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Mutational Analysis of the Varicella-Zoster Virus ORF62/63 Intergenic Region

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The varicella-zoster virus (VZV) ORF62/63 intergenic region was cloned between the *Renilla* and firefly luciferase genes, which acted as reporters of ORF62 and ORF63 transcription, and recombinant viruses were generated that carried these reporter cassettes along with the intact native sequences in the repeat regions of the VZV genome. In order to investigate the potential contributions of cellular transregulatory proteins to ORF62 and ORF63 transcription, recombinant reporter viruses with mutations of consensus binding sites for six proteins within the intergenic region were also created. The reporter viruses were used to evaluate ORF62 and ORF63 transcription during VZV replication in cultured fibroblasts and in skin xenografts in SCIDhu mice in vivo. Mutations in putative binding sites for heat shock factor 1 (HSF-1), nuclear factor 1 (NF-1), and one of two cyclic AMP-responsive elements (CRE) reduced ORF62 reporter transcription in fibroblasts, while mutations in binding sites for HSF-1, NF-1, and octamer binding proteins (Oct-1) increased ORF62 reporter transcription in skin. Mutations in one CRE and the NF-1 site altered ORF63 transcription in fibroblasts, while mutation of the Oct-1 binding site increased ORF63 reporter transcription in skin. The effect of each of these mutations implies that the intact binding site sequence regulates native ORF62 and ORF63 transcription. Mutation of the only NF-κB/Rel binding site had no effect on ORF62 or ORF63 transcription in vitro or in vivo. The segment of the ORF62/63 intergenic region proximal to ORF63 was most important for ORF63 transcription, but mutagenesis also altered ORF62 transcription, indicating that this region functions as a bidirectional promoter. This first analysis of the ORF62/63 intergenic region in the context of VZV replication indicates that it is a dual promoter and that cellular transregulatory factors affect the transcription of these key VZV regulatory genes.

Varicella-zoster virus (VZV) is a human alphaherpesvirus that causes varicella (chicken pox) and herpes zoster (2, 7). The pathogenesis of primary VZV infection involves mucosal inoculation of infectious particles, followed by a primary viremia with spread of virus by infected T cells to the skin, with the eventual establishment of latency in sensory ganglia (3, 21). As is characteristic of herpesviruses, VZV gene transcription is presumed to be temporally regulated, and VZV replication depends on the expression of putative immediate-early (IE) regulatory genes (7). Two of the expressed proteins, IE62 and IE63, are encoded by the duplicate ORF62 and ORF63 genes, which are transcribed in opposite directions from a 1.5-kb intergenic region that also contains the VZV origin of replication (32). IE62, like its herpes simplex virus type 1 (HSV-1) homolog ICP4, transactivates putative IE, early, and late gene promoters (14, 31), as well as its own promoter (12), and is required for viral growth and replication in cultured cells (34). The ORF63/70 gene product is a small 278-amino-acid protein that is a component of the virion tegument (11, 20) and can regulate viral gene transcription (5, 15, 16, 24, 35). Understanding the factors that influence IE63 expression is of particular interest because ORF63 appears to encode the predominant viral transcript and may be translated during latent infections of human ganglia (6, 8, 18, 23, 25).

The region between ORF62 and ORF63 contains two important TAATGARAT-like elements where the VZV ORF10 protein complexes with octamer-binding protein 1 (Oct-1) and host cell factor 1 to enhance ORF62 transactivation in plasmid reporter assays (28). The role of these elements in ORF63 transactivation has not been determined. The region also contains cyclic AMP (cAMP)-responsive elements (CRE), to which CRE-binding (CREB) proteins bind, and a GA-rich region that affect ORF62 transactivation in transient expression assays (28). Computational analysis suggests that the ORF62/63 intergenic region has binding sites for other cellular transregulatory proteins, including NF-κB, Elk-1, STAT, N-FAT, nuclear factor 1 (NF-1), and activator protein 2, and additional sites for octamer and CREB proteins.

In order to analyze the possible contributions of cellular transregulatory protein binding sites to the transcription of the key VZV regulatory genes, ORF62 and ORF63, the 1.5-kb region of DNA between ORF62 and ORF63 was cloned in either orientation between genes encoding the firefly and *Renilla* luciferases (Promega) so that the luciferase genes acted as reporters of ORF62 and ORF63 transcription (Fig. 1). ORF62/63 promoter-driven luciferase cassettes, with the intact ORF62/63 intergenic region, this region with targeted mutations, or a negative control cassette lacking the ORF62/63 intergenic region, were cloned into the AvrII site of the vOka Spe21 cosmid, and recombinants were generated by cosmid transfection (26). Insertion of the reporter cassettes into the VZV genome allowed analysis of the transcription of ORF62 and ORF63 during VZV replication, with the native ORF62 and ORF63 promoters remaining intact. Recombinant viruses, named rOkaF62/63RL, rOkaF63/62RL, and rOkaFRL (control), were made. Six putative cellular transregulatory protein binding sites

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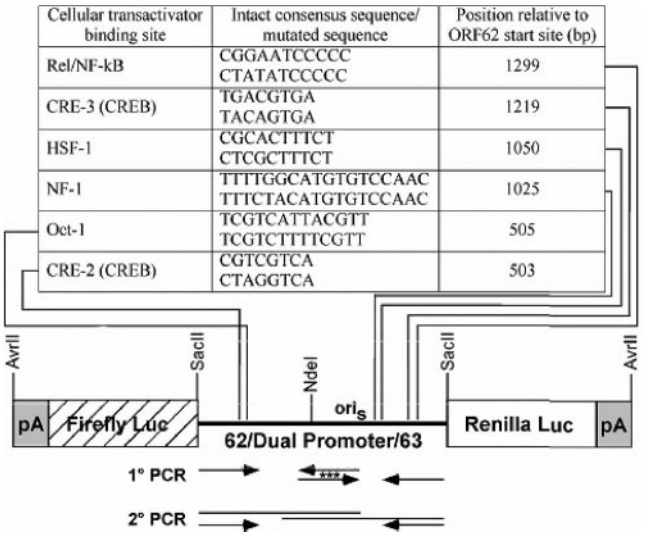

FIG. 1. Construction of VZV recombinants with ORF62/63 dual-promoter luciferase cassettes. The region between open reading frames 62 and 63 was cloned between the firefly and *Renilla* luciferase genes. PCR mutagenesis was used to make mutations in cellular trans-regulatory protein binding sites, as shown. These cassettes were cloned into the AvrII site in the Spe21 cosmid. Relevant restriction sites are shown.

were identified in the ORF62/63 intergenic region, using the MOTIF program (http://motif.genome.jp/), and mutations of these sites were made in the promoter-luciferase cassette by using a nested PCR mutagenesis scheme as described previously (4). All sites were present in both the vaccine Oka and parent Oka ORF62/63 intergenic regions. The mutated cassettes were used to generate the following recombinant viruses: rOk aCRE-2, rOkaCRE-3, rOkaHSF-1, rOkaNF-1, rOkaRel, and rOkaOct-1. The virus rOka63sub had the 445 bp closest to the ORF63 start site replaced by irrelevant DNA from the pLitmus28 vector. The cellular proteins and their consensus and mutated binding sites in the ORF62/63 intergenic region, along with the position of each site relative to the ORF62 translational start site, are shown in Fig. 1. The growth kinetics of each of the VZV recombinants containing a reporter cassette was examined in primary fibroblasts over 4 days and demonstrated that the insertions did not inhibit replication compared to recombinant vOka (data not shown). Furthermore, the orientation of the reporter cassette with respect to the flanking viral genes did not affect the growth of the reporter viruses in vitro (data not shown). All viruses used in the experiments presented had the reporter cassette in the same orientation, except rOkaHSF-1 and rOka63sub; in all viruses except these two, the cassettes were positioned such that the ORF63 reporter gene was closest to ORF65 and the ORF62 reporter gene was closest to ORF66.

To assess ORF62 and ORF63 transcription, human foreskin fibroblasts were grown in T25 flasks to approximately 85% confluence and inoculated with infected cell preparations of the VZV recombinants that had equivalent titers in an infectious focus assay. Fibroblasts were harvested 72 h later, when ~85 to 95% of the cells were infected. Human skin xenografts in SCID mice were infected with the luciferase reporter viruses in fibroblasts and harvested 21 days after infection, using our established methods (27). Protocols for animal studies were approved by the Stanford University Administrative Panel on Laboratory Animal Care; human fetal tissues were obtained with informed consent according to federal and state regulations. Infected skin xenografts were minced and homogenized (Tissue Tearor; Biospec, Inc.); half of each sample was used to determine viral titers by infectious focus assay, and half was set aside for use in the luciferase assay. No significant differences were found in the growth kinetics of the reporter viruses in fibroblasts or skin (data not shown); the amount of sample used in each luciferase assay was based on the viral titer (36). The luciferase assay was performed with a dual-luciferase reporter assay system (Promega, Madison, WI), using cells or tissues harvested in passive lysis buffer (500 μl) and tested in duplicate. In addition to calculating the amount of sample to be tested based on the viral titer, the detection of VZV glycoprotein E by Western blotting was used to evaluate the specimens based on viral protein synthesis as a second method to ensure comparability in the copies of luciferase genes in each sample and to allow the attribution of any differences observed in the reporter luciferase activity to promoter function alone. Luciferase measurements were made on a Lumat LB 9507 luminometer (EG&G Berthold). The linear range for the detection of firefly and *Renilla* luciferase activities was determined (data not shown); the values for all samples in every experiment fell within this range. The data from the fibroblast experiments represent the average luciferase activities (relative light units) normalized to that of the control, rOkaF62/63RL, from four independent assays done with each reporter virus, while the data from skin experiments represent the average luciferase activities normalized to that of the control (rOkaF62/63RL) from independent assays with three rOkaFRL-, rOkaF62/63RL-, rOkaF63/62RL-, rOka63sub-, rOkaHSF-1-, or rOkaRel- or two rOkaCRE 2-, rOkaCRE 3-, rOkaNF-1-, or rOkaOct-1-infected skin specimens. All standard errors were calculated using Kalidegraph (Synergy Software), and P values were derived using Student's t test. All mutations in the ORF62/63 intergenic region were confirmed by sequencing, including persistence of the expected changes after 21 days of replication in skin xenografts.

Using this approach, we were able to determine the relative transcription of ORF62 and ORF63 in cultured fibroblasts and skin xenografts in vivo. When the firefly and *Renilla* luciferase activities were compared in fibroblasts infected with rOkaF62/63RL and rOkaF63/62RL, the two viruses that had the intact intergenic region situated in either orientation between the reporter genes, the luciferase gene that was acting as the reporter for ORF63 had 20 to 50% higher activity than the luciferase gene that was acting as the reporter for ORF62, a finding that was highly significant ($P < 0.001$) (data not shown). Likewise, for skin, the ORF63 reporter had a >2-fold higher luciferase activity than the ORF62 reporter (data not shown). These results imply that more ORF63 transcripts than ORF62 transcripts were present in VZV-infected fibroblasts and skin tissue. This observation has also been made with VZV-infected BSC-1 cells (9). The biological significance of this observation is not known but is of interest because of the apparent relative increase in ORF63 transcription in autopsied human ganglia (8, 10, 17, 19, 38).

The effects of mutations in the ORF62/63 intergenic region were also assessed by measuring the luciferase activities of reporter viruses in fibroblasts and skin. The luciferase mea-

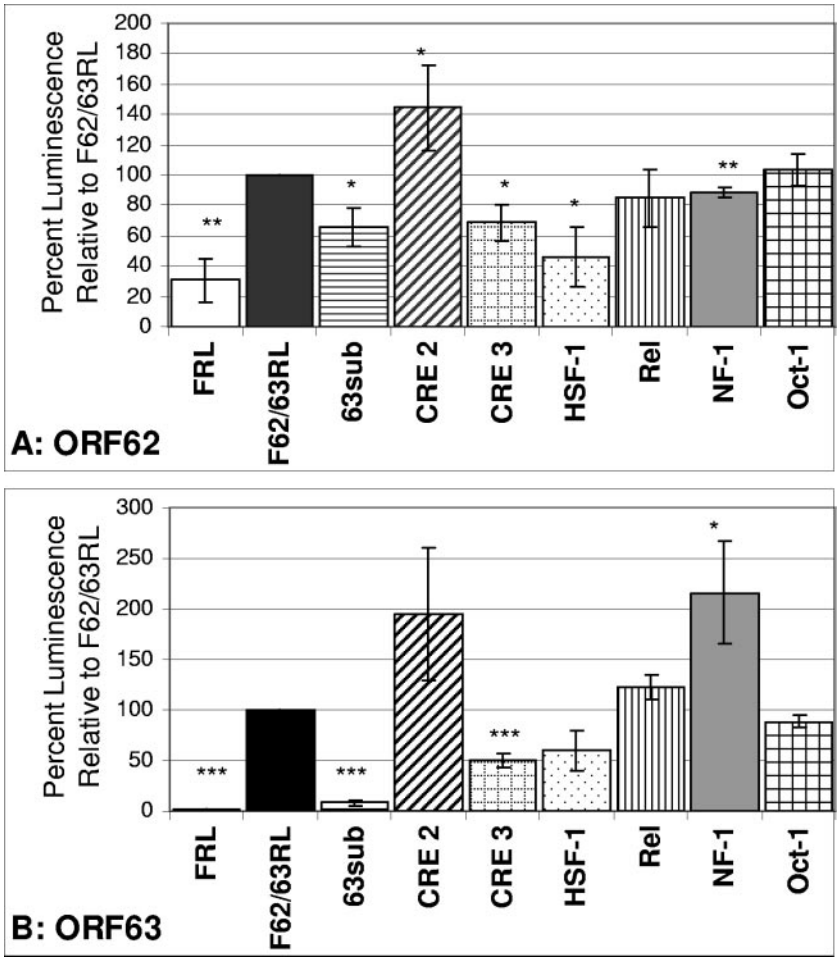

FIG. 2. Effects of mutations in cellular transactivator binding sites on transcription of the ORF62 and ORF63 reporter genes in primary human fibroblasts. Fibroblasts were infected and harvested as described in the text. The firefly (A) and *Renilla* (B) luciferase activities were normalized to those of the rOkaF62/63RL control, and results from four independent experiments were averaged. For panel A, firefly luciferase acted as a reporter for ORF62, and for panel B, *Renilla* luciferase acted as a reporter for ORF63. Standard errors and confidence levels are shown for the fibroblast experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

surements for fibroblasts and skin are shown in Fig. 2 and 3, respectively. Luciferase activity from the promoterless control reporter virus was very low and was significantly lower than those of the other reporter viruses in all experiments. Statistical analysis was used to determine whether changes in the activity of one or both reporters were significant by Student's *t* test. In these experiments, increased reporter activity suggests that a cellular protein, which is presumed to bind its consensus site in the intergenic region, may act as an inhibitor of ORF62 or ORF63 transcription, whereas decreased reporter activity suggests that the protein may function as a coactivator along with the major VZV IE62 protein.

Among the significant findings, fibroblasts infected with rOka63sub, the reporter virus with a large substitution in the intergenic region, demonstrated a reduction of ORF63 and ORF62 reporter luciferase activities compared to rOkaF62/63RL (Fig. 2). ORF63 transcription was decreased to almost none, while ORF62 transcription was about 62% that of the control. These results suggest that intergenic sequences near the ORF63 start site have predominant effects on ORF63 transcription but can also affect ORF62 transcription in fibroblasts and indicate that the ORF62/63 intergenic region functions as a true bidirectional promoter. The importance of this region for ORF63 transcription was evident in skin xenografts in vivo (Fig. 3).

Some mutations, like those in the NF-κB/Rel binding site, had no effect on the activity of either the ORF62 or ORF63 reporter in fibroblasts or skin, while other mutations appeared to have gene- or cell-specific effects (Fig. 2 and 3). For instance, ORF62 and ORF63 transcription was not sensitive to mutations of the cAMP-responsive elements, designated CRE-2 and CRE-3, in skin. In fibroblasts, the CRE-2 mutation resulted in significantly increased activity of the ORF62 reporter only, and CRE-3 mutations decreased the activities of both reporters. Mutations of the heat shock factor 1 and NF-1 sites decreased the activity of the ORF62 reporter in fibroblasts but increased the activity in the skin, indicating that these cellular proteins may down-regulate IE62 expression in vivo. In contrast, these mutations did not alter ORF63 reporter transcription in skin.

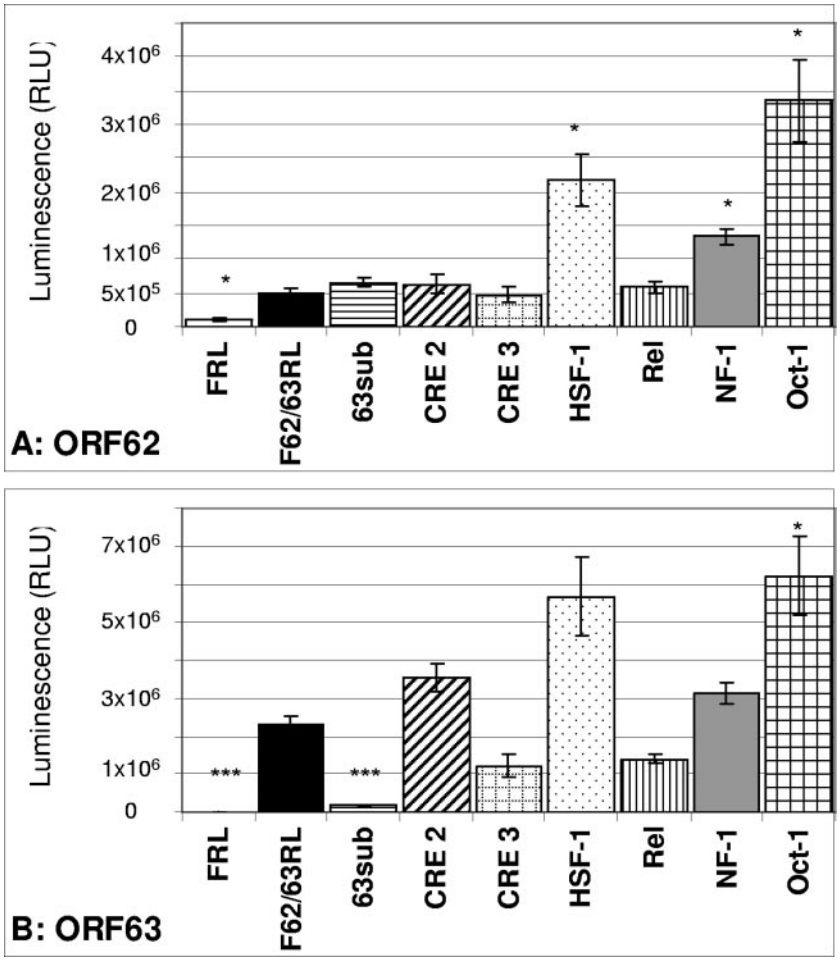

FIG. 3. Effects of mutations in cellular transactivator binding sites on transcription of the ORF62 and ORF63 reporter genes in SCIDhu skin xenografts. Skin grafts were infected and harvested as described previously. The firefly (A) and *Renilla* (B) luciferase activities were measured as the averages of two or three independently infected skin grafts in the same experiment. For panel A, firefly luciferase acted as a reporter for ORF62, and for panel B, *Renilla* luciferase acted as a reporter for ORF63. Standard errors and confidence levels are shown for the experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Interestingly, mutations of the two cAMP-responsive elements, CRE-2 and CRE-3, had some different effects on transcription of the ORF62 and ORF63 gene reporters. The CREB protein belongs to a large family of transcription factors that contain a basic region-leucine zipper motif which mediates both sequence-specific DNA binding and dimerization (33). These two cAMP-responsive elements are >700 bp apart in the ORF62/63 intergenic region and are on opposite sides of the ori_S sequence. It appears that the location of a particular cellular transactivator binding site in the dual promoter can determine its effect on viral gene transcription. In a previous study, mutation of CRE-2 caused a slight decrease in both the basal level of activity of a transiently transfected ORF62 reporter plasmid and ORF10 transactivation of the reporter plasmid in melanoma cells (28). Conversely, using our reporter virus construct, this mutation caused an increase in the activity of the ORF62 reporter, indicating an inhibitory role for CREB binding at this site in fibroblasts. The discrepancy between these results likely reflects the difference between measuring isolated reporter activity in vitro and measuring it in the context of viral replication.

The NF-κB family member RelA (p65) is important in herpesvirus biology. Many herpesviruses, including HSV-1, the human herpesvirus most closely related to VZV, induce a persistent nuclear translocation of NF-κB proteins, including Rel, and HSV-1 depends on NF-κB activation for optimal replication (1, 13, 29, 37). HSV-1 has at least four consensus NF-κB binding sites in various IE gene promoters, while VZV has only one homologous site, located in the ORF62/63 dual-promoter region. Mutation of this site did not significantly affect the transcription of either ORF62 or ORF63 in fibroblasts or skin. VZV infection is associated with cytoplasmic sequestration of NF-κB proteins (submitted for publication), so it is possible that NF-κB binding sites are not as important for the replication of VZV as they are for that of HSV-1.

Octamer proteins are members of the POU family of transcription factors that are important in the tissue-specific regulation of genes (22). In a previous study, mutation of an Oct-1

binding site different from the one that we studied was found to have little effect on ORF62 reporter activity in vitro (28). This result was similar to our findings concerning the potential effects of Oct-1 on ORF62 regulation in fibroblasts. However, we found that mutation of an Oct-1 binding site was associated with increased activities of both reporters in vivo, indicating that this Oct-1 site normally mediates inhibition of ORF62 and ORF63 transcription in skin. These findings highlight the importance of evaluating transcription in vivo, as regulatory proteins may vary in their effects in differentiated human cells located within the tissue microenvironment compared to cultured cells. The differences observed in vitro and in vivo may result from different octamer proteins binding to this site in the promoter. For instance, multiple isoforms of the closely related Oct-2 protein bind to the same sites as Oct-1 proteins and inhibit transcription from a VZV IE promoter construct in a cell type-specific manner (30). The contrasting effects of the heat shock factor 1 mutation in fibroblasts and skin may occur for similar reasons.

The VZV luciferase reporter cassette recombinants described here provide an important tool for the study of VZV IE gene transcription in the context of normal viral replication because the insertion of the ORF62/63-luciferase reporter cassettes did not affect VZV growth in vitro or in vivo. In this study, we described the various effects of cellular transregulatory protein binding site mutations on the transcription of ORF62 and ORF63 in primary fibroblasts in culture and skin tissue in vivo. These experiments warrant further investigation of the roles of particular cellular proteins in ORF62 and ORF63 regulation, including experiments which demonstrate binding to the consensus, but not mutant, sites in the ORF62/63 dual promoter. Other cellular transregulatory protein binding sites in the ORF62/63 dual promoter could also be examined using this intraviral reporter system, including TAATGARAT-like elements, GA-rich elements, N-FAT and STAT transcription factor binding sites, C/EBP binding sites, and other Oct-1 and CREB sites. Interactions of cellular proteins with many of these sites are probably important for regulating ORF62 and ORF63 transcription during VZV pathogenesis in vivo and are likely to have differential effects on VZV replication in skin, T cells, and neurons.

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