

# The Herpesvirus Saimiri ORF50 Gene, Encoding a Transcriptional Activator Homologous to the Epstein-Barr Virus R Protein, Is Transcribed from Two Distinct Promoters of Different Temporal Phases

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**The mRNA species encoding the herpesvirus saimiri (HVS) homolog of the Epstein-Barr virus R transcriptional activator (termed ORF50) have been identified and used to determine transcriptional start sites within the gene. The first transcript is spliced and starts from a promoter within ORF49 containing a single intron; the second is produced from a promoter within the second exon and is in the same reading frame. The spliced transcript is detected at early times during productive virus replication in OMK cells, whereas the nonspliced transcript is detected later. The spliced transcript is fivefold-more potent in activating the delayed-early ORF6 promoter; the function of the nonspliced transcript is unclear. Thus, the role of this protein in activating herpesvirus saimiri from the latent state may differ significantly from that of the Epstein-Barr virus R protein.**

Herpesvirus saimiri (HVS) establishes asymptomatic infections involving a subset of T lymphocytes in its natural host, the squirrel monkey (*Saimiri sciureus*), but causes fatal T-cell lymphomas and lymphoproliferative diseases in other species of New World primates (6). HVS has been classified as a gamma-2 herpesvirus and shares significant homology with Epstein-Barr virus (EBV) (1, 2, 9, 10, 16, 24). The genomes of HVS and EBV are generally colinear, in that homologous genes are found in approximately equivalent locations and in the same relative orientations in the two viruses (22). As in many other herpesviruses, gene expression during lytic infection occurs in three main temporal phases: immediate-early (IE), delayed-early (DE), and late (17). The major IE transcript in HVS is encoded by the *HindIII*-G IE gene (ORF14) (2, 25–27). Analysis of the sequence shows that it does not exhibit homology with any EBV-encoded proteins (25), but it does contain local homology with a putative superantigen (29); however, its function remains to be established. The 52-kDa product of the HVS IE gene (ORF57) is homologous to the EBV transactivator encoded by BMLF1 (also known as Mta, M-IE, or IE-2) and to the 27-kDa or IE 63-kDa protein encoded by UL54 of herpes simplex virus 1 and RF4 of varicella-zoster virus (4, 14, 21, 22, 24).

The *EcoRI*-D or HVS R protein transactivator (ORF50) is homologous to the BRLF1 gene product (also known as R or Rta) (23). BRLF1 is translated from a bicistronic mRNA which also encodes another IE gene product, BZLF1 (12, 20). These two EBV gene products function in activating expression from a number of viral (3, 5, 8, 13, 15, 18, 28) and perhaps cellular (7) promoters, thereby initiating the lytic cascade. However, HVS R RNA is expressed as an early transcript in HVS-infected cells (23, 25). The HVS R gene is proposed to be spliced with more than one exon, although the initiation codon has not been mapped. Despite the likelihood of a spliced transcript, there is evidence that a DNA sequence within the

identified exon is able to produce a functionally active protein capable of activating a DE ORF6 gene promoter, a component of the major DNA binding protein (23), which suggests that there may be more than one transcript produced. In this report we characterize the intron-exon structure of ORF50 and demonstrate that two transcripts are produced from two separate promoters which are active at different stages of the virus replication cycle.

In order to identify the transcriptional start site of ORF50, the 5'-end cDNA was analysed by 5' rapid amplification of cDNA ends (5' RACE). Total RNA was isolated from OMK cells infected with HVS (strain 11) at 5 PFU/cell at 8 h postinfection (hpi). First-strand cDNA was reverse transcribed with Superscript II reverse transcriptase (Life Technologies) and an ORF50 gene-specific antisense primer 5'-GGA ATA CAT ATA GTA GGA. The first-strand cDNA was treated with terminal deoxynucleotidyl transferase (Boehringer Mannheim) in the presence of dATP, and second-strand synthesis was completed with an oligo(dT) primer. PCR amplification was performed with a nested 3'-gene-specific primer, 5'-CCA GAT TCA TTA AGT GCT TGA, and the 5' oligo(dT) primer. The reaction (30 cycles: 1 min at 92°C, 1 min at 60°C, 1 min at 72°C) was performed with 4 U of *Taq* polymerase (Promega). The amplified 5' cDNA was cycle sequenced with the *fmol* DNA sequencing system (Promega). Analysis of the sequence illustrates that ORF50 is a spliced gene, with its putative initiation codon at nucleotide 69878 (of the published sequence), demonstrating that exon 1 of ORF50 lies within ORF49. The gene contains an intron of 960 bp with splice donor and acceptor sites at nucleotide 69898 and 70858, respectively (Fig. 1).

Analysis of the upstream region of the initiation codon reveals classic eukaryotic promoter motifs, putative TATA and CCAAT consensus sequences at –99 and –183 bp, respectively (relative to the translation start site). In order to demonstrate that this is a functional promoter, a fragment containing the sequence from –417 to –3 bp (relative to the putative initiation codon) was generated by PCR with primers 5'-GCG CTG CAG AAG TCC TTG TGT GCA TTC TAT and 5'-CGG GTC GAC TGT TGT TGC TTA AGA GCT CAG CTG. These oligonucleotides incorporated *Pst*I and *Sal*I re-

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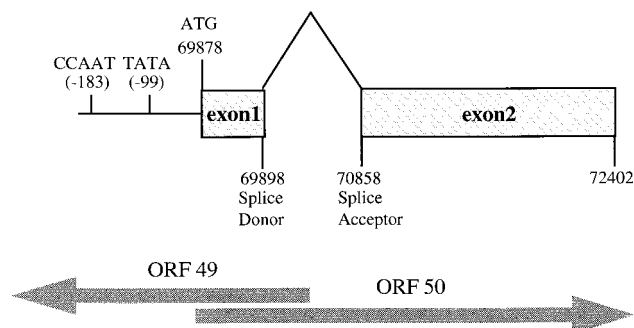


FIG. 1. Diagrammatic representation of the intron-exon structure of ORF50. 5' RACE identified ORF50 as a spliced gene, with its putative initiation codon at nucleotide 69878 in the virus DNA sequence on the antisense strand of ORF49. The gene contains an intron of 960 bp with splice donor and acceptor sites at nucleotide 69898 and 70858, respectively. The CCAAT and TATA box consensus sequences are indicated.

striction sites, for convenient cloning of the PCR product. This fragment was inserted upstream of the 5' chloramphenicol acetyltransferase (CAT) coding region in pCATBasic (Promega) to derive the reporter gene construct, pAWCAT1. The sequence was confirmed by DNA sequencing (data not shown). OMK cell monolayers were transfected with 2  $\mu$ g of pAWCAT1 by using DOTAP liposomal transfection reagent (Boehringer Mannheim), as directed by the manufacturer; cells subsequently remained uninfected or were superinfected with 50 PFU of HVS (strain 11) per cell at 24 h posttransfection. Cells were harvested at 48 h posttransfection, extracts were assayed for CAT activity by standard methods (11), and percentages of acetylation were calculated by scintillation counting of appropriate regions of the chromatography plate (Fig. 2). The sequences contained in pAWCAT1 were sufficient to express the heterologous reporter gene, and no additional expression was achieved in the presence of the superinfection.

Previously published transient-transfection experiments have demonstrated that a *HincII* fragment within the second exon of ORF50 is sufficient to transactivate the DE ORF6 promoter (23). We considered the possibility that, in addition to the promoter identified by 5' RACE, ORF50 contains a second transcriptional start site and promoter within the *HincII* fragment. Analysis of the 5' region of the *HincII* fragment identified an in-frame initiation codon at 71,188 bp and putative TATA and AP1-binding consensus sequences at -78 and -118 bp, respectively (upstream of this ATG). To determine if a second promoter functioned within exon 2, a deletion series of the 5' region of the *HincII* fragment was constructed and assayed for transactivating capacity of ORF6. The deletion series was constructed with the forward primers 5'-GCG CTG CAG CTG GTA CGG TCT ACT CAT, 5'-GCG CTG CAG GGA TGA TGT CCA AGT CAG, 5'-GCG CTG CAG TAA CCA GTC ACT AGT CAC GCC, 5'-GCG CTG CAG AGG GCG TGT CTA CAG ATT G, 5'-GCG CTG CAG TTG TAC ACT TTG TGG ACT, and 5'-GCG CTG CAG ACA AAA CAG CGA TCG CAG and the reverse primer 5'-GCG CTG CAG CCT TCA TCA TCT ACA TCA GTG; these oligonucleotides incorporated *PstI* restriction sites, for convenient cloning of the PCR products. Each fragment was inserted into the transfer vector pUC18, to derive pAWDel.1 through pAWDel.6 (Fig. 3). The DNA sequence containing the ORF6 promoter region was generated by PCR from viral DNA by using the primers 5'-GCG CTG CAG TAC CTC AAG TTG CGA ATG CGT ACG and 5'-CCG CTG CAG TGT TAG ATG TGG ATG CTT TGA GAG. These oligonucleotides

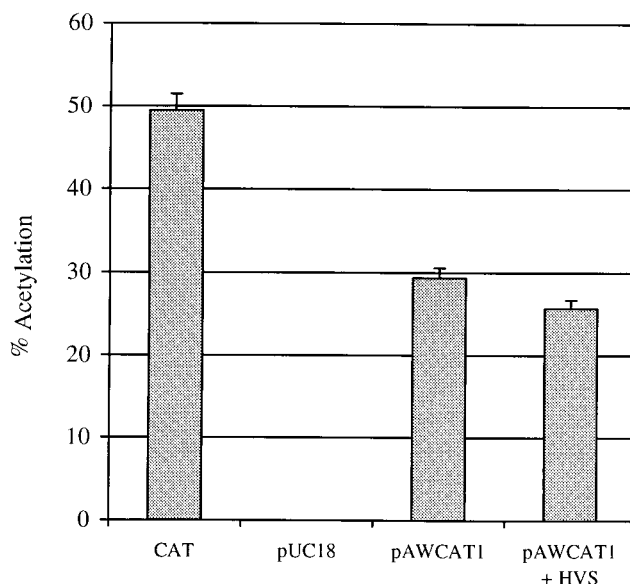


FIG. 2. Analysis of the ORF50 promoter. OMK cell monolayers were transfected with 2  $\mu$ g of pUC18 or pAWCAT1 by using DOTAP (Boehringer Mannheim), as directed by the manufacturer; cells containing pAWCAT1 subsequently either remained uninfected or were superinfected with 50 PFU of HVS (strain 11) per cell at 24 h posttransfection. Cells were harvested at 48 h posttransfection and extracts were assayed for CAT activity. Products from these assays were separated by thin-layer chromatography and detected by autoradiography. Percent acetylation was calculated by scintillation counting of the appropriate regions of the chromatography plate; error bars indicate variations between three replicate assays.

incorporated *PstI* restriction sites, for convenient cloning of the PCR product. To assay the transactivation of ORF6 by the deletion series, the promoter fragment was inserted 5' of the CAT coding region in pCATBasic to derive the reporter gene construct pAWCAT2. All constructs were confirmed by DNA sequencing. Each deletion plasmid was cotransfected with pAWCAT2, harvested after 48 h, and assayed for CAT activity (Fig. 4). Reduced CAT expression was observed when pAWDel.5 was used to transactivate pAWCAT2, and activation was completely lost when pAWDel.6 was used in the assay. pAWDel.5 corresponds to a deletion in the AP1-binding consensus sequence, and pAWDel.6 corresponds to removal of both the AP1-binding and TATA consensus sequences, thus indicating the presence of a second internal promoter. We therefore assumed that two transcripts of ORF50 are expressed individually from the promoter 5' to exon 1 and an internal promoter within exon 2 and propose that these be termed ORF50a and ORF50b, respectively.

To compare the transactivating capacity of ORF50a and ORF50b transcripts on the DE ORF6 promoter, the coding region of each transcript was cloned into a transfer vector. Both transcripts are contained in the *EcoRI*-D fragment; p*EcoRI*-D contains the *EcoRI*-D fragment of HVS L DNA cloned into the *EcoRI* site of pACYC184 (19). ORF50a and -b coding regions are contained in a *PstI* subfragment of *EcoRI*-D; however, only the coding region of ORF50b is contained in a *HincII* subfragment. These subfragments of *EcoRI*-D were generated by *HincII* and *PstI* restriction endonuclease digestion and cloned individually into pUC18, to derive pAW*HincII* and pAW*PstI*. However, pAW*PstI* contains both ORF50a and -b coding regions; therefore, to compare transactivation by the two transcripts, a cDNA construct of ORF50a was generated. Synthetic oligonucleotides encoding

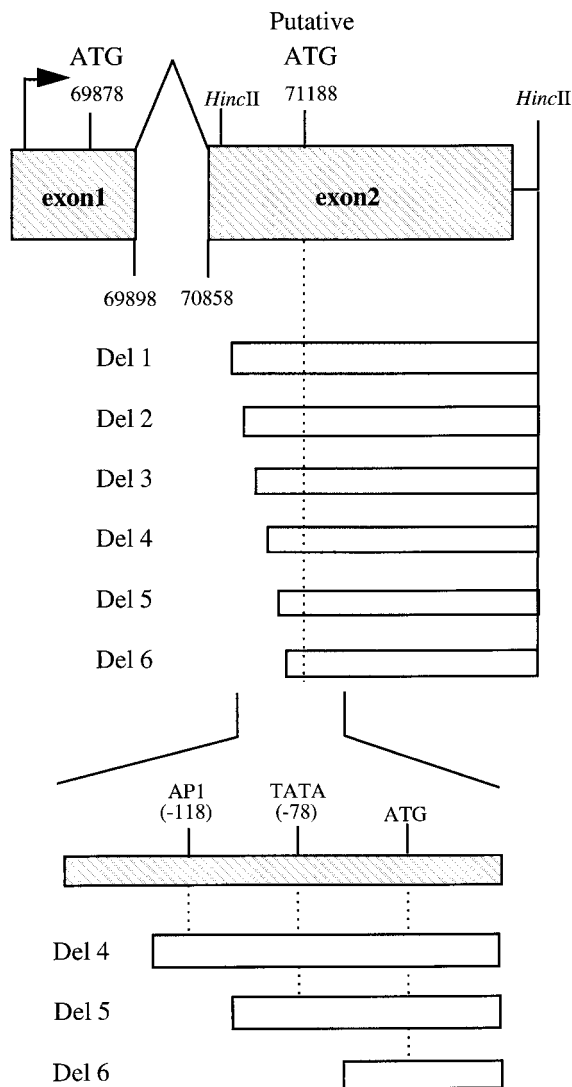


FIG. 3. Diagrammatic representation of the deletion series of the 5' region of the *HincII* fragment. Each deletion was PCR amplified and inserted into the transfer vector pUC18, to derive pAWDel.1 through pAWDel.6. An expanded view of the region of the *HincII* fragment containing the putative promoter and initiation codon is presented.

exon 1 and 78 bp of the 5' region of exon 2 up to the *PvuII* restriction site (nucleotide 70936 of the published sequence) were synthesized and purified by fast-performance liquid chromatography. These oligonucleotides incorporated *SalI* and *PvuII* restriction sites, for convenient cloning. These were annealed and ligated with a previously modified pUC18 transfer vector, which incorporated a *PstI-SalI-PvuII-HincII* polylinker. The remaining coding region of exon 2 was excised from p*EcoRI*-D as a *PvuII-HincII* fragment and cloned downstream of the synthetic oligonucleotide. The *PstI-SalI* fragment containing the ORF50 promoter identified by 5' RACE was cloned upstream of exon 1, to derive pORF50a. Each plasmid was cotransfected with pAWCAT2, harvested after 48 h, and assayed for CAT activity (Fig. 5). Each plasmid was also cotransfected with a plasmid expressing  $\beta$ -galactosidase under the control of the IE cytomegalovirus promoter, and no difference in expression of the reporter gene was observed (data not shown), demonstrating that the difference in CAT expres-

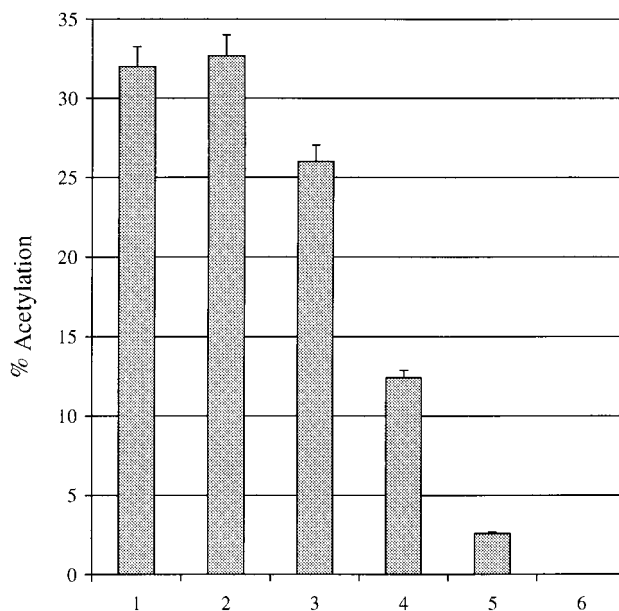


FIG. 4. Analysis of the 5' region of the *HincII* fragment of ORF50. OMK cells were transfected with 2  $\mu$ g of pUC18 or pAWCAT2 or cotransfected with pAWCAT2 plus each plasmid in the deletion series (pAWDel.1 through pAWDel.6 [bars 1 through 6]). Cells were harvested at 48 h posttransfection, and cell extracts were assayed for CAT activity as previously described. Percent acetylation was calculated by scintillation counting of the appropriate regions of the chromatography plate; error bars indicate the variations between three replicate assays.

sion is due to the difference in transactivating capabilities of the two transcripts rather than the presence of an intron in pAW*PstI*. It can be determined from these results that ORF50a is more efficient at activating the DE promoter, and it is presumed that this is likely to be the major early transcript.

In order to identify and determine the time of expression of the transcripts from both promoters, total RNA was isolated from OMK cells infected with HVS in the presence or absence of cycloheximide and harvested at 8, 24, and 48 hpi. cDNA was amplified with the ORF50 gene-specific oligonucleotide primer previously used in the 5' RACE and treated with terminal deoxynucleotidyl transferase in the presence of dATP. PCR amplification was performed with a nested 3'-gene-specific primer, 5'-CCA GAT TCA TTA AGT GCT TGA, and the 5' oligo(dT) primer and examined by gel electrophoresis. Two RNA species were identified by reverse transcription (RT)-PCR (Fig. 6). ORF50a transcripts were readily detectable with RNA harvested from cells at 8, 24, and 48 hpi in the absence of cycloheximide; however, no transcripts were detectable in the presence of cycloheximide, establishing that ORF50a is an early transcript. Similarly, no ORF50b transcripts were detected with RNA harvested from cells in the presence of cycloheximide; however, transcripts were identified at 24 and 48 hpi in the absence of cycloheximide, suggesting that ORF50b is a DE transcript possibly requiring transactivation from an IE gene product.

To quantify the transcripts produced by ORF50, Northern blot analysis was performed. RNA was isolated from OMK cells infected with HVS, harvested at 8, 24, and 48 hpi, separated by electrophoresis on 1% denaturing formaldehyde agarose gel, blotted onto a nylon membrane, and hybridized with radiolabelled antisense probes 5'-CTT AAC AAC CTT GTG TGT and 5'-CAA GGG AAG TGA GTA GGT, specific for exons 1 and 2, respectively (Fig. 7). Northern blot analysis

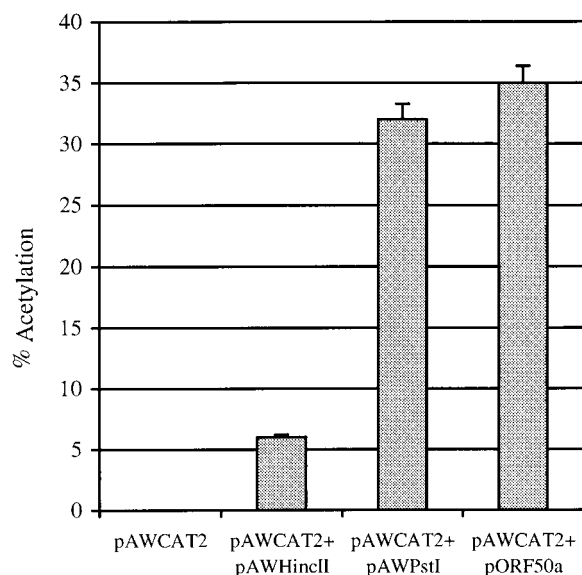


FIG. 5. Response of the DE ORF6 promoter to ORF50a and ORF50b transcripts. ORF50a and -b coding regions are contained in a *Pst*I subfragment of *Eco*RI-D; only the coding region of ORF50b is contained in a *Hinc*II subfragment. The coding region of each transcript was cloned into a transfer vector, pUC18, to derive pAWHincII and pAWPstI. A cDNA construct of ORF50a was generated by cloning the ORF50a promoter, exon 1, and exon 2 into a transfer vector to derive pORF50a. OMK cells were transfected with 2  $\mu$ g of pAWCAT2 or cotransfected with pAWCAT2 plus pAWHincII, pAWPstI, or pORF50a. Cells were harvested at 48 h posttransfection, and cell extracts were assayed for CAT activity as previously described. Percent acetylation was calculated by scintillation counting of the appropriate regions of the chromatography plate; error bars indicate the variations between three replicate assays.

confirmed the RT-PCR results, verifying that ORF50 produced two transcripts expressed at different times within the virus replication cycle. Comparison of the levels of the two transcripts shows that ORF50a was expressed at higher levels earlier, whereas ORF50b was expressed later, in relatively the same abundance as the earlier transcript; this suggests that ORF50b is not an artifact but has an as-yet-unknown function.

We have demonstrated that two distinct transcripts are produced from ORF50, the major transcriptional activating gene of HVS. These transcripts, termed ORF50a and ORF50b, are produced from different promoters. ORF50a is a spliced transcript with two exons, the first contained in the noncoding strand of ORF49. The transcripts are expressed at different temporal phases within the lytic cycle, ORF50a as an early

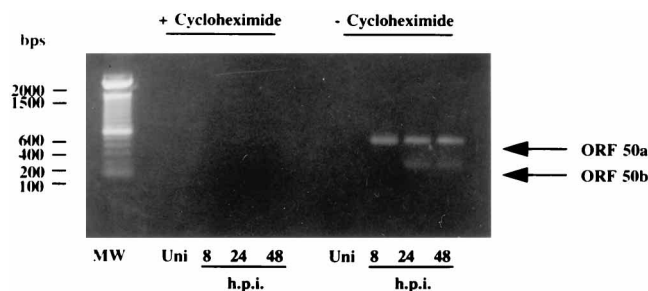


FIG. 6. Analysis of expression of the transcripts from both promoters. OMK cells were infected with HVS in the presence or absence of cycloheximide. Total RNA was harvested at 8, 24, and 48 hpi. By RT-PCR, cDNA was amplified with oligonucleotide primers previously used in the 5' RACE experiment and examined by gel electrophoresis.

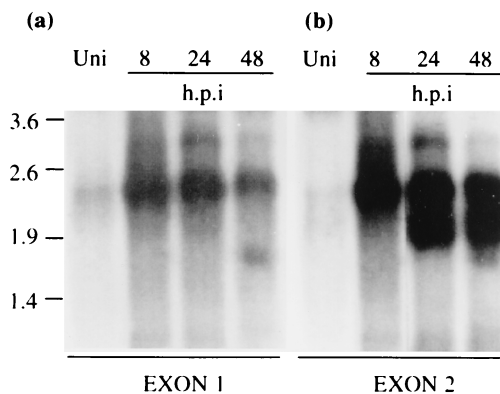


FIG. 7. Northern blot analysis of ORF50 transcripts. Total RNA was isolated from OMK cells infected with HVS, harvested at 8, 24, and 48 hpi, separated by electrophoresis on 1% denaturing formaldehyde agarose gels, blotted onto a nylon membrane, and hybridized with radiolabelled antisense primers specific for exon 1 (a) and exon 2 (b).

gene and ORF50b as a DE gene. We presume that ORF50a is the important transcript for transactivating the DE ORF6 promoter. The role, if any, of ORF50b has yet to be determined. It may be a consequence of the failure of RNA splicing during later stages of virus replication. However, as ORF50b has been shown to be expressed later in the replication cycle, it may have biological significance. ORF50b transcript has been shown to be expressed at relatively high levels, and it could be postulated that this transcript has a role separate from that of ORF50a, later in the infection. One possible model would be that ORF50 regulates itself. ORF50a is transcribed first, and the gene product transactivates DE gene promoters (such as ORF6); it may also activate its own internal promoter, enabling the later transcript to transactivate a range of late promoters. Alternatively, the ORF50b gene product may compete for binding to regulatory elements with the ORF50a product, thus acting as a negative regulator.

The levels of protein expression from the two transcripts have yet to be determined, and the production of antibodies to each protein is in progress. As this protein has only limited homology with better-characterized transcriptional activators, functional domains of the protein still need to be identified.

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