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Analysis of Ori-S sequence of HSV-1: identification of one functional DNA binding domain

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ABSTRACT

Using gel retardation assays, we have detected an Ori-S binding activity in the nuclear extract of HSV-1 infected Vero cells. The sequence-specific DNA binding activity seems to be identical to that described by Elias et al. (Proc. Natl. Acad. Sci. USA 83: 6322-6326, 1986). This activity fails to retard a mutant origin DNA that has a 5 bp deletion in the reported protein binding site along with an A to T substitution at a position 16 base-pairs away from the site. This mutant also failed to replicate in a transient replication assay, thus correlating binding of the factor on the origin to replication efficiency. Using crude nuclear extracts as the source of the factor and with the help of footprint and gel retardation analyses, we confirmed that protection is only observed on the preferred site of binding on and near the left arm of the Ori-S palindrome. In order to analyze the sequence specificity of the binding we have generated a set of binding site mutants. Competition experiments with these mutant origins indicate that the sequence 5'-TTCGCACTT-3' is crucial for binding.

INTRODUCTION

In contrast to the prokaryotic systems, the complexities of the size and organization of eukaryotic genomes and the general lack of genetic analysis pose difficulties in exploring the events of initiation of DNA replication. Very little is known about the structural features of the origins of replication and the events that take place during initiation in mammalian nuclei. However, studies of DNA replication of eukaryotic viruses and of prokaryotic systems suggest a general model that appears to be common to sequence-specific initiation of replication (3). In this model, one of the first events in initiation is the sequence-specific binding of an initiator protein to the origin of replication. This event is usually followed by the formation of a specialized nucleoprotein structure on the origin

(1,3,12-14,21), localized strand opening (2,28), and attachment of DNA polymerase and other factors initiating DNA replication in the leading strand (3).

To understand the initiation of DNA replication in a eukaryotic system we have chosen herpes simplex virus type 1 (HSV-1). Since HSV-1 encodes for a number of gene products necessary for its own DNA replication (5,27), the process of replication could in theory be analyzed using genetic as well as biochemical approaches. Electron microscopic studies of replicating viral genomes and analysis of defective interfering particles localized three presumptive replication origins on the viral genome (17-20,22,23,29). Plasmids bearing these origin sequences are amplified in transient replication assays if viral gene products are supplied in trans either by infection with wild-type virus (25,29,33) or by co-transfection with a set of plasmids harboring a defined set of viral DNA sequences (5,34). HSV-1 has one Ori-L and two Ori-S sequences that can serve as origins in these types of assays. The two Ori-S regions are of identical sequences residing on the 'c' repeats, while Ori-L lies on the middle of the U-L sequence (27). The Ori-S sequence contains a 45 bp long palindrome with an 18 bp long AT stretch at the center (32).

Elias et el. (15) identified a factor in HSV-1 infected nuclear extracts that can bind to a region on and near the left arm of the Ori-S palindrome. Although similar sequences are present on the right, the binding affinity for the site detected on the left is at least ten-fold higher than that on the right (16). Recently, Olivo et al. (26) have found evidence to identify the gene responsible for the expression of this origin binding protein. However, site-directed mutagenesis modifying the sequence at the binding site was not tested for DNA binding in order to look for essential sequence that is essential for the binding.

In the present communication, we detail our study for sequence requirement for protein-DNA interactions at the Ori-S sequence using HSV-1 infected nuclear extracts as a protein source and gel retardation as a method of analysis. We detected one prominent DNA-protein complex. DNaseI footprint analyses

indicated binding at a site similar to that as detected earlier (15) at the left end of the 45 bp palindrome. We were unable to detect any significant protection on the right side. An origin mutant, with a five base-pair deletion at the protected region along with a single A to T substitution at a position 16 base-pairs away from the left end of the binding site, lost capacity to bind to the factor present in the nuclear extract as well as to function as an origin in the *in vivo* replication assay. We have done systematic mutagenesis of the binding site and used the mutant origins as competitors in the gel retardation assay. These competition analyses of the binding site defined 5'-TTCGCACTT-3' as crucial for the binding domain. This domain includes 9 out of 11 base-pairs of the sequence 5'-CGTTCGCACTT-3' found also in the Ori-S region of varicella zoster virus (31).

MATERIALS AND METHODS

Plasmids and Recombinant Origins Constructions

Construction of Ori-S containing plasmids has already been described by Deb and Doelberg (8). In brief, origin sequences have been cloned in between HindIII and NcoI sites of a pOR vehicle (11) which consists of pML2 sequences 651 to 4361 with a HindIII linker added to the 651 site and a polylinker (NcoI, SalI, BamHI and XmaI) added to the EcoRI site at nucleotide 4363. The cloning of the recombinant origins, including the base-substitution mutants, was done using cassettes of overlapping oligonucleotides as described previously (6-9). Del-1 was obtained as an unplanned recombinant clone while screening for wild-type pOR-S clone (8). The plasmid clone H-62 has been generated by controlled Bal 31 deletion from the HindIII end of the origin mutant pOR-S1 following the methodology described in detail by Deb et al. (6). The plasmid DNAs were prepared and purified as described by Deb et al. (6-9).

Cells and Viruses

Vero cells were grown in Dulbecco modified Eagle medium supplemented with 5% newborn bovine serum, while BHK-C21 cells were grown in DME supplement with 10% calf serum and 10% tryptose phosphate broth. The virus strain used for preparation of infected cell extract was HSV-1 (HFEM).

Plasmid Amplification Assay. Transient plasmid amplification assays were carried out using BHK-C21 cells and HSV-1 (F) as described by Deb and Doelberg (8). Briefly, 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; this was followed by a dimethyl sulfoxide boost at 4 h. Incubations were carried out at 37°C. At 6h 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 HindIII and MboI. HindIII linearizes the plasmid and Mbol cleaves GATC sequences only when the adenine in the site remains unmethylated. Since our plasmids were grown in dam-positive DH5 bacteria, GATC sequences remained methylated at the adenines and hence uncleavable with MboI. Thus, MboI 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.

Preparation of HSV-1 Infected Cell Nuclear Extracts

HSV-1 infected nuclear extracts were prepared essentially as described by Elias et al. (15). In short, three roller bottles of confluent Vero cells were infected with the virus at 5-10 pfu/cell, and harvested at 18 hours post-infection. Nuclei were collected, suspended in 20 mM Hepes, pH 7.6, 0.5 mM dithiothreitol (DTT), 10 mM $NaHSO_3$, pH 7.0, 0.5 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.), 2 µg/ml leupeptin (Sigma Chemical Co.) and 10% sucrose. Nuclei were lysed by adding an equal volume of 3.4 M NaCl in the same buffer and swirling in a flask. The high salt extract was then separated from insoluble material by centrifugation. No ammonium sulfate precipitation was attempted at this stage. The supernatant was dialyzed for 12 hours against the same Hepes buffer as described above except that 10% glycerol was used instead of 10% sucrose. The dialyzed material was stored in aliquots at -80°C until use. For all our assays we used this nuclear extract as the source of infected cell nuclear protein.

Gel Electrophoresis DNA Binding Assay

Protein-DNA complexes were resolved on low ionic strength polyacrylamide gels as described by Carthew et al. (4) with slight modifications. Protein samples were incubated with 2000-3000 cpm (0.6 ng) of an end-labeled double-stranded DNA fragment in the presence of 1 µg poly(dI-dC).poly(dI-dC) (Pharmacia) in a final volume of 50-100 µl. Incubations were carried out on ice for 30-60 minutes in a binding buffer (50 mM Hepes adjusted to pH 7.5 with NaOH, 0.1 mM EDTA, 0.5 mM DTT, 10% (wt/vol) glycerol, and 100 mM NaCl). Samples were layered onto low ionic strength 4% polyacrylamide gels (acrylamide: bisacrylamide weight ratio 80:2). Gels were pre-electrophoresed for 3 hours at 4°C at 20 mA in a buffer consisting of 6.7 mM Tris-HCl (pH 7.9), 3.3 mM Na-acetate and 1 mM EDTA, which was recirculated between the compartments. Gels were electrophoresed at 30 mA at 4°C for different time intervals, depending on the length of the end-labeled fragments used. They were then transferred to Whatman 3MM filter paper, dried and autoradiographed.

DNaseI Footprinting

Protein samples were incubated with radiolabeled probe in the presence of poly(dI-dC).poly(dI-dC) as above in 100 µl for 30 to 40 minutes on ice. Samples were then treated with 5 µl of DNase I (Worthington) in 5 mM $MgCl_2$ and 2.5 mM $CaCl_2$ (final concentrations) at concentrations as indicated in the figure legend for 5 minutes on ice. Reactions were stopped by adding EDTA to a final concentration of 10 mM as described by Carthew et al. (4). Samples were filtered through nitrocellulose filters to remove unbound labeled DNA and washed with 15 ml of the binding buffer containing 10 mM EDTA. The protein bound DNA was eluted from the filter with 200 µl of 10 mM Tris-borate (pH 8.3), 0.2% sodium dodecyl sulfate (SDS), 10% glycerol and 1 mM EDTA for four hours at 45°C. Control end-labeled DNA samples were treated similarly except that they were incubated with equivalent amounts of bovine serum albumin (BSA) instead of nuclear extract, and the DNaseI digestion was stopped by the addition of 105 µl of 1 M ammoniumacetate, 0.2 % SDS, 0.1 mM EDTA at pH 7.0. The DNAs were ethanol precipitated and analyzed on an 8% urea-sequence gel as described previously (10).

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          10        20        30        40        50        60        70        80        90       100
GGCCGCCGGGTAAAAGAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCCAATATATATATATTATTAGGGCGAAGTGCGAGCACTGGCGCCGGCCCCGGG
CCGGCGGCCCATTTTCTTCACTCTTGCGCTTCGCAAGCGTGAAGCAGGGTTATATATATATAATAATCCCGCTTCACGCTCGTGACCGCGGCCGGGGCCC   pOR-S

GGCCGCCGGGTAATAGAAGTGAGAACGCG      TCGCACTTCGTCCCAATATATATATATTATTAGGGCGAAGTGCGAGCACTGGCGCCGGCCCCGGG
CCGGCGGCCCATTATCTTCACTCTTGCGC      AGCGTGAAGCAGGGTTATATATATATAATAATCCCGCTTCACGCTCGTGACCGCGGCCGGGGCCC   del-1

GGCCGCCGGGTAAAAGAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCCAATATATATATATTATTA
CCGGCGGCCCATTTTCTTCACTCTTGCGCTTCGCAAGCGTGAAGCAGGGTTATATATATATAATAAT                                   pOR-S1
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Figure 1. Ori-S-Derived Sequences. pOR-S contains the entire origin region (31). The region on the left protected by the Ori-S binding protein from DNaseI (15) is underlined. A similar sequence on the right is also underlined. The plasmid del-1 deletes the sequence from position 30 to 35 and has an A to T substitution at position 14. pOR-S1 contains the sequence from position 1 to 67. Nucleotide numbers are indicated as described earlier (8). The origin sequences are flanked by a HindIII site on the left and an NcoI site on the right.

RESULTS

Detection of DNA-protein complexes formed by the interaction of the Ori-S and HSV-1-infected cell nuclear extract

In this study we have used a modification of the gel electrophoresis DNA binding assay as described by Carthew et al. (4) to detect any factor(s) that can specifically bind to the Ori-S of HSV-1. To reduce the effect of nonspecific DNA binding proteins we have used poly(dI-dC).poly(dI-dC) as a nonspecific competitor in each assay system. Extracts made from HSV-1 infected Vero cell nuclei were incubated with the HindIII-EcoRI fragment of pOR-S (Figure 1) containing the Ori-S DNA, labeled at the HindIII end. In the experimental data depicted in Figure 2, identical amounts of end-labeled probe DNAs were incubated with increasing amounts of nuclear extract in the presence of a fixed amount of poly(dI-dC).poly(dI-dC) and then analyzed in the low-ionic strength polyacrylamide gel. In the absence of added protein, the end-labeled fragment migrated as a slightly diffused band of free DNA (first lane from the left). In the presence of 1 µg of nuclear protein, a definite retarded band was visible with some DNA migrating at the position of free DNA (second lane). Under longer exposures other less prominent bands became visible (data not shown). At a higher protein concentration of 4 µg, no free DNA was observed. Under these conditions the intensity of the discrete retarded band decreased (with smearing at the top)

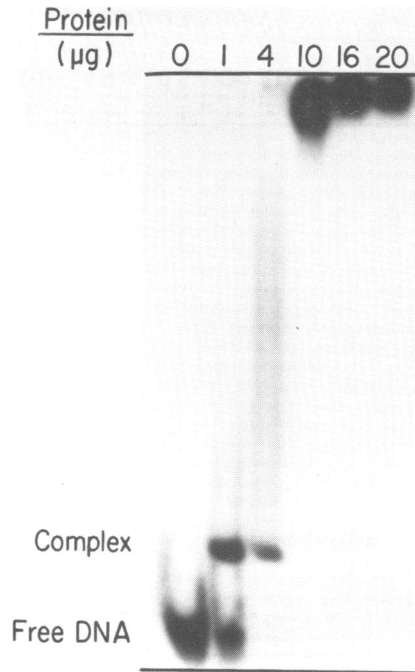

Figure 2. Detection of DNA-Protein Complex Between the Ori-S and HSV-1-Infected Cell Nuclear Extracts by the Gel Retardation Assay. The assay procedure is described in "Materials and Methods". Each lane contained 0.6 ng of isolated end-labeled origin sequence from pOR-S and 1 µg of poly(dI-dC).poly(dI-dC). Increasing amounts of extract were incubated with the fixed amount of the probe. Positions of the free DNA and complexed DNA are indicated.

and labeled DNA accumulated at the top of the lane (third lane). Further increases in the amount of extract resulted in the accumulation of the entire labeled DNA near the top of the lane with no free DNA or discrete retarded bands in the lanes (4th, 5th and 6th lanes). Thus, at a certain concentration of protein, end-labeled probe and non-specific competitor polymer, one major retarded band was observed (2nd lane), suggesting complex formation between Ori-S DNA and protein(s) present in HSV-1 infected cell nuclear extracts. For all our further work we have used the conditions employed for the 2nd lane (Figure 2) to analyze the Ori-S-protein complex, because these conditions

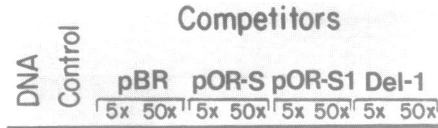

Figure 3. Effect of Competitor DNAs on the DNA-Protein Complex Formation. The general procedure for this gel retardation assay is described in the "Materials and Methods" and in the legend to Figure 2. Two different concentrations of the competitors are used in 5 and 50 molar excess in terms of total nucleotide equivalents. Competitors used were whole plasmid DNAs. Each incubation mixture also contained 1 µg of poly(dI-dC).poly(dI-dC), 1 µg of infected cell nuclear extract and 0.6 ng of end-labeled Ori-S probe. "DNA" indicated the lane containing the incubation system in the absence of nuclear extract. "Control" indicated the incubation system with probe and extract in the absence of any competitor DNA except poly(dI-dC).poly(dI-dC). All the other lanes contained competitor DNAs as indicated.

yielded optimum discrete complex formation. Although not shown, Vero cell extracts in the absence of HSV infection failed to produce specific retarded bands under similar conditions. This indicates that the Ori-S binding factor is a virus encoded gene product or a virally induced cellular factor.

Specific DNA sequences are involved in the formation of the retarded band

We then examined whether the retarded band was formed because of sequence-specific interaction of a protein(s) with the

Ori-S. For this we carried out the incubations of end-labeled wild-type origin with the infected nuclear extract in the presence of different competitor DNAs and a constant amount of 1 µg poly(dI-dC).poly(dI-dC). No attempt was made to linearize the competitor DNAs or to use isolated DNA fragments. All the competitors have the same vector sequences. Molarity of competitors used were 5x and 50x in terms of total nucleotide equivalents of that of the probe DNA fragment. Figure 3 shows one of the autoradiograms obtained after a gel retardation assay with the protein and end-labeled probe incubated with pBR322 (third and fourth lanes); pOR-S (Figure 1), which contains the entire wild type origin (fifth and sixth lanes); pOR-S1 (Figure 1), which contains the 67 bp long origin sequence with a deletion of the GC-rich part of the right arm of the Ori-S palindrome (seventh and eighth lanes); and del-1 (Figure 1), which internally deletes 6 bp from the binding site observed by others (15) (ninth and tenth lanes). The plasmid del-1 has also one A to T substitution at position 14. A visual examination of the intensities of the retarded bands clearly indicates that while pBR322 was the least effective competitor, both pOR-S and pOR-S1 were quite efficient. The competition exerted by del-1 was much less than that by pOR-S or pOR-S1. The results of our competition experiments strongly suggest that the Ori-S sequences 30 to 35 are involved in the DNA-protein interaction. This conclusion is in agreement with the results of others (15,16). The competitions exerted by the internal deletion mutant also indicate that the second binding site has less affinity for the protein.

DNaseI footprinting to localize the binding site

In order to localize the sequences on the Ori-S that are protected from DNaseI by the complex formation, we performed footprint analyses using end-labeled Ori-S fragments and infected Vero cell nuclear extracts. The binding was done under the same conditions as in the gel retardation assay. After 30-40 minutes on ice DNaseI was added. The DNaseI was inactivated five minutes later by adding EDTA. To decrease the background we separated the protein bound DNA by the nitrocellulose filtration procedure as described in "Materials and Methods". The

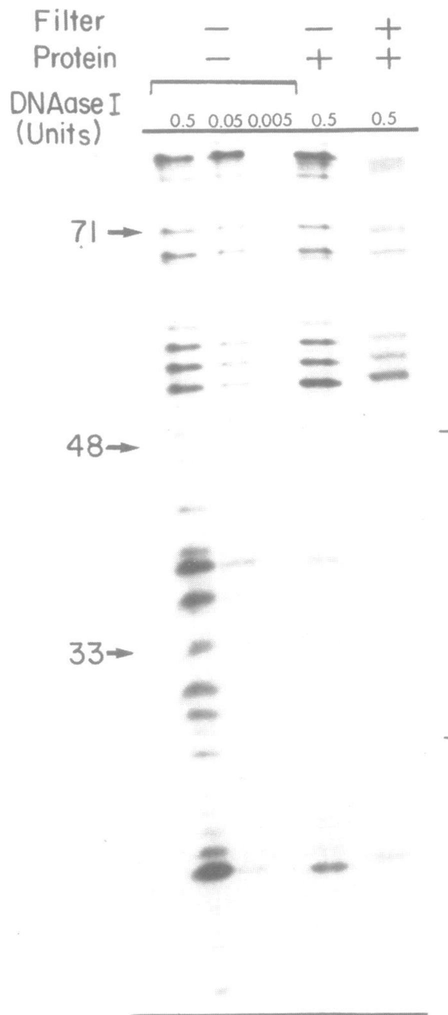

Figure 4. DNaseI Footprint of the Nuclear Extract. HindIII end-labeled HindIII-EcoRI (2.5 ng) fragments containing Ori-S from pOR-S were incubated with 4 µg of infected cell nuclear extract and 2.5 µg of poly(dI-dC).poly(dI-dC) on ice as described in "Materials and Methods". The mixtures were treated with the indicated amounts of DNaseI and either stopped by adding 105 µl of 1 M ammonium acetate, 0.2 % SDS, 0.1 mM EDTA at pH 7.0, or EDTA alone to a final concentration of 10 mM. In the latter case the protein-DNA complexes were isolated by nitrocellulose filtration. The samples were then analyzed as described in the text. Free DNA was treated with three different concentrations of DNaseI to generate an appropriate ladder. The footprint was visible in the lane where no filtration was used. However, the

protein-bound DNA was eluted from the nitrocellulose filter, analyzed on a sequencing gel and compared to extract-untreated DNA that was similarly DNaseI treated. As evident from Figure 4, a minimum protection of a region covering the sequence 5'-GGACGAAGTGCGAACGCT-3' of the lower strand (Figure 1) was observed. The extent of protection matches the protection observed by others on the left arm of the palindrome using more purified preparations of Ori-S binding protein (15,16). In fact, the protection observed by us was slightly more extensive than that observed by others, but that could reflect differences in experimental approaches and purity of the factor. This may also be indicative of our observation of multiple protein-DNA interaction at the Ori-S sequence. Although it is difficult to interpret the data because of the presence of gaps in part of the region in the DNaseI control reaction on protein-free DNA, our footprint analyses did not reveal any significant protection on or near the right arm of the palindrome where another similar sequence exists. This observation is in agreement with that reported by Elias et al. (15).

<u>The retarded band is formed by complex formation on the left side of the palindrome</u>

We took a two-fold approach to determine if the retarded band was formed due to complex formation at the protected site. First, we determined whether or not the origin fragment from the mutant del-1 (Figure 1), which deletes part of the DNaseI protected region, can be retarded by the infected Vero cell nuclear extract. Second, we examined whether or not the origin fragment from the mutant pOR-S1, which deletes the right arm of the palindrome but retains the intact DNaseI protected region, can form complexes effectively with the infected Vero cell nuclear extract.

The data presented in Figure 5a show that under the conditions of the gel retardation assay, while wild-type origin

footprint became most prominent in the lane containing isolated protein-DNA complex. The bracketed area represents the minimal area of protection, although the actual footprint visible is more extensive. The numbers on the left edge of the figure correspond to the nucleotide positions of pOR-S (Figure 1).

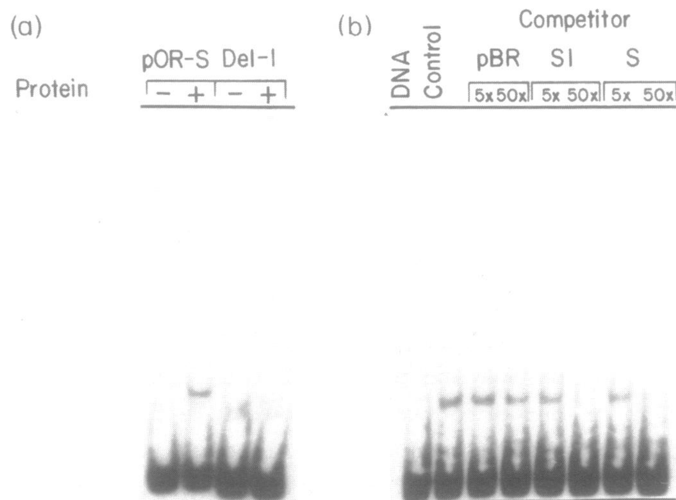

Figure 5. Gel Retardation Assay with Ori-S Mutant. (a) End-labeled HindIII-EcoRI origin fragments from pOR-S (first and second lanes from the left) and del-1 (third and fourth lanes) were analyzed by the gel retardation procedure described in "Materials and Methods" after incubation with nuclear extracts in the presence of poly(dI-dC).poly(dI-dC). The absence of a retarded band in the lane containing del-1 fragment and protein indicates failure of the del-1 origin to form an effective complex. (b) End-labeled HindIII-EcoRI origin fragments from pOR-S1 were assayed for complex formation. The presence of a specific retarded band in the control lane that contained pOR-S1 origin fragments incubated with the extract in the presence of poly(dI-dC). poly(dI-dC) indicates formation of a protein-DNA complex. The complex was specifically competed out by pOR-S1 or pOR-S DNA but not by pBR322 when incubations were done in the presence of 5 or 50 molar excess of competitor DNAs. "DNA" indicates the lane containing the incubation system in the absence of nuclear extract. "Control" indicates the lane containing the incubation system in the presence of the extract but in the absence of any competitor DNAs except poly(dI-dC).poly(dI-dC). "S1" and "S" indicate pOR-S1 and pOR-S respectively.

fragments from pOR-S can efficiently form the retarded band, the mutant origin fragment from del-1 cannot. The fact that the internal deletion mutant failed to form the complex suggests the importance of the deleted sequence 5'-ACGCTTC-3' in the binding, although theoretically one can still assume that the defect may be due to the A to T change at position 14 or the combination of substitution and deletion. The deleted sequence overlaps

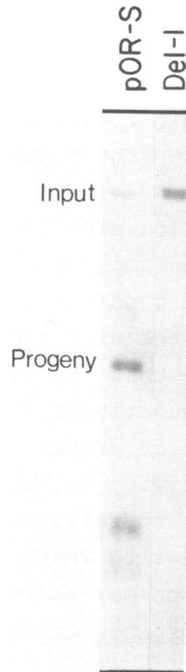

Figure 6. Transient Replication of del-1. The assay conditions have been described in the text. The absence of progeny bands in del-1 lane indicates partial or total failure of del-1 origin sequence to function as a HSV-1 activated origin.

partially with the protected region. Thus, retarded band formation requires the presence of sequences previously shown to be protected from DNaseI by the Ori-S binding protein (15,16). The absence of the formation of the retarded band with the del-1 origin fragment also shows that under the conditions of the assay the right hand binding site for the protein (15) is not significantly active assuming that binding at the left hand binding site has no effect on binding at the right hand binding site.

Figure 5b shows that the origin fragment from pOR-S1 is capable of forming a retarded band under the same conditions when the wild-type fragment retards but the internal deletion mutant del-1 does not. The complex formation can be efficiently

competed by the presence of pOR-S or pOR-S1 DNA but not by nonspecific pBR322 DNA. This shows sequence specificity of the complex formed between the factor(s) in the infected cell nuclear extract and the pOR-S1 origin fragment. Therefore, the complex formation can occur with one site present only.

Replication of del-1.

We tested the replication efficiency of the plasmid del-1 by performing transient plasmid amplification assay. As shown in Figure 6 this mutant origin containing plasmid showed little or no replication activity while the wild-type pOR-S replicated as usual (8). This result signifies that the sequences deleted internally in the plasmid and/or the substitution at position 16 not only are important for the formation of protein DNA-complex but also are crucial for replication activity as judged by this transient replication assay. Thus, a correlation between the binding of the factor to the origin at the site where the origin binding protein binds and the origin function may be assumed.

Evaluation of relative importance of the sequences in the binding site

The specificity of the sequences protected by the complex formation from DNaseI cleavage was evaluated by using competition experiments in the gel retardation assay. To achieve this objective we have synthesized binding site mutants as shown in Figure 6. Six of them are triple substitution mutants in which three consecutive base-pairs are substituted. In these cases, Ts were substituted by Gs, As were substituted with Cs and vice versa. We also used two other mutants del-31 and bs-1' whose sequences are also shown in Figure 7. To determine the relative importance of the nucleotides in binding, we incubated the end-labeled origin insert of pOR-S1 with the infected Vero cell nuclear extract in the presence of individual cold plasmid DNAs mutated at the binding site in 50 molar excess over the probe in terms of total nucleotide equivalents. As demonstrated in Figure 6 all the competitor DNAs except bs-9, -10, and -11 can compete with the end-labeled origin insert of pOR-S1 for the complex formation quite efficiently, suggesting that sites altered in them are not crucial for binding, while at least some of the

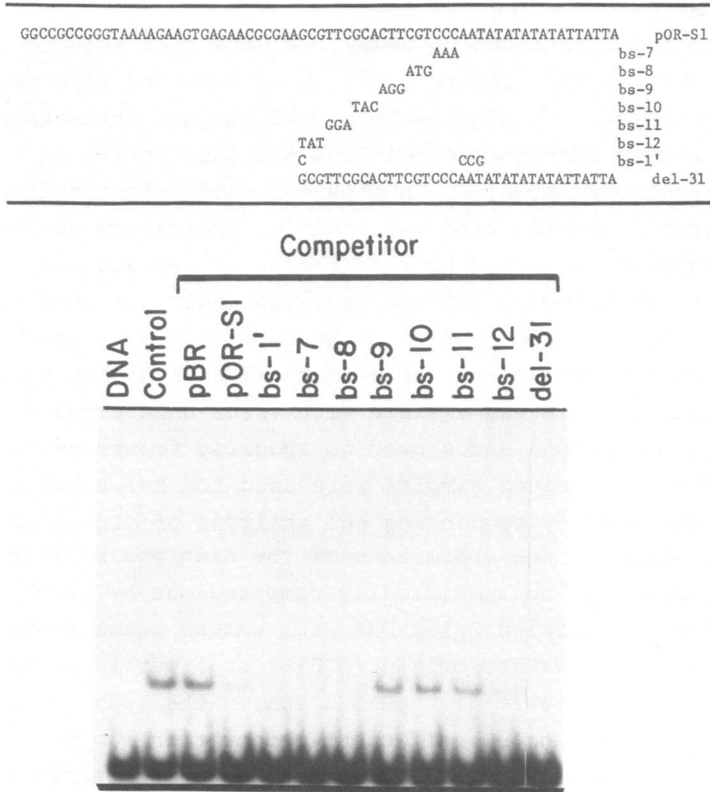

Figure 7. Sequence Specificity of the Binding Site. The various mutant origin sequences used to determine the binding site sequence-specificity are shown at the top of the figure. The HindIII-EcoRI origin fragment from pOR-S1 was used as a probe to determine the binding capacity of the nuclear extract in the presence and absence of different competitor DNAs. "Control" lane indicates incubation in the absence of any competitor except poly(dI-dC).poly(dI-dC). The conditions of the assay have been described in the text. The intensity of the retarded bands indicates the capacity of individual binding site mutants to compete the complex formation between wild-type pOR-S1 probe and the DNA binding activity in the extract. A 50-fold molar excess of the competitors over the probe in terms of total nucleotide equivalents was used.

nucleotides altered in bs-9, -10 and -11 are critical for efficient competition. Thus, these results indicate that at least part of the sequence 5'-TTCGCACTT-3' at the site protected from DNaseI is essential for efficient binding.

DISCUSSION

Using a gel retardation assay, we have been able to identify a specific Ori-S DNA binding activity in infected Vero cell nuclear extracts. Uninfected Vero cell nuclear extracts prepared under otherwise identical conditions are ineffective in Ori-S binding (data not shown). In this gel retardation analysis only one prominent retarded band was visible, indicative of one major type of complex formation between the Ori-S DNA and the factor present in the infected nuclear extracts under our conditions of analyses. We have also observed the formation of a larger protein-DNA complex at the top of the gel at a higher protein concentration. This was visible with virus-uninfected extracts also (data not shown) and showed no specific footprint when limited DNase I cleaved samples were used for gel retardation analysis followed by sequencing gel analysis of eluted complex (data not shown). The appearance of the discrete band (Figure 2, lanes 2 and 3) can be specifically competed out by incubating the extract and end-labeled Ori-S DNA with excess plasmid DNA containing the wild-type origin (pOR-S) or a partially deleted origin (pOR-S1) (Figure 3). pBR322 DNA is the least active in the competition assay while inefficient competition was also observed with del-1. The del-1 plasmid deletes sequences within the binding site identified by others (15,16) on and near the left arm of the palindrome and also has an A to T substitution at position 14. The inefficient competition tends to localize at least some part of the sequences required for binding within base-pairs 30-35. These observations demonstrate the sequence-specificity for the complex formation and localize one binding determinant near the left arm of the 45 bp palindrome in a region where others have found a binding site for a virus encoded protein (15,16, 26).

The mutant del-1 cannot compete efficiently with the wild-type origin for the complex formation (Figure 3) while pOR-S1, which retains the left half of the palindrome only, can. As explained above, this implicates the involvement of sequences at the left arm for DNA-binding. We verified our idea that del-1 cannot efficiently bind the factor while pOR-S1 can by performing gel retardation assays using these mutant origins as probes. Data presented in Figure 5 showed that while pOR-S1 was capable

of forming the complex, del-1 was not, showing the sequence contained in pOR-S1 has the ability to form a complex with the factor in the infected nuclear extract. It is interesting to note, in this regard, that pOR-S1 has the information to replicate in the transient replication assay (8) and has both the regions of sequence homology between varicella zoster virus (VZV) Ori-S and HSV-1 Ori-S (31). VZV Ori-S does not have any sequence homology with the right arm of the Ori-S palindrome of HSV-1 and can function as the origin of replication in the transient replication assay if superinfected with HSV-1 or VZV (31).

We have confirmed the binding site by performing DNaseI footprint analysis using crude nuclear extracts as the source of the DNA binding protein (Figure 4). Enrichment of protein-DNA complexes over unbound DNA was achieved by isolating the protein-bound DNA on a nitrocellulose filter. The footprint demonstrates a protection of at least 5'-GGACGAAGTGCGAACGCT-3' on the lower strand from position 31 to 48 (Figure 1). This matches with the data presented by Elias et al. (15).

Gel retardation assays in the presence of a planned set of binding site mutants showed the importance of the sequence 5'-TTCGCACTT-3' from position 35 to 43 (Figure 1) which includes the guanines at positions 39 and 41 whose methylations interfere with the binding (unpublished data). The exact sequences necessary and sufficient for efficient binding have yet to be determined. The second binding site for the Ori-S binding protein as identified by others (15,16) differs from this one at positions 33, 35 and 46, of which only the nucleotide at position 35 is included in the crucial sequence-specific domain identified by us. This suggests that high-affinity binding depends on the nucleotide at position 35, since the two binding sites differ in their affinities by about 10-fold for the Ori-S binding protein (16). An examination of the deleted sequence in del-1 (Figure 1) and a comparison of that with the domain 5'-TTCGCACTT-3' identified by us reveal that del-1 deletes the T at position 35. It is possible that this single base-pair deletion is enough to cause failure of binding capacity. It will be important to change one nucleotide at a time in the binding domain to determine which

nucleotides are crucial for high-affinity binding.

The factor responsible for the complex formation is most probably the same as detected by others (15,16,27). This conclusion is partly based on the similarity of the footprint data obtained by us and Elias et al. (15). Other strong evidence showing similarity of binding activity is our competition experiments. These experiments which use mutants where specific nucleotides in the footprinted region have been mutagenized prove that alteration of sequences in the specific areas of the footprinted region alters binding affinity. This not only shows similarity of binding activities found by us and Elias et al. (15) but also identifies a 9 bp motif that is crucial for binding. Both of our footprint data with and without isolated protein-DNA complex and the gel-retardation data indicate one major type of complex formation. We therefore believe that they represent one and the same complex, which is very similar to that observed by Elias et al. (15). The differences between our gel retardation experiments and that reported by Elias and Lehman (16) may be the result of the differing gel concentrations and fragment lengths and/or the fact that at a higher purified protein concentration they get protein binding on both arms of the palindrome. However, in the absence of purified protein, in our case, it is not possible to directly compare our mobility shift experiments with that done by Elias and Lehman (16).

While this manuscript was in preparation, Koff and Tegtmeyer (24) reported an elegant analysis of the binding site for the origin binding protein. Using methylation interference and DNaseI footprinting analyses they have arrived at a domain 5'-GTTCGCAC-3' very similar to us. Work from Mark Challberg's laboratory (26,34) has demonstrated that the UL9 gene product of HSV that probably codes for this origin binding protein is essential for DNA replication. It seems very likely that binding of the Ori-S binding protein to the Ori-S is necessary for replication, because from our data (Figure 5) it appears that del-1 may have completely failed to replicate with a concomitant failure to bind to the factor present in the infected cell nuclear extract. At this stage we are in the dark about the actual biochemical function of the Ori-S binding activity in DNA

replication. It will be imperative to purify the protein (16) and test for its possible function(s). Identification of this sequence-specific DNA binding protein which interacts with the HSV-1 origin of replication raises the possibility of it being the initiator protein. This initiator protein binding may trigger a specialized nucleoprotein structure formation at the origin as in the case of oriC, lambda and SV40 DNA replication (10,12,13,21). In both E. coli oriC and SV40 systems the initiator protein, in the presence of ATP, makes a wider complex at the origin and begins a localized unwinding there (2,10,12). In the case of lambda, the O protein starts a nucleoprotein structure and melts the origin locally in the absence of ATP (28). It will be important to investigate the functional aspects of the protein keeping these ideas in mind.

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