EBNA1 Distorts *oriP*, the Epstein-Barr Virus Latent Replication Origin

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The Epstein-Barr virus nuclear antigen 1 (EBNA1) protein binds and activates the latent replication origin (*oriP*) of the Epstein-Barr virus. We have been studying EBNA1 to determine how it activates replication at *oriP*. Here we demonstrate that upon binding of EBNA1 to *oriP*, two thymine residues become reactive to potassium permanganate (KMnO₄), indicating a helical distortion at these sites. The KMnO₄-reactive thymines are 64 bp apart in the region of dyad symmetry of *oriP*. Dimethyl sulfate protection studies indicated that EBNA1 binds on the opposite face of the helix from the reactive thymines. The nature of the helical distortion induced by EBNA1 and its possible significance to the initiation of replication are discussed.

During latent infection of human B lymphocytes, multiple copies of the Epstein-Barr virus (EBV) genome are maintained as extrachromosomal DNA plasmids which replicate once per cell cycle (1, 32). Replication of these EBV plasmids depends on only one viral protein, EBV nuclear antigen 1 (EBNA1) (25, 33). EBNA1 binds the latent origin of replication (*oriP*) of the viral genome (24). *oriP* contains two essential elements separated by 1 kb, the family of repeats (FR) and the region of dyad symmetry (DS) (16, 25, 30). The FR element consists of 20 tandem copies of a 30-bp sequence, each of which contains an 18-bp palindromic EBNA1-binding site (16, 25). The DS element contains four EBNA1-binding sites, two of which are located within a 65-bp region of dyad symmetry (25, 30). Increasing evidence suggests that replication initiates within the DS element of *oriP* and is activated by the multiple EBNA1 sites in the FR element (8, 14, 31).

The origin-binding proteins of *Escherichia coli* (7, 13), bacteriophage λ (10, 28, 34), simian virus 40 (3, 5, 9, 22, 23), herpes simplex virus type 1 (19), and plasmids R6K (11, 21) and pT181 (18) induce structural alterations in their respective origins. Such alterations include localized melting (5, 7, 28), bending (11, 21, 34), untwisting (5), and wrapping (10, 13) of the origin DNA. In some of these systems, the structural alteration of the origin has been correlated with its initiation function (7, 10, 19, 22, 28).

We previously overproduced EBNA1 in the baculovirus system (bEBNA1) and purified it to homogeneity (12). Here we examine bEBNA1 for ability to induce structural alterations in *oriP*. As a probe of DNA structure, we used potassium permanganate (KMnO₄), which oxidizes pyrimidine residues in regions of the DNA helix that have been structurally distorted (6, 27).

bEBNA1 alters the structure of the DS element. At physiological pH, KMnO₄ selectively oxidizes pyrimidines, especially thymine residues, in single-stranded DNA (15) and in helically distorted duplex DNA (6, 27). Oxidized pyrimidines prevent DNA synthesis (i.e., primer extension) beyond the modified residue by the large fragment of DNA polymerase

I (6, 11, 26). We used the KMnO₄ oxidation-primer extension assay to probe for helical distortions in *oriP* induced by EBNA1 (Fig. 1). The EBNA1 used in this study was expressed from a recombinant baculovirus in insect cells and purified to homogeneity as described previously (12). We refer to this protein as bEBNA1; it lacks six N-terminal amino acids and most of the nonessential (33) glycine-alanine repeat region. bEBNA1 was titrated into a solution of supercoiled plasmid containing *oriP* (pGEMoriP). The samples were treated with KMnO₄, and after removal of bEBNA1, both strands of *oriP* were analyzed for oxidized pyrimidines by primer extension. Analysis of the DS element is shown in Fig. 1A. Two residues (arrows) become reactive to KMnO₄ oxidation upon addition of bEBNA1; one is in the top strand and the other is in the bottom strand. Densitometric analysis of the bEBNA1 titration (Fig. 1A) showed that the extent of KMnO₄ oxidation of these residues increased as bEBNA1 was increased up to 4 to 6 pmol of bEBNA1 (1.1 to 1.7 dimers per binding site in *oriP*). The two KMnO₄-reactive residues map to a T residue in EBNA1-binding site 1 on the bottom strand and a T residue in binding site 4 on the top strand (circled Ts in Fig. 1B). These two EBNA1 sites are unique in having a T at the KMnO₄-reactive position (ATGCTTCCC); the other 22 EBNA1 sites in *oriP* have an A at this position.

Induction of these KMnO₄-reactive T residues by bEBNA1 was not affected by 4 mM ATP and 5 mM MgCl₂, by the absence of MgCl₂, or by NaCl concentrations between 50 and 300 mM. Similar results were obtained on linear DNA containing *oriP* (data not shown). Therefore, torsional stress is not required for bEBNA1 to induce these structural distortions in the DS element.

We designed four primers to examine both strands of the entire 600-bp FR element for bEBNA1-induced KMnO₄ oxidized residues. No bEBNA1-induced KMnO₄-reactive residues were detected in any of the 20 EBNA1-binding sites of the FR element (data not shown).

Initiation of replication from the DS element of *oriP* is activated by the FR element (8, 14, 31). We examined whether the FR element was required for the bEBNA1-induced structural alterations of the DS element. A plasmid (pGEMdyad) which contains the DS element but lacks the FR element was tested in the KMnO₄ assay with and without bEBNA1. bEBNA1 induced KMnO₄ reactivity to the same

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FIG. 1. bEBNA1 induces two KMnO₄-reactive sites within the DS element of *oriP*. (A) bEBNA1 (0 to 6 pmol as dimer) was incubated with 150 fmol (0.5 μ g) of supercoiled plasmid containing *oriP* (pGEMoriP [25]) for 10 min at 37°C in 30 μ l of 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.5)–300 mM NaCl–5 mM MgCl₂. KMnO₄ was added to 18 mM and incubated for 4 min at 37°C. The reaction was quenched by the addition of β -mercaptoethanol to 1.0 M. bEBNA1 was removed by phenol-chloroform (1:1) extraction in the presence of 2% SDS. Both strands of the DNA were analyzed for oxidized pyrimidines by extension of ³²P-end-labeled primers, using DNA polymerase I large fragment as described previously (16, 27). The 3' end of the primer (5'-AGAGGGCATTAGCAATAGTG-3') for analysis of the top strand hybridizes 29 bp 3' to EBNA1-binding site 1. The 3' end of the primer (5'-ATGGGGTGGGAGATATCGCT-3') for analysis of the bottom strand hybridizes 28 bp 3' to EBNA1 site 4. Extension products were separated on a 6% polyacrylamide–50% urea sequencing gel and visualized by autoradiography. Arrows indicate the bEBNA1-induced KMnO₄-reactive thymines. Densitometric quantitation of the KMnO₄-reactive thymines is shown on the right side of the figure. The positions of the four 18-bp EBNA1-binding sites, deduced from dideoxy sequencing ladders, are indicated along the sides of the autoradiograph. EBNA1 site 4 is proximal to the FR element. (B) Sequence of the DS element showing the four 18-bp EBNA1-binding sites (boxed) and the KMnO₄-reactive thymines (circled). The arrows above sites 3 and 4 mark the imperfect palindrome of the DS element.

two T residues in pGEMdyad that were induced in pGEM-oriP (data not shown). Therefore, the bEBNA1-induced structural alterations of the DS element do not require the FR element.

Work in other systems has shown that KMnO₄ can react with DNA which is melted, bent, or untwisted (3, 5, 6, 22, 23, 27). In melted DNA, pyrimidine residues on both strands of the DNA are reactive with KMnO₄ (3, 5, 22, 27). However, there are no pyrimidine residues on the strand opposite either KMnO₄-reactive thymine. Therefore, we employed the dimethyl sulfate (DMS)-S1 method of Siebenlist (29) to examine the possibility that bEBNA1 induces melting of DNA. DMS methylates the N-1 of adenosine and, to a lesser extent, the N-3 of cytosine, only in the context of single-stranded DNA (these positions are involved in hydrogen bonding in duplex DNA). Adenosine and cytosine methylated at these positions will not participate in formation of duplex DNA. Hence, if a DNA-binding protein induces melting of DNA, treatment of the DNA-protein complex with DMS and then extraction of the protein yields methylated DNA which will not renature. Subsequent treatment with the single-strand-specific S1 nuclease cleaves the DNA at N-1-methylated adenosine (29) and at N-3-methylated cytosine (5).

bEBNA1 was added to *oriP* DNA and then reacted with DMS. The bEBNA1 was removed by sodium dodecyl sulfate (SDS)-phenol extraction, and S1 nuclease was used to probe for potential melted DNA sequences stabilized in the melted state by the DMS treatment. No bEBNA1-induced S1 nuclease-sensitive sites were detected in the DS element by this method (data not shown), even though several A and C residues surround the KMnO₄-reactive thymines. Direct treatment of bEBNA1-*oriP* complexes with S1 nuclease also failed to reveal S1-sensitive sites (data not shown). These results indicate that bEBNA1 does not induce significant melting of the DS element.

bEBNA1 binds on the opposite face of the helix from the KMnO₄-reactive thymines. To gain further insight into how bEBNA1 interacts with the DS element, we performed methylation protection footprints. In duplex DNA, DMS methylates G at N-7 and A at N-3 (20). bEBNA1 was titrated into a solution of supercoiled pGEMoriP and then treated with DMS. The bEBNA1 was extracted from the methylated DNA, and the DNA was treated with piperidine, which breaks the DNA backbone at methylated G and, to a lesser extent, methylated A (20). Sites of cleavage were determined by primer extension analysis. Comparison of the G residues in *oriP* DNA, with and without bEBNA1, showed that 14 of the 31 G residues in the four EBNA1 sites of the DS element were protected by bEBNA1 (Fig. 2). The DMS protection pattern of site 1 matches that of a synthetic consensus EBNA1 site protected by a 28-kDa fusion protein containing the C-terminal third of EBNA1 (17).

The DMS protection analysis revealed three interesting features of the interaction between bEBNA1 and the DS element. First, the protected G residues are located mainly on one face of the DNA helix (Fig. 3). The protected face is opposite the helical face containing the KMnO₄-reactive T residues. Previous hydroxy radical footprinting (17) and methylation interference assays (2) of the 28-kDa EBNA1 fusion protein also indicated that EBNA1 interacts with only one helical face of the DS element. Second, densitometric analysis (Fig. 2) of G protection with increasing bEBNA1 indicates that the four EBNA1-binding sites in the DS element become bound by bEBNA1 simultaneously and plateau at 4 pmol of bEBNA1 (1.1 dimers per binding site). The EBNA1-binding sites in the DS element are predicted to have different affinities for EBNA1 (2). Hence, the simultaneous filling of these sites suggests that bEBNA1 binds cooperatively to the DS element. Third, bEBNA1 binding to

FIG. 2. Methylation protection footprints of bEBNA1 on the DS element of *oriP*. bEBNA1 was incubated with supercoiled pGEMoriP DNA as described in the legend to Fig. 1. DMS was added to 10 mM and incubated for 5 min at 37°C. The reaction was quenched with β -mercaptoethanol (0.67 M final), extracted with phenol-chloroform (1:1) in 2% SDS, and precipitated with ethanol. DNA was resuspended in 100 μ l of 1 M piperidine and heated to 90°C for 30 min. Samples were analyzed by primer extension as described in the legend to Fig. 1. Primer extension analysis was performed on the bottom (A) and top (B) strands of the DS element. Autoradiographs were scanned by a laser densitometer. Bands which decreased in intensity by 50% or more are numbered to the right of each autoradiograph. Band 9 is actually three bands which the densitometer did not resolve, but visual inspection showed that the two faster-migrating bands were clearly protected more than 50% by bEBNA1 and that the slowest-migrating band was not protected. The positions of the four EBNA1 sites are indicated beside each autoradiograph (boxes). For each EBNA1-binding site, densitometric analysis of the numbered bands within it is shown to the right of the autoradiograph. The arrow indicates the DMS-hyperreactive adenosine. (C) Sequence of the DS element showing the four EBNA1-binding sites (boxes), G residues protected by bEBNA1 (circles), and the DMS-hyperreactive adenosine (dot). The arrows above sites 3 and 4 mark the imperfect palindrome of the DS element.

oriP caused one adenosine between sites 3 and 4 of the DS element to become hyperreactive to DMS (Fig. 2A, arrow, and C, dot). The adenosine is presumably methylated at N-3 since methylation at N1 would have been revealed in the DMS-S1 nuclease assay. Hyperreactivity toward DMS has been reported to occur in regions of the DNA helix that are distorted (4, 6). This A residue is on the same face of the helix as the KMnO_4 -reactive T residues (Fig. 3).

Possible nature of the bEBNA1-induced helical distortion of *oriP*. EBNA1 is similar to other origin-binding proteins in that it induces a structural alteration of the DNA upon binding the origin. We would like to know the nature of the structural distortions induced by bEBNA1 in the DS element of *oriP*. In the *E. coli* (7, 13), bacteriophage λ (10, 28, 34), and simian virus 40 (3, 5, 9, 22, 23) systems, the origin-binding proteins induce, among other alterations, limited

melting of their respective origins. In such cases of protein-induced melting, several nucleotides on both strands become reactive to chemical reagents or single-strand-specific nucleases (4, 5, 22, 27–29). The inability to detect S1 nuclease cleavage in the DS element (even when DMS was used to stabilize melted DNA sequences after removal of bEBNA1) suggests that bEBNA1 does not induce DNA melting. However, these studies are complicated by DMS protection of residues near these sites by bEBNA1. Unlike DMS and S1 nuclease, KMnO_4 has been reported to react with distorted residues even when they are bound by protein (5). Inability to detect KMnO_4 reactivity in the T residue adjacent to the KMnO_4 -reactive T suggests that bEBNA1 does not melt the DNA. Furthermore, KMnO_4 oxidation of C residues has been detected upon distortion of DNA structure by protein binding (6, 11). In the DS element, multiple C residues are

FIG. 3. Summary of KMnO_4 and DMS protection assays. Computer model of the DS element DNA helix showing positions of bEBNA1-induced KMnO_4 -reactive thymines (red, and arrows), the DMS-hyperreactive adenosine (purple); and DMS-protected guanines (blue). The positions of the four EBNA1-binding sites are indicated beside the helix. The right side of the figure shows an enlargement of the reactive sections of the DS element. The two sections are slightly rotated (but to the same degree as one another) relative to the long helix on the left side of the figure.

adjacent to each KMnO_4 -reactive T, yet none were oxidized by KMnO_4 .

The chemical reactivity of the bEBNA1-*oriP* complex is consistent with the reactivity of bent DNA. For example, the λ O protein binds one face of its binding site and bends the DNA toward itself, opening the grooves on the opposite side

and exposing those bases to chemical attack (34). The *lac* transcription complex also binds one face of the helix and bends the DNA, with the result that nucleotides on one side become protected from attack by DMS, while those on the other face are hyperreactive (4). The two KMnO_4 -reactive thymines and the DMS-hyperreactive adenosine induced by bEBNA1 in *oriP* are all on the same face of the helix (Fig. 3, red and purple nucleotides, respectively). The G residues protected from DMS by bEBNA1 are largely confined to the opposite face of the helix (Fig. 3, blue nucleotides). This bilateral pattern of protected and reactive residues suggests that bEBNA1 binds to one face of the DNA helix and causes residues on the opposite face to become reactive to modification reagents, as is the case of protein-induced DNA bending. Bending of the DS element by bEBNA1 is consistent with electron microscopic observations of the bEBNA1-DS element complex, which appears as a ball with DNA entering and leaving the same side (12a). However, despite these correlations in reactivity of bent DNA and the bEBNA1-induced distortions in *oriP*, it is still possible that bEBNA1 melts, winds, unwinds, untwists, or otherwise distorts *oriP*.

The significance of the bEBNA1-induced distortions of the DS element to initiation of replication will require further studies, but their relevance is suggested by their occurrence within the site of initiation (8, 14, 31). The two sites of distortion are positioned on both ends of the DS element and on opposite strands of DNA. Hence, it is tempting to speculate that these may be sites at which two bidirectional replication forks are assembled. The localized distortion of the DNA helix induced by EBNA1 might be expanded to melted regions by cellular proteins as a first step in the process of initiation of replication.

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