The Herpesvirus Saimiri Open Reading Frame (ORF) 50 (Rta) Protein Contains an AT Hook Required for Binding to the ORF 50 Response Element in Delayed-Early Promoters

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The herpesvirus saimiri open reading frame (ORF) 50 encodes two proteins, which activate transcription directly, following interactions with delayed-early (DE) promoters containing a specific motif. In this report, we demonstrate that ORF 50 contains a DNA binding domain that has homology to an AT hook DNA binding motif. Deletion analysis of this domain reduces ORF 50-mediated transactivation of the DE ORF 6 and ORF 57 promoters by 100 and 90%, respectively. Furthermore, gel retardation experiments demonstrated that the AT hook motif is required for binding the ORF 50 response element in the promoters of DE genes. Single site-directed mutagenesis of the AT hook revealed that mutation of the glycine residue at position 408 to an alanine reduces ORF 50 transactivation of the ORF 57 promoter by 40%. Moreover, the mutation of multiple basic residues in conjunction with the glycine residue within the core element of the AT hook abolishes ORF 50-mediated transactivation. In addition, p50GFPAT-hook is capable of functioning as a trans-dominant mutant, leading to a reduction in virus production of approximately 50% compared to that for wild-type ORF 50.

Herpesvirus saimiri (HVS) is the prototype gammaherpesvirus of the Rhadinovirus genus that persistently infects squirrel monkeys (Saimiri sciureus) without causing an overt manifestation of disease. However, experimental HVS infection of New World primate species can result in fulminant polyclonal T-cell lymphomas and lymphoproliferative diseases (7). In addition, HVS is capable of transforming simian and human lymphocytes to continuous growth in vitro, where they harbor the virus genome as a high-copy-number, nonintegrated episome without production of virus particles (5). The genome of HVS (strain A11) consists of a unique, internal, low-G+C DNA segment (L-DNA) of approximately 110 kbp flanked by tandem repetitions of 1,444-bp, high-G+C tandem repetitions (H-DNA) (2). Sequence analysis indicates that HVS shares significant homology with other herpesviruses of oncogenic potential, including Epstein-Barr virus (EBV), Kaposi’s sarcoma associated herpesvirus (or human herpesvirus 8) (KSHV), and murine gammaherpesvirus 68 (MHV-68) (1, 2, 18, 19, 25, 26). The genomes of these viruses are generally colinear, with large blocks of conserved genes interspersed by relatively small regions of sequences unique to each virus (2, 6, 25, 26). Like all Rhadinoviruses, the HVS genome encodes a number of cellular homologues whose products may have a role in transformation, immune evasion, and long-term persistence of the viral episome (2).

Gene expression in HVS is modulated by the two major transcription-regulating genes carried on open reading frame (ORF) 50 and ORF 57 (20, 21, 27–29). The ORF 57 gene encodes a multifunctional protein capable of both transactivation and repression of viral gene expression at a posttranscriptional level (28, 29). Recent analysis has demonstrated that ORF 57 encodes a nucleocytoplasmic shuttle protein which mediates the nuclear export of late virus transcripts (8), whereas repression of gene expression is due to the presence of introns in target genes and may correlate with the ability of ORF 57 to redistribute splicing factors into distinct aggregations (28, 29).

The ORF 50 gene produces two transcripts, termed ORF 50a and b. The first is spliced, contains a single intron, and is detected at early times during the productive cycle, whereas the second is expressed later and is produced from a promoter within the second exon. The spliced transcript is fivefold more potent in activating the delayed-early (DE) ORF 6 and ORF 57 promoters. However, the function of the spliced transcript is unclear (20, 27, 28). Further analysis of the ORF 50 gene products has demonstrated that they activate transcription directly, following interactions with promoters containing a specific sequence motif. Deletion and gel retardation analyses have identified a consensus ORF 50 recognition sequence required for ORF 50 binding, CCN9GG, termed the ORF 50 response element (RE) (30). This RE has significant homology to the EBV Rta RE consensus sequence, GNCCN9GGNG. Guanine methylation studies have shown that the CCN9GG motif is essential for EBV Rta binding and suggest that Rta binds to adjacent major grooves of the DNA (11–13). Once bound to the recognition sequence, HVS ORF 50 recruits and interacts with the TATA binding protein (14) via a carboxy-terminal transactivation domain, which suggests that HVS ORF 50 recruits components of the TFIID complex, allowing the initiation of transcription by RNA polymerase II.

Furthermore, studies utilizing a human lung carcinoma cell line latently infected with HVS demonstrated that overexpres-

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sion of the ORF 50a gene, under the control of the constitutively active cytomegalovirus immediate-early promoter, results in the production of infectious virions via the complete lytic cascade. This finding has implicated the ORF 50a gene product as the latent-lytic switch protein (9).

**HVS ORF 50 encodes an AT hook required for DE promoter transactivation.** At present it is unknown which domains within ORF 50 are responsible for the recognition and binding of ORF 50 REs in the promoters of DE genes. To identify potential DNA binding domains within ORF 50, the ORF 50 sequence was analyzed by using PROSITE (www.expasy.ch/prosite/). The results identified a motif within ORF 50 which contained a high degree of homology to the DNA binding domain carried by mammalian high mobility group A (HMG A) chromosomal proteins. This motif, known as an AT hook, has a consensus sequence, T-P-K-R-R-P-R-G-R-P-K-K (24). Comparison of the HVS ORF 50 putative binding domain shows a consensus motif, T-P-R-P-R-G-R-P-K-G, between bp 72000 and 72030 of the published sequence (Fig. 1).

To elucidate the role of the putative ORF 50 AT hook on the transactivation capability of ORF 50, a deletion construct removing this domain was produced by a PCR-based method (Fig. 2a). The wild-type ORF 50 second exon was PCR amplified in two fragments, thereby removing the putative DNA binding domain by using the following sets of forward and reverse primers: primer A, 5'-CTG GAA TAG TCT CTA CAA CAT TAG CA-3'; primer B, 5'-CCG CTC GAG GTT TGG ATC TAT GTT GCG ACT CTG-3'; primer C, 5'-CCG CTC GAG CGC CCT ACA TGC TCT CCT G-3'; and primer D, 5'-AGT GTA TGG AAG CAA TTG CAC A-3'. Oligonucleotides B and C incorporated XhoI restriction sites, and D incorporated an MunI site. Oligonucleotide A contained no restriction site, as the Pmel site at bp 71470 of the published sequence was utilized for cloning purposes. The PCR (5 min at 95°C; 30 cycles of 1 min at 95°C, 1 min at 55°C, and 2 min at 68°C; and a final 10 min at 68°C) was performed using 1U of Pfx polymerase (Life Technologies). The PCR fragments were then cloned into the vector pUC50 (27), previously digested with EcoRI and ApaI, to derive the vectors p50GFP and p50GFP ΔAT-hook, respectively. Sequencing of these constructs confirmed the integrity of the PCR products (data not shown).

In order to confirm that p50GFP and p50GFP ΔAT-hook produce stable protein products, immunofluorescence and Western blot analysis were performed. Initially, 293T monolayers were transfected with 2 μg of pEGFP, p50GFp, or p50GFP ΔAT-hook, using Lipofectamine 2000 (Life Technologies), and the subcellular localization of GFP was observed by fluorescence microscopy. Both p50GFP and p50GFP ΔAT-hook were shown to produce a stable protein product that localized within the nucleus, with a similar nuclear, speckled pattern distinct from that of pEGFP, which is consistent with previous results (14) (Fig. 2b). In addition, transfected cellular extracts were resolved on a sodium dodecyl sulfate–10% polyacrylamide gel and soaked for 10 min in transfer buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol, 0.1% sodium dodecyl sulfate). The proteins were transferred to nitrocellulose membranes by electroblotting for 3 h at 250 mA. After being transferred, the membranes were soaked in phosphate-buffered saline (PBS) and blocked by preincubation with 2% [wt/vol] nonfat milk powder for 2 h at 37°C. The membranes were incubated for 2 h with a 1/1,000 dilution of the anti-GFP monoclonal antibody (Clontech), washed with PBS, and incubated for 1 h at 37°C with a 1/2,000 dilution of anti-mouse immunoglobulin conjugated with horseradish peroxidase (Dako) in blocking buffer. After five washes with PBS, the nitrocellulose membranes were developed by using enhanced chemiluminescence (Pierce) according to the manufacturer’s directions. The results shown in Fig. 2c demonstrate that both p50GFP and p50GFP ΔAT-hook produce protein products of approximately 80 kDa, encompassing ~50 kDa of the ORF 50 protein and ~28 kDa of the GFP tag.

To determine whether the putative ORF 50 AT hook is required for the DNA transactivation capacity of ORF 50, reporter gene-based transient transfection assays were performed. 293T cells were transfected with 1 μg of either pEGFP, p50GFP, or p50GFP ΔAT-hook in the presence of 1 μg of pAWCAT2 (30) or pORF57CAT1 (28), using Lipofectamine 2000. These reporter constructs contained the chloramphenicol acetyltransferase (CAT) coding region under the control of the ORF 50-responsive ORF 6 and ORF 57 promoters, respectively (28, 30). Cells were harvested after 36 h and assayed for CAT activity by standard methods (10) (Fig. 2d). p50GFP was shown to transactivate the ORF 6 and ORF 57 promoters to levels previously reported. All CAT assays throughout this report were controlled to be in the linear range of enzymatic activity (28, 30). However, dramatically reduced CAT activity was observed when p50GFP ΔAT-hook was assessed for transactivation capability of the ORF 6 or ORF 57 promoters. The results demonstrated that deletion of the ORF 50 AT hook reduced CAT activity by 100% for the ORF 6 promoter and approximately 90% for the ORF 57 promoter. To confirm that the transfection efficiency of these experiments was normalized, immunofluorescence and Western blot analysis were performed as mentioned previously to assess the expression of p50GFP and p50GFP ΔAT-hook (data not shown). The results

\[ \text{T-P-K-R-R-P-R-G-R-P-K} \]

**AT-hook consensus**

\[ ^{295}\text{DFNT-T-P-R-P-R-G-R-P-K-G}_{142} \]

**ORF 50**

FIG. 1. Amino acid sequence alignment of the consensus and ORF 50 AT hook motif.
demonstrated that both constructs expressed at similar levels, suggesting that the putative ORF 50 AT hook situated between bp 72000 and 72030 is required for ORF 50-dependent transactivation of the DE ORF 6 and ORF 57 promoters.

The ORF 50 AT hook is required for DNA binding to ORF 50 REs. We have previously shown that the ORF 50 proteins activate transcription directly following interactions with promoters containing a specific sequence motif, termed the ORF 50 RE (28, 30). To assess if the deleted AT hook is required for ORF 50 binding to the RE, gel retardation experiments were...
performed using a set of oligonucleotides which spanned the ORF 6 promoter RE, which have previously been shown to specifically bind both ORF 50a and b proteins (30). Two oligonucleotides encoding the ORF 50 REs, 5'-TTA AAA ATT TCC TGT CAA TGT GGT TTG CTT GG-3' and 5'-CCA AGC AAA CCA CAT TGA CAG GAA ATT TTT AA-3', were annealed and labeled by using T4 polynucleotide kinase in the presence of [γ-32P]dATP. The radiolabeled oligonucleotides were incubated for 20 min with nuclear extracts of untransfected 293T cells or 293T cells transfected with pEGFP, p50GFP, or p50GFPΔAT-hook by the method of Andrews and Faller (3). The binding reactions were performed in 20 μl of binding buffer (100 mM KCl, 20 mM HEPES [pH 7.3], 1% glycerol, 0.2 mM EDTA, 5 mM MgCl2, 4 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) with 1 μg of poly(dI-dC) as an unspecific competitor. The protein-nucleic acid complexes were separated on a 5% polyacrylamide gel, run in 1% Tris-borate-EDTA buffer, and detected by autoradiography (Fig. 3). The results show the formation of a retarded complex with the p50GFP-transfected cell extracts. No other complex was identified in the mock, pEGFP, or p50GFPΔAT-hook extracts, indicating that the ORF 50 AT hook, deleted in p50GFPΔAT-hook, is required for ORF 50 binding to the RE.

**Mutational analysis of the ORF 50 AT hook.** To assess the importance of the ORF 50 AT hook for ORF 50 transactivation, site-directed mutational analysis on a range of amino acid residues within the AT hook was undertaken by a PCR-based method. The following six residues, which lie within the putative DNA binding domain, were all individually mutated to alanine: T402, R404, P406, R407, G408, and R409 (Fig. 4a). Moreover, a seventh mutation, which alters the five residues between R404 and G408 to alanine, was produced. The ORF 50 mutations were created by utilizing a Bsu36I restriction site

| Mutant | Reverse Primer |
|--------|----------------|
| T402A | 5'-AGA GCC CTT AGG TCT GCC TCT TGG CCG CCT TGG TCG CCT TGG AGC-3' |
| R404A | 5'-AGA GCC CTT AGG TCT GCC TCT TGG CCG CCT TGG TCG CCT TGG AGC-3' |
| P406A | 5'-AGA GCC CTT AGG TCT GCC TCT TGG CCG CCT TGG TCG CCT TGG AGC-3' |
| R407A | 5'-AGA GCC CTT AGG TCT GCC TCT TGG CCG CCT TGG TCG CCT TGG AGC-3' |
| G408A | 5'-AGA GCC CTT AGG TCT GCC TCT TGG CCG CCT TGG TCG CCT TGG AGC-3' |
| R409A | 5'-AGA GCC CTT AGG TCT GCC TCT TGG CCG CCT TGG TCG CCT TGG AGC-3' |
| pR404-G408A | 5'-AGA GCC CTT AGG TCT GCC TCT TGG CCG CCT TGG TCG CCT TGG AGC-3' |

FIG. 3. Gel retardation analysis. The ORF 6 promoter oligonucleotides were incubated with cellular extracts prepared from untransfected 293T cells (I), pEGFP-transfected 293T cells (II), p50GFP-transfected 293T cells (III), or p50GFPΔAT-hook transfected cells (IV).

FIG. 4. (a) Amino acid sequence alignment of the single site-directed mutations generated for characterization of the ORF 50 AT hook. (b) Reverse PCR primer sequences used in the generation of the single site-directed mutations.
that lies downstream of the AT hook. PCR products were amplified, using the forward primer E and a set of reverse primers (Fig. 4b), which incorporated this Bsu36I restriction site and the desired mutation. The generated PCR products were then digested with EcoRI and Bsu36I and subsequently cloned into p50GFP, which had previously been digested with the respective enzymes, to generate GFP fusions of these site-directed AT hook mutations. Sequencing of these constructs confirmed the integrity of the PCR products (data not shown). In addition, to confirm that the mutated constructs produced stable protein products, immunofluorescence and Western blot analysis were performed. All the mutated constructs produced a stable protein product that localized within the nucleus and produced a protein of approximately 80 kDa on a Western blot (data not shown).

To determine the effect of these mutations on the transactivation capability of ORF 50, reporter gene-based transient transfection assays were performed. 293T cells were transfected with 1 µg of either pEGFP, p50GFP, or one of the site-directed mutated sequences in the presence of 1 µg of pORF57CAT1 (28), using Lipofectamine 2000, and CAT assays were performed as previously described. The results demonstrated that the majority of the site-directed mutations (T402, R404, P406, R407, and R409) had no effect on ORF 50 transactivation. However, the mutation of G408 to alanine reduces ORF 50 transactivation by approximately 50% compared to wild-type levels, suggesting that this residue is important for ORF 50 transactivation. Moreover, the mutation of all five residues between R404 and G408 within the AT hook abolished ORF 50-mediated transactivation (Fig. 5a). The results further demonstrate that the ORF 50 AT hook is required for ORF 50-dependent transactivation of DE promoters. To confirm that the transfection efficiency of these experiments was normalized, Western blot analysis was performed as mentioned previously to assess the expression of p50GFP and each site-directed mutation. The results demonstrated that all constructs were expressed at similar levels (Fig. 5b).

The ORF 50 AT hook mutation can function as a trans-dominant mutant to reduce virus replication. Infection of owl monkey kidney (OMK) cells with HVS leads to expression of viral lytic proteins and production of infectious viruses. Since the AT hook domain within ORF 50 is required for transactivation of the ORF 6 and ORF 57 DE promoters, we examined whether introduction of this mutated ORF 50 could affect lytic replication and therefore virus production. OMK cells were mock transfected or transfected with 1 µg of pEGFP, p50GFP, or p50GFPΔAT-hook, using an integrin-targeting peptide combined with lipofectin (Invitrogen), as described previously (15). This reagent was used to enhance transfection efficiency in OMK cells. Cells were exposed to lipid-peptide-DNA complexes for 4 to 6 h in serum-free Dulbecco modified Eagle medium. At 24 h posttransfection, a similar percentage of approximately 50 to 60% of cells were expressing both p50GFP and p50GFPΔAT-hook, indicating that there was no difference in transfection efficiency. The OMK cells were subsequently infected with HVS-GFP at a multiplicity of infection of 1 and incubated at 37°C for 5 days until destruction of the cell sheet had occurred. The supernatants were then harvested from each well, and the viral titers were measured by plaque assay (Fig. 6). The results demonstrated that the viral titer produced from untransfected cells and that from cells pretransfected with pEGFP were approximately the same. However, the virus titer from p50GFP pretransfected cells increased by approximately 30%. The virus titer from p50GFPΔAT-hook pretransfected cells was reduced by approximately 50% compared to the titer from p50GFP pretransfected cells. We believe that the modest reduction by the trans-dominant mutation is due to the poor transfection efficiency of the OMK cells, as Western blot analysis demonstrated that there were similar levels of wild-type proteins and proteins encoded by the mutated ORF 50 (data not shown). However, the experiment does suggest that the ORF 50 AT hook mutation can act as a trans-dominant mutant to inhibit virus replication and thereby virus production.

Previous studies have shown that the ORF 50 proteins activate transcription directly, following interactions with promoters containing a specific sequence motif, termed the ORF 50 RE (28, 30). In this study, the ORF 50 DNA binding domain responsible for the interaction with the ORF 50 RE was iden-
hook is a small DNA binding protein motif that was first described in the nonhistone chromosomal protein HMG A and allows binding to the minor groove of short stretches of AT-rich DNA. The AT hook has a core consensus sequence of Pro-Arg-Gly-Arg-Pro (with R-G-R-F being invariant), flanked on either side by a number of positively charged lysine or arginine residues. The core of the AT hook peptide motif is highly conserved in evolution from bacteria to humans and is found in one or more copies in a large number of other, non-HMG A proteins, many of which are transcription factors or components of chromatin remodeling complexes (reviewed in references 4, 22, and 23).

We constructed a mutant with a deletion in this region (p50GFPΔAT-hook), which was unable to transactivate the HVS ORF 6 and ORF 57 promoters. Moreover, gel retardation analysis demonstrated that this region was required for ORF 50 DNA binding to the ORF 50 RE. Single site-directed mutations within the ORF 50 DNA binding domain helped characterize specific amino acids that are important for ORF 50 transactivation. The majority of the single-point mutations had no effect on ORF 50 transactivation; however, mutation of the glycine residue at position 408 to alanine reduced ORF 50 transactivation. The majority of the single-point mutations within the ORF 50 RE. The ORF 50 homologue of EBV has been shown to function in this manner (17). However, it has not been shown whether wild-type or mutated HVS ORF 50 is capable of forming dimers. Alternatively, mutated ORF 50 may reduce viral replication by competing with wild-type ORF 50 for essential transcription factors within the nucleus and hence reduce wild-type ORF 50 transactivation.

Sequence analysis of the gamma-2 herpesvirus ORF 50 homologues has revealed that HVS strain C488 and herpesvirus ates contain putative AT hook DNA binding domains. However, we have not identified a similar motif in other gamma-2 herpesvirus ORF 50 homologues, such as KSHV or MHV-68. The AT hook-containing HMG A proteins are DNA binding proteins which promote gene activation by facilitating the formation of stereo-specific complexes called enhanceosomes on promoter and/or enhancer regions as a consequence of both protein-DNA and protein-protein interactions. It has been demonstrated that KSHV ORF 50 can transactivate viral gene expression via direct and indirect mechanisms. Therefore, it will be of interest to determine whether HMGA cellular proteins and specifically their AT hooks play a significant role in ORF 50-mediated transactivation in both direct and indirect transactivation mechanisms. Preliminary analysis suggests that this is indeed the case (Ren Sun, University of California, Los Angeles, personal communication). They may promote the formation of a stereo-specific complex on KSHV DE promoters or remodel the chromatin-DNA structure of DE promoters, allowing active gene expression through the recruitment of transcription factors. In summary, we have demonstrated that the prototype gamma-2 herpesvirus ORF 50 protein contains a DNA binding motif reminiscent of an AT hook, which is required for DNA binding and transactivation. We believe that the AT hook motif of HVS ORF 50 is the first identification of a functional AT hook DNA binding motif in a viral transactivating protein.

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