The carboxy terminus of the herpesvirus saimiri ORF 57 gene contains domains that are required for transactivation and transrepression

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Herpesvirus saimiri (HVS) ORF 57 is homologous to genes identified in all classes of herpesviruses. We have previously shown that ORF 57 encodes a multifunctional protein, responsible for both transactivation and repression of viral gene expression at a post-transcriptional level. This suggests that the ORF 57 protein shares some functional similarities with the herpes simplex virus IE63/ICP27 and Epstein–Barr virus Mta proteins. However, little is known about the functional domains responsible for the properties of ORF 57 due to the limited homology shared between these proteins. In this report, we have identified the functional domains responsible for transactivation and repression by the ORF 57 protein. We demonstrate that the carboxy terminus is required for ORF 57 transactivation, repression and an intense SC-35 nuclear spotting. This region contains two highly conserved motifs amongst its homologues, a zinc finger-like motif and a highly hydrophobic domain. We further show that the hydrophobic domain is required for transactivation and is also involved in nuclear localization of the ORF 57 protein, whereas the zinc finger-like domain is required for transactivation, repression and the intense SC-35 nuclear spotting.

Introduction

Herpesvirus saimiri (HVS) is the prototype γ2-herpesvirus, or rhadinovirus, and shares significant homology with the human gammaherpesviruses Kaposi’s sarcoma-associated virus (KSHV) and Epstein–Barr virus (EBV) and the murine gamma-herpesvirus MHV-68 (Albrecht et al., 1992; Neipel et al., 1997; Russo et al., 1996; Virgin et al., 1997). HVS was originally isolated from its natural host, the squirrel monkey (Saimiri sciureus), in which it causes a persistent asymptomatic infection. However, HVS infection of other New World primates including cottontop tamarins (Saguinus oedipus) and common marmosets (Callithrix jacchus) results in acute T cell lymphoma within a few weeks (Fleckenstein & Desrosiers, 1982).

Gene expression during the HVS lytic replication cycle is regulated by the products of the two major transcriptional regulating genes encoded by open reading frames (ORFs) 50 and 57 (Nicholas et al., 1991; Whitehouse et al., 1997a, 1998a, b). The ORF 50 or R gene encodes two proteins which are homologous to the EBV BRLF1 protein (Rta), which is sufficient for disruption of latency in EBV-permissive B cells and epithelial cells (Ragoczy et al., 1998; Zalani et al., 1996), and the KSHV R protein, which induces lytic replication in transformed B cells (Lukac et al., 1998; Sun et al., 1998). The HVS ORF 50 proteins activate transcription directly following interactions with promoters containing a specific sequence motif, the consensus ORF 50 recognition sequence, CCN₉GG (Whitehouse et al., 1997b). Furthermore, ORF 50 contains a conserved carboxy-terminal activation domain required for ORF 50 transactivation and for the interaction between the ORF 50 proteins and the general cellular transcription factor TATA-binding protein (Hall et al., 1999).

The second transcriptional activator encoded by HVS is ORF 57, which is homologous to genes identified in all classes of herpesviruses, including the EBV Mta protein transactivator encoded by BMLF1, ORF 57 of KSHV, IE63 or ICP27 of herpes simplex virus (HSV-1), ORF 4 of varicella-zoster virus (VZV) and UL69 in human cytomegalovirus (Albecht et al., 1992; Bello et al., 1999; Kenney et al., 1989; Nicholas et al., 1988; Perera et al., 1994; Russo et al., 1996; Winkler et al., 1994). ORF 57 is a 52 kDa multifunctional trans-regulatory
protein. Transactivation of late viral genes by ORF 57 occurs independently of target gene promoter sequences and appears to be mediated at a post-transcriptional level (Whitehouse et al., 1998a). Recent analysis has demonstrated that the ORF 57 protein has the ability to shuttle between the nucleus and cytoplasm and is required for efficient cytoplasmic accumulation of virus mRNA, suggesting that ORF 57 plays a role in mediating the nuclear export of viral transcripts (Goodwin et al., 1999).

In addition to its transactivation properties, ORF 57 is responsible for repression of viral gene expression, which appears to correlate with the presence of introns within the target gene (Whitehouse et al., 1998a,b). ORF 57 also redistributes both U2 and SC-35 splicing factors during an HVS infection into intense distinct nuclear aggregations. ORF 57 causes a more intense SC-35 staining with larger more intense nuclear spotting resulting in a less diffuse SC-35 background staining in the nucleus as compared with control cells (Cooper et al., 1999). These observations further suggest that ORF 57 plays a role in RNA processing and shares some common properties with the IE63 and Mta proteins (Phelan & Clements, 1998; Ruvolo et al., 1998; Semmes et al., 1998). In addition, the more widely studied IE63 protein also contributes to the shut-off of host-cell protein synthesis and to a decrease in cellular mRNA levels during infection (Hardwicke & Sandri-Goldin, 1994; Hardy & Sandri-Goldin, 1994).

Analysis of IE63 has shown that it contains a number of functional domains including an RGG box required for RNA binding (Mears & Rice, 1996), an amino-terminal nuclear localization signal (NLS) (Hibbard & Sandri-Goldin, 1995; Mears et al., 1995), a leucine-rich nuclear export signal (NES) (Sandri-Goldin, 1998), and carboxy-terminal transactivation and repression domains (Sandri-Goldin et al., 1995; Sandri-Goldin & Hibbard, 1996). However, although the HVS ORF 57 protein has shown to possess some common properties with IE63, there is a limited degree of sequence homology between these proteins. As such, there is little information regarding the functional domains contained within the ORF 57 protein. At present, a leucine-rich NES has been identified which is required for the nuclear cytoplasmic shuttling of ORF 57. Moreover, utilizing ORF 57 fusion proteins, we have demonstrated that the RNA-binding determinant is contained within the amino terminus of ORF 57 (Goodwin et al., 1999).

In this report, we have further investigated the properties of the ORF 57 protein and identified the functional domains responsible for the transactivation and repression properties of ORF 57. We demonstrate that the carboxy terminus is required for ORF 57 transactivation, repression and the more intense SC-35 nuclear spotting. This region contains two highly conserved motifs amongst its homologues, a zinc finger-like motif and a hydrophobic GLFF domain. Moreover, we show that the GLFF domain is required for transactivation, repression and the intense SC-35 nuclear spotting.

Methods

Plasmid constructs. The 3’ deletion series of the ORF 57 gene was constructed by PCR amplification from pUC57 (Whitehouse et al., 1998b) using as forward primer 57FOR, and a series of reverse primers, full-length 57REV and the deletion primers Δ1–Δ3REV (Fig. 1a). These oligonucleotides incorporated PstI and BamHI restriction sites for the convenient cloning of the PCR products. Each fragment was inserted into the eukaryotic expression vector pcDNA3.1 (Invitrogen) to derive the control plasmid, p57, and the deletion series, p57A1–3.

A range of mutants containing site-directed alterations within the conserved hydrophobic GLFF region of the ORF 57 carboxy terminus was generated by a PCR-based method. Carboxy-terminal 278 bp fragments were amplified which incorporated the alteration of each conserved GLFF residue to alanine (underlined) in the 3’ primer. The fragments were generated using the CFOR forward primer and a series of reverse primers, GLFF1–4REV (Fig. 1a). These fragments incorporated EcoRV and BamHI restriction sites, allowing the convenient cloning of each PCR product into p57 previously digested with EcoRV and BamHI, thereby replacing the wild-type sequence with the site-directed mutated sequence, deriving the GLFF mutations, pGLFF1–4.

Site-directed mutants containing alterations in elements of the zinc finger-like motif were produced by a method similar to that above. Histidine and cysteine residues were altered to phenylalanine and serine, respectively (underlined), using the forward primer CFOR2 and a series of reverse primers, ZF1–7REV (Fig. 1a). These 500 bp fragments incorporated MluI restriction sites, allowing the convenient cloning of each PCR product into p57 previously digested with MluI, deriving the zinc finger-like mutations, pZF1–7.

Viruses, cell culture and transfections. HVS (strain A11) was propagated in Owl Monkey kidney (OMK) cells which were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies) supplemented with 10% foetal calf serum (FCS). Cos-7 and 293T cells were also maintained in DMEM supplemented with 10% FCS. Plasmids used in the transfections were prepared using Qiagen plasmid kits according to the manufacturer’s directions. Cells were seeded at 5 × 10⁴ cells per 35 mm diameter Petri dish 24 h before transfection. Transfections were performed using Lipofectamine (Life Technologies) as described by the manufacturer, using 2 μg of the appropriate DNAs.

CAT assay. Cell extracts were prepared 48 h after transfection and incubated with [14C]chloramphenicol in the presence of acetyl coenzyme-A as described previously (Gorman et al., 1982). The percentage acetylation of chloramphenicol was quantified by scintillation counting (Packard) of appropriate regions of the thin-layer chromatography plate.

RNA extraction. Total RNA was isolated from control and transfected cells using Trizol reagent (Life Technologies). Chloroform (0.2 ml) was then added, and the solution vortex-mixed for 20 s and stored at 20 °C for 15 min. Samples were centrifuged for 15 min at 4 °C, and the aqueous phase containing nucleic acids was precipitated using 0.5 ml of isopropanol. After this, the pellet was washed with 70% ethanol, resuspended in 50 μl of water and stored at −70 °C.

RT–PCR. Total RNA (0.1 or 1 μg) was reverse transcribed with Superscript II (Life Technologies) for 1 h at 42 °C using an oligo(dT) primer. cDNA was then amplified by PCR using ORF 57 gene-specific primers, 57FOR and Δ3REV. As a RNA control GAPDH specific primers were also utilized. The amplification conditions were: 1 cycle of 5 min
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Fig. 1. (a) Primer sequences used in construction of the ORF 57 deletion series and mutational analysis.
(b) Schematic representation of the carboxy terminus deletion series of the ORF 57 gene. A series of 3' mutants was constructed by PCR amplification and ligated into the eukaryotic expression vector pCDNA3.1 to derive p57\(\Delta\)1–3.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>57FOR</td>
<td>5'-AAACTGGAGTTGCAAGCTATGCGG</td>
</tr>
<tr>
<td>57REV</td>
<td>5'-CCGGATCCGCTGAGCTATGCGG</td>
</tr>
<tr>
<td>(\Delta)1REV</td>
<td>5'-CCGGATCCGCTGAGCTATGCGG</td>
</tr>
<tr>
<td>(\Delta)2REV</td>
<td>5'-CCGGATCCGCTGAGCTATGCGG</td>
</tr>
<tr>
<td>(\Delta)3REV</td>
<td>5'-CCGGATCCGCTGAGCTATGCGG</td>
</tr>
<tr>
<td>CFOR1</td>
<td>5'-CTGTAAAGATATCAACAGCAGG</td>
</tr>
<tr>
<td>GLFF1REV</td>
<td>5'-CCGGATCCGCTGAGCTATGCGG</td>
</tr>
<tr>
<td>GLFF2REV</td>
<td>5'-CCGGATCCGCTGAGCTATGCGG</td>
</tr>
<tr>
<td>GLFF3REV</td>
<td>5'-CCGGATCCGCTGAGCTATGCGG</td>
</tr>
<tr>
<td>GLFF4REV</td>
<td>5'-CCGGATCCGCTGAGCTATGCGG</td>
</tr>
<tr>
<td>CFOR2</td>
<td>5'-AAACAAATTTATCTGAGCTATCAATCAGG</td>
</tr>
<tr>
<td>ZF1REV</td>
<td>5'-GTATAAACAGAAGTCAGTACAG</td>
</tr>
<tr>
<td>ZF2REV</td>
<td>5'-GTATAAACAGAAGTCAGTACAG</td>
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<tr>
<td>ZF3REV</td>
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<td>ZF4REV</td>
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<td>ZF5REV</td>
<td>5'-GTATAAACAGAAGTCAGTACAG</td>
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<td>ZF6REV</td>
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<tr>
<td>ZF7REV</td>
<td>5'-GTATAAACAGAAGTCAGTACAG</td>
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95°C, 30 cycles of 1 min 95°C, 1 min 55°C and 1 min 72°C, followed by 10 min at 72°C.

Polyclonal antibody generation. Polyclonal antiserum was raised against a portion of recombinant ORF 57 protein. The ORF 57 fragment was expressed as a GST fusion protein in E. coli DH5\(\alpha\) and purified from crude lysates by affinity chromatography with glutathione–Sepharose 4B according to the manufacturer's specification (Pharmacia), as previously described (Goodwin et al., 1999). The purified recombinant protein was used to generate a polyclonal antibody in New Zealand White rabbits using standard protocols.
**Immunofluorescence analysis.** Cells were fixed with 4% formaldehyde in PBS, washed in PBS and permeabilized in 0.5% Triton X-100 for 5 min. The cells were rinsed in PBS and blocked by preincubation with 1% (w/v) non-fat milk powder for 1 h at 37 °C. A 1:50 dilution of anti-ORF 57 antibody was layered over the cells and incubated for 1 h at 37 °C. Fluorescence-conjugated anti-rabbit immunoglobulin (Dako), 1:50 dilution, was added and the mixture incubated for 1 h at 37 °C. For SnRNP redistribution experiments, colocalization studies were performed using a 1:1000 dilution of anti-SC-35 MAb (Sigma) and 1:250 dilution of Texas red-conjugated anti-mouse immunoglobulin (Vector Laboratories). After each incubation step, cells were washed extensively with PBS. The immunofluorescence slides were observed using a Zeiss Axiovert 135TV inverted microscope with a Neofluar 40× oil-immersion lens.

**Results**

**Deletion analysis of the carboxy terminus of ORF 57**

In order to analyse the sequences which are required for transactivation, repression of intron-containing viral genes and SnRNP redistribution by the ORF 57 protein, a 3′ deletion series of the ORF 57 coding region was produced by a PCR-based method deleting larger portions of the carboxy terminus. The carboxy terminus was chosen as this region contains a greater degree of similarity amongst the ORF 57 homologues (Brown et al., 1995). Each fragment was inserted into the transfer vector pcDNA3.1 (Invitrogen) to derive the control plasmid, p57, and p57Δ1–3 (Fig. 1b). All constructs were confirmed by DNA sequencing (data not shown).

The carboxy terminus is required for the transactivation and repression properties of the ORF 57 protein

We have previously shown that ORF 57 transactivates a range of HVS promoters, including those driving expression of the gB and capsid genes, but does not significantly alter the level of mRNA, suggesting that ORF 57 acts by a post-transcriptional mechanism. To identify the sequences which are required for ORF 57 transactivation activity, 1 µg of each deletion plasmid was cotransfected with 1 µg pgBCAT2. This plasmid contains the CAT coding region under the control of the HVS gB promoter (Whitehouse et al., 1998a). Plasmid p57 was also used in the assay as a positive control. Both the HVS permissive OMK and the highly transfectable 293T cell lines were used. Cells were harvested after 48 h and assayed for CAT activity by standard methods (Gorman et al., 1982) (Fig. 2a). Severely reduced CAT activity was observed when each deletion construct was utilized in the assay. However, p57 was shown to transactivate the gB promoter to levels previously shown (Whitehouse et al., 1998a). This suggested that the sequences contained within the ORF 57 carboxy terminus are required for transactivation by the ORF 57 protein.

In addition to the transactivation capabilities of ORF 57, it is also responsible for the repression of intron-containing viral genes. We have previously shown that ORF 57 has a regulatory effect on the spliced ORF 50a transcriptional activator (Whitehouse et al., 1998a). To determine if the carboxy terminus contains sequences responsible for ORF 50a regulation, cotransfection assays were performed as previously described (Whitehouse et al., 1998a). The HVS permissive OMK cells and the highly transfectable 293T cells were transfected with 1 µg pAWCAT2 and pAWPst in the absence or presence of 1 µg of p57 and each deletion plasmid, p57Δ1–3. Cells were harvested at 48 h post-transfection and cell extracts were assayed for CAT activity. (b) 293T cell (shaded) and OMK cell (unshaded) monolayers were transfected with 1 µg pAWCAT2 and pAWPst in the absence or presence of 1 µg of p57 and each deletion plasmid, p57Δ1–3. Cells were harvested at 48 h post-transfection and cell extracts were assayed for CAT activity. For each experiment, percentage acetylation was calculated by scintillation counting of the appropriate regions of the chromatography plate and is shown in graphical format; the variations between three replicated assays are indicated.
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The ORF 57 carboxy-terminal deletions are transcribed at similar levels to the wild-type ORF 57 and produce nuclear proteins

In order to determine whether the lack of transactivation and repression by the ORF 57 deletion series was due to the removal of the ORF 57 carboxy terminus, or whether the removal of these sequences affected ORF 57 transcription or protein stability, immunofluorescence and RT–PCR were performed. Initially, to determine whether each deletion construct produced a stable protein, Western blot and immunofluorescence analysis were attempted. Previously, we have studied the expression and cellular localization of the ORF 57 protein using the SB monoclonal antibody (Randall et al., 1983). However, this protein did not react with any of our deletion constructs using immunofluorescence, suggesting that the SB reactive epitope is contained within the carboxy portion of the ORF 57 protein (unpublished observations). Therefore, to analyse the ORF 57 carboxy terminus deletion series a polyclonal antiserum was raised against the amino portion of recombinant ORF 57 protein (Goodwin et al., 1999).

Unfortunately, the ORF 57 polyclonal antiserum did not react on a Western blot of HVS-infected or ORF 57-transfected cells (data not shown). Therefore, to determine if each deletion produced a protein product, immunofluorescence analysis of HVS-infected (m.o.i. of 1) and transiently transfected cells was performed using the polyclonal 57 antibody. Immunofluorescence analysis of HVS-infected cells resulted in a strong fluorescence of the nuclei of infected cells (data not shown). Similar results were observed with p57- and p57Δ1–3-transfected cells (Fig. 3a); no reaction was observed with cells that had not been transfected (data not shown). This suggested that each deletion construct produced a protein which localized to the cell nucleus.

Fig. 3. The ORF 57 carboxy-terminal deletions produce nuclear proteins which do not increase the intensity of nuclear spotting of spliceosome components but are transcribed at similar levels to the wild-type ORF 57. (a) COS-7 cells were transfected with 2 µg of p57 (panels i–v), p57Δ1 (panels ii–vi), p57Δ2 (panels iii–vii), p57Δ3 (panels iv–viii) and 24 h later were fixed as described. The cells were dual labelled with a 1:50 dilution of the ORF 57 polyclonal antibody (panels i–iv) and a 1:1000 dilution of MAb SC-35 (panels v–viii). (b) Total RNA [(i) 0.1 µg and (ii) 1 µg] was isolated from untransfected, p57-transfected or p57Δ1–3-transfected COS-7 cells. First strand cDNA was reversed transcribed using reverse transcriptase and RT–PCR amplification was performed using (i + ii) ORF 57-specific primers or (iii) GAPDH gene-specific primers: lane 1, untransfected cells; lane 2, p57; lane 3, p57Δ1; lane 4, p57Δ2; lane 5, p57Δ3.
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**Fig. 4.** Mutational analysis of the ORF 57 carboxy terminus. (a) Amino acid sequence alignment of the carboxy termini of ORF 57 and a number of its homologues in other herpesviruses. As highlighted, the carboxy terminus of the ORF 57 homologues contains two positionally conserved motifs: a zinc finger-like domain and a hydrophobic GLFF domain. (b) A range of site-directed GLFF domain mutations was constructed in which each conserved hydrophobic residue was substituted with an alanine residue (shown in bold). (c) A range of site-directed zinc finger-like mutations was constructed in which one or multiple conserved histidine or cysteine residues were substituted with phenylalanine and serine residues, respectively (shown in bold).

Furthermore, although we could not determine the protein levels produced from each deletion construct, because of lack of reactivity of the polyclonal antibody using Western blot analysis, we determined if each deletion construct expressed the ORF 57 transcript at similar levels to the wild-type using RT–PCR. Total RNA was isolated from untransfected, p57- or p57\(^{\Delta}1–3\)-transfected cells. First strand cDNA was reversed transcribed using reverse transcriptase. In order to ensure that the RT–PCR was in a linear range serial dilutions of the total input total RNA were utilized. RT–PCR amplification was performed using ORF 57 gene-specific primers and, as a control, GAPDH-specific primers. The results (Fig. 3 b) demonstrate that RT–PCR products of the correct size were amplified from RNA isolated from p57- and p57\(^{\Delta}1–3\)-transfected cells. In addition, the products from the two dilutions amplified in similar quantities to the wild-type protein, suggesting that deletion of the carboxy terminus does not affect the level of expression or stability of the ORF 57 mutant transcripts.

**The ORF 57 carboxy terminus is also required for an increase in the intensity of SC-35 nuclear spotting**

We have previously demonstrated that the ORF 57 protein is also responsible for the redistribution of spliceosome components leading to intense spotting during HVS infection. ORF 57 causes a more intense SC-35 staining with larger more intense spotting, resulting in a less diffuse SC-35 background in the nucleus compared with control cells (Cooper et al., 1999). This redistribution correlates with the impairment of splicing by ORF 57. However, it is believed that splicing inhibition involves mechanisms more complex than simply redistributing spliceosome components. To further investigate this link we utilized members of the ORF 57 carboxy deletion series to determine if they maintained the ability to redistribute spliceosome components. Immunofluorescence was performed on untransfected, p57-transfected or p57\(^{\Delta}1–3\)-transfected cells. Cells were incubated for 24 h before being fixed and dual labelled with the polyclonal ORF 57 and SC-35 antibodies. Cells expressing the full-length ORF 57 protein resulted in a more intense nuclear spotting of the SC-35 spliceosome factor as previously described (Cooper et al., 1999), whereas untransfected and p57\(^{\Delta}1–3\)-transfected cells showed no SC-35 redistribution, suggesting that the carboxy terminus is also required for the intense nuclear spotting of the SC-35 spliceosome component (Fig. 3 a).

**Mutational analysis of the ORF 57 carboxy terminus**

The above results indicate that the carboxy terminus is
required for both the transactivation and repression properties of the ORF 57 protein. Fig. 4(a) shows amino acid sequence alignments of the carboxy termini of ORF 57 and a number of its homologues. As highlighted, the carboxy termini of the ORF 57 homologues contains two positionally conserved motifs: a zinc finger-like domain conserved in all herpesviruses (Vaughan et al., 1992), and a hydrophobic GLFF domain highly conserved in gammaherpesviruses, except in both cases for the MHV-68 ORF 57 gene. To determine the importance of these conserved motifs for ORF 57 transactivation and repression activity, a range of site-directed mutations within the GLFF (Fig. 4b) and elements of the zinc finger-like (Fig. 4c) motifs were constructed. These were generated by a PCR-based method which incorporated the alteration of one or more of the conserved residues. All constructs were confirmed by DNA sequencing (data not shown).

The GLFF domain is required for ORF 57 transactivation not repression

To determine if the residues contained within the highly conserved hydrophobic GLFF domain are required for the transactivation or repression properties of ORF 57, CAT assays were performed as described above. Initially, to identify if these residues are required for ORF 57 transactivation activity 1 µg of each pGLFF1–4 was cotransfected with 1 µg pgBCAT2. Plasmid p57 was also used in the assay as a positive control. Cells were harvested after 48 h and assayed for CAT activity (Fig. 5a). Results show that the mutations of all four GLFF residues encoded at 79601–79613 bp of the published sequence were highly detrimental to ORF 57 transactivation, reducing CAT activity by approximately 70–80% in each case. This demonstrates that these conserved residues are required for ORF 57 transactivation activity.

Secondly, to determine if the GLFF residues are responsible for downregulation of intron-containing genes, cotransfection assays were performed, again as described above. Cells were transfected with pAWCAT2 and pAWPst, in the absence or presence of pGLFF1–4. Cells were harvested after 48 h and assayed for CAT activity (Fig. 5b). Reduced CAT activity was observed in cells transfected with p57 and the GLFF mutants, suggesting that these residues have a limited role, if any, for the repression of intron-containing genes by the ORF 57 protein.

The ORF 57 GLFF mutations are transcribed at similar levels to the wild-type ORF 57 and produce nuclear proteins

In order to determine whether the mutation of these residues affected the expression level or protein stability, RT–PCR and immunofluorescence were again performed as described above. Firstly, RT–PCR was performed using two serial dilutions of input RNA isolated from p57- or pGLFF1–4-transfected cells. The results (Fig. 6a) demonstrate that RT–PCR products of the correct size and in similar quantities to the wild-type protein were amplified, suggesting that alteration of these residues did not affect ORF 57 transcription or RNA stability.

Furthermore, to demonstrate that each GLFF mutation produces a stable protein and to determine the subcellular localization, immunofluorescence was performed. Results showing the subcellular localization of each GLFF mutation are...
The ORF 57 GLFF mutations are transcribed at similar levels to the wild-type ORF 57 and produce nuclear proteins which increase the intensity of nuclear spotting of spliceosome components. (a) Total RNA [(i) 0.1 µg and (ii) 1 µg] was isolated from untransfected, p57-transfected or pGLFF1–4-transfected COS-7 cells. First strand cDNA was reversed transcribed using reverse transcriptase and RT–PCR amplification was performed using (i–ii) ORF 57-gene specific primers or (iii) GAPDH gene-specific primers: lane 1, untransfected cells; lane 2, p57; lane 3, pGLFF1; lane 4, pGLFF2; lane 5, pGLFF3; lane 6, pGLFF4. (b) COS-7 cells were transfected with 2 µg of p57 (panels i–vi), pGLFF1 (panels ii–vi), pGLFF2 (panels iii–vii), pGLFF3 (panels iv–ix) or pGLFF4 (panels v–x), and 24 h later were fixed as described. The cells were dual labelled with a 1:50 dilution of the ORF 57 polyclonal antibody (panels i–v) and a 1:3000 dilution of MAb SC-35 (panels vi–x).

Fig. 6. The zinc finger-like domain is required for ORF 57 transactivation and repression. (a) 293T cell monolayers were transfected with 1 µg pgBCAT2 in the absence or presence of 1 µg of p57 and each ZF mutation plasmid, pZF1–7. Cells were harvested at 48 h post-transfection and cell extracts assayed for CAT activity. (b) 293T cell monolayers were transfected with 1 µg of pAWCAT2 and pAWPst1 in the absence or presence of 1 µg of p57 and each ZF mutation plasmid, pZF1–7. Cells were harvested at 48 h post-transfection and cell extracts assayed for CAT activity. For each experiment percentage acetylation was calculated by scintillation counting of the appropriate regions of the chromatography plate and is shown in graphical format; the variations between three replicated assays are indicated. Shown in Fig. 6(b). Mutation of the phenylalanine residues in particular demonstrated that the majority of the ORF 57 protein is again present in the nucleus; however, the mutant proteins were also in the cytoplasm, suggesting that these residues may have a role in the nuclear localization of the ORF 57 protein.
The ORF 57 GLFF residues are not required for the intense SC-35 nuclear spotting

To determine if the GLFF residues are required for the ability of ORF 57 to redistribute spliceosome components, immunofluorescence was performed on untransfected, p57-transfected or pGLFF1–4-transfected cells. Cells were incubated for 24 h before being fixed and dual labelled with the polyclonal ORF 57 and SC-35 antibodies. Cells expressing the full-length ORF 57 protein but also the GLFF mutations resulted in a more intense nuclear spotting of the SC-35 spliceosome factor (Fig. 6b), suggesting that these residues are not required for redistribution of the SC-35 spliceosome component into the intense nuclear spots.

The zinc finger-like domain is required for both transactivation and repression

As previously mentioned, all ORF 57 homologues, except that of MHV-68, contain conserved residues which constitute a zinc finger-like domain (Vaughan et al., 1992). In order to investigate the role of this domain in the properties of the ORF 57 protein the histidine residue was changed to phenylalanine and the cysteine residues altered to serines, thereby introducing minimal overall structural or conformation perturbations in the polypeptide. To determine if the residues are required for the transactivation or repression properties of ORF 57, CAT assays were performed as described above. Initially, to identify if these residues are required for ORF 57 transactivation activity, 1 µg of each pZF1–7 was cotransfected with 1 µg of pgBCAT2. Plasmid p57 was also used in the assay as a positive control (Fig. 7a). Results show that single mutations at the conserved histidine at 79526 bp and cysteines at 79538 and 79555 bp of the published sequence proved highly detrimental to ORF 57 transactivation, reducing CAT activity by approximately 90, 70 and 87%, respectively. Furthermore, multiple substitutions of these residues completely abrogated biological activity, showing that these conserved residues, which constitute elements of the zinc finger-like domain, are required for ORF 57 transactivation activity.

Secondly, to determine if the zinc finger-like domain is responsible for downregulation of intron-containing genes, cotransfection assays were performed, again as described above. Cells were transfected with pAWCAT2 and pAWPshI, pZF1–7-transfected COS-7 cells. First strand cDNA was reverse transcribed using reverse transcriptase and RT–PCR amplification was performed using (i + ii) ORF 57 gene-specific primers or (iii) GAPDH gene-specific primers: lane 1, untransfected cells; lane 2, p57; lane 3, pZF1; lane 4, pZF2; lane 5, pZF3; lane 6, pZF4; lane 7, pZF5; lane 8, pZF6; lane 9, pZF7. (b) COS-7 cells were transfected with 2 µg of p57 (panels i–v), pZF1 (panels ii–vi), pZF2 (panels iii–vii) or pZF3 (panels iv–viii), and 24 h later were fixed as described. The cells were dual labelled with a 1:50 dilution of the ORF 57 polyclonal antibody (panels i–v) and a 1:3000 dilution of MAb SC-35 (panels v–viii).

Fig. 8. The ORF 57 ZF mutations are transcribed at similar levels to the wild-type ORF 57 and produce nuclear proteins which do not increase the intensity of nuclear spotting of spliceosome components. (a) Total RNA [(i) 0.1 µg and (ii) 1 µg] was isolated from untransfected, p57-transfected or
in the absence or presence of each pZF1–7. Cells were harvested after 48 h and assayed for CAT activity (Fig. 7b). Reduced CAT activity was observed in cells transfected with p57; however, no or very limited repression of ORF 50a transactivation was observed in cells transfected with the zinc finger-like mutations. This suggested that the sequences contained within the zinc finger-like domain are also required for repression of intron-containing viral genes by the ORF 57 protein.

The zinc finger-like mutations are expressed at similar levels to the wild-type ORF 57 and produce nuclear proteins

In order to determine whether the mutation of these residues affected expression levels or protein stability, RT–PCR and immunofluorescence were again performed as described. Firstly, RT–PCR was performed using two serial dilutions of input RNA isolated from p57- or pZF1–7-transfected cells. The results (Fig. 8a) demonstrate that RT–PCR products of the correct size and in similar quantities to the wild-type protein were amplified, suggesting that the mutation of these residues does not affect RNA stability or transcription.

Furthermore, to demonstrate that each ZF mutation produces a stable protein and to determine the subcellular localization, immunofluorescence was performed. Results showing the subcellular localization of ZF1–3 mutations are shown in Fig. 8(b). Mutation of the ZF residues did not affect the subcellular localization of the ORF 57 protein; similar results were observed for all the zinc finger mutations (data not shown).

The ORF 57 zinc finger-like domain is required for the intense SC-35 nuclear spotting

To determine if the zinc finger-like domain is required for the ability of the ORF 57 protein to redistribute spliceosome components, immunofluorescence was performed on untransfected, p57-transfected or p57ZF1–7-transfected cells. Cells were incubated for 24 h before being fixed and dual labelled with the polyclonal ORF 57 and SC-35 antibodies. As shown in Fig. 8(b), ZF mutations 1 and 3 showed no intense nuclear spotting suggesting, that this domain is required for spliceosome component redistribution. Similar results were observed for the remaining zinc finger-like mutations, ZF4–7 (data not shown). It was noted that ZF2, which mutated the cysteine residue at 79538 bp of the published sequence, resulted in what we have termed a partial spotting as shown in Fig. 8(b) (panel vii).

Discussion

In this report, we have identified two functional domains within the carboxy terminus responsible for the transactivation and repression properties of the HVS ORF 57 protein. Interestingly, both the hydrophobic GLFF and zinc finger-like domains are involved in the transactivation properties of ORF 57. Moreover, these domains may be involved in a common transactivational or a structural role in all herpesvirus ORF 57 homologues. This is supported by analysis of ORF 57 homologues, including mutant viruses defective in the activation of late genes and deletion constructs. It has been demonstrated that the IE63 transactivation domain maps to the carboxy-terminal portion of the protein (Hardwicke et al., 1989; McMahan & Schaffer, 1990). Furthermore, extensive analysis has been performed by transient transfection assays using a range of site-directed mutations within the conserved carboxy terminus residues, including the zinc finger-like mutations highlighted in this report (Brown et al., 1995). Results demonstrated that these residues were required for the transactivation by IE63 and closely correlate to loss of function mutants which are blocked in immediate-early mRNA shutoff and late-gene mRNA accumulation (Rice & Lam, 1994). Furthermore, equivalent mutations within the VZV ORF4 protein also abrogate the transactivation activity of the polypeptide (Perera et al., 1994).

Moreover, mutations within the GLFF hydrophobic domain result in a distinct subcellular localization. The majority of the hydrophobic GLFF proteins were observed in the nucleus; however, a proportion of the protein is present in the cytoplasm. This subcellular localization has never been observed with the wild-type ORF 57 protein or any other mutants tested to date. At present the implication of this result is yet to be elucidated. It is possible that these carboxy-terminal hydrophobic residues are involved in protein–protein interactions with a nuclear cellular protein which retains the ORF 57 protein in the nucleus, or that this hydrophobic domain is itself a novel nuclear localization or retention signal. Further analysis of this domain is now required to fully determine its role in the multifunctional properties of the ORF 57 protein.

In addition to the role in transactivation, the zinc finger-like domain is required for the repression of intron-containing viral genes by ORF 57. Similar observations that the carboxy terminus has a role in repression of IE63 have been reported. Mutant viruses defective in the repression of an early target plasmid have been mapped to within the carboxy-terminal 78 amino acids of IE63 (Hardwicke et al., 1989; Smith et al., 1991). In addition, Brown et al. (1995) observed that the mutations within the carboxy terminus conserved residues, which abrogated transactivation, were also responsible for the repression properties of the IE63 protein. However, in contrast, other studies utilizing IE63 proteins containing mutations within the carboxy terminus maintain the ability to repress gene expression whereas amino-terminal deletions were deficient in the repression function (Rice et al., 1993; Rice & Lam, 1994). At present, we have not performed mutational analysis on the amino-terminal portion of the ORF 57 protein and it cannot be excluded that this may also include additional repression domains. The amino-terminal similarity between
ORF 57 and its homologues is very limited, however; they all contain a cluster of acidic residues, which suggests that this acidic region has a common function. We have also demonstrated that the ORF 57 zinc finger-like domain is required for the intense nuclear spotting of the SC-35 spliceosome component. This increase in spotting may correlate with the impairment of splicing by ORF 57; however, it is believed that splicing inhibition involves mechanisms more complex than simply redistributing spliceosome components. Similar results have been observed in IE63, demonstrating that the carboxy terminus is required for interaction with SnRNPs and the redistribution of SnRNPs and SC-35, but does not inhibit host cell splicing (Sandri-Goldin et al., 1995; Sandri-Goldin & Hibbard, 1996). Interestingly, analysis of the zinc finger-like mutant 2, which alters the cysteine at 79738 of the published sequence to a serine, results in what we have termed a partial spotting effect. Moreover, this mutation results in a limited repression of intron-containing genes which correlates with the partial spotting effect. These results suggest that this is the least important residue contained with the zinc finger domain mutated in this report and draws to a parallel with the conservation in this domain. This residue is the only one not totally conserved: as highlighted in Fig. 4, the MHV-68 carboxy terminus contains a lysine residue in this position. It would be of interest to determine if these domains have similar functions in other gammaherpesvirus ORF 57 homologues.

At present we cannot determine the exact role of these domains in the transactivation and repression properties of ORF 57 protein. These functional domains within the carboxy terminus of the ORF 57 gene may have several functions. (i) They may be required for the self-interaction of the ORF 57 protein, enabling the ORF 57 protein to function as a multimer. Analysis of HSV-1 IE63 has shown that this region is involved in the self-interaction of the protein, suggesting that IE63 acts as a multimer in infected cells (Zhi et al., 1999; Wadd et al., 1999). At present studies are being undertaken to determine if ORF 57 self-interacts. (ii) These domains are required for the proper folding or protein stability of the ORF 57 protein. However, the limited analysis we could perform using our ORF 57 reagents suggest that these mutations have a limited effect on RNA transcription or protein stability. (iii) These domains are required for the binding of RNA by ORF 57. Although, we believe the ORF 57 RNA binding determinant is contained within the amino-terminal portion (Goodwin et al., 1999), it cannot be excluded that ORF 57 binds directly to RNA via its zinc finger-like domain. (iv) These domains are required for specific protein–protein interactions with cellular proteins which are required for the multifunctional properties. For example, the EBV Mta protein interacts with exportin 1, which mediates the function and intracellular localization of the Mta protein (Boyle et al., 1999). Moreover, HSV IE63 has been shown to interact with the heterogeneous ribonucleoprotein K, a multifunctional protein with the ability to shuttle between the nucleus and cytoplasm, and casein kinase 2 (Wadd et al., 1999). At present, we are determining if ORF 57 interacts with any cellular proteins and it will be of interest to establish if the functional domains identified in this report are important for these interactions.

In summary, we demonstrate that the carboxy terminus is required for ORF 57 transactivation, repression and the intense spotting of the SC-35 spliceosome component. This region contains two highly conserved motifs amongst its homologues, a hydrophobic GLFF domain and a zinc finger-like motif. We further show that the hydrophobic domain is required for transactivation and is also involved in nuclear localization of the ORF 57 protein, whereas the zinc finger-like domain is required for transactivation, repression and the intense SC-35 nuclear spotting.

This work was supported in part from grants from the Medical Research Council, Candlelighters Trust, Wellcome Trust and Yorkshire Cancer Research. D.J.G. and A.W. are recipients of an MRC studentship and MRC fellowship, respectively. We thank Dr P. Robinson and Dr P. L. Coletta (Molecular Medicine Unit) for helpful discussions.

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Received 12 January 2000; Accepted 26 May 2000