A 67-Base-Pair Segment from the Ori-S Region of Herpes Simplex Virus Type 1 Encodes Origin Function

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The Ori-S segment of herpes simplex virus type 1 contains a 45-base-pair-long imperfect palindrome with an AT segment at its center. We cloned Ori-S into a poisonless plasmid to investigate the role of the palindromic components in DNA replication. Neither a large insertion within the AT segment nor a deletion of the right side of the palindrome significantly inhibited DNA replication under our conditions of analysis. These findings argue against the necessity for a specific cruciform structure in the initiation of replication. We scanned the entire AT segment with triple tandem-base-pair substitutions to pinpoint essential functional sequences. Only the first 3 base pairs at the left end of the segment are absolutely essential for replication in the presence of the remaining AT sequences.

Herpes simplex virus type 1 (HSV-1) encodes a number of proteins essential for the replication of viral DNA (7, 22). One or more of these proteins may interact with specific viral DNA sequences to initiate replication by an unknown mechanism (6). Current evidence indicates a rolling circle mechanism of replication, at least at the late stage of infection (22). The viral DNA has three presumptive origins of replication. Two small origins (Ori-S) have the same sequence and reside in the C repeats of the genome; the third and larger origin (Ori-L) is located in the middle of the unique large segment of viral DNA (22).

Localization of the origins of replication on the viral genome has been accomplished by electron microscopy of replicating viral DNA (9, 12) and by studying the replication of defective interfering particle DNAs (7-10, 15, 16, 25) and cloned viral DNA segments (20, 25, 31) in the presence of helper virus. Stow and McMonagle (28) cloned a 90-base-pair (bp) viral DNA fragment of Ori-S that is sufficient for the initiation of replication. Replicated Ori-S-containing DNA was shown to form tandem head-to-tail repetitions of monomeric units (26), which is reminiscent of replicated DNA of wild-type HSV-1 (1, 13, 14). Thus, the replication of Ori-S-containing DNA appears to proceed by a rolling circle mechanism similar to that of authentic viral replication.

Like many other defined origins, the HSV origins have palindromic regions and clusters of G+C-rich and A+T-rich regions. The Ori-S sequence is shown in Fig. 1. It has a 45-bp-long palindrome with an 18-bp AT stretch at the center. Part of the AT sequence consists of alternating adenine and thymine residues, which are characteristic of DNA with a chemically reactive and torsionally deformable structure (19). The Ori-S sequences from HSV-1 and HSV-2 are virtually identical (17). The left half of Ori-S is also very homologous to the corresponding regions of palindromes in the Ori-L sequences of HSV-1 and HSV-2 as well as to that in the origin of varicella-zoster virus (17, 27). Elias et al. (6) have isolated a protein from infected cells that binds specifically to the left arm of the Ori-S palindrome but not to its right arm (Fig. 1).

We cloned the wild-type Ori-S segment shown in Fig. 1 into a poisonless plasmid for a genetic analysis of the role of the palindromic components in DNA replication. We synthesized the entire length of the 100-bp DNA fragment shown in Fig. 1 in six overlapping cassettes of oligonucleotides which, after proper hybridization, should generate a *Hind*III site overhang at one end and a *Nco*I site overhang at the other. These oligonucleotides were kinased and hybridized to each other in a manner similar to that described by Deb et al. (2-4). The hybridized oligonucleotides were then ligated to the pOR vector (5), a pML2 derivative. The pOR vehicle, as described previously (5), was constructed by replacing the sequences from *Eco*RI (bp 4361) to *Acc*I (bp 651) of pML2 with a polylinker sequence comprising restriction sites *Nco*I, *Sal*I, *Bam*HI, *Sma*I, *Xma*I, and *Eco*RI, with concomitant insertion of a *Hind*III linker at the *Acc*I site at position 651. After ligation, competent DH5 cells were transformed with the ligation mixture to ampicillin resistance. Recombinant clones were then screened by a dideoxy sequencing technique, using supercoiled plasmids as templates (5, 30). Mutant origins were constructed by replacing two of the six wild-type oligonucleotides with complementary mutant oligonucleotides in the hybridization and ligation procedures outlined above.

Cesium chloride-ethidium bromide gradient-purified recombinant plasmid DNA (pOR-S) that had the correct sequence was then tested for replication ability in BHK-C21 cells, as described by Stow and McMonagle (28). In short, 50%-confluent BHK-C21 cell monolayers in 100-mm-diameter plastic petri dishes were transfected with 750 ng of supercoiled plasmid DNA and 5 µg of calf thymus DNA by the calcium phosphate precipitation technique (11); this was followed by a dimethyl sulfoxide boost at 4 h (29). Incubations were carried out at 37°C. At 6 h posttransfection, cells were infected with wild-type HSV-1 (strain F) at about 5 to 10 PFU per cell. The cells were incubated for 24 h to reach 100% cytopathic effect, at which time whole-cell DNAs were isolated. Sample of the DNA solution was digested to completion with *Hind*III and *Mbo*I. *Hind*III linearizes the plasmid and *Mbo*I cleaves GATC sequences only when the adenine in the site remains unmethylated (21). Since our plasmids were grown in dam-positive DH5 bacteria, GATC sequences remained methylated at the adenines and hence

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FIG. 1. Ori-S sequence. The 45-bp-long palindrome is shown by the long inverted arrows. Major sequence homology with varicella-zoster virus (VZV) origin of replication (27) is shown by underlines. The region where the Ori-S-binding protein identified by Elias et al. (6) binds is indicated by the lowest bar. For convenience the sequence is numbered from left to right.

uncleavable with *Mbo*I. Thus, *Mbo*I treatment should digest only replicated DNA which remains unmethylated in animal cells while the input DNA remains intact. Digested DNAs were electrophoresed in a 1.2% agarose gel. This gel was then blotted, and the blot was hybridized with nick-translated pBR322 DNA.

Figure 2 shows an analysis of replication of plasmid DNAs containing wild-type Ori-S and mutant Ori-S with insertions and deletions. The wild-type origin replicated well, whereas pBR322 and a mutant that deletes sequences 26 to 73 from Ori-S did not replicate at all. These findings verify the authenticity of the assay and demonstrate the importance of a portion of the palindromic sequences in replication. While screening for the wild-type pOR-S clone, we identified an unplanned recombinant clone (pOR-S-ins) (Fig. 2). The clone contains a 1-bp insertion near the right end and a 34-bp insertion near the left end of the AT segment. Unexpectedly,

this plasmid replicated almost as well as the wild-type pOR-S. This finding could be explained in two ways. Origin function might not require DNA on one side of the insertion, or precise spacing between distinct origin domains might not be crucial for function. To distinguish between the possibilities, we constructed clone pOR-S-1 (Fig. 2), which deletes all the sequences to the right of the AT segment. This clone replicates with an efficiency similar to that of the wild-type pOR-S (Fig. 2). Therefore, under the conditions of the assay, the minimal origin of replication does not require most of the right arm of the palindrome and consists of 67 or fewer bp. It is, however, difficult to totally exclude the possibility of recombination with helper viral DNA during the assay.

Replication of the clone with an insertion in the AT stretch also suggested that the sequence requirements of that segment are not stringent. Thus, we made systematic base substitutions across the AT region. To make significant

FIG. 2. Replication analysis of wild-type and deletion mutants of Ori-S-containing plasmids. Sequences of the different mutants and wild-type origins are shown at the top. The extended black bar represents the deleted DNA sequence in mutant Del 26-73 compared with wild-type pOR-S. The palindrome is indicated by arrows. At the bottom is an autoradiogram showing replication efficiency of different constructs. The positions of one replicated progeny DNA fragment and of unreplicated input DNA are indicated. Lanes: wt, wild type; ins, pOR-S-ins; dl, Del 26-73; pBr, pBR322; S1, pOR-S-1.

FIG. 3. Replication analysis of wild-type (wt) and base-substitution mutants. The wild-type DNA used is that of pOR-S-1, whose sequence is shown at the top. Consecutive base substitutions are indicated below the wild-type sequence. Base-substitution mutations are labeled bs 1 through 6 according to the position of the mutagenized triplet. Below the sequences is an autoradiogram showing the replication efficiency of different constructs. Lanes 1 through 6, Base-substitution mutants; Wt., wild type.

changes in the sequence environment, we substituted AT with CG base pairs in a triple tandem arrangement. Figure 3 shows a replication analysis of the mutants. The substitution of CCG for AAT at the first 3 bp of the left end of the AT sequences caused a dramatic loss of origin function. In contrast, 3-bp substitutions in the five remaining positions did not reduce replication significantly.

Our findings demonstrate that the HSV-1 minimal origin of replication consists of 67 or fewer bp. The large G+C-rich segment of the right arm of the origin palindrome is not essential for replication, and a large insertion into the palindrome has no detectable effect on origin function. These findings argue that the formation of a cruciform structure in the origin is not necessary for the initiation of DNA synthesis. Furthermore, this conclusion is consistent with the finding that an apparent viral protein binds to the left arm but not to the right arm of the origin palindrome (6). We also show that the sequence of the first 3 bp of the AT segment is far more crucial than the sequence of the remainder of the segment. It is interesting that these 3 bp are adjacent to a protein-binding site. They are probably not part of the protein recognition sequence, because protein binding does not protect them from DNase (6). They may be part of a nicking site for an endonuclease that starts DNA replication via a rolling circle mechanism, or they may be essential for the initiation of melting of the DNA double helix.

This work was supported by Public Health Service grants CA-18808, CA-38146, and CA-09176 from the National Cancer Institute to Peter Tegtmeier and partly by New Faculty Awards from the Research Affairs Committee and the Cancer Center of the Medical College of Wisconsin to Sumitra Deb.

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