Mutations in a Herpes Simplex Virus Type 1 Origin That Inhibit Interaction with Origin-Binding Protein Also Inhibit DNA Replication

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The herpes simplex virus type 1 genome contains three origins of replication: Ori_L and a diploid Ori_S. The origin-binding protein, the product of the UL9 gene, interacts with two sites within Ori_S, box I and box II. A third site, box III, which is homologous to boxes I and II, may also be a binding site for the origin-binding protein. Mutations in these three sites significantly reduce Ori_S-directed plasmid replication measured in transient replication assays. The reduction in replication efficiency of the mutants correlates well with the decrease in the ability to bind to the origin-binding protein, as determined by Elias et al. (P. Elias, C. M. Gustafsson, and O. Hammarsten, J. Biol. Chem. 265:17167–17173, 1990). The effect of multiple mutations in boxes I, II, and III on plasmid replication suggests that there are multiple binding sites in Ori_S for the origin-binding protein. These studies indicate that proper interaction of the origin-binding protein with the Ori_S sequence is essential for Ori_S-directed DNA replication.

Herpes simplex virus type 1 (HSV-1) encodes a protein that binds specifically to the HSV-1 origins of replication, Ori_S and Ori_L (7, 8, 20). This origin-binding protein, the product of the UL9 gene (20), is essential for viral DNA replication. Like the simian virus 40 large T antigen (1, 25) the HSV-1 origin-binding protein possesses helicase activity (2) and may unwind the DNA duplex at the origin, thereby providing an entry site for the complex of enzymes that initiates DNA replication (5, 11).

Two sites within the Ori_S sequence that bind specifically to the origin-binding protein have been identified (7, 8, 20). These sites, boxes I and II, show significant sequence homology (Fig. 1). A third potential binding site, box III, is homologous to boxes I and II. However, in contrast to boxes I and II, which interact strongly with the origin-binding protein (6, 7), no strong interaction with the box III sequence has been observed. Nevertheless, deletions in box III block the initiation of DNA replication at the HSV-2 Ori_S (15).

Mutations in boxes I, II, and III had previously been investigated by Elias et al. for their ability to interact with the origin-binding protein (6). In this study, we tested plasmids containing these Ori_S mutations for their ability to replicate in transient replication assays. We found that the reduction in the replication efficiency of box I and box II mutants correlates well with the decrease in their binding activity, suggesting that proper interaction of the origin-binding protein with Ori_S is essential for DNA replication. We also found that a box III mutant is deficient in replication, supporting the hypothesis that this site plays a role in the initiation of DNA replication. Finally, our data suggest that there may be multiple binding sites within Ori_S for the origin-binding protein.

Recently, Weir and Stow (30) have reported that boxes I and II are required for efficient Ori_S activity.

The plasmids constructed by Elias et al. (6) contain the Ori_S region cloned into the vector pTZ18r at the unique *Bam*HI site. Dinucleotide substitutions were made in boxes I, II, and III to generate the mutants shown in Fig. 1. The plasmids were propagated in *Escherichia coli* DH5α by standard methods, harvested by alkaline lysis, and purified further by centrifugation in CsCl gradients (16). To determine the ability of the mutant plasmids to replicate, actively growing Vero cells were transfected by electroporation (19) with either 10 or 20 μg of the test plasmid and 10 or 20 μg of pBR322, which served as a nonreplicating control. The cells were allowed to recover for 36 to 48 h and then were either infected with HSV-1 RΔ305 (21) at 10 PFU per cell or mock infected. Eighteen hours after infection, the DNA was harvested by whole-cell DNA extraction (16). Replication of plasmid DNA was measured by resistance to digestion by the restriction enzyme *Dpn*I. The input plasmid DNA grown in *dam*⁺ *E. coli* is methylated at adenine residues within the sequence GATC (13). *Dpn*I cleaves DNA with methylated adenine (on both strands) but not unmethylated or hemimethylated DNA (14). Consequently, a plasmid that has undergone replication should contain unmethylated DNA and is resistant to *Dpn*I cleavage. Cellular DNA (10 μg) was incubated with 10 U of *Eco*RI in *Eco*RI restriction buffer in a volume of 0.2 ml for 12 to 16 h at 37°C. The DNA was precipitated with 0.3 M sodium acetate and 2 volumes of ethanol. The pellet was washed with 80% ethanol and redissolved in 0.2 ml of a Tris-acetate buffer, pH 7.7, composed of 33 mM Tris-acetate, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, 100 μg of bovine serum albumin per ml, and 0.2% Triton X-100. The DNA was incubated with 5 U of *Dpn*I in 0.2 ml for 12 h at 37°C, precipitated with ethanol, and washed with 80% ethanol; the pellet was resuspended in 20 μl of sample loading buffer (5% glycerol, 0.05% bromophenol blue, 0.1% sodium dodecyl sulfate). The DNA samples were electrophoresed through a 0.8% agarose gel in TAE buffer (16) for 6 to 12 h until the bromophenol blue dye reached the end of the gel. The gels were stained with ethidium bromide, photographed
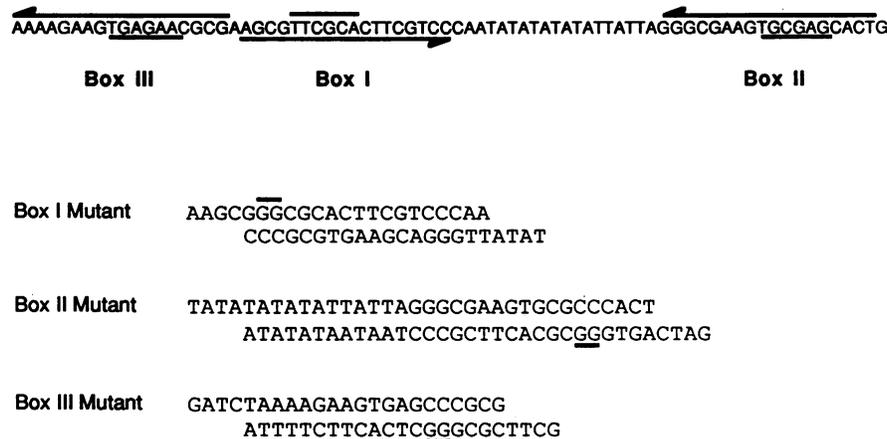
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AAAAGAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCCAATATATATATATTATTAGGGCGAAGTGCGAGCACTG

Box III   **Box I**   **Box II**

Box I Mutant   AAGCGGGCGCACTTCGTCCCAA
                       CCCGCGTGAAGCAGGGTTATAT

Box II Mutant   TATATATATATTATTAGGGCGAAGTGCGCCCACT
                       ATATATAATAATCCCGCTTCACGCGGGTGACTAG

Box III Mutant   GATCTAAAAGAAGTGAGCCCGCG
                       ATTTTCTTCACTCGGGCGCTTCG

FIG. 1. Minimal HSV-1 Ori_S sequence. The arrows indicate the sequences corresponding to boxes I, II, and III. The orientations of the sites are indicated by the directions of the arrowheads. The 6-bp binding site (TTCGCA in box I) in each box is indicated by a line over or under the sequence. The duplex oligonucleotides used to construct the mutant Ori_S sequences are shown, with the dinucleotide substitutions (GG) highlighted by a horizontal line.

under UV radiation, and then Southern blotted onto nitrocellulose or modified nitrocellulose filters (16). The filters were probed (16) with EcoRI-linearized pTZ18 and pBR322 (50 ng) that had been labeled with ^{32}P by using random oligonucleotide primers (9) and the large fragment of E. coli DNA polymerase I. The filters were exposed to preflashed Kodak XAR film with a Lightning-Plus intensifying screen (Du Pont) at $-70°C$. The autoradiographs were scanned by a Molecular Dynamics model 300A computing densitometer; ImageQuant software (Molecular Dynamics) was used to analyze the intensity of the bands. A set of autoradiographed ^{32}P-labeled DNA standards was also analyzed to ensure a linear response between optical density and radioactivity in a given band. The value for replicated DNA is given as the ratio of the optical density of DpnI-resistant pCG or pTZ18 DNA to that of DpnI-sensitive pBR322 DNA.

As shown in Fig. 2, plasmid replication required both the Ori_S sequence and superinfection with HSV-1. Plasmids pTZ18 and pBR322, which do not contain a herpesvirus origin, did not replicate even in the presence of HSV-1 infection. Replication of Ori_S-containing plasmids was readily apparent after infection but was undetectable in the absence of HSV-1 infection. Consistent with the finding that concatemers are produced before they are cleaved and packaged as monomers (22), replicating plasmid DNA was found to exist primarily in a high-molecular-weight form and migrated in a 0.8% agarose gel at a position corresponding to structures greater than 12 kb in length (data not shown). Unit-length plasmid DNA could be detected only after digestion with EcoRI, which introduces a single cut in pTZ18 and its derivatives.

The binding of origin-binding protein to the Ori_S sequence has been analyzed by DNase I protection analysis and has revealed two protected sites, box I and box II (Fig. 1) (7, 8, 20). As shown in Fig. 2 and Table 1, mutations at these sites caused decreases in replication to 6 and 15% of the wild-type replication level, respectively. A mutation in a third site, box III, caused a decrease to 20% of the wild-type level.

To test for the possibility that multiple binding sites are required for the initiation of DNA replication, plasmids containing the Ori_S sequence with mutations in boxes I, II, and III were examined (Fig. 1). Mutations in boxes I and II

(pCG42) or in boxes II and III (pCG35) reduced replication to levels below those seen with the single mutations (Fig. 2 and Table 1). In contrast, the plasmid with mutations in both boxes I and III (pCG28) showed approximately the same extent of replication as the plasmid containing the single box I mutation (pCG11). Finally, pCG40, which contains mutations in all three sites, showed little if any replication. DpnI-resistant DNA could not be detected, even after considerable overexposure of the autoradiographs (Fig. 2).

Analysis of the HSV-1 Ori_S sequence by a number of investigators has defined a minimal origin of approximately 80 bp (18, 26, 28, 29). This sequence consists of the two binding sites for the origin-binding protein, boxes I and II; a potential binding site, box III; and a stretch of alternating A and T residues (7, 8, 15, 20, 28) (Fig. 1).

Box I contains the consensus sequence, CGTTCGCATT, which has homologs in boxes II and III (Fig. 1). The box I sequence is the most highly conserved, as judged by sequence comparisons between HSV-1 Ori_S and the presumed origins of other herpesviruses (7, 10, 12, 15, 23, 24, 27, 28, 31). By mutational analysis, Elias et al. have shown this consensus sequence to be critical for binding of the origin-binding protein (6). Methylation interference studies have confirmed that residues contained within this sequence are critical for binding (12).

Mutations in box I (pCG11) or in box II (pCG16) both reduced the extent of replication; however, the greatest effect was seen with pCG11. Similarly, a box I mutation had a greater effect on the binding of the origin protein (a 500-fold reduction in binding affinity) than a box II mutation had (6). Both sets of results are consistent with the finding that the origin-binding protein has an approximately 5- to 10-fold higher affinity for box I than it does for box II (7).

The role of box III is still unclear. In contrast to boxes I and II, footprint analyses and filter-binding measurements have failed to reveal a stable interaction between the origin-binding protein and box III (6, 7, 20). However, a box III mutant, pCG20, shows a significantly reduced level of replication, suggesting that sequences within box III are indeed important for replication. Martin et al. (16a) have also found that substitution mutations within box III can reduce the replication of Ori_S plasmids in transient assays. Finally,
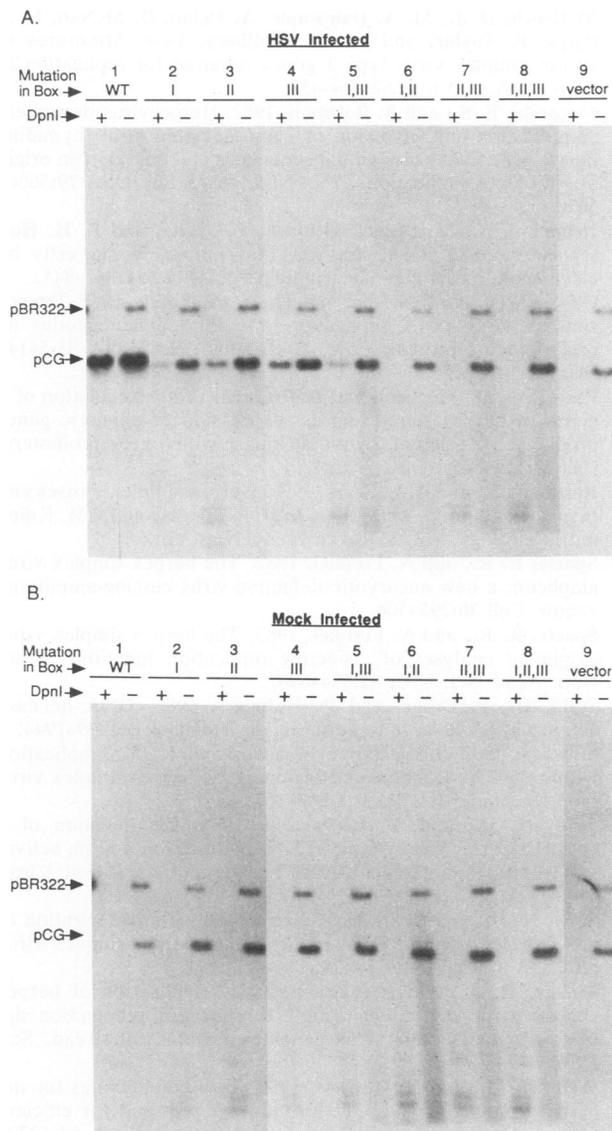
A.

HSV Infected

B.

Mock Infected

FIG. 2. Southern blot analysis of transient DNA replication. Cells transfected with the indicated plasmids were either infected with HSV-1 (A) or mock infected (B). After 18 h, the DNA was isolated, and 10 μg of DNA was linearized with EcoRI. To measure replicated DNA, a second aliquot of each sample was digested with EcoRI and DpnI. The addition of DpnI is indicated by a plus or minus. The blot was probed with ^{32}P-labeled pBR322 and pTZ18, washed, and autoradiographed. The positions of the linear forms of pBR322 and pCG are indicated. Lanes 1 through 8 correspond to the Ori$_S$-containing plasmids pCG5, pCG11, pCG16, pCG20, pCG28, pCG42, pCG35, and pCG40, respectively. The sites containing mutations in each plasmid are indicated. WT is wild-type Ori$_S$. Lane 9 corresponds to the vector pTZ18.

Lockshon and Galloway have identified the core Ori$_S$ sequence in HSV-2 and showed that sequences analogous to HSV-1 box III are essential for efficient replication (15).

The conservation of box III in the Ori$_L$ and Ori$_S$ sequences of HSV-1 and HSV-2 (15, 28, 31) and its homology to boxes I and II strongly suggest that it functions in binding of the origin-binding protein to Ori$_L$ and Ori$_S$. Possibly the origin-binding protein binds box III only weakly under our

TABLE 1. Replication of Ori$_S$-containing plasmids

Plasmid	Mutation(s)[a]	Relative replication (%) in expt no.[b]:	
		1	2
pCG5	Wild type	100	100
pCG11	I	6	7
pCG16	II	17	13
pCG20	III	13	29
pCG42	I, II	3	<1
pCG35	II, III	1	1
pCG28	I, III	14	5
pCG40	I, II, III	3	<1
pTZ18	Vector	1	<1

[a] Roman numerals refer to the binding sites for the origin-binding protein within Ori$_S$.

[b] Values given are relative to plasmid pCG5, which contains wild-type Ori$_S$.

conditions, so that DNase I footprinting and filter-binding measurements do not detect their interaction. Alternatively, the origin-binding protein may interact with box III only in the presence of another factor (viral or host). Recently, Dabrowski and Schaffer have found that binding of purified HSV-1 origin-binding protein to box I can be enhanced by additions of extracts from uninfected Vero cells (5a), suggesting that the origin-binding protein–box I complex may be stabilized by a host factor.

A single mutation in box I or box II significantly decreases replication. Both sites must therefore be required for replication. A further reduction in the level of replication by the introduction of mutations in both boxes I and II (or in box III) establishes the need for multiple binding sites for the origin-binding protein within the Ori$_S$ sequence. The exception is pCG28, which contains mutations in both boxes I and III. Replication of this plasmid was not significantly different from replication of the plasmid containing only the box I mutation. It is, however, worth noting that the box I mutation leads to an altered DNase footprint in the box III region (6).

A difficulty in correlating changes in the binding of origin-binding protein to Ori$_S$ directly with decreases in replication is that binding of the origin-binding protein is only one step in a series of strictly controlled events that lead to the initiation of DNA replication at the origin. An altered Ori$_S$ sequence may permit binding of the origin-binding protein but not allow the correct assembly of the protein-DNA complex that is required for initiation. The simian virus 40 large T antigen binds in a sequence-dependent manner to the simian virus 40 origin, forming a well-defined nucleoprotein structure (1). In the presence of ATP, the T antigen opens and unwinds the duplex DNA at the origin (1, 5, 11), providing access for the replication enzymes and thereby permitting them to initiate DNA replication.

Although the HSV-1 origin-binding protein has not yet been shown to be directly involved in the initiation of HSV-1 DNA replication, it shares several key features with the simian virus 40 large T antigen. It binds to the origin in a sequence-dependent manner (7, 8), it has an ATP-dependent helicase activity (2), and it is essential for the replication of both HSV-1 and Ori$_S$-containing plasmids (3, 4, 17, 20, 32).

Our studies of the replication of plasmids containing the Ori$_S$ sequence have revealed essential elements in Ori$_S$ that are required for replication of the HSV-1 genome. However, an understanding of the events that mediate initiation of replication at Ori$_S$, and presumably at Ori$_L$, will require a detailed biochemical analysis of this complex process.

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