGGGCGTGACATTGTATTAAGT... 3'
	Mutagenic USF	114347	5'... AATGGGGCGTTCCTCTGTATTA... 3'
	Mutagenic Sp1/USF	114347	5'... GTTGTAAATGGTTCGTTCCTCTGTATTA... 3'
2	Reverse primer	111800	5'... ACCCTCACTAAAGGGAACAAA... 3'
3	Sp1	114374	5'... AAGGATAAAACACGGGATCATT... 3'
	AP-1	114366	5'... ATTACAACAAGGATAAAACACG... 3'
	USF	114369	5'... ACAACAAGGATAAAACACGGGA... 3'
	Sp1/USF	114374	5'... AAGGATAAAACACGGGATCATT... 3'
4	Reverse primer	115133	5'... AGATCAAGCATATGTTGAAACA... 3'

^a Underlining indicates positions of mutagenesis.

isolated. A triple ligation was set up using the isolated, digested PCR products and the *Kpn*I-digested pSac6A to create plasmid clones that had substitutions in the AUS. The mutated plasmids were designated pSac6A/Sp1, pSac6A/AP-1, pSac6A/USF, or pSac6A/Sp1-USF. The 4.5-kb *Avr*II/*Sgr*AI fragments from these mutated plasmids were used to replace the original 4.5-kb *Avr*II/*Sgr*AI fragment in pVSpe21. Two repaired cosmids were made from the mutated Sp1/USF cosmid, generating pVSpe21:rep-Sp1/USF @*Avr*II, as described for construction of rOKAΔgI-gI@*Avr*II, which is a gI deletion mutant in which ORF67 has been reinserted into the *Avr*II site (16).

The 29RE region of the gI promoter is illustrated in Fig. 1D. Mutations replacing each of four 29RE regions with GATAACTACA were transferred from vectors pCAT-Basic/29RE-1, 2, 3, and 4 (Fig. 1D) (10). The same 10-bp substitution, GATAACTACA, was used to replace each of four 10-bp blocks in the 29RE region, as shown in boldface type. The rationale was that examination of the 29RE region identified two CCAAT-box like elements within the element. Our hypothesis was that this known transcriptional element might play a role in enhancement mediated by the ORF29 protein. The GATAACTACA sequence was chosen to replace each of four 10-bp blocks in the 29RE region because the TAACT core of this sequence is, in effect, an inverse CCAAT box with a thymidine substituting for one of the two cytosines and was predicted to be nonfunctional.

All AUS and 29RE mutations in pVSpe21 were sequenced to verify substitutions. Cosmid preparation, transfections of melanoma cells, growth curve assays, and plaque size determinations were done as described previously (16, 22, 35). In growth curve assays, cells from four replicate wells were trypsinized on days 1 to 6 after inoculation, and titers were determined at each time point. Statistical comparisons were done using Student's *t* test.

PCR and sequencing. DNA was isolated from melanoma cells with DNAzol reagent (Gibco BRL, Inc., Grand Island, N.Y.) and from implant tissues using a DNeasy tissue kit (Qiagen, Inc., Chatsworth, Calif.). PCR was performed using Elongase enzyme mix (Gibco BRL, Inc.). Solubilized DNA was used as a template for PCR primers that annealed within ORF66 (annealing site, 113354) and ORF68 (annealing site, 117112), producing a 3,758-bp product (data not shown). Primers for human β-globin gene were included as a positive control for xenograft specimens (33). PCR products were cloned into pCR4-TOPO (Invitrogen, Inc.) and sequenced by the Stanford Protein and Nucleic Acid facility. Primers located at positions 116194 and positions 117317 were used to define insertions at *Avr*II. Sequencing reactions were primed by using T7 primer contained in the pCR4-TOPO vector and five custom primers to cover the promoter and ORF67 coding region at the *Avr*II site.

Infection of human xenografts in SCIDhu mice. Skin or thymus-liver implants were made in homozygous *CB-17^{pcid/scid}* mice (22, 23), using human fetal tissues obtained with informed consent according to federal and state regulations. Animal use was in accordance with the Animal Welfare Act and approved by the Stanford University Administrative Panel on Laboratory Animal Care. VZV recombinants, passed three times in primary human lung (HEL) cells, were used to inoculate xenografts; infectious virus titers were determined for each inoculum at the time the implants were injected (22). Skin xenografts were harvested after 14, 21, and 28 days and analyzed by infectious focus assay, immunoblotting, immunohistochemistry, and PCR and sequencing. For immunoblotting and immunohistochemistry, tissue extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 7.5% gels and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, Mass.); VZV proteins

were detected with high-titer polyclonal human anti-VZV immunoglobulin G (IgG) and secondary goat anti-human IgG-horseradish peroxidase conjugate (22). Skin implant sections were stained with anti-VZV IgG, biotinylated anti-human IgG, and 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, Calif.). Infected thymus-liver implants were collected after 7, 14, and 21 days and tested by infectious focus assay and PCR and sequencing.

RESULTS

Generation of rOKA recombinants with gI promoter mutations. Two independently derived VZV recombinants were made from two separately constructed pVSpe21cosmids carrying each AUS mutation and the double Sp1/USF mutation; these viruses were designated rOKAgI-Sp1, rOKAgI-AP-1, rOKAgI-USF, and rOKAgI-Sp1/USF. Two independently derived recombinants were also made with the 29RE-1 and 29RE-2 substitutions, and one 29RE-3 mutant was generated; these viruses were designated rOKAgI-29RE-1, rOKAgI-29RE-2, and rOKAgI-29RE-3. The mutated pVSpe21 cosmids were sequenced, and the presence of the expected mutations in all VZV recombinants was verified by sequencing all viruses that were recovered after transfection of mutant cosmids with the three intact cosmids. Two independently derived repaired viruses, designated rOKAgI:rep-Sp1/USF, were made from cosmids that had the intact gI promoter and ORF67 inserted into the *Avr*II site in the pVSpe21-Sp1/USF cosmid. The expected increases in fragment sizes from 1.1 kb in rOKA to 2.5 kb in rOKAgI:rep-Sp1/USF and preservation of Sp1 and USF mutations in the native gI promoter were confirmed by PCR and sequencing. Transfections using pVSpe21gI-29RE-4 yielded no infectious virus in three experiments, nor did insertion of intact gI promoter and ORF67 coding sequence into the *Avr*II site in the pVSpe21-29RE-4 cosmid, suggesting that substitution of GATAACTACA for native 29RE-4 was lethal.

Effects of gI promoter mutations on VZV replication in vitro. Growth kinetics of all AUS mutants were evaluated over 6 days and were equivalent to those of rOKA in melanoma cells (Fig. 2A). The rOKA titers were significantly higher than those of rOKAgI-29RE-1, 29RE-2, and 29RE-3 mutants at days 1 ($P < 0.0005$), 2 ($P < 0.005$), and 3 ($P < 0.004$) but were equivalent at days 4 to 6 (Fig. 2B). All gI promoter mutants produced significantly higher titers than rOKAΔgI ($P < 0.001$). Plaque size was not affected by individual mutation of Sp1 or USF sites, the putative AP-1 site, or 29RE regions,

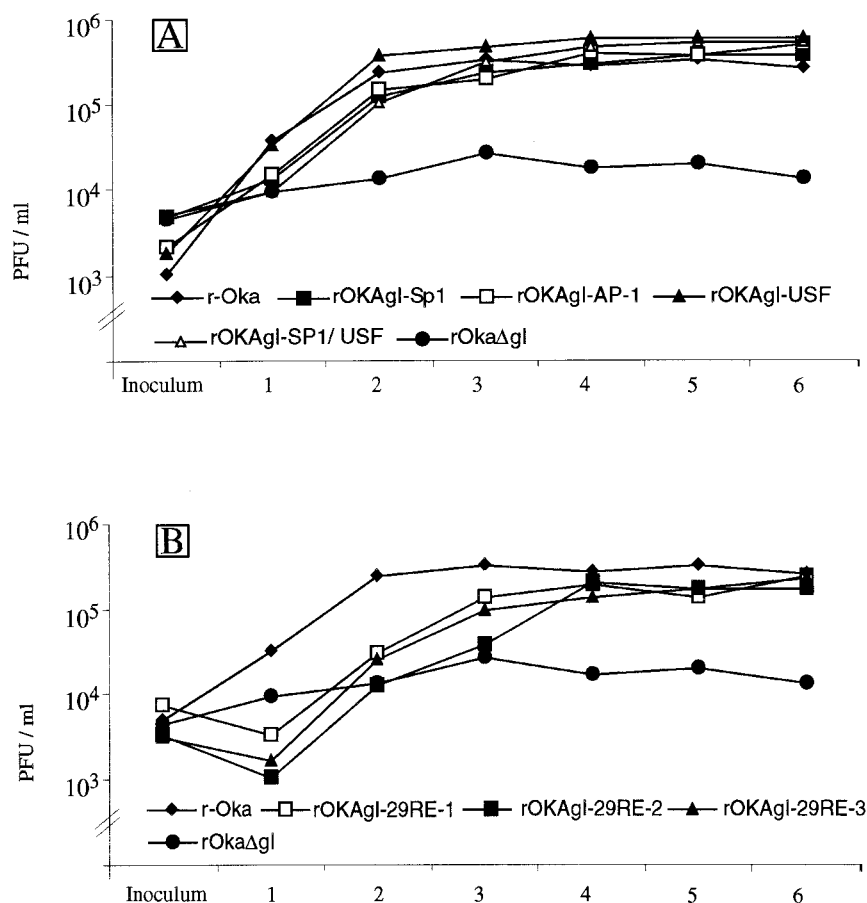


FIG. 2. Effects of gI promoter mutations on VZV replication in vitro. Yields of infectious virus were determined for 6 days after inoculation of melanoma cells with 10^3 to 10^4 PFU of AUS mutants and controls (A) and 29RE mutants and controls (B); titers are expressed as means for quadruplicate wells at each time point. The x axis shows the days after inoculation when infected cell monolayers were harvested, and the y axis shows PFU as determined by infectious focus assay.

compared to rOKA. However, rOKAgI-Sp1/USF mutants exhibited a small-plaque phenotype in initial transfections, which persisted upon passage. Plaque sizes (mean \pm standard deviation) for both of the rOKAgI-Sp1/USF mutants were smaller than those of rOKA (0.77 ± 0.10 mm versus 1.08 ± 0.16 mm; $P < 0.0001$), but larger than those of rOKAΔgI (0.40 ± 0.08 mm; $P < 0.0001$). Plaque sizes of rOKAgI:rep-Sp1/USF mutants were equivalent to those of rOKA and larger than those of rOKAgI-Sp1/USF ($P < 0.0001$). Thus, disrupting both Sp1 and USF binding sites inhibited polykaryocyte formation by VZV in vitro, but to a lesser extent than the complete deletion of ORF67 in rOKAΔgI. The lack of any effect of the AP-1 mutation on growth kinetics or plaque size was expected, because AP-1 does not bind to gI promoter DNA in reporter construct experiments (10). It is theoretically possible that the two nucleotide substitutions in the USF site could also alter this putative AP-1 site, but AP-1 does not bind to the intact DNA sequence, and the present experiments with rOKAgI-AP-1 mutants confirm that its interaction with the gI promoter is not required in vitro. In contrast, USF does bind to the AUS region, and USF binding is disrupted by replacing the TC nucleotides with GA (Fig. 1C). Therefore, the recombinants with changes in the USF binding site were considered to be rOKAgI-USF mutants.

Effects of AUS mutations on VZV replication in skin in vivo.

Replication of rOKAgI-AP-1 mutants was equivalent to that of rOKA at days 14 and 21 and less than 1.0 log lower at day 28 (Fig. 3). These experiments further demonstrate that the putative AP-1 site is not important for gI transcription. Inoculation of rOKAgI-USF yielded less virus than rOKA at days 14, 21, and 28, but all differences were < 1.0 log. Infectious virus was recovered at days 14, 21, and 28 from 75, 100, and 80% of implants inoculated with rOKAgI-AP-1 and from 100% of those infected with rOKAgI-USF, which was equivalent to the growth pattern of vaccine OKA (22, 23). In contrast, rOKAgI-Sp1 exhibited substantially lower titers than rOKA at days 14 and 21 ($P = 0.001$) and limited duration of skin replication, with no virus detected at day 28 (Fig. 3). The rOKAgI-Sp1/USF double mutants did not replicate in any of 29 skin implants harvested at days 14, 21, or 28 (Fig. 3), whereas repaired rOKAgI:rep-Sp1/USF was as infectious as rOKA. Comparisons with rOKAΔgI and rOKAΔgI-gI@AvrII confirmed that gI is essential in skin (Fig. 3) (20).

VZV DNA and viral proteins were detected in all skin implants that yielded infectious virus at all time points after inoculation of rOKAgI-Sp1, rOKAgI-USF, rOKA, rOKAgI:rep-Sp1/USF, and rOKAΔgI-gI@AvrII mutants (Fig. 4). Figure 4A shows representative immunoblots of lysates from im-

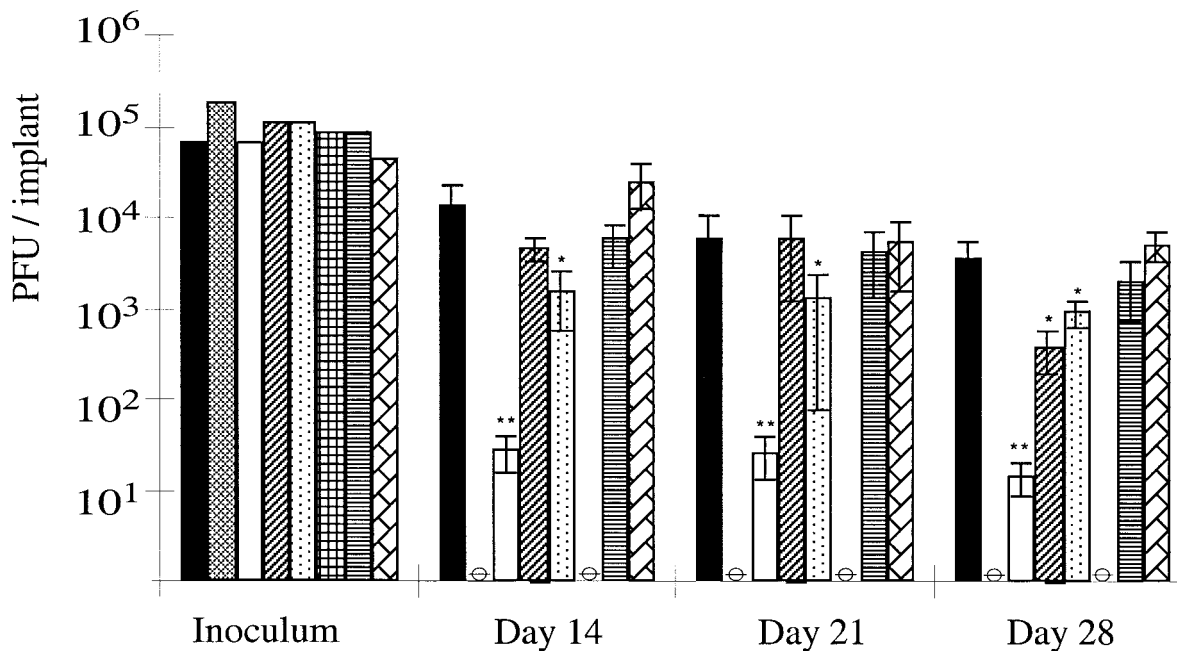


FIG. 3. Effects of mutations in the AUS region of the gI promoter on VZV replication in skin xenografts in SCIDhu mice. Skin xenografts were inoculated with rOKA (■), rOKAΔgI (▨), rOKAgI-Sp1 (□), rOKAgI-AP1 (▩), rOKAgI-USF (▤), rOKAgI-Sp1/USF (▥), rOKAgI:rep-Sp1/USF (▦), or rOKAΔgI-gI@AvrII (▧). Each bar represents the mean titer of infectious virus (error bars, standard deviations) recovered from six to eight implants harvested at 14, 21, or 28 days after inoculation. Inoculum titers are shown at the left. The symbol ⊖ indicates no viral growth in any implants. One asterisk above the error bar indicates that titers were significantly lower than those of rOKA ($P < 0.05$) at the same time point; two asterisks indicates a difference with a P of < 0.01 .

plants infected with these recombinants and implants inoculated with rOKAgI-Sp1/USF or rOKAΔgI. Figure 4B shows the results of PCR analysis for VZV DNA in the same specimens, and Fig. 4C indicates detection of the β -globin gene control. No viral DNA or protein was present in 29 specimens inoculated with rOKAgI-Sp1/USF or in 23 implants injected with rOKAΔgI (Fig. 4A and B); β -globin gene was detected in all samples (Fig. 4C). Sequencing showed that the gI promoter genotypes of rOKAgI-Sp1, rOKAgI-AP-1, rOKAgI-USF, and rOKA were as designed (Fig. 1C) and were identical to the inoculum virus, for both of two recombinants recovered after 28 days of replication in skin. Insertion of the gI promoter and ORF67 at the *AvrII* site (Fig. 4B) and preservation of Sp1/USF native promoter mutations were confirmed by PCR and sequencing of two isolates of the repaired, rOKAgI:rep-Sp1/USF recombinants recovered from skin implants at day 28.

Effects of AUS mutations on VZV replication in T cells in vivo. The rOKAgI-AP-1 and rOKAgI-USF mutants exhibited growth characteristics typical of rOKA in thymus-liver implants (21) (Fig. 5A). Infectivity of rOKAgI-Sp1 was decreased significantly in T cells compared to rOKA, rOKAgI-AP-1, and rOKAgI-USF, as it was in skin. Titers of rOKAgI-Sp1 were low at days 7 and 14 but were higher than those of rOKA at day 21, because rOKA depletes T cells by day 21 (22). Although the double mutation was lethal in skin, rOKAgI-Sp1/USF retained a limited capacity to replicate in T cells (Fig. 5A). Mean peak titers of rOKAgI-Sp1/USF were 1.1×10^2 PFU at day 21, whereas rOKA reached peak titers of 3.1×10^4 PFU by day 7 in T-cell implants. The rOKA titers had declined markedly by day 21, when few intact T cells remained in the implants; in

contrast, the delayed replication of rOKAgI-Sp1/USF at early time points allowed survival of T cells in the implants, which could sustain replication at day 21. Sequencing of two isolates recovered at day 21 showed that the Sp1/USF mutations were intact in both rOKAgI-Sp1/USF recombinants. Infectivity was restored in experiments with rOKAgI:rep-Sp1/USF mutants, compared with rOKA (Fig. 5B); the expected pattern of high initial titers which decreased at later time points was observed for rOKAgI:rep-Sp1/USF and rOKA viruses. As expected, VZV rOKA-ΔgI did not replicate, but rOKA-ΔgI-gI@AvrII replication was equivalent to that of rOKA (Fig. 5) (20).

VZV DNA could not be detected by PCR analysis of T-cell xenografts infected with rOKAgI-Sp1/USF on day 7 or with rOKAΔgI mutants at any time point, whereas all other specimens were positive; β -globin was detected in all samples (data not shown). The gI promoter genotypes of two isolates of each recombinant recovered from infected T cells after 14 to 21 days of replication were identical to those of the input viruses. These sequencing data, along with the restored virulence of the rOKAgI:rep-Sp1/USF mutants, indicate that the altered virulence of the rOKAgI-Sp1/USF was due to the mutations that had been introduced into the gI promoter.

Effects of 29RE mutations on VZV replication in skin and T cells in vivo. Replication of rOKAgI-29RE-1, 29RE-2, or 29RE-3 mutants was demonstrated in 55 of 56 skin implants (99%) at days 14, 21, or 28, whereas rOKAΔgI was not infectious at any time point in 29 implants (Fig. 6A). The infectious virus yield of rOKAgI-29RE-1 was marginally lower than that of rOKA at day 28 ($P = 0.05$). The effect of the 29RE-3 mutation was more significant, with titers lower than those of

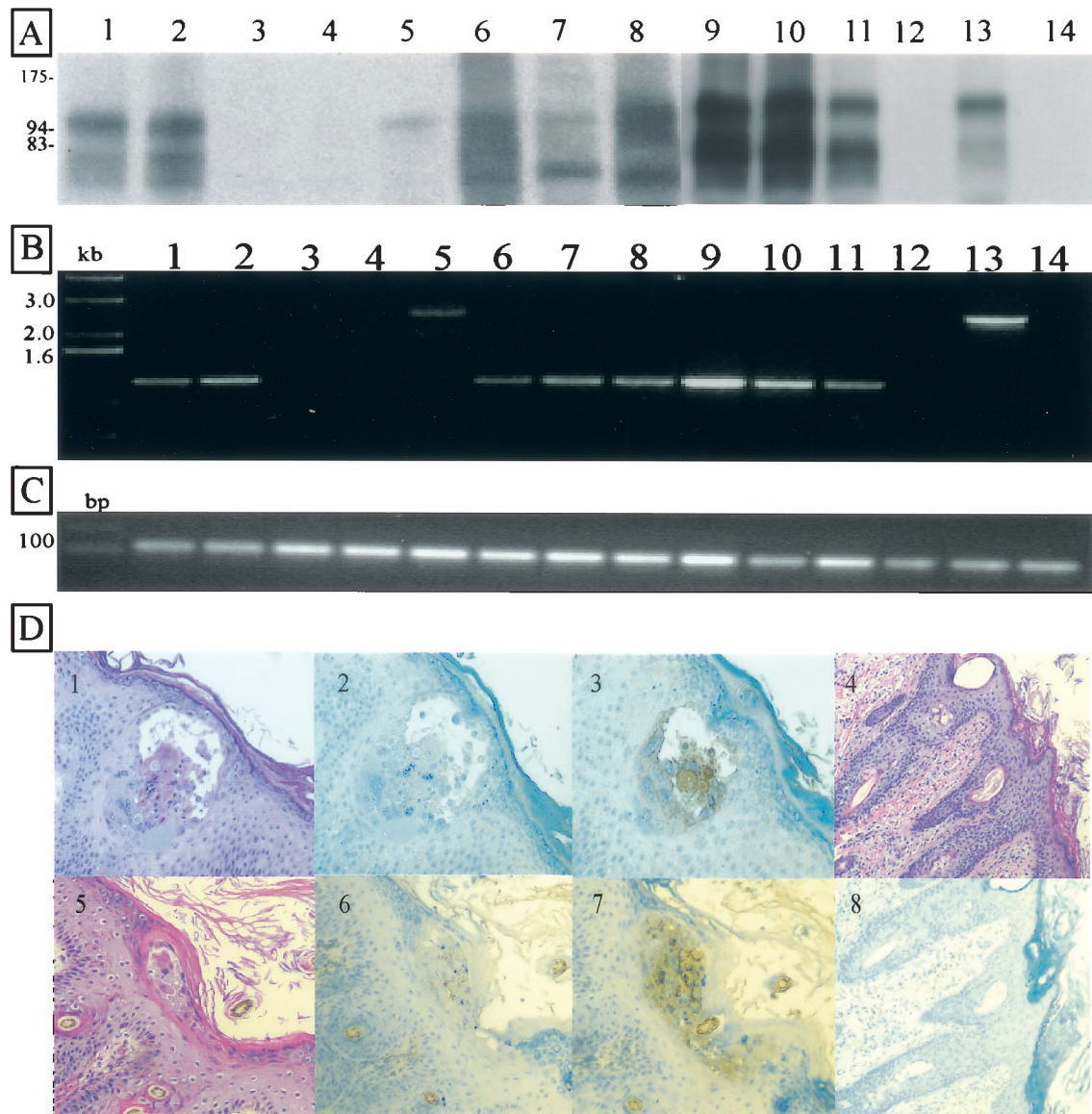


FIG. 4. Detection of VZV DNA and protein synthesis in skin xenografts in SCIDhu mice inoculated with gI promoter mutants. All skin implants inoculated with gI promoter mutants and controls were tested for VZV DNA by PCR and for VZV proteins in extracts of infected cells by immunoblot. Representative results are shown for each recombinant: rOKA (lanes 1 and 2), rOKAgI-Sp1/USF (lanes 3 and 4), rOKAgI-R-Sp1/USF (lane 5), rOKAgI-Sp1 (lane 6), rOKAgI-API (lane 7), rOKAgI-USF (lane 8), rOKAgI-29RE-1 (lane 9), rOKAgI-29RE-2 (lane 10), rOKAgI-RE-3 (lane 11), rOKA Δ gI (lane 12), rOKA Δ gI-gI@AvrII (lane 13), or uninfected HEL cells (lane 14). (A) By immunoblotting, VZV proteins in the 70- to 110-kDa range were detected using a high-titer polyclonal human anti-VZV IgG. No VZV protein synthesis was detected in implants infected with rOKAgI-Sp1/USF (lanes 3 and 4) or rOKA Δ gI (lane 12) or in mock-infected implants (lane 14). (B) PCR products of the expected size were detected using primers for ORF67 (lanes 1, 2, and 6 to 11), and primers spanning the AvrII site were used to detect PCR products showing insertions (lanes 5 and 13). (C) Human β -globin primers were used as a positive PCR control. (D) For histological analysis, skin implants infected with rOKA (panels 1, 2, and 3), rOKAgI-Sp1 (panels 5, 6, and 7), or uninfected HEL cells (panels 4 and 8) were fixed in paraformaldehyde, paraffin embedded, and cut into 3- μ m-thick sections before staining with hematoxylin and eosin (panels 1 and 5) or high-titer polyclonal human VZV IgG (panels 3, 7, and 8) or VZV negative IgG (panels 2 and 6) as a primary antibody. VZV-infected cells were detected in the epidermal layer at 28 days after inoculation with rOKA (panels 1 and 3) or rOKAgI-Sp1 (panels 5 and 7). Mock-infected skin had a normal appearance (panel 4) with no VZV IgG staining (panel 8).

rOKA at day 21 ($P = 0.01$) and at day 28 ($P = 0.001$). Viral protein synthesis was detected by immunoblotting of all 18 skin implants inoculated with rOKAgI-29RE-1, all 18 inoculated with rOKAgI-29RE-2, 19 of 20 infected with rOKAgI-29RE-3, and 17 of 18 inoculated with rOKA; no viral protein was detected in any of 29 skin specimens inoculated with rOKA Δ gI

(Fig. 4). VZV DNA was detected by PCR of implants inoculated with all 29RE mutants and rOKA, but not in those inoculated with rOKA Δ gI; β -globin gene was detected in all samples. Sequencing showed that the gI promoter mutations were preserved as designed (Fig. 1D) in 29RE mutants after replication in skin for 28 days.

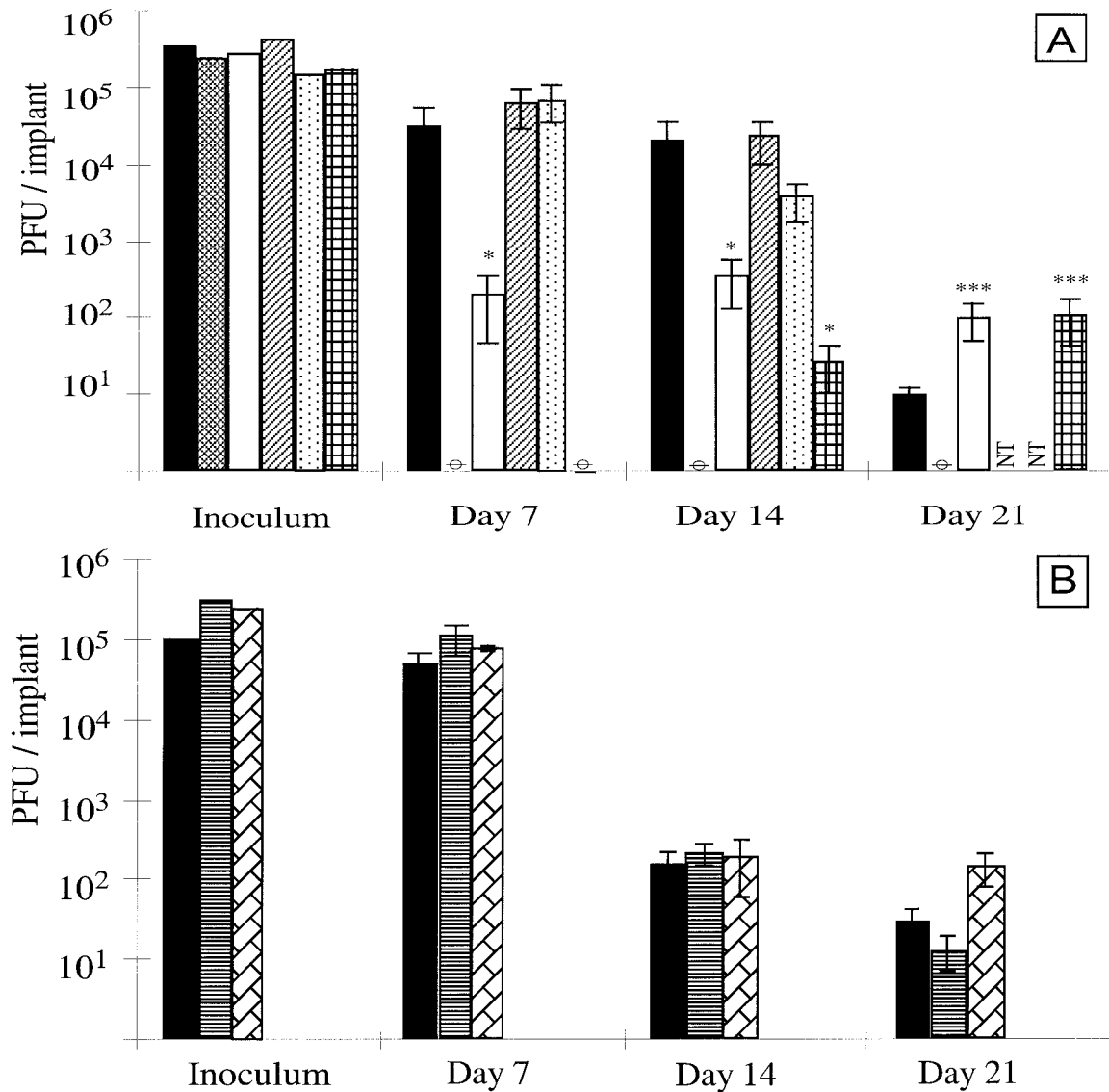


FIG. 5. Effects of mutations in the AUS region of the gI promoter on VZV replication in T-cell xenografts in SCIDhu mice. Thymus-liver implants were inoculated with rOKA (■), rOKAΔgI (▨), rOKAgI-Sp1 (□), rOKAgI-AP1 (▩), rOKAgI-USF (▧), or rOKAgI-Sp1/USF (▦) (A) and with rOKA (■), rOKAgI:rep-Sp1/USF (▨), or rOKAΔgI-gI@AvrII (▩) (B). Each bar represents the mean titer of infectious virus (error bars, standard deviations) recovered from six to eight implants (A) or three to five implants (B), harvested at 7, 14, or 21 days after inoculation. Inoculum titers are shown at the left. The symbol ⊖ indicates no viral growth in any implants; NT means not tested. One asterisk indicates that titers were significantly lower than those of rOKA ($P < 0.05$) at the same time point; three asterisks indicates a difference with a P of < 0.001 .

Growth of rOKAgI-29RE-1 and rOKAgI-29RE-2 mutants was comparable to that of rOKA in thymus-liver implants, but rOKAgI-29RE-3 titers were significantly lower ($P = 0.05$) (Fig. 6B). The rOKAgI-29RE-2 mutant appeared to yield lower titers at day 14, but the difference was not significant compared to titers of the intact rOKA control ($P = 0.07$). In contrast, growth of rOKAgI-29RE-3 was reduced significantly at both day 7 and day 14. All of the 29RE mutants and rOKA were detected by PCR analysis of infected T-cell implants; rOKAΔgI was not detected (data not shown). The gI promoter sequences of each of two 29RE mutants and rOKA recovered from thymus-liver implants at day 14 were identical to those of the respective inoculum virus.

The 29RE region overlaps with the region of ORF66 encod-

ing the N terminus of ORF66 protein (Fig. 1). Previous work by the Cohen laboratory showed that introducing a stop codon to block expression of ORF66 did not alter VZV replication in vitro (5). The 29RE-1 sequence substitution is predicted to function as a stop codon, deleting only the last 9 amino acids of the ORF66 protein. The 29RE-2 mutation changes 3 amino acids, amino acids 389 to 391, from Met-Glu-Val to Ile-Thr-Thr. Our experiments suggest that alterations in the ORF66 protein that might result from the substitutions in 29RE-1 and 29RE-2 regions have no significant consequences for VZV replication in vitro or in vivo. The 29RE-3 mutation changes amino acids 392 to 393 and eliminates the ORF66 stop codon, which could permit the addition of 17 amino acid residues to the ORF66 protein. Our previous evaluation of the ORF66

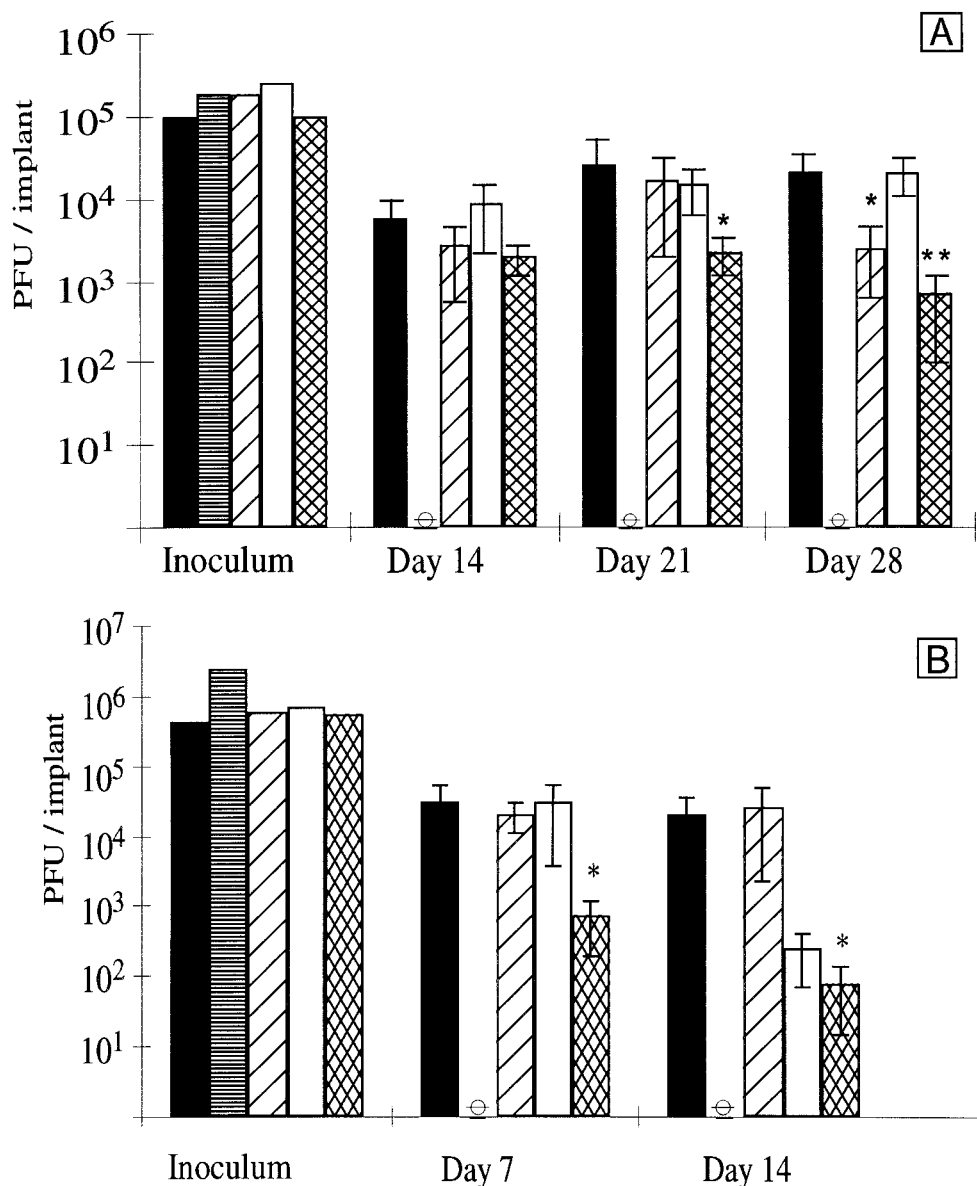


FIG. 6. Effects of mutations in the 29RE region of the gI promoter on VZV replication in skin and T-cell xenografts in SCIDhu mice. Skin and thymus-liver implants were inoculated with rOKA (■), rOKAΔgI (▨), rOKAgI-29RE-1 (▧), rOKAgI-29RE-2 (□), or rOKAgI-29RE-3 (▩). Skin implants were harvested at 14, 21, or 28 days (A) and T-cell implants were harvested at days 7 and 14 (B). Each bar represents the mean titer of infectious virus (error bars, standard deviations) recovered from six to eight skin implants (A) or four to six T-cell implants (B), harvested at 7, 14, or 21 days after inoculation. Inoculum titers are shown at the left. The symbol ⊖ indicates no viral growth in any implants. One asterisk indicates that titers were significantly lower than those of rOKA ($P < 0.05$) at the same time point; two asterisks indicates a difference with a P of < 0.01 .

stop codon mutant in the SCIDhu model showed no effect on virulence in skin but replication was reduced in T cells in thymus-liver implants (23). In the present experiments, the 29RE-3 mutant showed reduced infectivity in T-cell implants, but the effect was not as marked as we observed when ORF66 expression was blocked in the ORF66 stop codon mutant (23). The regions of the ORF66 gene that encode the kinase motifs are distant from all of these 29RE-1, 29RE-2, and 29RE-3 mutations. The 29RE-4 mutation is outside of the ORF66 coding region.

Immunohistologic analysis of skin implants infected with gI

promoter mutants. AUS and 29RE mutants that replicated in skin caused the typical lesions observed in xenografts inoculated with rOKA derived from vaccine OKA cosmids, as shown in representative sections of implants infected with rOKAgI-Sp1 (Fig. 4D, panels 5, 6, and 7) and rOKA (Fig. 4D, panels 1, 2, and 3) with polyclonal anti-VZV IgG or controls (22). The infectious process was confined to the epidermis in two to four implants examined for each recombinant. No lesions were visible in tissue sections from skin xenografts inoculated with rOKAgI-Sp1/USF or rOKAΔgI, all of which were also negative by virus titration and PCR; all of these implants showed stain-

ing patterns indistinguishable from mock-infected controls (Fig. 4D, panels 4 and 8).

DISCUSSION

The construction of VZV recombinants with mutations in the gI promoter and assessment of the consequences for pathogenesis in the SCIDhu model provides the first evidence that interactions between cellular transcription factors and viral promoter sequences can determine the virulence of a herpesvirus in differentiated host cells *in vivo*. Mutations in particular motifs within the AUS region of the gI promoter revealed a hierarchy of contributions by cellular transcription factors to VZV replication in human epidermal cells and T cells *in vivo*. In contrast, no differences in VZV replication were detectable when AUS mutations were introduced into the context of the viral genome and assessed for effects in cell culture, demonstrating the need to define genetic determinants of VZV virulence within differentiated human cells *in vivo*.

As a single mutation, substituting two nucleotides in the Sp1 site caused the most-significant decrease in VZV infectivity for skin and T cells. Disrupting the USF binding site resulted in some reduction of replication in skin but not in T cells, suggesting a differential dependence on USF-enhanced gI expression which was related to the host target cell. Altering the AP-1 site within the AUS had no effect *in vivo*, correlating with failure of AP-1 to bind to gI promoter DNA. These experiments with the AP-1 mutants are important for documenting that changes can be made in the AUS region of the gI promoter sequence without impairing virulence. Notably, the genotypes of two isolates of all AUS mutants were stable after prolonged replication in skin and T cells *in vivo*. In contrast to the modulating effects of the single Sp1 mutation and, to a lesser extent, the effect of the single USF mutation on VZV virulence, the simultaneous mutation of the Sp1 and USF sites, which changed only four nucleotides in the gI promoter, was lethal for VZV growth in skin. The dual Sp1/USF mutation was compatible with some replication in T-cell implants, but only small quantities of virus were produced. That these dramatic effects were the result of blocking Sp1- and USF-mediated transactivation of ORF67 was confirmed by demonstrating the restoration of virulence when repaired rOKAgI:rep-Sp1/USF recombinants were evaluated in skin and T-cell xenografts.

Mutations in the 29RE-1, 29RE-2, and 29RE-3 regions of the gI promoter, which mediate enhancement of gI transcription by the ORF29 single-stranded DNA binding protein, had much less effect on VZV replication *in vivo* than the Sp1 or Sp1/USF mutations. However, the intact 29RE-3 sequence was required for optimal VZV infection of skin and T cells. These observations indicate that regulatory functions of viral DNA binding proteins, like ORF29 protein, may contribute to efficient viral replication in differentiated host cells *in vivo*. Enhancer elements within the gI promoter appear to be useful but are not essential determinants of VZV virulence *in vivo*. Like AUS mutations, sequencing showed that the substitutions in the 29RE region of the gI promoter were maintained over several weeks of replication of the 29RE mutants in skin and T-cell implants.

The consequences of gI promoter mutations may reflect

interference with unique functions of gI. Alternatively, disrupting ORF67 transcription and reducing gI synthesis could affect VZV virulence in critical host target cells *in vivo* through effects on gE (1, 16, 19, 24, 25, 39, 40, 42, 43). Current models of VZV assembly suggest that gE must localize to the *trans*-Golgi network; VZV gI facilitates gE trafficking, and virion synthesis is reduced in the absence of gI (16, 19, 40). The gI homologues of HSV and pseudorabies virus, while dispensable *in vitro*, are required for optimal replication in animal models, which may also be due to gE-gI interactions (4, 7, 11, 38). Like the full deletion of gI, disrupting both Sp1 and USF binding sites inhibited the polykaryocyte formation which is characteristic of VZV replication *in vitro* (16). Since skin infection involves cell-cell spread, it is of interest that the small-plaque phenotypes of both rOKAgI-Sp1/USF and rOKAΔgI mutants correlated with a failure of the recombinant virus to replicate in skin (20).

In contrast to skin, cell fusion is not observed in VZV-infected thymus-liver implants; VZV appears to spread within these implants by release of infectious virus from infected T cells (21). The replication of rOKAgI-Sp1/USF in T cells indicates that the Sp1/USF mutation did not result in a complete block of gI expression, because experiments showing that rOKAΔgI cannot replicate in thymus-liver implants show that some gI synthesis is essential for T-cell infection (20). Viral transactivating proteins or an alternative cellular transactivator must compensate for blocking Sp1 and USF functions in T cells by a mechanism that does not occur in skin, yielding the minimal amount of gI required for VZV replication in T cells.

Sp1, and related mammalian cell proteins Sp2 to Sp4, bind to GC box and GT/CA box motifs (14, 37). Although the Sp1 motif in the gI promoter is an atypical GT/CA box, Sp1 also binds to this sequence (10). With respect to possible mechanisms of effects on gI expression, Sp1 exhibits heterotypic interactions with mammalian cell nuclear proteins and could complex with VZV IE62 in the nucleus since this major regulatory protein undergoes rapid nuclear translocation upon virion entry (13). While IE62 protein does not attach directly to the AUS promoter region, recent experiments by the Ruyechan and Hay laboratories indicate that IE62 protein binds to Sp1 in the absence of viral or cellular proteins. Sp1 may be particularly important for VZV replication because the gE promoter contains Sp1 motifs and VZV gE is essential *in vivo* (19, 29, 30).

Like other bHLHZ proteins, USF activates transcription through binding to a consensus E box, binding to target DNA as a dimmer and interacting with the general transcription factor, TFIID (15). The USF binding site in the gI promoter is an alternative sequence uncommon in promoters of mammalian cell genes (10). USF transactivates promoters of ORF4, ORF10, ORF28, and ORF29 genes in conjunction with IE62 protein in reporter assays (13, 17, 18). The contribution of USF as a cellular transactivator could be more important for expression of these VZV proteins than for gI. However, mutation of the USF site in AUS further enhanced the impact of the Sp1 mutation on VZV virulence, suggesting that these two cellular transactivators act in concert to regulate gI expression *in vivo*. The observation that rOKAgI-Sp1/USF could replicate in T cells, but not in skin, suggests that interactive effects of cellular transactivators on the viral gene promoter can be ei-

ther important or absolutely essential *in vivo*, depending upon the host cell type.

From a broader perspective, the construction and characterization of these gI promoter mutants *in vivo* indicates that binding sites for cellular transactivators and for enhancer elements in promoters of nonessential viral genes may be useful targets for mutagenesis. Altering promoter sequences should allow the construction of mutants that are differentially attenuated in various types of host target cells *in vivo*. Recombinant viruses with promoter changes that are specifically designed to modulate viral gene expression in particular host tissues have potential value as herpesviral gene therapy vectors and as genetically engineered, attenuated viral vaccines.

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