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A DNA binding protein specific for an origin of replication of herpes simplex virus type 1

PER ELIAS*, MICHAEL E. O'DONNELL*, EDWARD S. MOCARSKI†, AND I. R. LEHMAN*

Departments of *Biochemistry and †Medical Microbiology, Stanford University School of Medicine, Stanford, CA 94305

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ABSTRACT **We have identified a protein that binds specifically to an origin of replication (ori_S) of the herpes simplex virus type 1 genome. The ori_S binding protein, detectable only in nuclear extracts of infected cells, shows the same time course of appearance as the herpesvirus-induced DNA polymerase and the DNA binding protein ICP8. The partially purified ori_S binding protein generates a DNase I "footprint" that spans 18- of the 90-base-pair minimal ori_S sequence. The ori_S binding protein may, therefore, be analogous to other origin-specific binding proteins that are required for the initiation of viral and chromosomal DNA replication.**

The initiation of DNA replication occurs at well-defined sequences termed origins that act to regulate initiation as well as to facilitate assembly of the multienzyme complex required for semiconservative DNA replication (1). To understand the molecular mechanisms that underlie the initiation of DNA replication in eukaryotic cells, we have chosen to study herpes simplex virus type 1 (HSV-1). The herpesvirus family, whose members are highly cell-associated, appears to have genomic signals for lytic replication as well as for maintenance during latency. It is likely that they may encode functions that regulate the decision between these alternatives (2).

The 150-kilobase genome of HSV-1 contains elements, ori_S and ori_L, that can serve as origins of replication when isolated from the viral genome and are believed to be responsible for the initiation of DNA replication during lytic growth of the virus (Fig. 1) (2). The genome has two copies of ori_S, which are present within the reiterated *c* sequence of the S component, and one copy of ori_L, which maps in the unique sequences (U_L) of the L component (Fig. 1) (3–6). Plasmids containing cloned ori_S or ori_L sequences can replicate in mammalian cells superinfected with HSV-1 (4, 5, 7, 8). Analysis of these sequences has demonstrated that a 90-base-pair (bp) region of the ori_S sequence is sufficient to function as a minimal origin *in vivo* and that the nucleotide sequences of ori_S and ori_L show substantial homology in this region (5, 8, 9).

Our approach has been to identify viral proteins that specifically recognize the ori_S sequence and could, as a consequence, function in the initiation of HSV-1 DNA replication. Here we report on the identification and partial purification of a virus-induced DNA binding protein that is specific for the ori_S sequence.

MATERIALS AND METHODS

Cells and Viruses. RΔ305 (10), a thymidine kinase-deficient mutant of HSV-1 [F], was used to infect roller bottle cultures of Vero cells using a multiplicity of infection of 5–10 plaque-forming units per cell.

Construction of ori_S-Containing Plasmids. The plasmid pON103 was constructed by inserting a 294-bp *Ava* I fragment spanning ori_S from pRB370 (11) into the plasmid vector pMT11 (12). This fragment, whose nucleotide sequence is identical to the comparable region of HSV-1 [17] (13), was positive for origin function by cotransfection with intact HSV-1 [F] into Vero cells (data not shown) as described (6). A 360-bp *Eco*RI–*Hin*dIII fragment from pON103 containing the ori_S sequence was introduced into M13 mp18 (Fig. 1) (14). The M13 mp18 ori_S phage were grown under standard conditions (15), and closed-circular duplex DNA was prepared by the alkaline lysis method (16).

Labeling of ori_S-Containing Fragment. The *Eco*RI–*Hin*dIII ori_S-containing fragment was labeled using the large fragment of DNA polymerase I and [α-^{32}P]dTTP (800 Ci/mmol; 1 Ci = 37 GBq) or [α-^{32}P]dCTP (3000 Ci/mmol) (16).

Construction of ori_S Deletion Mutant. The oligonucleotide CGCGAAGCGTGCACTGGCGC synthesized by the phosphoramidate method (17, 18) using a 380 A DNA synthesizer (Applied Biosystems, Foster City, CA) was phosphorylated at the 5′ end by treatment with T4 polynucleotide kinase and [γ-^{32}P]ATP and purified by Sep-Pak chromatography (19). A 100-fold excess of the labeled oligonucleotide was annealed to M13 mp18 ori_S single-stranded DNA in 10 μl of 0.01 M Tris·HCl (pH 8.0)/0.01 M $MgCl_2$/0.05 M NaCl (buffer A). An equal volume of 1 mM ATP/0.25 mM each of the four dNTPs/10 mM dithiothreitol in buffer A containing 5 units of the large fragment of DNA polymerase I and 1 unit of T4 DNA ligase was added. Incubation was for 12 hr at 12°C. The product closed-circular duplex DNA was isolated by electrophoresis in a 0.8% SeaPlaque agarose gel. It was cut out of the gel (which was melted), diluted with 2 vol of 20 mM Tris·HCl (pH 7.5), 1 mM EDTA, and used to transform competent *Escherichia coli* MC1061 cells (20). The M13 phage were grown in *E. coli* TG1 (21), and the single-stranded DNA was isolated by standard procedures. Dideoxy sequencing (22) of the mutant phage obtained by this procedure confirmed that the entire 45-bp inverted repeat present in the ori_S sequence had been deleted; it was designated M13 mp18Δori$_S$ (Fig. 1).

Partial Purification of ori_S Binding Protein from HSV-1-Infected Cells. HSV-1-infected cells from 50 roller bottles (20–30 g of cells), grown as described above, were harvested 18 hr after infection by shaking vigorously or by scraping with a rubber policeman and then collected by centrifugation. The cells were washed in 250 ml of cold 20 mM Hepes adjusted to pH 7.6 with NaOH/150 mM NaCl/0.5 mM dithiothreitol and subsequently lysed in 200 ml of 20 mM Hepes adjusted to pH 7.6 with NaOH/0.5 mM dithiothreitol/10 mM $NaHSO_3$, pH 7.0/0.5 mM phenylmethylsulfonyl fluoride/leupeptin at 2 μg/ml (buffer B) using a Dounce homogenizer with a tight fitting pestle. Nuclei were collected by low-speed centrifugation and washed with buffer B. The nuclear pellet was finally suspended in 40 ml of buffer B containing 10%

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Abbreviations: bp, base pairs: HSV-1, herpes simplex virus type 1; RF, closed-circular duplex DNA.

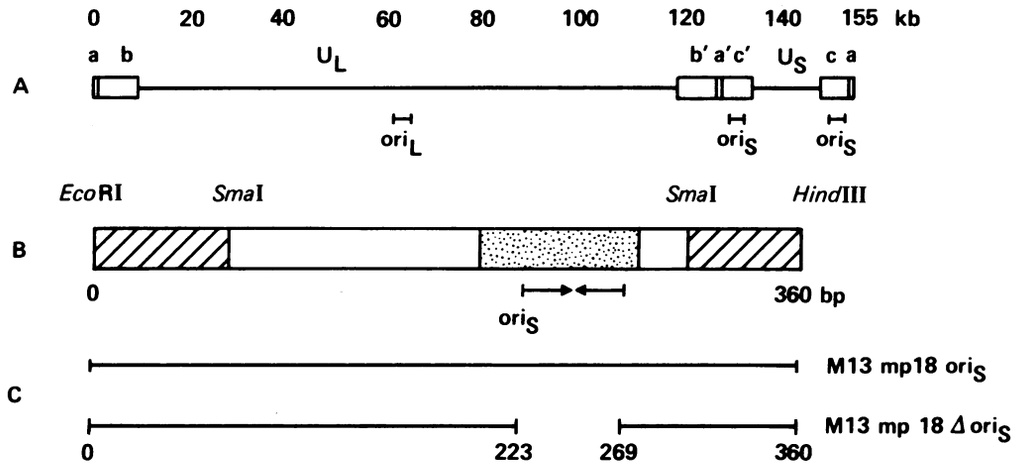

FIG. 1. Diagram of the HSV-1 genome, ori_S region, and M13 constructions. (*A*) The HSV-1 genome showing the location of the putative origins of replication. (*B*) Cloned fragment containing the ori_S sequence. The hatched area indicates poly-linker sequences bordering the HSV-1 *Sma* I–*Sma* I fragment (235 bp). The minimal origin is shown as a stippled box, and the arrows indicate the 45-bp inverted repeat. (*C*) Structure of the M13 mp18 derivatives used in this study. M13 mp18Δori_S has a 45-bp deletion of the entire inverted repeat.

(wt/vol) sucrose and stored at −80°C. Four such nuclear preparations were thawed and pooled by swirling in a large flask with an equal volume of 3.4 M NaCl in buffer B. The highly viscous extract was centrifuged at 27,000 rpm in a Beckman SW28 rotor for 2 hr at 4°C. Solid $(NH_4)_2SO_4$ (50 g/100 ml) was added to the pooled supernatants. The precipitate was collected by centrifugation for 1 hr at 20,000 × *g* at 4°C and dissolved in a minimal volume of buffer B containing 10% (wt/vol) glycerol, 0.5 mM EDTA and dialyzed for 12 hr against the same buffer. The precipitate remaining after dialysis was removed by centrifugation, and the supernatant was adjusted to approximately 0.05 M NaCl by dilution with the dialysis buffer. This fraction (100–250 mg of protein in approximately 100 ml) was then applied to a phosphocellulose column (2.5 × 15 cm) equilibrated with 20 mM Hepes adjusted to pH 7.6 with NaOH/0.5 mM dithiothreitol/0.5 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride/10% (wt/vol) glycerol (buffer C). The column was eluted with a linear gradient from 0.1 to 0.7 M NaCl in a total volume of 1 liter of buffer C. Fractions (5 ml) were collected and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (23). Assays for DNA polymerase and ori_S binding protein are described below. Fractions containing the ori_S binding protein were pooled and dialyzed against buffer C. The precipitate formed during dialysis that contained the ori_S binding protein was collected by centrifugation and dissolved in 2 ml of 0.5 M NaCl in buffer C. This fraction, which could be stored at −80°C for at least 2 months without significant loss of activity, was used for the experiments described below unless otherwise indicated.

Assays. ICP8 was identified as the predominant band with an apparent molecular weight of 130,000 by $NaDodSO_4$/polyacrylamide gel electrophoresis (24). It was quantitated by scanning Coomassie blue stained gels with a GS300 scanning densitometer (Hoefer, San Francisco).

The HSV-1 DNA polymerase was assayed in a reaction mixture (25 μl) containing 20 mM Tris·HCl (pH 7.5); 0.1 mM EDTA; bovine serum albumin at 0.04 mg/ml; 4% (wt/vol) glycerol; 3 mM $MgCl_2$; 5 mM dithiothreitol; 0.06 mM each of dATP, dGTP, and dCTP; 0.005 mM [^{3}H]dTTP (40 Ci/mmol); 150 mM $(NH_4)_2SO_4$; and activated calf thymus DNA at 0.09 mg/ml (25). Incubation was for 10 min at 37°C, and the incorporation of labeled nucleotide into trichloroacetic acid-precipitable material was monitored as described (26).

The ori_S binding protein was assayed by nitrocellulose filter binding. The reaction mixture (25 μl) contained 50 mM Hepes adjusted to pH 7.5 with NaOH, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, 10% (wt/vol) glycerol, 100 mM NaCl, and 20 fmol of the ^{32}P-labeled *Eco*RI–*Hin*dIII ori_S-containing fragment. Sonicated calf thymus DNA (4 mg/ml, average size 600–900 bp) was added as a competitor. After incubation for 10 min at 25°C, the reaction mixture was diluted with 1 ml of 50 mM Hepes adjusted to pH 7.5 with NaOH/5 mM $MgCl_2$/0.1 mM EDTA/0.5 mM dithiothreitol/10% (wt/vol) glycerol (buffer D), and immediately filtered through 0.45-μm Millipore filters type HA. The filters were dried, and their radioactivity was determined using a toluene-based scintillation fluid. A background, which ranged from 2000 to 6000 cpm due to nonspecific retention of the labeled fragment, was subtracted.

DNase I "Footprinting." The ori_S binding protein was incubated with the ^{32}P-labeled *Eco*RI–*Hin*dIII fragment under conditions identical to those used in the standard binding assay except that the concentration of competitor DNA was reduced to 0.04 mg/ml. Following incubation for 10 min at 25°C, DNase I was added to a final concentration of 4 μg/ml. Digestion was interrupted after 1 min at 25°C by the addition of 1 ml of buffer D. DNA–protein complexes were isolated on Millipore filters as described above. The DNA fragments retained were eluted as described by Fuller *et al.* (27), extracted with phenol/chloroform, 1:1 (vol/vol), and analyzed on 6%, 0.2-mm sequencing gels (28). The positions of the DNase I cuts were identified by means of Maxam–Gilbert G and G+A reactions (29) as well as with standard restriction fragments.

RESULTS

Identification of ori_S Binding Protein. We have used a nitrocellulose filter assay to detect proteins that bind specifically to the restriction fragment containing the ori_S sequence. To eliminate the competitive effect of other DNA binding proteins a large excess of sonicated calf thymus DNA was added. As shown in Fig. 2, nuclear extracts from cells infected with HSV-1 contained an activity that promoted retention of the labeled ori_S fragment on nitrocellulose filters even in the presence of a 10,000-fold excess of calf thymus DNA. To reduce further the background due to nonspecific DNA binding proteins, we partially purified the ori_S binding activity from nuclear lysates of cells 18 hr after infection by phosphocellulose chromatography. As shown in Fig. 3, ICP8, the major DNA binding protein, eluted at about 0.15 M NaCl, and the virus-encoded DNA polymerase was eluted at 0.3 M

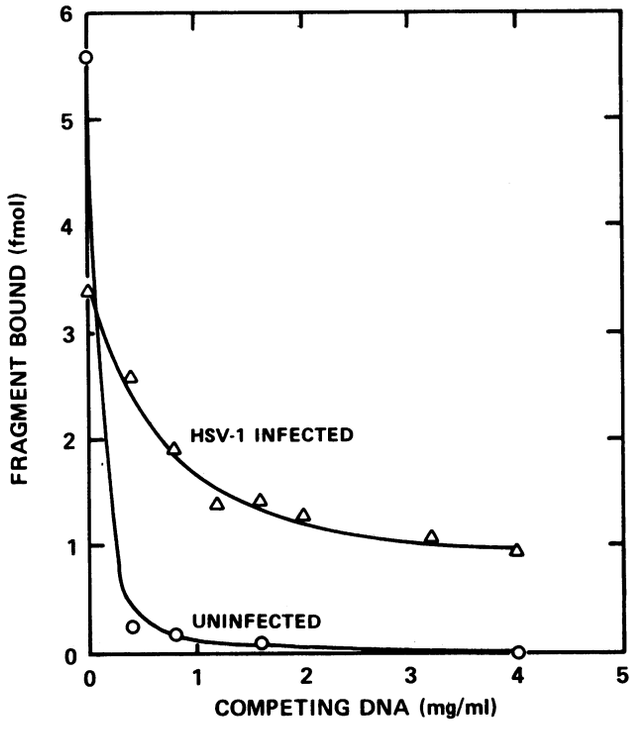

FIG. 2. Retention of the ori_S fragment on nitrocellulose filters. Nuclear extracts were prepared from uninfected cells (○) and from cells 12 hr after HSV-1 infection (△). Equivalent amounts of extract (5 μl, 2 mg/ml) were used in the filter binding assay.

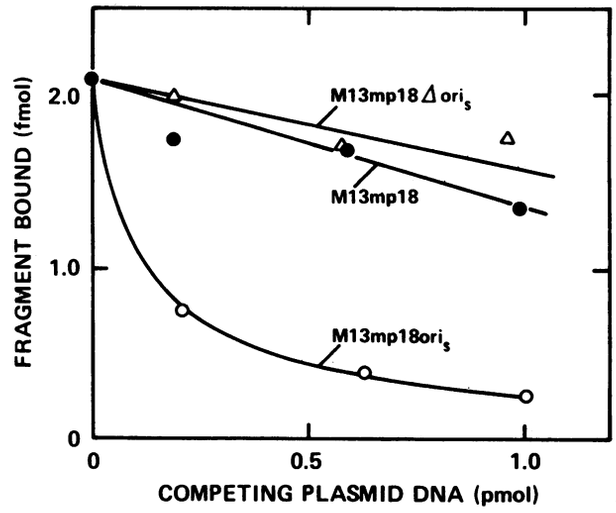

FIG. 4. Failure of ori_S deletion mutant to compete for binding to ori_S sequence. Phosphocellulose peak fraction (5 μg) (Fig. 3) was used as the source of ori_S binding protein in the nitrocellulose filter binding assay. M13 mp18 RF DNA (●), M13 mp18ori$_S$ RF DNA (○), and M13 mp18Δori$_S$ RF DNA z(Δ) were added as indicated.

NaCl. The ori_S binding protein eluted at 0.5 M NaCl. The ori_S binding fractions were pooled and purified further.

Specificity of ori_S Binding Protein. The ori_S binding protein was specific for the 360-bp *Eco*RI–*Hin*dIII fragment containing ori_S. As shown in Fig. 4, M13 mp18 RF DNA lacking this sequence failed to compete for binding. Furthermore, M13 mp18Δori$_S$ RF DNA, in which the 45-bp dyad within the origin was deleted, showed little if any competition for binding with the wild-type ori_S sequence, indicating that critical binding sites are located within the dyad.

The specific sequence with which the ori_S binding protein interacts was determined by means of the DNase I "footprinting" technique (30). A complex was formed under conditions identical to those used in the binding assay except that the 360-bp fragment was end-labeled at its *Hin*dIII site with [α-^{32}P]dCMP. Following brief digestion with DNase I the complex was isolated and subjected to electrophoresis on a 6% sequencing gel. As shown in Figs. 5 and 6, a stretch of 18 bp was protected from DNase I digestion. The protected region starting at adenosine-219 and ending at cytidine-236 includes a portion of one of the inverted repeats and a short sequence immediately adjacent to its border. There was no evidence of protection of the remaining inverted repeat nor of any other sequence in this region.

Time Course of Appearance of ori_S Binding Protein. The time course of appearance of the ori_S binding protein following HSV-1 infection of Vero cells is shown in Fig. 7. ori_S binding activity was absent from nuclear extracts made from uninfected cells. Activity was, however, detectable at 8 hr after infection and reached a maximum at 18 hr. As shown in Fig. 7, the appearance of the ori_S binding protein coincided with two other proteins associated with HSV-1 DNA replication, the HSV-1 DNA polymerase and ICP8.

DISCUSSION

We have identified and partially purified a protein that specifically binds an origin of replication, ori_S, of the HSV-1 genome. This ori_S binding protein is detectable only in

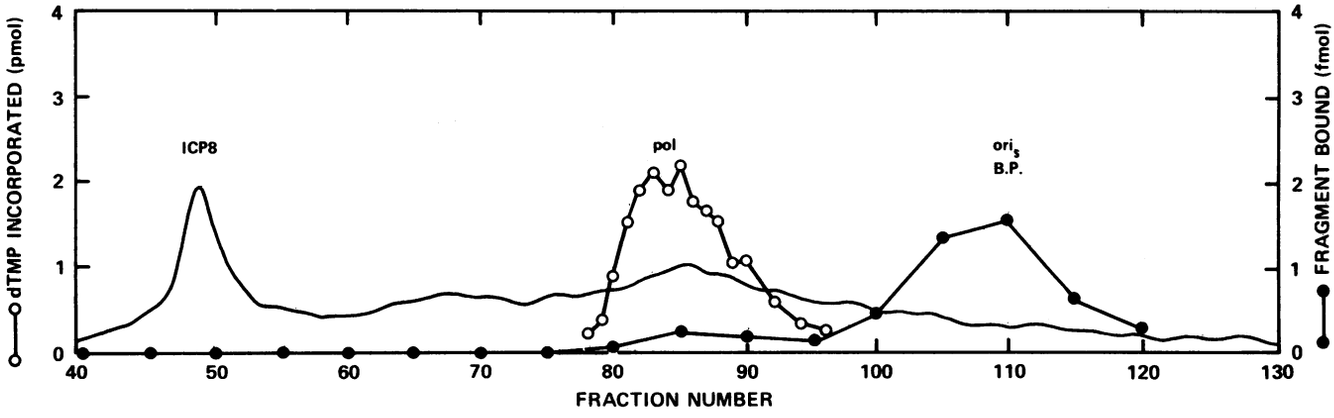

FIG. 3. Separation of ICP8, HSV-1 DNA polymerase, and ori_S binding protein by phosphocellulose chromatography. The solid line indicates the UV profile recorded continuously by an absorbance monitor. Solid line only, ICP8; open circles, HSV-1 DNA polymerase; and closed circles, ori_S binding protein (ori_S B.P.).

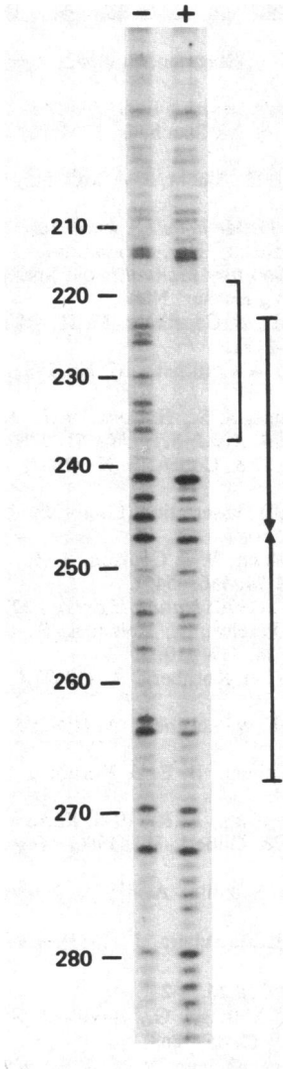

FIG. 5. "Footprint" of the ori_S binding protein on ori_S-containing fragment. DNase I digest of the complex between the ori_S binding protein and the *Eco*RI–*Hin*dIII ori_S-containing fragment was carried out. −, Control without protein; +, "footprint" obtained with 10 μg of ori_S binding protein.

nuclear extracts from infected cells and accumulates during infection coincident with the HSV-1-induced DNA polymerase and the DNA binding protein ICP8. It may, therefore, be analogous to the sequence-specific binding proteins that are essential for the origin-dependent initiation of DNA synthesis in a variety of eukaryotic and prokaryotic cells (27, 31–34). The direct involvement of the ori_S binding protein in the initiation of DNA replication remains, however, to be established.

The partially purified ori_S binding protein produces a DNase I "footprint" covering 18 bp of the ori_S sequence.

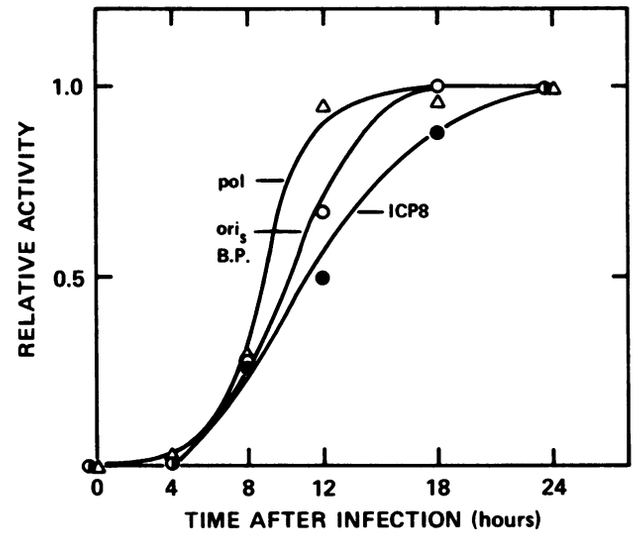

FIG. 7. Time course of appearance of ori_S binding protein following HSV-1 infection. Nuclei were prepared from Vero cells at various times after infection. The amount of ICP8 (●) was determined by densitometric scanning of Coomassie blue-stained $NaDodSO_4$/polyacrylamide gels. HSV-1 DNA polymerase (pol) (Δ) was assayed in the unfractionated nuclear extract. ori_S binding protein (ori_S BP) (○) was assayed after chromatography on a 0.2-ml phosphocellulose column using a step elution between 0.15 and 0.7 M NaCl in buffer C. Activities were normalized to the total activity found in nuclear extracts of cells harvested 24 hr after infection.

Fourteen of these are present in one part of the 45-bp palindrome that is a characteristic feature of the ori_S sequence (5). We have not observed protection of the other arm of the palindrome indicating that there is only a single binding site in the ori_S sequence. Since the two potential binding sites within ori_S differ at only three positions, it will be informative to determine whether point mutations introduced by site-directed mutagenesis can convert the active to an inactive site, or alternatively, produce two active binding sites within ori_S. Stow (35) has shown that an ori_S-containing plasmid with a 23-bp deletion in the center of the dyad is unable to replicate in HSV-1-infected cells. The fact that this plasmid lacks four of the nucleotides that were found to be protected in our "footprinting" experiment further supports a role for the ori_S binding protein in HSV-1 replication.

The minimal sequence required for replication of plasmid DNA in cells superinfected with HSV-1 (i.e., ori_S) consists of 90 nucleotides (5). It is noteworthy that one of the nonfunctional plasmids (S13) used in defining the minimal origin lacks two nucleotides of the sequence protected by the ori_S binding protein but includes the entire region of dyad symmetry (5). Inasmuch as this plasmid is unable to replicate, sequences immediately bordering the palindrome may be essential because of their interaction with the ori_S binding protein. The finding that sequences immediately outside the 45-bp region of dyad symmetry of ori_S are required for an interaction with the ori_S binding protein is also consistent

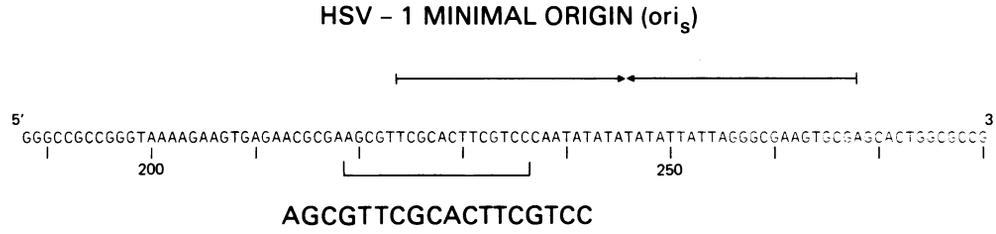

FIG. 6. Sequence of the minimal ori_S. The position of the inverted repeat as well as the protected region is indicated.

with our observation that a synthetic 20-bp duplex DNA fragment containing one-half of the dyad cannot compete with the 360-bp ori_S-containing restriction fragment in the nitrocellulose filter-binding assay (P.E., M.E.O'D., and I.R.L., unpublished observations).

The precise recognition site for the ori_S binding protein is still a matter of speculation. However, it is worth noting that an element of 2-fold symmetry exists within the protected sequence; as shown in Fig. 6, part of the sequence CGAAGCG occurs as an inverted repeat within this region.

In addition to ori_S, the HSV-1 genome contains another origin of replication, ori_L. The nucleotide sequence of ori_L is homologous to ori_S, and contains two equivalent sites within the 177-bp region of dyad symmetry that differ from the protected region of ori_S at only one position. These sites should endow ori_L with a 2-fold symmetry of binding that may be related to the bidirectional initiation of DNA replication (8, 9).

Finally, a comparison of the origins of replication in the HSV-1 and HSV-2 genomes reveals that the potential binding sites for the ori_S binding protein are completely conserved (36, 37).

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