Characterization of the herpesvirus saimiri ORF73 gene product

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The herpesvirus saimiri (HVS) gene product encoded by ORF73 shares a limited homology with the ORF73 encoded protein of Kaposi's sarcoma-associated herpesvirus (KSHV). It has recently been shown that the KSHV ORF73 protein is expressed during a latent infection and co-localizes with host cell chromosomes, suggesting that it plays a role in episomal maintenance by tethering viral genomes to host cell chromosomes. At present the role of the HVS ORF73 gene product is unknown. However, the expression of HVS ORF73 in a stably transduced human carcinoma cell line, where the HVS genome persists as a non-integrated circular episome, has recently been shown. In this report, the characterization of the HVS ORF73 protein and the mapping of its functional domains are described. The results suggest that the HVS ORF73 gene encodes a 64 kDa nuclear protein. Moreover, the amino terminus contains two functional nuclear localization signals, whereas the carboxy terminus is required for the distinctive speckled nuclear distribution pattern as observed with both the HVS and KSHV ORF73 proteins.

The herpesvirus saimiri (HVS) is the prototype gamma-2 herpesvirus. It shares significant homology with the human gammaherpesviruses, Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein–Barr virus, as well as murine gammaherpesvirus-68 (MHV-68) (Albrecht et al., 1992; Neipel et al., 1997; Russo et al., 1996; Virgin et al., 1997). HVS infects its natural host, the squirrel monkey, without causing any obvious symptoms of disease. However, HVS infection of other New World primates results in a range of lymphoproliferative diseases (Fleckenstein & Desrosiers, 1982). HVS is also capable of inducing the transformation of simian and human T lymphocytes to continuous growth in vitro (Biesinger et al., 1992).

There is considerable interest in the role of the ORF73 gene products in the life-cycle of gamma-2 herpesviruses. It has recently been shown that a cluster of genes, ORF71, ORF72 and ORF73, are expressed in the latent stage of a KSHV infection (Dittmer et al., 1998; Sarid et al., 1998; Talbot et al., 1999). In addition, when HVS DNA is stably maintained as a non-integrated circular latent episome in a human lung carcinoma cell line, Northern blot analysis has demonstrated the expression of ORF71 to ORF73. Moreover, these genes are transcribed from a polycistronic mRNA species produced from a common promoter upstream of ORF73 (Hall et al., 2000).

Previous analysis has shown that in both HVS and KSHV, ORF71 encodes an anti-apoptosis protein (v-FLIP) and that ORF72 encodes a v-cyclin D homologue (Chang et al., 1996; Jung et al., 1994; Thome et al., 1997). However, the function of the HVS ORF73 gene product is unknown. The KSHV ORF73 gene encodes the latency-associated nuclear antigen (LANA) (Kedes et al., 1997; Kellam et al., 1997; Rainbow et al., 1997). LANA is a large nuclear protein (222–234 kDa) that has three distinct domains: a proline-rich amino-terminal domain, a long acidic internal repeat domain containing a leucine zipper motif and a carboxy-terminal domain containing a putative nuclear localization signal (NLS) (Rainbow et al., 1997; Russo et al., 1996). It has recently been shown that KSHV ORF73 interacts with RING3, a homologue of the Drosophila female sterile homeotic (fsh) gene (Platt et al., 1999), and p53 (Friborg et al., 1999). Moreover, it has been shown that in cells harbouring KSHV episomes, LANA and KSHV DNA co-localize at discrete points in interphase nuclei and along mitotic chromosomes. This suggests that KSHV ORF73 is involved in episomal maintenance by tethering viral genomes to host cell chromosomes (Ballestas et al., 1999; Cotter & Robertson, 1999). In support of this hypothesis is the observation that KSHV ORF73 can bind the histone H1 protein in immunoprecipitation assays (Cotter & Robertson, 1999).

Although HVS ORF73 shares only very limited homology with KSHV ORF73, both proteins share the common feature of...
a large central acidic repeat domain. Moreover, it is interesting to note that the ORF73 gene is poorly conserved between other gammaherpesviruses. Equine herpesvirus-2, for example, lacks an ORF73 homologue (Telford et al., 1995), and the ORF73 homologues of bovine herpesvirus-4 and MHV-68 do not contain this internal acidic repeat region (Lomonte et al., 1995; Virgin et al., 1997). This limited similarity in primary structure prompted us to perform an initial characterization of the HVS ORF73 protein. In this report, we show that the HVS ORF73 gene encodes a 64 kDa protein. In addition, by using deletion analysis we demonstrate that the amino terminus of ORF73 contains two NLS domains, whereas the carboxy terminus is required for the distinctive nuclear speckling pattern observed with both the HVS and KSHV ORF73 proteins.

In order to determine the molecular mass and subcellular localization of the ORF73 protein, a polyclonal antiserum was raised against the full-length recombinant protein. The ORF73 gene was amplified by PCR (1 cycle of 5 min at 95 °C; 30 cycles of 30 s at 95 °C, 30 s at 60 °C, 2 min at 72 °C; 1 cycle of 10 min at 72 °C) using the primer pair 5′ CGGGATCC-GTTGCAATTATAGATGGCACC 3′ and 5′ CGGGGAT-CCTATAGGCAGGCTTTTGCT 3′. These primers incorporated BamHI restriction sites to facilitate cloning into the bacterial expression vector pGEX-2T (Pharmacia), to produce pGEX73. The ORF73 gene was expressed as a glutathione S-transferase fusion protein in E. coli strain DH5α and purified from crude lysates by incubation with glutathione-sepharose 4B affinity beads as specified by the manufacturer. The purified recombinant protein was used to generate a polyclonal antibody in New Zealand White rabbits using standard protocols.

To ascertain the molecular mass of the ORF73 gene product, Western blot analysis of transiently transfected and infected cells was performed. Control untransfected owl monkey kidney (OMK) cells were compared to OMK cells transfected with 2 µg pcDNA73 (a eukaryotic expression vector expressing the full-length coding region of ORF73) and HVS-infected cells (strain A11; m.o.i. of 1). Cells were harvested after 24 h and protein extracts were separated on a 7.5% SDS–PAGE gel. After electrophoresis the proteins were transferred to nitrocellulose membranes by electroblotting. The membranes were then blocked by preincubation in 2% (w/v) non-fat milk powder for 2 h at 37 °C. Membranes were incubated with a 1:400 dilution of ORF73 polyclonal antibody, washed and then incubated with a 1:2000 dilution of anti-rabbit immunoglobulin conjugated with horseradish peroxidase. After washing, the nitrocellulose membranes were developed using ECL (Pierce) according to the manufacturer’s directions. Western blot analysis demonstrated that ORF73 encodes a 64 kDa protein (Fig. 1a).

To determine the subcellular localization of ORF73, indirect immunofluorescence analysis was performed. Control untransfected OMK cells were compared to OMK cells transfected with 2 µg pcDNA73 and HVS-infected cells (strain A11; m.o.i. of 1). After 24 h, cells were fixed with 4% formaldehyde, permeabilized in 0.5% Triton X-100 for 10 min and blocked by preincubation with 1% (w/v) non-fat milk powder for 1 h at 37 °C. A 1:250 dilution of ORF73 polyclonal antibody was layered over the cells and incubated for 1 h at 37 °C. FITC-conjugated anti-rabbit immunoglobulin (Dako) at a 1:500 dilution was added for 1 h at 37 °C. After each incubation step, cells were washed thoroughly with PBS. The immunofluorescence slides were examined using a Zeiss Axiovert 135TV inverted microscope with a Neofluar 40 × oil immersion lens. This revealed a distinct nuclear speckling pattern in ORF73-transfected and HVS-infected cells (Fig. 1b). This staining resembles that of KSHV ORF73 in the nuclei of KSHV-infected cells. However, this staining pattern has not been observed in the absence of the KSHV genome.

To characterize the individual functional domains within ORF73, a deletion series of the ORF73 coding region was produced by a PCR-based method, deleting the amino terminus, the central acidic domain or large portions of the carboxy terminus (Fig. 2a). Each deletion construct was ampliﬁed by PCR using the primer sets shown in Fig. 2(b).

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**Fig. 1.** The HVS ORF73 encodes a 64 kDa nuclear protein. (a) OMK cells: untransfected control (lane 1); transfected with pcDNA73 (lane 2); and HVS-infected (strain A11; m.o.i. of 1) (lane 3). After 24 h, cells were harvested and protein extracts were separated by 7.5% SDS–PAGE, then transferred to a nitrocellulose membrane. The membrane was incubated with ORF73 polyclonal antiserum, washed and then incubated with anti-rabbit immunoglobulin conjugated with horseradish peroxidase. After five washes the nitrocellulose membrane was developed using ECL. (b) OMK cells: untransfected control (panel i); transfected with pcDNA73 (panel ii); and HVS-infected (strain A11; m.o.i. of 1) (panel iii). Cells were incubated with ORF73 polyclonal antiserum and then stained with FITC-conjugated anti-rabbit immunoglobulin.
Terminal oligonucleotides incorporated BamHI and XhoI restriction sites, whereas the internal oligonucleotides incorporated EcoRI restriction sites for the convenient cloning of the PCR products. The resulting ORF73 deletion fragments were inserted into the eukaryotic expression vector pcDNA73 (Invitrogen) to yield p73Δ1 to p73Δ6 respectively. These constructs contained a carboxy-terminal green fluorescent protein (GFP) fusion tag, which allowed direct visualization of the ORF73 deletion proteins using fluorescence microscopy. As a control, the complete coding region of ORF73 was excised from pcDNA73 and cloned into pcDNA73 to produce p73GFP. The integrity of all constructs was confirmed by DNA sequencing.

To determine the subcellular localization of each ORF73 deletion mutant, transient transfections were performed and the resulting fluorescence pattern was subsequently evaluated. OMK cells were transfected with 2 µg p73Δ1 to p73Δ6, respectively and incubated for 24 h. Cells were then fixed and examined using an Zeiss Axiovert 135TV inverted microscope with a Neofluar 40 × oil immersion lens (Fig. 2c). pcDNA73GFP was used as a control and displayed, as expected, a fluorescent signal throughout the cell nucleus and cytoplasm.

In contrast, transfection with p73GFP resulted in a distinct nuclear speckling pattern resembling the pattern previously observed with HVS-infected and ORF73-transfected cells using ORF73 polyclonal antiserum. Furthermore, deletion of the amino terminus in p73Δ1 abolished this nuclear speckling pattern and all fluorescence was restricted to the cytoplasm. This result indicates that the amino terminus contains a potential NLS. This conclusion was further supported by the result obtained with p73Δ5, which only contains the amino terminus of ORF73. Here, fluorescence was only observed in the nucleus. Moreover, this amino-terminal fusion resulted in a strong fluorescent signal in nuclear compartments that resembled nucleoli. The p73Δ2 and p73Δ3 plasmids, in which larger portions of the carboxy terminus were removed, produced a similar general nuclear fluorescence, but without the distinctive nuclear speckling pattern. Furthermore, deletion
of the entire carboxy terminus resulted in a strong fluorescent signal in the nucleoli, as observed with p73Δ5. Overall, this suggests that the carboxy terminus may be involved in a protein–protein interaction(s), which leads to the distinctive nuclear speckling. Also supporting this conclusion was the result obtained with p73Δ6, containing only the carboxy terminus, which was itself sufficient to produce the nuclear speckled pattern. The nuclear localization of this construct suggests that an additional NLS is present in the carboxy terminus. Analysis using p73Δ4, in which the acidic central domain was removed, resulted in a nuclear speckling pattern reminiscent of the wild-type protein. At present we are therefore unable to draw firm conclusions regarding the role of the acidic central domain.

To further elucidate the NLS contained within the amino terminus, three further deletion mutants were produced. Analysis of the amino-terminal sequence identified two putative lysine/arginine-rich domains, which may each function as an NLS (Fig. 3). To determine whether these regions are involved in nuclear localization, mutant proteins containing deletions in each, or both, putative NLS domains were constructed by the PCR-based method, as previously described (Fig. 3a). Each deletion construct was amplified by PCR using the primer sets shown in Fig. 3(b). The terminal oligonucleotides incorporated BamHI and XhoI restriction sites, whereas the internal oligonucleotides incorporated EcoRI restriction sites, for convenient cloning of the PCR products. The resulting ORF73 NLS deletion fragments were again inserted into pcDNAGFP to yield p73NLSΔ1, p73NLSΔ2 and p73NLSΔ1 + 2, respectively. The integrity of these constructs was confirmed by DNA sequencing.

To determine the subcellular localization of each NLS mutant, transient transfections were performed and the fluorescence pattern was evaluated. Both p73NLSΔ1 and p73NLSΔ2 constructs resulted in the distinct nuclear speckling pattern, with the absence of any cytoplasmic staining, similar to that observed with wild-type protein (Fig. 3c). However, when both NLS domains were specifically deleted in p73NLSΔ1 + 2, all fluorescence was restricted to the cytoplasm. This indicates that both putative NLS domains contained within the amino terminus are able to independently direct the ORF73 protein to the nucleus.

In this report we have demonstrated that the HVS ORF73 encodes a 64 kDa nuclear protein that generates a speckled nuclear staining pattern in immunofluorescence studies, similar to that observed for the KSHV ORF73 protein. In contrast to the predicted molecular mass of 46-6 kDa from DNA sequencing (Albrecht et al., 1992), ORF73 has an apparent molecular mass of 64 kDa when analysed by Western blot analysis. This is presumably due to the highly negatively charged set of amino acids contained within the internal repeat. Our initial characterization of the ORF73 domains has identified two functional NLS domains, which could function independently. They are located in the amino terminus and are composed of an abundance of basic lysine/arginine residues. This may be in contrast to the KSHV ORF73 where a putative NLS is located in the carboxy terminus (Russo et al., 1996). However, when the carboxy terminus of HVS ORF73 alone...
was fused to GFP, this construct resulted in a nuclear speckling pattern. This suggests that an additional NLS is present in the carboxy terminus resembling the carboxy terminus of KSHV ORF73. Analysis of the HVS ORF73 sequence highlights a putative lysine-rich NLS at aa 381–398. However, this putative NLS is only functional when both the amino terminus and the acidic domains are removed.

When the carboxy terminus of HVS ORF73 is deleted, the protein is still able to localize to the nucleus but without the nuclear speckling pattern. It is interesting to note that this speckling pattern is observed in transfected cells alone and does not require the presence of the HVS genome. From these observations we infer that the carboxy terminus of HVS ORF73 may be involved in an interaction with a host cell nuclear protein that leads to this distinctive staining pattern. Although the HVS and KSHV ORF73 genes share very limited similarity, there is a higher degree of similarity in their carboxy termini. Recent work has identified a number of cellular proteins that interact with the carboxy terminus of KSHV ORF73. Platt et al. (1999) demonstrated an interaction with RING3, a member of the Drosophila fsh family of proteins. In addition, KSHV ORF73 interacts with p53, repressing its transcriptional activity, and thus contributing to virus persistence and oncogenesis (Friborg et al., 1999). It will be of interest to determine if the HVS ORF73 protein also interacts with these cellular proteins.

In summary, HVS ORF73 encodes a 64 kDa nuclear protein. The amino terminus contains two functional NLS domains, whereas the carboxy terminus is required for the distinctive nuclear speckling pattern observed with the HVS and KSHV ORF73 proteins.

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