COOPERATIVE ASSEMBLY OF EBNA1 AND INDUCED DNA DISTORTION
CHARACTERIZATION OF EBNA1 COOPERATIVE INTERACTIONS
AND EBNA1-INDUCED ORIGIN DNA DISTORTION

By
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A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree
Master of Sciences

McMaster University
September 1996
Characterization of EBNA1 cooperative interactions and EBNA1-induced origin DNA distortion

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ABSTRACT

Epstein-Barr virus nuclear antigen 1 (EBNA1) is the only viral product needed for replication of the latent Epstein-Barr virus genome. The latent origin of replication, oriP, consists of two cis-acting elements, the family of repeats (FR) containing twenty EBNA1 recognition sites, and the dyad symmetry element (DS) containing four recognition sites. Bidirectional DNA replication is known to occur within or near the DS. Previous studies have suggested that EBNA1 binds cooperatively to its recognition sites in the DS and have shown that EBNA1 binding induces DNA distortion within site 1 and site 4 of the DS. I have used EBNA1 mutants in electrophoretic mobility shift assays, methylation protection footprinting, and potassium permanganate reactivity analysis to examine EBNA1 assembly on the DS and the requirements for DNA distortion. I have found that: 1) EBNA1 has a 10-11 fold higher affinity for the outer two sites of the DS than the inner two sites due to DNA sequence variation, 2) the minimum region of EBNA1 necessary for site specific binding is contained within amino acids (a.a.) 470-607 but a.a. 459-470 greatly affect binding affinity, 3) EBNA1 dimers bind cooperatively on
adjacent binding sites and the region responsible for this interaction is also contained between a.a. 470-607. I have also shown that EBNA1 binding to a single DS site 1 recognition site is sufficient to induce DNA distortion within that site and this distortion can be caused by a truncation mutant spanning a.a. 463-607 but not a.a. 468-607. Finally, although wild type spacing between recognition sites of the DS is critical for replication it is not crucial for EBNA1 binding or EBNA1 induced DNA distortion.
ACKNOWLEDGEMENTS

I would like to thank Dr. Lori Frappier for her support, patience and help throughout my studies and in the writing of this thesis. Patricia Vinton's help in the preparation of the figures was invaluable and greatly appreciated. My introduction to the lab was greatly aided by Jean Barwell's guidance and patience, her support throughout my studies has been immensely appreciated. I would also like to thank the members of the Edwards and Frappier labs, especially, Steve Orlicky, Sally Hemming, Richard Pfuetzner, Derek Ceccarelli, Angela Fleming, Kathy (BOSS) Shire, and Carrie (C.R.) Rosenberger, for their support, instruction, laughter and continued friendship. And lastly, I thank my family and Dave Kundapur for the support and understanding that was instrumental in the completion of this thesis.
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ABBREVIATIONS

A  adenosine
a.a. amino acid
Ala alanine
BAP bovine alkaline phosphatase
bp base pair
DMS dimethyl sulfate
DNA deoxynucleic acid
dNTPs deoxynucleic triphosphates
DS dyad symmetry element
DTT dithiothreitol
EBNA1 Epstein-Barr virus nuclear antigen 1
EBV Epstein-Barr virus
E. coli Escherichia coli
EDTA ethylenediaminetetraacetic acid
EM electron microscopy
EMSA electrophoretic mobility shift assay
FR family of repeats
G guanine
HEPES N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC high pressure liquid chromatography
<table>
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<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tr>
<td>IPTG</td>
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</tr>
<tr>
<td>Kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>$K_d$</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>KMnO$_4$</td>
<td>potassium permanganate</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>NEB</td>
<td>New England Biolabs</td>
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<tr>
<td>OBP</td>
<td>origin binding protein</td>
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<tr>
<td>OBR</td>
<td>origin of bidirectional replication</td>
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<tr>
<td>O.D.</td>
<td>optical density</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>Phe</td>
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</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
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<td>simian virus 40</td>
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<tr>
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<td>thymine</td>
</tr>
<tr>
<td>TB</td>
<td>terrific broth</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
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I. Introduction

I.1. Epstein-Barr Virus and Associated Diseases

Greater than 95% of the entire human adult population is infected with Epstein-Barr Virus (EBV) (reviewed in Rogers et al., 1992). EBV is a double stranded DNA herpesvirus. Initially the virus infects, and replicates in, epithelial cells of the oropharynx. Shortly after primary infection the virus will infect B lymphocytes where it generally does not produce infectious particles, but remains in a latent state (Kieff and Liebowitz, 1990). EBV is able to immortalize B lymphocytes that it infects, in that it induces and maintains the proliferation of the infected B lymphocyte (reviewed in Middleton et al., 1991). The virus has been associated with several diseases including infectious mononucleosis, nasopharyngeal cancer, Burkitt's Lymphoma, Hodgkin's disease and various lymphomas found in immunocomprised individuals such as AIDS patients (reviewed in Miller, 1990). The virus consists of a 172 kilobase (Kb) linear genome which circularizes upon release into the nucleus where it is maintained in the latent state as a circular episome (Shaw et al., 1979; Baer et al., 1984).
I.2. Latent EBV DNA Replication

EBV is maintained latently in vivo as an extrachromosomal nuclear episome. The viral genome is replicated once per cell cycle during S phase (Adams, 1987; Yates and Guan, 1991). Bidirectional DNA replication of the latent EBV genome occurs from the viral latent origin of replication, oriP (Yates et al., 1984; Gahn and Schildkraut, 1989). Only one viral product, Epstein-Barr Virus Nuclear Antigen 1 (EBNA1), is necessary for replication of the latent viral genome (Reisman et al., 1985; Yates et al., 1985). EBNA1 is the viral origin binding protein (OBP) which binds to specific recognition sites within oriP (Rawlins et al., 1985) and has no intrinsic enzymatic activity (Frappier and O'Donnell, 1991a); all remaining replication functions are provided by the host cell (Yates et al., 1985). Replication of the latent EBV genome is a good model for studying cellular replication mechanisms because latent EBV DNA replication proceeds from a known origin, requires only one viral product with all other replication functions provided by host cellular machinery, is stably maintained, and undergoes replication once per cell cycle.
I.3. EBV Latent Origin of Replication, oriP

EBV has a well defined latent origin of replication, oriP. The origin consists of two cis-acting functional elements separated by approximately 960 base pairs (bp) of DNA, the family of repeats (FR) and the dyad symmetry element (DS) (Figure 1). The FR contains twenty tandem 30 bp repeats each containing an 18 bp EBNA1 binding site, while the DS contains four EBNA1 binding sites. The DS is so named because two of the four binding sites lie in a 65 bp region of dyad symmetry (Reisman et al., 1985; Jones et al., 1989).
Figure 1. The latent origin of replication, oriP. Model of oriP displaying the FR and DS. The exact sequences of binding sites within the DS are shown as well as the spacing between the sites. The circled Ts represent thymine residues which become reactive to potassium permanganate upon EBNA1 binding.
oriP

Family of Repeats

Dyad Symmetry

EBNA-1 Binding Site

Dyad Symmetry

Site 4

CGATAGCATATGCT10CCC
SCTACGTATACGAAAGG

Site 3

GGTAAACATATGCTATTG
CCCTTGTATAGATAAC

Site 2

GGATGTATATACTA
CCTATGATGAT

Site 1

GGGAGCATATGCTACCC
CCCTTGATACGATGGG

~1 Kb
Bidirectional DNA replication appears to initiate within or near the DS element (Gahn and Schildkraut, 1989; Niller et al., 1995). This initiation is activated by the FR (Wysokenski and Yates, 1989). The four EBNA1 binding sites within the DS are separated into two pairs, sites 1 and 2 and sites 3 and 4. There are 3 bp of DNA separating each site within a pair and 15 bp separating sites 2 and 3 (Ambinder et al., 1990). Previous experiments by Ambinder et al. predicted that EBNA1 would have a higher affinity for sites 1 and 4 than sites 2 and 3 due to sequence variations between the sites (Ambinder et al., 1990). Recent evidence from Harrison et al. has suggested that only one pair of EBNA1 binding sites (sites 1 and 2 or 3 and 4) is necessary for initiation of DNA replication (Harrison et al., 1994).

Many studies have attempted to define the minimal regions of oriP required to sustain effective replication of the EBV genome. Experiments have determined that the 960 bp region of DNA separating the FR and DS can be deleted with no effect on replication (Reisman et al., 1985). The FR is required, in a non-orientation dependent manner, for replication in some cell lines (Reisman et al., 1985; Wysokenski and Yates 1989). Of the 20 tandem EBNA1 binding sites within the FR; however, only 6-8 are required for replication (Chittenden et al., 1989; Wysokenski and
Yates, 1989). The FR can be replaced by two or more copies of the DS, but replication from such an origin is less effective than when the FR is present and the plasmids are not effectively or stably maintained (Wysokenski and Yates, 1989; Platt et al., 1993).

I.4. Epstein-Barr Nuclear Antigen 1

I.4.i EBNA1 Characteristics

The EBNA1 construct typically depicted in the literature is composed of 641 amino acids (a.a.) in vivo; however, the size of the protein varies due to variation in the length of the gly-ala repeat region of the protein depending on the viral isolate (Baer et al., 1984; Hennessy et al., 1983). Several regions within the protein have been identified: the gly-ala repeat region between a.a. 90-327; a highly basic region between a.a. 327-383; an acidic carboxyl tail between a.a. 619-641; a nuclear localization signal between a.a. 379-386 (Ambinder et al., 1991); and a DNA binding and dimerization region between a.a. 459-607 (Rawlins et al., 1985; Milman and Hwang, 1987; Ambinder et al., 1991; Shah et al., 1992; Chen et al., 1993; Summers et al., 1995). Figure 2 shows a schematic representation of known features of the EBNA1 protein. In latently infected B lymphocytes EBNA1 is present as a phosphoprotein, phosphorylated on serine residues but not tyrosines or threonines (Hearing and Levine, 1985). No relationship has
yet been shown between phosphorylation and EBNA1 function. EBNA1 exists as a dimer both in solution and when bound to its 18 bp DNA recognition site (Ambinder et al., 1991; Frappier and O'Donnell, 1991a; Chen et al., 1993).

I.4.ii. EBNA1 DNA Binding and Dimerization Region

EBNA1's DNA binding and dimerization region have been extensively investigated. Initial studies showed that the carboxy terminal region between a.a. 450-641 was responsible for the site specific binding of EBNA1 both to binding sites in the EBV genome and a synthetic consensus binding site (Rawlins et al., 1985; Milman and Hwang, 1987). Recent studies have refined the region, showing that the domain consisting of a.a. 459-607 is a stable DNA binding region and that a.a. 459-617 form a protease resistant domain when bound to DNA (Ambinder et al., 1991; Shah et al., 1992). The region responsible for EBNA1 dimerization also resides within the DNA binding region and dimerization is a prerequisite for DNA binding (Chen et al., 1993). Footprinting studies have shown that EBNA1 remains bound to the DS and FR throughout the cell cycle (Hseih et al., 1993). EBNA1 binds primarily on one face of the DNA helix. This was demonstrated by methylation protection footprinting demonstrating that EBNA1 binding will protect G residues on one face of the helix from dimethyl sulfide (DMS) methylation while nucleotides on
the opposite strand become hypersensitive (Frappier and O'Donnell, 1992).

Recently the crystal structures of a single EBNA1 dimer (a.a. 470-607) and a EBNA1 dimer (459-607) bound to a consensus EBNA1 binding site were solved (Bochkarev et al., 1995; Bochkarev et al., 1996). The information obtained from the structure has greatly increased present understanding of EBNA1's DNA binding and dimerization region. The structure showed that EBNA1's dimerization function is mediated by an eight stranded antiparallel $\beta$ barrel where each monomer contributes four $\beta$ strands. The DNA binding region is composed of two distinct domains, the core domain (a.a. 504-604) and the flanking domain (a.a. 470-503). The core domain has a highly homologous structure to the E2 protein of bovine papilloma virus (Hegde et al., 1992) while the flanking domain contains a unique region (a.a. 461-469) with the polypeptide chain lying along the minor groove of the DNA (Bochkarev et al., 1996).

**I.4.iii Cooperative Assembly of EBNA1 on the DS**

There are two pieces of evidence suggesting that EBNA1 binds cooperatively to sites within the DS. One indication came from experiments which footprinted EBNA1 on the DS. When titrated onto the DS EBNA1 appeared to bind all four sites of the DS at the same protein concentration (Frappier
and O'Donnell, 1992; Harrison et al., 1994), even though earlier predictions suggested that the outer two sites (sites 1 and 4) had a higher affinity for EBNA1 than the inner two sites (sites 2 and 3) due to nucleotide sequence variations (Ambinder et al., 1990). Additional evidence was provided by the finding that point mutations in the binding sites which disrupt binding to either sites 1 or 4 also abrogate binding to sites 2 and 3 respectively (Harrison et al., 1994).

EBNA1 bound to the DS alone has been visualized by electron microscopy and it was noted that the protein and DNA form a roughly spherical shape from which the protruding DNA ends exit at sharp angles (Frappier and O'Donnell, 1991b). This evidence suggests that the DNA may be folded or wrapped around the protein complex. The cooperative binding data and EM evidence suggest that EBNA1 is forming a higher ordered structure at the DS, the EBV origin of DNA replication.

I.4.iv. EBNA1 Induced DNA Distortion

EBNA1 binding has been shown to induce distortion within the DS both in vitro and in vivo (Frappier and O'Donnell, 1992; Hearing et al., 1992; Hsieh et al., 1993). This distortion is demonstrated by the induction of reactivity to potassium permanganate to two particular thymine (T) residues. Potassium permanganate will oxidize
pyrimidines, especially T, in single stranded DNA or duplex DNA which has been distorted (Boroweic et al., 1987; Sasse-Dwight and Gralla, 1989; Iida and Hayatsu, 1991). One potassium permanganate reactive T is located in site 1 of the DS and the other is on the opposite strand in site 4. DNA distortion is not detected upon EBNA1 binding to sites within the FR (Hearing et al., 1992). The distortion induced by EBNA1 binding must be more localized than DNA melting or unwinding because T residues adjacent to the permanganate-reactive T do not react and the binding does not induce regions of single stranded DNA (Frappier and O'Donnell, 1992; Hearing et al., 1992; Hsieh et al., 1993). The mechanism by which EBNA1 induces such distortion and the function the distortion plays in DNA replication is unknown.

I.4.v. EBNA1 Interactions at a Distance

EBNA1 dimers bound at the FR will interact with EBNA1 dimers bound at the DS resulting in a looping out of the intervening 960 bp of DNA between the two elements (Frappier and O'Donnell, 1991b; Su et al., 1991). This protein-protein interaction stabilizes EBNA1 binding to the DS and can be visualized by the EM (Frappier et al., 1991b; Su et al., 1991, Frappier et al., 1994). By creating chimeric proteins consisting of the GAL4 DNA binding domain and different fragments of EBNA1, Laine and
Frappier showed that two fragments of EBNA1 could interact at a distance. The two fragments (a.a. 320-355 and a.a. 351-377) are distinct from the DNA binding and dimerization region and mainly contain an eight a.a. sequence repeat rich in arginines. The fact that these regions of EBNA1 do not interact in solution suggests that DNA binding affects the structure of the looping region, allowing it to interact (Laine and Frappier, 1995). Recently, a third region has been identified as being able to mediate interactions at a distance. The region is located between a.a. 54-89. This region was fused to a GAL4 DNA binding and dimerization domain and shown to mediate looping in a ligation enhancement assay (Mackey et al., 1995). The role that the formation of this looped, higher ordered structure plays in replication from oriP is unclear.

I.4.iv. Additional EBNA1 Functions

EBNA1 is involved in mechanisms other than DNA replication. In order for the EBV genome to persist in a latent state while replicating once per cell cycle, it must be able to stably segregate to daughter cells in a manner analogous to cellular chromosomes. EBNA1 and the FR are required for maintenance and stable segregation of plasmid DNA (Krysan et al., 1989). Using plasmids which contained incomplete oriPs, capable of binding EBNA1 but not replicating, Middleton and Sugden showed that EBNA1 bound
to DNA will cause a statistically significant amount of plasmid DNA to be retained when compared to plasmids which did not contain EBNA1 binding sites. Only two EBNA1 binding sites will function almost equally as well retaining plasmids as the twenty found in the FR. They also showed that the DNA binding and dimerization domain and nuclear localization signal are needed (Middleton and Sugden, 1994).

EBNA1 bound to the FR has the ability to enhance expression of genes located in cis to the FR (Reisman and Sugden, 1986). The EBNA1-FR complex has been shown to enhance transcription, both from heterologous promoters and the Cp promoter within the EBV genome (Reisman and Sugden, 1986; Sugden and Warren, 1989). One of the proteins synthesized from the Cp promoter is EBNA1, thus, EBNA1 may play a role in regulating its own synthesis. It has been shown that EBNA1 must be bound to DNA to be able to act as a transcriptional activator (Polvino-Bodnar and Shaffer, 1992). The region between a.a. 450-641, comprising the DNA binding and dimerization domain and the acidic tail contain activation domains; however, the acidic tail can be deleted from the full length protein and transactivation function remains (Ambinder et al., 1991). Middleton and Sugden used a chimeric protein consisting of the DNA-binding domain of EBNA1 in the place of the activation domain of the estrogen
receptor to show that, although this region is sufficient to support transactivation it is not sufficient for DNA replication (Middleton, and Sugden, 1992).

EBNA1 has also been found to be capable of binding non-specifically to RNA, including EBER transcripts from the EBV genome (Snudden et al., 1994; D. Ceccarelli and L. Frappier, personal communication). The role that this non-specific mechanism may play \textit{in vivo} is not yet understood. Levikskaya et al., have also proposed that EBNA1 may be involved in cellular immune response evasion. They suggest that gly-ala repeats within the N-terminus of the protein are involved in the down regulation of anti-EBNA1 immune responses by preventing MHC class 1 antigen presentation of EBNA1 polypeptides (Levikskaya et al., 1995). However, this theory requires continued investigation.

\noindent \textbf{I.5 OBPs Role in Initiation of Replication}

In known replication systems in bacteria, animal virus and yeast, it has been shown that initiation of replication is dependent upon origin binding proteins (OBPs). OBPs bind to specific genomic sequences located within origins of DNA replication (reviewed in Marx, 1995). A common feature of the OBPs is that they induce some form of structural alteration on the DNA that they bind (reviewed in Bramhill and Kornberg 1988). The structural alterations may take the form of localized melted, bent, or untwisted
DNA. Such distortions may be induced either simply by the binding of the OBP or the formation of a protein-DNA complex in which the DNA is wrapped around the OBP (Borowiec and Hurwitz, 1988; Bramhill and Kornberg, 1988; Flashner and Shafferman, 1990; Fuller et al., 1984; Mukerjee et al., 1985). OBPs have been identified for many replication systems: dnaA protein from *E. coli*, λO protein from bacteriophage λ, RepA protein from the Phage plasmid P1, ORC from yeast, T-antigen from simian virus 40 (SV40), the E1 protein from bovine papilloma virus, and UL9 from HSV-1 (Koff et al., 1991; reviewed in DePamphilis, 1993; Kelman and O'Donnell, 1994; Stillman 1994). Although each of these proteins are regulated by different factors, they each bind to multiple copies of recognition sites within their respective origins to form specialized nucleoprotein structures (snup). The assembly of a snup results in the formation of an open complex containing distorted or melted DNA (reviewed in Kelman and O'Donnell, 1994). The OBP from *E. coli*, dnaA, binds four recognition sites within the origin, oriC. This binding results in wrapping of the DNA around the dnaA protein core. The wrapped complex will then result in the melting of three 13mer repeats that are AT-rich (reviewed in Bramhill and Kornberg, 1988). The SV40 OBP, T antigen, does not wrap the DNA around itself but still causes DNA distortion at the origin. T antigen
binds, in an ATP dependent manner, to the origin DNA as two hexamers. These hexamers form a protein complex which completely surrounds the DNA. T antigen binding results in 50% of the core origin DNA sequences being melted or severely distorted. Subsequent to the formation of this distortion T antigen's helicase activity initiates bidirectional unwinding of the DNA (reviewed in Boroweic et al., 1990). The complex nucleoprotein formation, consisting of OBP and origin sequences, can then be used as a "landing pad" (Stillman, 1994) to recruit other proteins such as single stranded DNA binding proteins and DNA helicases. The recruited proteins then create enough single stranded DNA to allow the primase, polymerase and accessory factors access to initiate DNA synthesis (reviewed in Stillman, 1994).

EBNA1 appears to provide mechanisms for DNA replication initiation of the EBV genome similar to those provided by OBP's from other systems. It binds multiple copies of a specific recognition site within the origin and induces DNA distortion. Since EBNA1 appears to be bound to the origin throughout the cell cycle and since DNA replication occurs only once during S phase, there must be regulatory factors which act upon the EBNA1-DNA complex to control initiation of replication. These regulatory factors have yet to be identified and the exact mechanism
by which EBNA1 binding and induced DNA distortion participates in the initiation of DNA synthesis remains elusive. Further insights into this system will provide a clearer understanding of initiation of DNA synthesis and suggest possible interventions which could aid in controlling EBV associated diseases.

My study has focussed on the formation of the EBNA1-DNA nucleoprotein complex formed on the dyad symmetry element of oriP. I have used EBNA1 and dyad symmetry element mutants in conjunction with electrophoretic mobility shift assays, methylation protection footprinting and potassium permanganate reactivity assays in an attempt to define and map regions of EBV. I have identified regions responsible for cooperative assembly of EBNA1 on the DS and characterized the requirements for EBNA1 induced DNA distortion.
II. Materials and Methods

II.1 DNA Manipulations

II.1.i Cloning of EBNA1 Truncation Mutants

EBNA1 truncation mutants comprising amino acids 463-607 and 468-607 were cloned using Vent Polymerase (New England Biolabs) in the Polymerase Chain Reaction (PCR) to amplify the region of the EBNA1 gene found on plasmid p205 (Yates et al., 1985). The following PCR primers were used for the amplification:

463-607 5'primer-5'CGTCGACATATGGGTGGTTTGAAAGCAT3'
3'primer-5'CGTGCAGGATCCTCAAGGCAAATCTACTCCATC3'

468-607 5'primer-5'CGTCGACATATGCATCGTGGTCAAGGAGGT3'
3'primer-5'CGTGCAGGATCCTCAAGGCAAATCTACTCCATC3'

Each 5' primer included a tail containing an Nde I site while the 3' primer included a Bam HI site for cohesive end ligation into the vector. Following amplification, DNA products were visualized on a 1% agarose 1X TBE (90 mM Tris, 90 mM Borate, 2mM EDTA) gel, stained with 5 µg/ml ethidium bromide and purified using a Qiaex kit (Qiagen) according to the manufacturer's instructions. Purified DNA fragments were resuspended in double distilled water (ddH2O) and subjected to digestion by 20 units Nde I (New England Biolabs (NEB)) at 37°C for 4 hours. Following the Nde I digestion, 100mM NaCl and 10 units Bam HI (NEB) were
added and the digestion allowed to continue for an additional 2 hours. The DNA was purified from solution using Qiaex (Qiagen) to remove the digested ends. The vectors, pET15b (463-607) and pET11a (468-607) (Novagen) were similarly digested with Nde I and Bam HI and purified from a 1% agarose gel.

Ligations were performed by incubating 100 fmol of prepared PCR product with 25 fmol of digested pET15b or pET11a using 800 units of ligase (NEB) in a final volume of 20 µl, at 15°C for at least 8 hours. Following incubation, 10 µl of the ligation mixture was used to transform 100 µl of a competent HB101 strain of Escherichia coli (E. coli) cells. After a 1 hour incubation in 1 ml of Luria Broth (LB) (10 g/L bactotryptone, 5 g/L yeast extract, 10 g/L NaCl) at 37°C, the cells were plated on LB plates containing 10 g/L agar and 100 µg/ml ampicillin and placed at 37°C overnight. Colonies were picked and screened using a previously published method of mini-preparation of the DNA (Sambrook et al., 1989). The presence of an insert in the plasmid was confirmed following digestion with Hind III and Xba I (NEB) and visualization on an ethidium bromide stained agarose gel.
II.1.ii Cloning of pGEM2xs1[wt] and pGEM2xs1[2] Plasmids

The vector pGEM2 (Promega) was used as a backbone to develop a plasmid containing two adjacent high affinity dyad symmetry site 1 sites with both wild type (pGEM2xs1[wt]) spacing (3 bp) and decreased spacing (2 bp) (pGEM2xs1[2]) between the two sites. The vector was digested with 10 units of Sma I (NEB) for 1 hour at 37°C followed by the addition of another 10 units of Sma I and a further 1 hour incubation. This digestion resulted in the formation of a linearized plasmid which was subsequently purified using the Qiaex kit (Qiagen). The plasmid was treated with bovine alkaline phosphatase (BAP from BRL). A 160 µl reaction containing 105 units BAP, 3 µg DNA, and 10 mM Tris-HCl pH 8.0 was incubated at 65°C for 1 hour. The reaction was stopped following the addition of 4.5 mM EDTA and a 10 min incubation at 50°C. The plasmid was again purified from solution using the Qiaex kit. The insert was prepared by radiolabelling the Site 1 top oligo (as described in II.1.iii) and adding an unlabelled 5' phosphate to the Site 1 bottom oligo (Site 1 top 5'-CCGGGAAGCATATGCTACCCG-3'; Site 1 bottom 5'-GGGTAGCATATGCTTCCCGG-3'). The oligos were hybridized (as described in section II.1.iv) and the double stranded Site 1 oligos ligated to one another using 800 units T4 DNA ligase (New England Biolabs) at 15°C for 2 hours. Ligation
products were separated on a 15% 1X TBE polyacrylamide gel run at 4°C. The products were visualized using autoradiography, the band containing a double site was excised, and the DNA eluted by shaking in TE (10 mM Tris pH 7.5, 1 mM EDTA (ethylenediaminetetraacetic acid)) at 37°C overnight. The DNA was ethanol precipitated and resuspended in TE. Insert (100 fmol) and vector (25 fmol) were ligated using 800 units of T4 DNA ligase (NEB) at 15°C overnight. Competent DH5α E. coli cells were transformed, grown, and screened as described previously except that the presence of insert was confirmed following NdeI (NEB) digestion. Spacing between the two adjacent sites was confirmed by sequencing using the Pharmacia Biotech T7 Sequencing Kit as per manufacturer's instructions.

II.1.iii Radiolabelling DNA Oligomers

DNA oligos were end labelled using γ32P-ATP. A 20 μl reaction mixture containing 10 pmol oligo, 30 μCi γ32P-ATP (Dupont), 50 mM Tris-HCl (pH 7.5), 5 mM dithiothreitil (DTT), 10 mM MgCl2, 1 mM spermidine and 10 units of T4 polynucleotide DNA kinase (NEB) was incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.5 mM EDTA (pH 8.0). A small amount (0.5 μl) of the sample was spotted on DE81 filter paper for future quantification. The removal of unincorporated nucleotides was accomplished by applying the reaction to a 0.8 ml G-25 spun column.
(Boehringer Mannheim) according to manufacturer's instructions and was followed by a 20 µl TE (pH 7.5) wash. Two 0.5 µl samples of each of the first and second washes were spotted on separate DE81 filter papers and one of each with the original filter was washed several times with 300 mM ammonium formate and 10 mM sodium pyrophosphate. This was followed by ddH₂O, then 100% EtOH rinses. The filters were dried and immersed in toluene for ³²P quantification using a Beckman LS 1801 scintillation counter.

II.1.iv Preparation of DNA for Electrophoretic Mobility Shift Assays

Electrophoretic Mobility Shift Assays (EMSAs) were performed on 18 bp double stranded oligomers comprising either site 1 or site 2 from the DS, a 56 bp fragment of DNA containing both site 1 and site 2 (site 1/2) or a 65 bp fragment containing two high affinity site 1 sites separated by either 3 bp (2xs1[wt]) or 2 bp (2xs1[2]). Site 1 was formed using the oligomers Top 5'CGGGAAGCATATGCTACCCG3' and Bottom 5'CGGGTAGCATATGCTTCCCG3' and the site 2 oligomers used were Top 5'GGGTAGTAGTATATATCCCG3' and Bottom 5'GGATAGTATATATCCCG3'. One oligo from each pair was end labelled with γ³²P-ATP as described above. The oligos were hybridized by combining 400 fmol of ³²P-labelled oligo with 800 fmols of the complementary unlabelled oligo, 50 mM
Tris-HCl pH 7.5, 1 mM DTT and 5 mM MgCl\(_2\) in 20 μl. These mixtures were placed in a Perkin Elmer Cetus thermocycler, heated to 92°C in 2 min, cooled to 50°C over 4 hours, maintained at 50°C for 1 hour, and cooled to room temperature over 1.5 hours.

The site1/2 fragment was amplified from the plasmid pGEM1/2 (contains only site 1 and 2 from the DS) using PCR and primers 5'CGGAATTCGGGTTAGTCTGGATAGT3' and 5'AGAGGGCATATTAGCAATAGTG3'. Once amplified, the fragment was digested with 20 units Hpa I (NEB) at 37°C for 2 hours to produce a 56 bp fragment. This fragment was purified from an ethidium bromide stained 12% 1xTBE polyacrylamide gel. The crushed polyacrylamide gel slice containing the desired DNA fragment was immersed in 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA (pH 8.0), 0.1% SDS and maintained in a shaking water bath at 37°C for 6 hours. All liquid was removed from the remaining polyacrylamide gel and the DNA was ethanol precipitated and resuspended in ddH\(_2\)O. The 56 bp fragment was then radiolabelled as described above. The 65 bp fragments 2xs1[wt] and 2xs1[2] were removed by endonuclease digestion from their respective plasmids (pGEM2xs1[wt] and pGEM2xs1[2]). A total of 5 μg plasmid was incubated with 20 units each of Hinc II and Sac I (NEB) for 2 hours at 37°C. The desired fragment was purified and radiolabelled as described for
II.2 Overexpression of EBNA1 Truncation Mutants

Both the 463-607 clone in pET15b and the 468-607 clone in pET11a were used to transform a competent BL21(DE3)pLysS strain of *E. coli* (Studier et al., 1990) and incubated at 37°C in a shaker for 1 hour. The cells were plated and incubated overnight at 37°C on LB plates containing 100 mg/ml ampicillin and 34 mg/ml chloramphenicol. A single colony was used to inoculate a culture (100 ml LB for 468-607 and 300ml LB for 463-607) containing 100 mg/ml ampicillin and was incubated at 37°C until it reached an approximate optical density (O.D.) of 0.8 at 600nm. These cultures were then used to inoculate a 6 L culture of Terrific Broth (TB) (12 g/L bactotryptone, 24 g/L yeast extract, 4 ml/L glycerol, 0.017 M KH₂PO₄, 0.074 M K₂HPO₄) (Sambrook et al., 1989) containing 100 mg/ml ampicillin for the 468-607 clone and 30 L of TB/ampicillin for the 463-607 clone. Both cultures were incubated with shaking (240 RPM) at 37°C to an OD of 0.6-0.8. At this time expression of the truncation mutant was induced by the addition of 1 mM isopropyl-B-D-thiogalactopyranoside (IPTG). Three hours following induction the cells were harvested by centrifugation in a Beckman GS6KR centrifuge for 15 min at 6000 RPM (6869 x g) at 4°C, resuspended in 20 mM Tris pH 7.5 and 10% sucrose, and frozen at -70°C.
II.3 Purification of EBNA1 Truncation Mutants

Frozen cells were thawed on ice in the presence of 1.0 mM benzamidine, 1.0 mM phenylmethylsulfonyl fluoride (PMSF) and 1.0 mM EDTA (1 ml per gram of cells). The cells were then lysed by passage through a french press at 1000 lbs/sq in and NaCl was added to a final concentration of 350 mM. Following a 20 min incubation on ice, the cell debris was removed from the lysate by centrifugation in a Beckman L8-55 ultracentrifuge at 20 000 rpm for 30 min. The supernatant was then heated at 60°C for 5 min, followed by a 10 min incubation on ice, and clarified by centrifugation at 5 000 rpm for 15 min. From this point on, all samples were maintained on ice and all purification steps occurred at 4°C. For the 463-607 clone the supernatant was passed through a 60 ml DE52 (Whatman) column previously equilibrated with 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) pH 7.5, 350 mM NaCl, 10 mM DTT and 10% glycerol. The flow-through collected from the DE52 column was diluted to a final NaCl concentration of 200 mM with 50 mM HEPES pH 7.5. The diluted DE52 eluant was applied to a 120 ml heparin-agarose column equilibrated with buffer A (50 mM HEPES pH 7.5, 200 mM NaCl, 10 mM DTT, 1 mM PMSF, 1 mM benzamidine and 10% glycerol). The column was washed with 4 column volumes of buffer A and the protein was eluted with buffer A containing 750 mM NaCl.
The protein-containing fractions as determined by Coomassie stained SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis (SDS PAGE), were pooled. The eluant was dialyzed against buffer B (50 mM HEPES pH 7.5, 750 mM NaCl, 10% glycerol). The sample was then loaded at 5 ml/min onto a 5 ml HPLC metal-chelating column (Perseptive Biosystems) charged with nickel and equilibrated with buffer B containing 5 mM imidazole. The protein was eluted at 2 ml/min using a 20 ml linear gradient of 5 mM to 1 M imidazole containing buffer B. A final concentration of 1 mM of both DTT and EDTA was added to the protein-containing fractions immediately following elution. These fractions were pooled and the N-terminal histidine tag of the protein, added by the pET15b vector, was removed by specific cleavage with thrombin. The protein was incubated with 10 units/mg of thrombin in buffer C (50 mM HEPES pH 7.5 750 mM NaCl, 10 mM DTT, 10% glycerol and 2.5 mM CaCl₂) at 37°C for 1 hour; then another 5 units thrombin per mg of protein was added and the mixture was incubated for another 2 hours. The protein was then diluted using buffer A lacking NaCl to a final NaCl concentration of 200 mM and loaded onto a 1 ml HPLC Mono S column (Perseptive Biosystems) equilibrated with buffer A containing 200 mM NaCl. The protein was eluted at 0.5 ml/min from the column with buffer A containing 750 mM NaCl. A protein
concentration of 11.1 mg/ml was calculated using $A_{280}$ and judged to be approximately 90% pure, as determined by Coomassie stained SDS PAGE.

The 468-607 clone was flowed over a 5 ml DE52 column equilibrated with buffer A containing 350 mM NaCl. The eluant was diluted using HEPES pH 7.5 to a final NaCl concentration of 200 mM and was loaded onto a 30 ml heparin-agarose column, equilibrated with buffer A containing 200 mM NaCl. The column was washed with 4 column volumes buffer A (200 mM NaCl) and the protein was eluted with buffer A containing 750 mM NaCl. The protein-containing fractions were pooled and diluted to 200 mM NaCl with salt free buffer A. The protein was then loaded on a 1 ml HPLC Mono S column (Perseptive Biosystems) and eluted with a 20 ml linear gradient of buffer A from 200 mM NaCl to 1 M NaCl over a 20 minute period. The resulting fractions contained 5.1 mg/ml of protein (calculated using $A_{280}$), and were determined to be approximately 90% pure by Coomassie stained SDS PAGE. All protein-containing fractions were aliquotted, flash frozen in liquid nitrogen, and stored at -70°C.
II.4 Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSAs) were performed using both the 463-607 and 468-607 clones as well as clones 459-619, 459-607 and 470-607 gratefully received from Jean Barwell and Richard Pfuetzner (Barwell et al., 1995). Pure protein (pmols dimer as calculated by A_{280}) was titrated onto 10 fmols of a ^32P-end-labelled DNA fragment (site 1, site 2, site1/2, 2xs1[w]t or 2xs1[2]) with 10 mM HEPES pH 7.5, 300 mM NaCl, 5mM MgCl₂, and 1 µg herring sperm DNA in a 20 µl reaction at room temperature for 10 min. Glycerol was added to a final concentration of 15% and the sample was subjected to electrophoresis on a 15% polyacrylamide gel in 0.5X TBE (45 mM Tris, 45 mM borate, 1 mM EDTA) at 4°C. Bound and unbound DNA bands were visualized by autoradiography and quantified with a Molecular Dynamics PhosphorImager and Imagequant software (Molecular Dynamics).

II.5 Methylation Protection Footprinting

bEBNA1, EBNA_{459-619}, EBNA_{459-607}, EBNA_{463-607}, EBNA_{468-607}, EBNA_{470-607} and the EBNA_{452-641} trp/phe mutant provided by Angela Fleming were titrated onto 0.5 µg of supercoiled DNA, in 20 µl of 250 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂. After a 10 min incubation at room temperature, dimethyl sulfate (DMS) was added to 10 mM and incubated at 37°C for 5 min. The reaction was stopped with the addition of β-
mercaptoethanol to 640 mM and SDS to 2%. Incubation at room temperature for 10 min was followed by the addition of TE to a final volume of 100 µl. The reaction was extracted with 120 µl phenol:chloroform (1:1) and the DNA was ethanol precipitated after the addition of NaAc pH 5.2 to 300 mM and 5 µg of glycogen. The dry pellets were resuspended in 100 µl of 1M piperidine and incubated at 90°C for 30 min. The reactions were cooled on ice and the DNA was ethanol precipitated as described above, but without the addition of glycogen. DNA pellets were washed with 70% ethanol, and dried pellets were resuspended in 21 µl ddH₂O. Each reaction was divided into 10 µl aliquots in two new eppendorf tubes. The samples were then analyzed using primer extension of DNA ladders (see II.7).

**II.6 Potassium Permanganate Reactivity Assays**

Pure protein was titrated onto 5 µg of supercoiled DNA in a 20 µl reaction containing 250 mM NaCl, 50 mM HEPES pH 7.5 and 5 mM MgCl₂ at room temperature for 10 min. Potassium permanganate (KMnO₄) was added to 20 mM and the reaction incubated at 37°C for 4 min. The reaction was quenched by the addition of β-mercaptoethanol to 1.3 M and SDS to 2% followed by a 10 min incubation at room temperature. The total volume was brought to 100 µl with TE and extracted with phenol-chloroform as with the DMS footprints. DNA was ethanol precipitated as in DMS footprints.
footprints except that the initial precipitation was followed by a 70% ethanol wash and the dry pellets were immediately resuspended in 21 μl ddH$_2$O and separated into 2 samples. Again the DNA was analyzed using primer extension of DNA ladder (see II.7).

**II.7 Primer Extension of DNA Ladder**

The 10 μl sample from either the DMS footprinting or KMnO$_4$ reactions was mixed with 250 000 cpm of $^{32}$P-end labelled primer and 0.9 mM NaOH in a final volume of 12.2 μl and heated at 80°C for 2 min to denature the DNA. The reactions were extended with 0.5 units of DNA Polymerase I Large Fragment (Klenow) from Pharmacia in the presence of 0.5 mM deoxynucleotide triphosphates (dNTPs) at 50°C for 10 min. The reactions were stopped with the addition of NH$_4$Ac to 1 M and the DNA was ethanol precipitated. The dried pellets were resuspended in 4.5 μl sequencing dye (98% deionized formamide, 10 mM EDTA, 0.025% xylene cyanol FF, 0.025% bromophenol blue). Extension products were separated on a 6% polyacrylamide-50% urea sequencing gel and visualized using autoradiography.

**II.8 Plasmids and Primers used in DMS and KMnO$_4$ Assays**

The following table outlines and describes the plasmids used for DMS footprinting and KMnO$_4$ reactivity assays and the primers subsequently used in the extension of the resulting DNA ladder. All pGEM plasmids were originally
derived from pGEM II (Promega) and the remaining plasmids were derived from pBluescript KS (Stratagene).

Table 1. Plasmids and Primers used in Footprinting Assays

<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Plasmid Description</th>
<th>Primer Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEMoriP (Frappier and O'Donnell, 1991a)</td>
<td>contains the entire oriP region, FR and DS</td>
<td>5'CGCTGTTCCCTTA GGACCC7TT3'</td>
</tr>
<tr>
<td>pGEMdyad (Frappier and O'Donnell, 1991b)</td>
<td>contains only the DS region of oriP</td>
<td>DyadUp2</td>
</tr>
<tr>
<td>pGEMS1 (Goldsmith et al., 1993)</td>
<td>contains only site 1 from the DS element of oriP</td>
<td>5'GTCGTTAGAACG CGGCTACAA3'</td>
</tr>
<tr>
<td>pGEM2xs1[wt]</td>
<td>contains 2 DS site 1 binding sites separated by 3 bp of DNA (as site 1 and 2 would in wt DS)</td>
<td>pGEMprimer</td>
</tr>
<tr>
<td>pGEM2xs1[2]</td>
<td>same as pGEM2xs1[wt] except there are only 2 bp separating the two sites</td>
<td>pGEMprimer</td>
</tr>
<tr>
<td>in1/2[5]-(Harrison et al., 1994)-Gift of J. Hearing-Stoney Brook University</td>
<td>contains the DS region with an additional 5 bp of DNA between sites 1 and 2</td>
<td>DyadUp2</td>
</tr>
<tr>
<td>in1/2[10]-(Harrison et al., 1994)-Gift of J. Hearing-Stoney Brook University</td>
<td>contains the DS region with an additional 10 bp of DNA between sites 1 and 2</td>
<td>DyadUp2</td>
</tr>
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III. Results

III.1. EBNA1 Mutants

In order to characterize EBNA1's cooperative assembly on the DS a number of truncation mutants were examined. A number of EBNA1 truncation mutants which spanned the region of EBNA1 already known to participate in EBNA1-EBNA1 interactions at a distance (DNA looping region), EBNA<sub>351-641</sub>, EBNA<sub>363-641</sub> and EBNA<sub>451-641</sub>, previously footprinted on the DS element filled all four DS sites simultaneously (Summers et al., 1996). Because the outer sites (sites 1 and 4) are predicted to have a higher affinity for EBNA1 than the inner two sites (sites 2 and 3), due to sequence variations, the simultaneous filling suggests that all EBNA1 truncation mutants were binding cooperatively to the DS (Ambinder et al., 1991). Therefore, in order to study the cooperative binding of EBNA1, different EBNA1 mutants were developed. These mutants span the DNA binding and dimerization domain and are outlined in Figure 2. Each of the mutants were amplified from the plasmid p205 and all mutants except EBNA<sub>468-607</sub> were inserted into the Nde I and Bam HI sites of the T7 polymerase expression vector pET15b (Novagen). The EBNA<sub>468-607</sub> truncation mutant was inserted into the Nde I and Bam HI sites of the T7 expression vector pET11a (Novagen). Each of the proteins expressed from
pET15b included N-terminal fusions of a 6 histidine tag and a thrombin site. The addition of the histidine tag allowed for purification using a nickel-charged metal-chelating column. The removal of the histidine tag from the pure protein was accomplished by thrombin digestion, however, four residues (glycine, serine, histidine and methionine) remained on the N-terminus of the proteins post digestion. The pure EBNA\textsubscript{468-607} mutant does not contain the additional four residues and its binding affinity was compared to an EBNA\textsubscript{468-607} protein expressed from pET15b to show that the additional residues did not affect binding to DNA.

The EBNA\textsubscript{1} mutants were purified to homogeneity as determined by Coomassie stained SDS PAGE (Figure 3). All protein concentrations were calculated using A\textsubscript{280} as protein concentrations determined by Bradford (Biorad) were unreliable and varied as much as 2.5 times depending on the mutant.
Figure 2. EBNAl mutants. EBNAl mutants are shown as well as known features of the entire EBNAl protein. The $\text{EBNA}_{452-641} \text{trp/phe}$ mutant was a gift of Angela Fleming. The $\text{EBNA}_{459-619}$, $\text{EBNA}_{459-607}$ and $\text{EBNA}_{470-607}$ truncation mutants were a gift of Jean Barwell and Richard Pfuetzner (Barwell et al., 1995).
EBNA1

1 90 327 376 459 607 619 641

Gly-Ala repeat  DNA looping  nuclear localization  DNA binding & dimerization  acidic tail

EBNA 452-641 (Trp/Phe)

EBNA 459-619

EBNA 459-607

EBNA 463-607

EBNA 468-607

EBNA 470-607

(Trp 464 mutated to Ala)  (Phe 465 mutated to Ser)
Figure 3. SDS PAGE of EBNA1 truncation mutants.

1.0 μg of purified protein was subjected to electrophoresis on a 15% polyacrylamide gel stained with Coomassie blue. Low molecular weight markers (Biorad) and their molecular weights in kilodaltons are shown on the left side of the gel.
II.2. Characterization of EBNA1 Mutant Binding to oriP

DMS footprinting was performed by titrating pure EBNA1 truncation mutants (EBNA\textsubscript{459-619}, EBNA\textsubscript{459-607}, EBNA\textsubscript{463-607}, EBNA\textsubscript{468-607} and EBNA\textsubscript{470-607}) onto 0.5 μg of supercoiled pGEMoriP. Binding to the four sites within the DS was examined by extending the DNA ladder through this region (see II.7) and separating the resulting products on a 6% polyacrylamide-50% urea sequencing gel, followed by visualization by autoradiography (Figure 4). DMS will methylate guanine (G) residues at N-7 and adenosine (A) at N-3 in duplex DNA (Maxam and Gilbert, 1980) that are not protected by specific contact with a bound protein. Subsequent treatment with piperidine will cleave methylated G residues and, to lesser extent, methylated A residues. Binding, therefore, is indicated by decrease in band intensity or complete disappearance of the band.
Figure 4. Methylation protection footprinting of EBNAl mutants on pGEMoriP. Pure EBNAl mutants (labelled above) were titrated onto 0.5 μg of supercoiled pGEMoriP and subjected to DMS and piperidine treatment followed by primer extension of the DNA ladder through the DS region. DNA products were separated by electrophoresis on a 6% polyacrylamide 50% urea sequencing gel and visualized by autoradiography. The numbered boxes on the right hand side indicate the four binding sites within the DS. Arrows on the left side indicate bands which become protected upon EBNAl binding.
pmol EBNA1 : (as dimer)

<table>
<thead>
<tr>
<th>pmol</th>
<th>0</th>
<th>7</th>
<th>14</th>
<th>28</th>
<th>56</th>
<th>85</th>
<th>113</th>
<th>142</th>
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<th>pmol</th>
<th>0</th>
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<th>45</th>
<th>90</th>
<th>135</th>
<th>240</th>
<th>360</th>
<th>480</th>
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<td>463-607</td>
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<td>470-607</td>
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The binding of the two largest mutants (EBNA$_{459-619}$ and EBNA$_{459-607}$) to the DS region of oriP resembles that seen previously with full length bEBNA1 (Frappier and O'Donnell, 1992). All four sites of the DS become protected at the same protein concentration. The EBNA$_{463-607}$ mutant displays a slightly different pattern of filling on the four DS binding sites. With this mutant, binding to the two outer or higher affinity sites is seen at 120-240 pmol of EBNA1 dimer, whereas, filling of the inner, low affinity, sites 2 and 3 does not occur until 480-960 pmol dimer. The smallest mutants, EBNA$_{468-607}$ and EBNA$_{470-607}$ had yet a different binding pattern. Binding of these mutants to the outer, higher affinity sites, could be detected (EBNA$_{468-607}$ at 86-172 pmol and EBNA$_{470-607}$ at 120-240 pmol); however, binding could not be detected on the inner, low affinity sites 2 and 3, even when the concentration of the protein was greatly increased to 0.75 and 1.6 nmol of dimer respectively (data not shown). The differences in the filling pattern of the four binding sites within the DS displayed by the various mutants suggested two possibilities. We had either localized the region responsible for cooperative binding required to fill the four sites simultaneously, or we had removed a region of the DNA binding and dimerization domain that greatly affects binding affinity.
III.3. Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSAs) were used to examine the binding affinity of each truncation mutant for site 1 and site 2 of the DS (Figure 5A/B). EMSAs were performed by titrating the various EBNA1 truncation mutants onto 10 fmol of $^{32}$P-end-labelled DNA fragments containing either site 1 of the DS, site 2 of the DS, or both site 1 and 2 (site 1/2) (Figure 5). Bound and unbound DNA fragments were separated on a 15% 0.5x TBE polyacrylamide gel. Dissociation constants ($K_d$s) (nM) were calculated from phosphorimager quantification of EMSAs shown in Figure 5A, B and C (Table 2). Protein-bound DNA versus EBNA1 protein was plotted and $K_d$s calculated from the point of half-maximal binding. The EMSAs show that all of the truncation mutants were able to bind specifically to both sites 1 and 2 of the DS. The two smallest truncation mutants, EBNA$_{468-607}$ and EBNA$_{470-607}$, bound very weakly to site 2 alone and the binding was barely detectable by EMSA. There was insufficient binding of EBNA$_{468-607}$ and EBNA$_{470-607}$ to site 2 to allow a reliable calculation of a $K_d$. 
Figure 5. EMSAs of EBNA1 truncation mutants on EBNA1 recognition sites of the DS element (Summers et al., 1996). Pure EBNA1 truncation mutants (as labelled) were titrated onto $^{32}$P-end-labelled site 1 alone (A), site 2 alone (B), or both site 1 and site 2, site1/2 (C). Bound and unbound DNA fragments were separated on 15% 0.5x TBE polyacrylamide gel and visualized by autoradiography.
Analysis of the various $K_d$s allowed several conclusions to be drawn. The difference between the affinity for site 1 and site 2 for the two largest mutants (EBNA$_{459-619}$ and EBNA$_{459-607}$) proves that EBNA1 binds site 1 with a 10 to 11-fold higher affinity than site 2, confirming the earlier suggestion that sequence variation between the two sites would result in different affinities (Ambinder et al., 1990). The $K_d$s also show that the smallest region of EBNA1 required to bind site specifically is located between amino acids 470-607, however, the region between amino acids 459-470 increases binding affinity, especially to lower affinity sites (site 2).
EBNA1 Truncation Mutant | Site 1 | Site 2 | Site 1/2
--- | --- | --- | ---
459-619 | 65 | 675 | 19
459-607 | 70 | 800 | 33
463-607 | 110 | 1,610 | 98
468-607 | 125 | ND\textsuperscript{a} | 1,360
470-607 | 240 | ND\textsuperscript{a} | 6,050

Table 2. \(K_d\)s for EBNA1 truncation mutants on EBNA1 recognition site 1, site 2 and site 1/2. \(K_d\)s listed are nM quantities. \(K_d\)s calculated from multiple experiments differed by less than twofold for a given truncation mutant. (Summers et al., 1996).

\textsuperscript{a} There was insufficient binding to site 2 alone with these clones to allow for the calculation of a reliable \(K_d\).

The cooperative interactions occurring between EBNA1 dimers bound at adjacent recognition sites was assessed using EMSAs and a 56 bp fragment of DNA containing both site 1 and site 2 (site 1/2) of the DS, separated by 3 bp of DNA (same spacing as in DS). The \(K_d\)s for site1/2 were calculated from the second site shift on the EMSAs. The \(K_d\)s for site1/2 are particularly revealing with respect to EBNA1's cooperative interactions. The fact that the
second shift $K_d$s for the three largest truncation mutants were lower than those for site 2 alone demonstrates that these mutants are binding cooperatively and the presence of an EBNA1 dimer bound at an adjacent binding sites aids EBNA1 binding to a second site. With the two smallest mutants it was impossible to determine a $K_d$ for binding to the low affinity site (site 2) due to insufficient binding; however, in the presence of two sites, binding to the second site improved such that a $K_d$ could be calculated. This illustrates that even the smallest mutants are binding cooperatively and the region responsible for cooperative interactions between adjacent EBNA1 dimers is located between amino acids 470-607.

III.3. Distortion of DS by EBNA1.

EBNA1 binding has previously been shown to distort the DNA within the DS both in vitro and in vivo (Frappier and O'Donnell, 1992; Hearing et al., 1992; Harrison et al., 1994; Hsieh et al., 1993). This distortion has been demonstrated by the presence of two thymine (T) residues (one in site 1 and one on the opposite strand in site 4) which become reactive to KMnO$_4$ upon EBNA1 binding (see Figure 1). KMnO$_4$ will oxidize pyrimidines, especially T, in single stranded DNA or duplex DNA which has been distorted (Borowec et al., 1987; Sasse-Dwight and Gralla, 1989; Iida and Hayatsu, 1991). The oxidized pyrimidine blocks DNA
synthesis by the large fragment of DNA polymerase I (Klenow) (Rouet and Essigman, 1985; Boroweic et al., 1987; Flashner, and Shafferman, 1990). To further characterize the requirements needed for DNA distortion, KMnO₄ assays and DMS footprinting were performed using EBNA₄₅₉-₆₁₉, EBNA₄₆₃-₆₀₇, and EBNA₄₆₈-₆₀₇. Each of the mutants was titrated onto 0.5 μg of pGEMdyad and assayed with either KMnO₄ or DMS (see Figure 6).
Figure 6. KMnO4 assays and DMS footprinting of EBNA1 mutants on pGEMdyad. Each of bEBNA1, EBNA459-619, EBNA463-607 and EBNA468-607 were titrated onto 10 fmols of supercoiled pGEMdyad and assayed for KMnO4 reactivity (A) or footprinted with DMS (B). The boxes on the right indicate the four EBNA1 binding sites within the DS. In panel A the arrow to the left indicates the T residue which becomes reactive to KMnO4 upon EBNA1 binding. In panel B the arrows on the left indicate G residues which become protected by EBNA1 binding thus causing the bands to lose intensity. The asterisk indicates a DMS-hyperreactive adenosine.
With the exception of EBNA\textsubscript{463-607}, all mutants showed a similar binding pattern on the DS region of pGEM\textsubscript{dyad} which lacks the FR region of oriP as they did on pGEM\textsubscript{oriP} which contains the entire oriP region. The EBNA\textsubscript{463-607} mutant appears to bind similarly to EBNA\textsubscript{459-619} when in the presence of DS alone, in that it will fill all four sites at the same protein concentration. This is likely due to the lack of competition for EBNA1 binding that the twenty higher affinity EBNA1 binding sites in the FR instill in the context of the entire oriP (as in pGEM\textsubscript{oriP}).

All EBNA1 mutants, except EBNA\textsubscript{468-607}, distorted the DNA within site 1 of DS as demonstrated by the KMnO\textsubscript{4} reactive band appearing upon EBNA1 binding. Binding of the EBNA\textsubscript{468-607} mutant did not cause the T in site 1 to become reactive. At this point, it was unclear whether the requirement for DNA distortion was binding to adjacent EBNA1 binding sites or whether the region between amino acids 463-468 was responsible. To further characterize the requirements for EBNA1 induced DNA distortion, the methylation protection footprinting and KMnO\textsubscript{4} reactivity assays were repeated using a plasmid which contained a single DS site 1 EBNA1 binding site (pGEM\textsubscript{s1}). The KMnO\textsubscript{4} reactivity assays were also performed on a linearized plasmid (pGEM\textsubscript{s1}) to determine if the DNA distortion was associated with the supercoiled nature of the DNA (Figure 7).
Figure 7. KMnO$_4$ reactivity assays and methylation protection footprinting of EBNA1 mutants on pGEMs1. KMnO$_4$ reactivity assays were performed as described in Materials and Methods on supercoiled pGEMs1 (A left side) or pGEMs1 linearized with Pvu I (NEB) (A right side) using EBNA1 mutants indicated above. Methylation protection footprinting was performed using the same mutants titrated onto supercoiled pGEMs1 (B). The box on the left side of the panels indicates the single DS site 1 EBNA1 binding site. The arrows to the right side of the panels indicate the KMnO$_4$ reactive T in site 1 (A) or the band within site 1 which becomes protected during DMS footprinting upon EBNA1 binding (B).
BA

pmol EBNA1:
(as dimer)
Binding to site 1 by all mutants was demonstrated with the methylation protection footprinting. Once again, however, all mutants except EBNA\textsubscript{468-607} induced DNA distortion, as indicated by the T in site 1 which becomes reactive to KMnO\textsubscript{4} upon EBNA1 binding. The EBNA\textsubscript{459-619} mutant was also seen to induce distortion on the linearized pGEM\textsubscript{s1} plasmid, illustrating that the distortion is not dependent upon supercoiled plasmid. These results clearly show that two adjacent EBNA1 binding sites are not required for EBNA1 induced DNA distortion and that this DNA distortion requires amino acids 463-468.

In order to continue to characterize the region responsible for DNA distortion, both KMnO\textsubscript{4} reactivity assays and DMS footprinting were repeated on pGEM\textsubscript{s1} using the EBNA1 mutant encompassing amino acids 452-641 with Trp 464 and Phe 465 point mutated to Ala and Ser, respectively (EBNA\textsubscript{452-641}(Trp/Phe)) (Figure 8). Structural information provided by the co-crystallization of EBNA\textsubscript{459-607} on an EBNA1 consensus binding site (Bochkarev et al., 1996) suggested that these two aromatic residues lie within the minor groove of the DNA and are aligned such that they may play a role in separating the two phosphate backbones.
Figure 8. KMnO₄ assay and DMS footprinting of EBNA_{452-641} (Trp/Phe) on pGEMs1. The EBNA_{452-641} (Trp/Phe) mutant was titrated (see amounts above) onto supercoiled pGEMs1 and subjected to KMnO₄ assays (left side) or DMS footprinting (right side). The box on the left indicates the DS site 1 EBNA1 binding site and the arrows on the right indicate the KMnO₄ reactive T or the band within site 1 which becomes protected during DMS footprinting upon EBNA1 binding.
The DMS footprinting showed that the EBNA\textsubscript{452-641} (Trp/Phe) mutant will bind to site 1 and KMnO\textsubscript{4} assays demonstrated that this mutant also induces DNA distortion within site 1 of the DS upon binding. This indicates that the amino acids Trp\textsubscript{464} and Phe\textsubscript{465} are not necessary to induce DNA distortion.

**III.4. Spacing Requirements of Adjacent EBNA1 Binding Sites**

EMSAs were performed using an oligo consisting of two adjacent site 1 sequences with either wt (3bp) (2xs1\textsubscript{[wt]}) spacing or decreased spacing of 2bp (2xs1\textsubscript{[2]}) to examine space constraints between EBNA1 binding sites and the effect of having two adjacent site 1 (high affinity) sequences (see Figure 9). The EMSAs showed that the EBNA\textsubscript{459-619} mutant was capable of binding both high affinity sites simultaneously with either wt or 2 bp spacing.

The plasmids pGEM2xs1\textsubscript{[wt]} and pGEM2xs1\textsubscript{[2]} and bEBNA1 were used to examine the effects of placing two high affinity sites adjacently with both wild type and decreased spacing (2 bp). KMnO\textsubscript{4} assays were again performed and DNA distortion examined (Figure 10).
Figure 9. EMSAs of EBNA\textsubscript{459-619} on oligos 2xsl[wt] and 2xsl[2]. EMSAs were performed by titrating EBNA\textsubscript{459-619} onto either 2xsl[wt] or 2xsl[2]. Samples were subjected to electrophoresis on a 5\% 0.5xTBE polyacrylamide gel and separated products were visualized by autoradiography. Brackets on the left indicate unbound DNA, oligos with only 1 site bound and oligos with both high affinity sites bound.
pmol EBNA 456-619 (as dimer)

2 sites bound
1 site bound
unbound
Figure 10. KMnO₄ assays binding bEBNA1 onto pGEM2xs1[wt] and pGEM2xs1[2]. bEBNA1 was bound to two adjacent high affinity binding sites (site 1 from DS) separated by either wild type spacing (pGEM2xs1[wt]) or decreased spacing of 2bp (pGEM2xs1[2]) and subjected to KMnO₄ reactivity assays followed by primer extension. The arrows on the left side indicate the T in both site 1 sequences which become reactive to KMnO₄ upon EBNA1 binding.
pmol bEBNA1 (as dimer)
bEBNA1 induced KMnO₄ reactivity of both Ts in each of the site 1 sequences on plasmids displaying both wt and decreased spacing further demonstrates that the spacing between adjacent EBNA1 binding sites is not critical for EBNA1 induced DNA distortion or binding. It is also possible for EBNA1 to bind to adjacent high affinity sites (site 1 of the DS) and induce DNA distortion in the same manner in both sites, simultaneously. Therefore, although wild type spacing between sites 1 and 2 is critical for replication (Harrison et al., 1994) it is not critical for EBNA1 binding or EBNA1 induced DNA distortion.

To further characterize EBNA1 binding and spacing constraints, methylation protection footprinting and KMnO₄ reactivity assays were performed on plasmids with increased spacing between sites 1 and 2. In the wild type DS there are 3 bp between sites 1 and 2 and sites 3 and 4, and 15 bp between sites 2 and 3 (see Figure 1). Only 1 pair of sites is necessary to support replication (Harrison et al., 1994); therefore, we focussed on the spacing between the high affinity site 1 and low affinity site 2. Plasmids obtained from Janet Hearing (Stony Brook University) contained an extra 5 bp (in1/2[5]) or 10 bp (in1/2[10]) of DNA between sites 1 and 2. EBNA459-619 was used in both DMS footprinting and KMnO₄ assays with these plasmids to examine both binding and DNA distortion (Figure 11).
Figure 11. DMS and KMnO₄ assays of EBNA₄₅₉₋₆₁₉ on in1/2[5] and in1/2[10]. EBNA₄₅₉₋₆₁₉ was titrated onto supercoiled in1/2[5] (A) or in1/2[10] (B) and subjected to DMS footprinting (left side of A and B) or KMnO₄ reactivity assays (right side of A and B). The boxes to the left of each panel indicate the four DS EBNA1 binding sites and their corresponding arrows indicate bands which become protected during DMS footprinting upon EBNA1 binding. The arrows on the right side indicate the T in site 1 which becomes reactive to KMnO₄ upon EBNA1 binding.
DMS footprinting and KMnO₄ assays show that even with increased spacing EBNA₄₅₉-₆₁₉ can bind to both site 1 and 2 within the DS and the two sites appear to become bound simultaneously. This suggests that the cooperative interaction between the two dimers is occurring over a greater distance than normally found between the two sites. The same T residue within site 1 still becomes reactive to KMnO₄ when the extra spacing is present. This indicates that site 1 is probably still being bound in the same fashion as seen when site 1 and 2 are separated by wild type spacing. The extra band in site 2 which becomes reactive to KMnO₄ upon EBNA₁ binding is likely due to the extra strain placed on the DNA due to cooperative interactions occurring between EBNA₁ dimers bound at site 1 and 2.
IV. Discussion

EBNA1 and its interactions with EBV's oriP play an integral role in initiation of bidirectional replication of the latent EBV genome (Reisman et al., 1985; Yates et al., 1985). This role appears to be similar to that played by OBPs in many other systems. As in the case of the lambda phage 0 protein, dnaA from *E. coli*, and RepA of the P1 plasmid, initiation of DNA synthesis from oriP involves assembly of EBNA1 onto multiple recognition sites. The arrangement of a higher-ordered structure on the origin requires homotypic interactions between the OBPs and involves bending or wrapping of origin DNA sequences around the protein complex (reviewed in Kelman and O'Donnell, 1994; Stillman, 1994). The experiments I have conducted were designed to characterize the formation of a higher order structure on EBV's DS region of the latent origin of replication, oriP.

Using EMSAs I have proven that EBNA1 has a 10-11 fold higher affinity for site 1 of the DS as compared to site 2. This confirms earlier predictions that sequence variations between the two sites would result in different affinities (Ambinder et al., 1990). The EMSAs also demonstrated that the smallest region of EBNA1 needed to bind site specifically was located between amino acids 470-607. The
region between a.a. 459-468; however, is important for binding, especially to lower affinity sites. The increase in binding affinity provided by this region was explained by information obtained from the structure of EBNA1 bound to DNA (EBNA$^{459-607}$ bound to a consensus EBNA1 binding site) (Bochkarev et al., 1996). The structure showed that the DNA binding and dimerization region is composed of two domains, a core domain and a flanking domain. In the flanking domain (a.a. 461-504), a.a. 461-469 lie in the minor groove of the DNA and make a series of contacts with the DNA (Bochkarev et al., 1996).

As well as being responsible for site specific binding, the region between a.a. 470-607 also contains amino acids responsible for cooperative interactions between EBNA1 dimers on adjoining binding sites. These cooperative interactions occur between dimers bound at adjacent binding sites such as site 1 and 2 or sites 3 and 4, which are separated by 3 bp of DNA. It is unknown whether cooperative interactions are occurring between dimers bound to sites 2 and 3, which are separated by 15 bp, or between dimers bound to adjacent sites within the FR. Increasing the spacing between adjacent EBNA1 binding sites by up to 10 bp or decreasing the spacing by 1 bp does not affect EBNA1's ability to bind simultaneously to adjacent binding sites. This indicates that cooperative
interactions between dimers bound at sites 2 and 3 or on adjacent sites in the FR could occur.

In order to identify potential contacts mediating cooperative interactions two EBNA1 dimers, bound to DNA, were modelled on adjacent EBNA1 binding sites with wild type (3 bp) spacing between them (Bochkarev et al., 1996). Initially two dimers could not be positioned on adjacent binding sites without the dimers physically colliding with each other. In order to accommodate binding of two EBNA1 dimers the DNA had to be distorted by unbending it 20° and unwinding it 20°. With the DNA in this new position the dimers could bind adjacent binding sites. Two helices on each dimer were identified (a.a. 477-490 and 568-584) that were aligned such that one helix may interact with the opposite helix on an adjacent dimer (Bochkarev et al., 1996). This model suggested a mechanism for the EBNA1 induced DNA distortion observed in the DS. The accommodation of EBNA1 dimers on adjacent binding sites DNA would cause DNA distortion. This distortion is not detected by KMnO₄ reactivity assays; however, as EBNA1 binding to a single recognition site is sufficient to induce reactivity and therefore distortion. There are no new T residues which become reactive to KMnO₄ when dimers bind on adjacent sites therefore the distortion that this may induce is not detectable using KMnO₄.
Methylation protection footprinting and KMnO₄ reactivity assays using EBNA1 truncation mutants EBNA_{459-619}, EBNA_{463-607} and EBNA_{468-607} and a plasmid containing the entire DS initially seemed to support the suggestion that binding to adjacent dimers was responsible for DNA distortion. The only mutant which did not induce DNA distortion upon binding was EBNA_{468-607}. Binding of EBNA_{468-607} could only be detected on site 1 and 4, not site 2 or 3. This continued to support the hypothesis that EBNA1 dimers bound on adjacent binding sites are the cause of EBNA1 induced DNA distortion. To confirm that binding to two sites was necessary to induce distortion, methylation protection footprinting and KMnO₄ reactivity assays were performed with the same mutants and a plasmid which contained only the DS site 1 EBNA1 recognition site. Binding could be detected on site 1 with all three mutants and EBNA_{459-619} and EBNA_{463-607} displayed KMnO₄ reactivity, indicating DNA distortion. This proved that EBNA1 binding to a single site could induce DNA distortion and binding of dimers to adjacent sites was not required. This new data shows that either residues in the region between amino acids 463-468 are responsible for EBNA1 induced DNA distortion or the extra binding affinity imparted by this region allows for DNA distortion. Recent data has also suggested that this region is responsible for replication fork pausing and inhibition of helicase
activity when EBNA1 is bound to the FR, but again, the mechanism is unclear (Olga Ermanov, Lori Frappier and Carl Schildkraut, submitted).

Between a.a. 463-468 two particular residues, Trp_{464} and Phe_{465}, were examined to elucidate their role in EBNA1 induced DNA distortion. These two aromatic residues, visualized in the EBNA/DNA co-crystal, were seen lying in the minor groove of the DNA, poised like hands pushing apart the phosphate backbones (Bochkarev et al., 1996). When these two residues were point mutated to ala and ser respectively, in the context of EBNA_{452-641}, binding and distortion of site 1 could still be detected. This showed that the Trp/Phe residues are not necessary to induce DNA distortion.

The finding that EBNA1 induced DNA distortion when bound to a single site 1 EBNA1 recognition site contrasted with the structure of EBNA1 bound to a consensus, palindromic binding site. The structure showed the DNA bound smoothly to the protein without any major perturbations. There are two possible explanations for this inconsistency. First, EBNA1 might bind the sites differently due to their sequence variations and thus cause distortion in the two DS recognition sites but not others. Secondly, the distortion which allows thymines to become reactive to potassium permanganate may not be detected in
the structure because it is only a minute distortion of the DNA capable of allowing KMnO₄ access to the thymine.

Harrison et al. had previously reported that only one pair of sites (sites 1 and 2 or sites 3 and 4) of the DS was necessary to support replication, but that the wild type spacing of 3 bp between either sets of sites was essential (Harrison et al., 1994). Plasmids and binding sites which had a decreased spacing of 2 bp between sites 1 and 2 or an additional 5 or 10 bp were used to examine space constraints on EBNA1 binding. In both cases EBNA1 was able to bind both sites simultaneously and DNA distortion was still detected in site 1. When two site 1 recognition sites from the DS were adjacent the same thymine in each site became reactive, indicating that EBNA1 is binding both sites in the same manner. This demonstrated that the spacing between the two EBNA1 binding sites within the DS required to permit replication is not crucial for EBNA1 binding and distortion of DNA. The wild type spacing between binding sites may be necessary for replication, to ensure that the two dimers are aligned in a manner that allows for interactions with other proteins required in the initiation complex. EBNA1 is known to interact with the human single-strand DNA binding protein (RPA) and may participate in other interactions necessary to complete an initiation complex (Dan Zhang, Lori
Frappier, and Mike O'Donnell submitted). The higher-ordered structure that EBNA1 forms on the DS incorporating EBNA1 interactions at a distance, cooperative assembly of dimers on the DS, and EBNA1 induced DNA distortion within the DS may participate in DNA replication initiation by providing a stable complex that will recruit proteins such as RPA, licensing factors and helicases needed for the assembly of an initiation complex.

Although valuable information has been provided by examining the structure of a single EBNA1 dimer (EBNA470-607) and a dimer (EBNA459-607) bound to DNA (Bochkarev et al., 1995; Bochkarev et al., 1996), characterization of both cooperative interactions and EBNA1 induced DNA distortion remains complicated. These properties have been localized to specific regions; however, their mechanism remains unclear. Future work in vitro will aid in understanding each of these mechanisms and progress in vivo will hopefully elucidate the roles these mechanisms play in initiation of DNA replication.
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