Studies on the Mechanism of DNA Linking by Epstein-Barr Virus Nuclear Antigen 1*

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Epstein-Barr virus nuclear antigen 1 (EBNA1) can both bind to and link DNA. Dimers of EBNA1 bind specific sites, two clusters of which, the FR and DS, comprise the necessary cis-acting elements of the Epstein-Barr viral origin of plasmid replication. EBNA1-dimers can link FR and DS, looping out the intervening DNA. EBNA1 can also intermolecularly link DNAs to which it binds. Residues of EBNA1 that can mediate linking have been mapped to at least three, non-overlapping domains. Two disparate mechanisms could underlie self-association of linking domains: 1) linking domains could associate with other linking domains directly, or 2) linking domains could associate indirectly by binding to a common nucleic acid intermediate. We have found that EBNA1 can link DNA by each of these mechanisms, however, the linking domains associate directly with a greater apparent affinity than through a nonspecific nucleic acid intermediate.

The Epstein-Barr virus (EBV) infects human B-lymphocytes and usually establishes a latent infection in them. In vivo and in vitro, the latently infected cells are induced to proliferate. Interestingly, the viral genome is maintained in these cells as a plasmid which is both replicated conservatively during S-phase and maintained efficiently at a stable copy number (1–4). Only one of the latent viral gene products, EBNA1, and a small (1.8 kbp) cis-acting element (oriP) are required to recapitulate faithful plasmid replication in human and some other cells (5–7). Replication of oriP plasmids provides a useful model for studying control of initiation of replication, segregation of replicated DNAs, and maintenance of those DNAs in the mammalian nucleus.

Dimers of EBNA1 bind specifically to degenerate 20-bp sequences of DNA (8). The carboxyl-terminal one-third of EBNA1 contains the residues sufficient for both dimerization and DNA binding (Fig. 1) (9–12). (In this report dimerization of EBNA1 contains 26 identified sites to which EBNA1 binds (13). Twenty-four of these sites are within two clusters which comprise oriP (5). Twenty sites with a high affinity for EBNA1 are embedded within a series of 30-bp repeats, termed the family of repeats (FR). The dyad symmetry element (DS), which is located 1 kbp away from FR contains 4 binding sites for EBNA1 with lower affinity than those in FR, two of which are part of a 65-base pair dyad (5, 8, 14, 15). EBNA1, when bound to FR, can activate transcription of two viral promoters, one of which is 10 kbp away (16–18). The ability of EBNA1 to bind to DNA is essential for its activation of replication and transcription through oriP (19, 20).

In addition to binding to FR and DS, EBNA1 can also link them, forming a loop of the intervening DNA (21, 22). Activities of EBNA1 other than DNA binding and DNA linking have not been identified. EBNA1 purified from insect and mammalian cells lacks detectable helicase or ATPase activity (23, 24). EBNA1’s apparent lack of enzymatic activities led several labs to search for proteins with which EBNA1 can interact. The most intriguing candidate thus far reported is EBNA1 itself. Does the ability of EBNA1 to link DNA contribute to its activation of transcription and replication? One study demonstrated that no small deletion within EBNA1, other than those which affect DNA-binding, abrogates the ability of EBNA1 to activate transcription or replication (19). The authors interpreted this finding to indicate that EBNA1 contains redundant activating domains. The linking domains of EBNA1 are redundant and therefore are reasonable candidates for its activating domains. Findings in another study support this contention (25). A derivative of EBNA1 lacking one of the three linking domains can activate transcription 9–28% and replication 4–28% as well as wild-type EBNA1. Derivatives of EBNA1 lacking all three linking domains fail to activate transcription or replication. DNA linking by EBNA1 is therefore likely to contribute to its activation of transcription and replication.

EBNA1 can form intramolecular loops between FR and DS and can also intermolecularly link DNAs to which it binds (21, 22, 24, 26–28). We have studied linking primarily in electrophoretic mobility shift assays (gel shifts) (28). Multiple derivatives of EBNA1 behave similarly in gel shift assays and three other described linking assays (26, 28). In a gel shift assay, DNAs linked by EBNA1 do not migrate appreciably into a 4% polyacrylamide gel. The efficiency with which a DNA is incorporated into a linked complex increases with the number of EBNA1-binding sites it contains, but linking of DNAs containing only one site can be detected. We have identified three regions of EBNA1, amino acids 54–89, 331–361, and 372–391, which mediate DNA linking. When fused to the dimerization and DNA-binding domain of GAL4 these domains of EBNA1 mediate linking of DNAs containing five GAL4-binding sites. We also observed that increasing concentrations of linking protein decrease the efficiency of linking. This phenomenon, reminiscent of dissolution of antibody-antigen complexes by
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Excess antigen, indicates that protein-protein interactions between the linking domains may underlie linking. We have tested this hypothesis.

Several mechanisms could explain how the DNA-linking domains of EBNA1 associate and thereby link DNA. Two possible mechanisms are: the linking domains associate through direct protein-protein interactions; and the linking domains associate indirectly by protein-nucleic acid interactions with common intermediates. The first putative mechanism invokes only protein-protein interactions. Because linking domains of EBNA1 function when fused to the exogenous DNA-binding protein, GAL4, models in which a linking domain interacts specifically with a DNA-binding domain cannot be supported. Therefore, if linking is mediated by protein-protein interactions, these interactions are likely to be between the linking domains themselves. The different linking domains of EBNA1 could associate heterotypically with one another or each could associate only homotypically. Linking domains could also associate by binding to nonspecific nucleic acid intermediates. In addition to the DNA bound site specifically by EBNA1, our linking assays find that with five GAL4-binding sites is the 109-bp fragment of DNA polymerase I in the presence of dATP, dGTP, dTTP, and [α-32P]dCTP (Amersham), and precipitated three times to remove unincorporated label (32).

The 41-bp DNA with two EBNA1-binding sites (41bp) was formed by annealing two oligonucleotides with the sequences 5'-ACGATAGCATTTGCTCAGTGCTGGAATATACTATCCCA-3' and 5'-TGGTAGTTATAGTTGTTATCCACTGGGTAGCATATGCTATCGT-3'. Prior to annealing, the oligonucleotides were 5'-end-labeled with [γ-32P]ATP (NEN Life Science Products) and T4 polynucleotide kinase (New England Biolabs). The double-stranded 41-mer was purified from a 15% polyacrylamide gel (32).

**Proteins**—The derivatives of EBNA1 (bEBNA1, N3630, and N3389) and the GAL4 derivatives (GAL4-1–94, GAL4(1–94)Æ54–89, GAL4(1–94)Æ31–361, and GAL4(1–94)Æ372–391) used in this study were all produced and purified as described previously (28). Briefly, bEBNA1 lacks amino acids 2–7 and all but 15 amino acids of the Gly-Gly-Ala repeats and was expressed in SF21 cells (23). N3630 and N3389 lack the first 360 and 389 amino acids, respectively, and were expressed in Escherichia coli. GAL4(1–94) contains amino acids 1–94 of the yeast trans-activator GAL4 plus six carboxyl-terminal amino acids coded by the poly-cloning region. The fusions designated GAL4(1–94)Æ4–# contain the indicated residues from EBNA1 cloned in-frame into the poly-cloning region.

**RESULTS**

**Effect of Linking Domains on the Efficiency of DNA Linking**—DNA linking by EBNA1 is inhibited at increasing concentrations of EBNA1 (28). Fusions of the DNA-linking domain of GAL4 and individual linking domains of EBNA1 also link DNAs containing GAL4-binding sites less efficiently as their concentration is increased beyond that which required to yield maximal linking. We tested whether each type of linking protein contributes to DNA linking by the other type. The derivative of GAL4(1–94)Æ54–89 (Fig. 1) to link DNAs containing three EBNA1-binding sites was tested in the absence of any competitor and in the presence of a 2.5-fold molar excess of three derivatives of EBNA1 (Fig. 1). Derivatives of EBNA1 which contain linking domains, bEBNA1 and N3360, inhibited linking by the fusion protein (Fig. 2A). N3389, which cannot link DNA, failed to inhibit linking by the fusion protein (Fig. 2A). The converse...
experiment was also conducted (Fig. 2B). The ability of NΔ360 to link DNAs containing two EBNA1-binding sites was tested in the absence of any competitor and in the presence of a 4.5-fold molar excess of four GAL4 derivatives (Fig. 1). Derivatives of GAL4 fused to linking domains of EBNA1, GAL4(1–94) & 54–89, GAL4(1–94) & 331–361, and GAL4(1–94) & 372–391, inhibited linking by NΔ360. Unfused GAL4(1–94) did not inhibit linking by NΔ360. In these experiments there was a direct correlation between proteins that can inhibit DNA linking and proteins that contain DNA-linking domains.

Effect of Peptides Containing Linking Domains on DNA Linking by EBNA1—The observation that proteins with linking domains could inhibit DNA linking led us to test whether linking domains alone would be sufficient to inhibit DNA linking by EBNA1. For most experiments, NΔ360, which contains only one linking domain, was studied to facilitate detection of inhibition of linking. Two DNAs encoding fragments of EBNA1 (residues 40–89 and 331–391, Fig. 1) were cloned into plasmids allowing efficient protein expression in bacteria. These fragments include all the amino acids from EBNA1 which are likely to contribute to DNA linking (28, 34). The fragments of EBNA1 are fused at their amino terminus to an epitope and at their carboxyl terminus to 6 histidines at the carboxyl terminus. The ability of NΔ360-mediated DNA linking to be specifically to the labeled DNA (data not shown). Inhibition of DNA linking by the peptides therefore does not result from a direct correlation between proteins that can inhibit DNA linking and proteins that contain DNA-linking domains.

The efficiency with which NΔ360 and NΔ389 to inhibit DNA linking, we sought a control peptide with a similar charge per molecule. Polysine (Sigma) with a mass distribution of 1000 to 4000 daltons (Da) can be estimated to have an average mass of 2500 Da and an average charge per molecule of +16 at neutral pH. The ability of pollysine, RNase A, and BSA to inhibit linking of DNAs containing two EBNA1-binding sites by NΔ360, a derivative of EBNA1 with one linking domain, was determined (Table I). The concentration of pollysine required to inhibit linking was less than that of BSA or RNase A. Pollysine, however, was less effective than A.A. 331–391 and A.A. 40–89 at inhibiting linking of DNAs containing two binding sites by NΔ360. The Kᵢ of pollysine was 35- and 60-fold greater than that for A.A. 40–89 and A.A. 331–391, respectively (Table I). These experiments demonstrate that the inhibition of DNA linking by A.A. 331–391 and A.A. 40–89 is not mediated by their charge alone.

Linking of DNAs containing two EBNA1-binding sites by bEBNA1 is more resistant to competition than is linking by NΔ360. bEBNA1, which has all three linking domains, links DNAs containing two EBNA1-binding sites almost twice as efficiently as does NΔ360, which has only one linking domain (28). Linking of DNAs containing two EBNA1-binding sites by bEBNA1 was inhibited by A.A. 40–89 and A.A. 331–391 (Table I). The Kᵢ values of A.A. 40–89 and A.A. 331–391 for linking by bEBNA1 were, respectively, 140- and 110-fold higher than for linking by NΔ360. Even at the highest concentrations tested, the peptides did not displace bEBNA1 from binding site specifically to the labeled DNA (data not shown). Inhibition of DNA linking by the peptides therefore does not result from a competition for DNA binding. The linked complexes formed by...
bEBNA1 and the two binding site DNAs were also 6-fold more resistant to polylysine than similar complexes formed by NΔ360 (Table I). bEBNA1-mediated linking of DNAs with two binding sites was insensitive to RNase A and BSA in the range of concentrations tested (Table I).

Effect of Nonspecific DNA on the Efficiency of DNA Linking by EBNA1—To determine whether the poly(dI)poly(dC) used as a nonspecific competitor DNA contributes to linking in these assays, reactions were conducted in its absence. NΔ360 linked DNAs containing two EBNA1-binding sites were combined with 150 fmol of dimers of NΔ360 and 675 fmol of dimers of the indicated derivatives of GAL4. For both A and B, the reactions were manipulated and analyzed to determine the percentage of maximum DNA linked as in Fig. 3. The amount of DNA linked in the absence of competitor protein is set to 100% and was 15% for NΔ360 and 22% for GAL4(1–94) & 54–89. The percentage linked of this maximum is shown for the various competitors. The standard deviations are from two separate experiments.

FIG. 2. Inhibition of DNA linking is mediated by linking domains. EBNA1 derivatives that can link DNA inhibit linking by GAL4(1–94) & 54–89, those which cannot link DNA do not inhibit linking by GAL4(1–94) & 54–89. GAL4(1–94) does not inhibit linking by NΔ360; derivatives of GAL4(1–94) fused to three separate linking domains of EBNA1 can inhibit linking by NΔ360. A, 10 fmol of a DNA with five GAL4-binding sites were combined with 400 fmol of dimers of GAL4(1–94) & 54–89 and 1000 fmol of dimers of the indicated competitor. In one experiment the reactions contained 10 fmol of an unlabeled DNA containing 10 EBNA1-binding sites. The presence of this DNA makes no detectable difference. B, 20 fmol of a DNA with two EBNA1-binding sites were combined with 100 fmol of dimers of NΔ360 and 200 fmol of dimers of the indicated derivatives of GAL4. For both A and B, the reactions were manipulated and analyzed to determine the percentage of maximum DNA linked as in Fig. 3. The amount of DNA linked in the absence of competitor protein is set to 100% and was 15% for NΔ360 and 22% for GAL4(1–94) & 54–89. The percentage linked of this maximum is shown for the various competitors. The standard deviations are from two separate experiments.

FIG. 3. Fragments of EBNA1 that contain linking domains can inhibit DNA linking by NΔ360. The concentrations of the peptides required to inhibit DNA linking are significantly lower than the concentrations at which the peptides bind to DNA. A, 20 fmol of a DNA with two EBNA1-binding sites were combined with the indicated concentrations of dimers of NΔ360 and A.A. 331–391 prior to separation by electrophoresis through a 4% polyacrylamide gel. Linked DNA is incorporated into complexes too large to migrate significantly into the gel. The positions of linked DNA, unbound DNA, and DNA with one and two sites occupied by NΔ360 (1 and 2, respectively) are indicated. Phosphorimager analysis was used to calculate the percentage of linked DNA by dividing the amount of linked DNA by the total DNA in each lane, and this percentage is displayed at the bottom. The percentage bound is the fraction of the total DNA in the lane which is shifted above the bracket for unbound DNA. B and C, graphical representations of the ability of A.A. 331–391 (B) and A.A. 40–89 (C) to inhibit linking by 6.5 nm dimers of NΔ360 and to bind to DNA in the absence of NΔ360. The concentrations of A.A. 331–391 and A.A. 40–89 required to reduce linking to 50% of that in the absence of competitor are 2 and 1% of the concentration of each required to bind 50% of the DNA, respectively. The standard deviations are from three separate experiments.
The ability of 6.5 nM dimers of either NΔ360 or bEBNA1 to link 20 fmol of a DNA with two EBNA1-binding sites was measured in the absence of competitor as well as in the presence of a range of concentrations of the indicated competitors. The concentration of competitor which inhibits linking to 50% of that in its absence (Kᵢ) was determined. The highest concentrations of RNase A and BSA tested were 42,000 and 8,800 nM, respectively.

| Competitor | Kᵢ (nM)  | Kᵢ (nM) |
|------------|----------|----------|
| A.A. 40–89 | 55       | 7,600    |
| A.A. 331–391 | 30   | 3,300    |
| Polysine   | 1,900    | 11,000   |
| RNase A    | 30,000   | >42,000  |
| BSA        | >8,800   | >8,800   |

The ability of 6.5 nM dimers of either γΔ360 or bEBNA1 to link 20 fmol of a DNA with two EBNA1-binding sites was measured in an otherwise identical reaction, this complex was not observed (data not shown). We interpret this complex to be DNA bound site specifically at both EBNA1-binding sites and non-site specifically elsewhere. bEBNA1 also linked DNAs containing two EBNA1-binding sites similarly in the presence and absence of poly(dI)poly(dC) (data not shown). These experiments demonstrate that the presence of nonspecific competitor DNA is not required for DNA linking by EBNA1.

**Effect of Nucleic Acids Other Than EBNA1-binding Sites on the Efficiency of DNA Linking by EBNA1**—Nucleic acids other than those bound site specifically by EBNA1 were introduced into the linking reactions from several sources. Poly(dI)poly(dC) added to standard reactions was not required for linking (Fig. 4). DNA flanking the EBNA1-binding sites and any DNA or RNA contaminating the protein preparations were also added to linking reactions. The ability of NΔ360 which had been treated with RNase A and/or DNase I to link DNA in the absence of the other nonspecific nucleic acids was measured. First, poly(dI)poly(dC) was excluded from the reactions. Second, a 41-bp DNA containing two EBNA1-binding sites, but lacking sequences flanking those sites (41BP) was used as the probe. This DNA is expected from x-ray crystallographic studies to be coated by EBNA1 with little or no uncovered flanking DNA (12). Third, the NΔ360 was treated with DNase I or RNase A or both prior to conducting the linking assay (treatments described under “Experimental Procedures”). NΔ360 tested under these conditions linked DNA (data not shown). The percentage of DNA linked increased then decreased with increasing protein concentration. Nuclease-treated and mock-treated NΔ360 did not differ in their activity. DNA linking mediated by NΔ360 is not substantially affected by treatment to exclude nucleic acids, other than those bound by EBNA1, from our assays.

The ability of NΔ360 to link 41BP in the absence and presence of poly(dI)poly(dC) was measured (Fig. 5). 41BP behaved differently than the 131-bp DNA containing two EBNA1-binding sites and flanking sequences used formerly. In the absence of poly(dI)poly(dC) the average maximum percentage of 41BP linked was 32%, approximately three times more than that of the 131-bp DNA with flanking sequences. In the absence of poly(dI)poly(dC), linking of 41BP and of the 131-bp DNA was inhibited as the concentration of NΔ360 increased (Figs. 5 and 4). In the presence of poly(dI)poly(dC) (130 ng/μl; 3 μg/reaction), 41BP was linked extremely efficiently, increasing to 80% at the highest concentration of NΔ360 tested (Fig. 5). Linking of 41BP in the presence of poly(dI)poly(dC) differed from that of a similar DNA with nonspecific flanking sequences because it was not competed by excess NΔ360. The enhanced linking of 41BP by high concentrations of NΔ360 in the presence of poly(dI)poly(dC) was out of the range of the sensitivity of our assay.

**Table I**

| Competitor | Kᵢ against NΔ360 | Kᵢ against bEBNA1 |
|------------|------------------|-------------------|
| A.A. 40–89 | 55               | 7,600             |
| A.A. 331–391 | 30   | 3,300             |
| Polysine   | 1,900            | 11,000            |
| RNase A    | 30,000           | >42,000           |
| BSA        | >8,800           | >8,800            |

**Fig. 4.** DNA linking by NΔ360 does not require poly(dI)poly(dC). The efficiency with which NΔ360 links DNAs containing two binding sites in the presence or absence of poly(dI)poly(dC) is similar. A, 20 fmol of a DNA with two EBNA1-binding sites were combined with a range of concentrations of NΔ360 prior to separation by electrophoresis through a 4% polyacrylamide gel. The positions of linked DNA, unbound DNA, DNA with one and two sites occupied (1 and 2, respectively), and a slower mobility complex (3) are indicated. PhosphorImager analysis was used to calculate the percentage of linked DNA of the total DNA in each lane, and this percentage is displayed at the bottom. B and C, DNA linking by NΔ360 in the absence (B) and presence (C) of 3 μg of poly(dI)poly(dC) per reaction was measured. Each graph plots the percentage of DNA with two EBNA1-binding sites linked against the concentration of dimers of NΔ360. Standard deviations are from four or five experiments in B and C, respectively. The data in C is a composite of that published previously (28) and additional new measurements.
We have used gel shift assays to study the mechanism of DNA linking by EBNA1 and its derivative Na360. We had shown previously that EBNA1 in excess of that required to bind all the EBNA1-binding sites in a reaction inhibited linking in that reaction (28). Competition by excess EBNA1 could be detected in the presence of a 40-fold excess of competitor DNA fragments. The competing protein was in excess relative to the DNA-bound protein, but not relative to the competing DNA. This finding indicated that protein-protein interactions could mediate DNA linking. The competition was hypothesized to be mediated by the linking domains of proteins not bound to DNA interacting with the linking domains of proteins bound to DNA, thereby disrupting links between DNA-bound proteins. One prediction of this hypothesis is that only proteins with linking domains would compete with DNA linking. An extension of this proposal is that linking domains alone would compete with DNA linking.

These predictions have been tested and confirmed. Only proteins which contained linking domains could inhibit DNA linking when provided in excess (Fig. 2). DNA linking by Gal4(1–94)&54–89 could be inhibited by hEBNA1 and Na360, but not by Na389. DNA linking by Na360 could be inhibited by Gal4(1–94)&54–89, Gal4(1–94)&331–361, and Gal4(1–94)&372–391, but not by Gal4(1–94). Because proteins with DNA-linking domains inhibit linking and derivatives without linking domains do not inhibit linking, it is likely that the linking domains themselves are mediating this inhibition. Proteins competed similarly whether they contained the same or different linking domain(s) than that of the protein with which they were competing. hEBNA1, which contains amino acids 54–89, and Na360, which lacks amino acids 54–89, could compete similarly for linking by Gal4(1–94)&54–89 (Fig. 2A). Gal4 fused to linking domains of EBNA1 competed equally well with DNA linking by Na360 whether the fusion contained the same or a different linking domain than found in Na360 (Fig. 2B). These observations indicate that EBNA1’s linking domains can interact heterotypically with one another.

Two peptides (A.A. 40–89 and A.A. 331–391, Fig. 1) which together contain all three of the identified linking domains inhibit DNA linking by EBNA1. Each of these peptides can also bind nonspecifically to DNA. The percentage of a DNA with two EBNA1-binding sites linked by Na360 could be halved by a 5-fold excess of A.A. 331–391 relative to Na360 (Fig. 3, A and B). At this concentration of A.A. 331–391 (30 nM), the poly(dI)·poly(dC) used as a competitor DNA was present in greater than 10-fold molar excess. Similarly, A.A. 40–89 could mediate a 50% inhibition of linking at 55 nM. The peptides can efficiently inhibit linking at concentrations within 10-fold of the concentration of Na360–peptide concentrations far less than that of the competitor DNA. These results indicate that inhibition of linking by the peptides is mediated by interactions with Na360 rather than nucleic acids.

We compared the ability of control proteins (BSA, RNase A, and polylysine) to inhibit DNA linking by Na360 (Table I). The ability of Na360 to link DNAs containing two EBNA1-binding sites was reduced only to 80% by 8.8 μg BSA. Linking could be inhibited to 50% by 30 μg RNase A. Approximately 1000-fold more RNase A than A.A. 331–391 was required to reduce linking by 50%. A.A. 40–89 and A.A. 331–391 each are basic peptides with predicted net charges of +10 and +15 at neutral pH, respectively. The ability of polylysine with a similar charge per peptide to compete with linking mediated by Na360 was significantly greater than BSA or RNase A, but significantly less than A.A. 40–89 or A.A. 331–391 (Table I). Linking could be inhibited to 50% by 1.9 μg polylysine, a 35- or 60-fold higher concentration than that required for similar inhibition by A.A. 40–89 or A.A. 331–391, respectively. This result demonstrates that the inhibition of Na360-mediated DNA linking by A.A. 40–89 and A.A. 331–391 may be mediated in part by nonspecific affects of their charge, but specific affects of their sequence or structure also contribute to their inhibitory activity.

The efficiency with which DNAs are linked by EBNA1 likely reflects the valency of the complexes formed by the DNA and EBNA1. Na360, which has one of three linking domains of EBNA1, links greater than 95% of DNAs with 10 EBNA1-binding sites (28), but only 12% of DNAs with two EBNA1-binding sites (Fig. 3). A.A. 331–391 competes for linking by Na360 of the 10 binding site DNAs approximately 0.3% as effectively as for linking of the two binding site DNAs (data not shown). hEBNA1, which has all three linking domains, links DNAs containing two EBNA1-binding sites approximately twice as efficiently as Na360. A.A. 40–89 and A.A. 331–391 compete approximately 1% as effectively as linking of a DNA with two binding sites by hEBNA1 as they do for linking of it by Na360 (Table I). The greater valency of contacts between linking domains within efficiently linked complexes presumably underlies the greater resistance of these complexes to being dissolved by peptides containing linking domains. The estimated concentration of EBNA1 in a nucleus with a 5-μm diameter is 1 mM (35). Assuming looping of orfP is important for EBNA1-dependent activities of orfP, the large number of EBNA1-binding sites in FR may be necessary to stabilize looping to DS in the presence of such high concentrations of EBNA1.
The high positive charge of the linking domains permits them to associate with nucleic acids at neutral pH. That such binding is unessential for linking was demonstrated by treatment of protein with nucleases prior to testing that protein in linking assays from which all nucleic acids, other than those bound site specifically by EBNA1, had been excluded. Experiments with a DNA that contains two binding sites for EBNA1 but lacks flanking sequences, 41BP, demonstrated that sufficiently long, nonspecific DNAs can contribute to linking (Fig. 5). We hypothesize that this contribution can be masked by nonspecific DNA flanking the binding sites for EBNA1, because the high local concentration of this flanking DNA favors its association with the linking domains of bound EBNA1.

The apparent affinity of protein-protein interactions between linking domains is greater than the apparent affinity of the linking domains for nonspecific DNA. A large difference exists between the concentration of nonspecific DNA required to enhance linking of 41BP (40–800 nM) and the concentration of N360 required to link 41BP in the absence of nonspecific DNA (5 nM). Interaction of linking domains with DNA may occur in vivo. However, because the apparent affinity of the linking domains for other linking domains is significantly higher than for DNA (or for RNA (29)), we predict that protein-protein interactions predominate in vivo.

Observations described in the Introduction support the assertion that DNA linking contributes to the activation of transcription and replication by EBNA1. That EBNA1 is likely to link DNA in vivo at oriP also supports this contention. Evidence for linking in vivo is indirect but strong. The length of oriP (1.8 kbp or 0.6 μm for B-form DNA) dictates that both FR and DS, and the EBNA1 bound to them, are confined to a maximum volume of approximately 0.1 femtoliters. Therefore, the concentration of EBNA1-binding sites at oriP is minimally 300 nM. EBNA1 occupies all of its binding sites at oriP for at least the majority of the cell cycle (36). In vitro, DNAs bound by EBNA1 are linked at concentrations far less than 300 nM. In Fig. 4, lane 4, 7 fmol of DNA is bound by EBNA1; therefore, the maximum concentration of occupied EBNA1-binding sites is 0.6 nM, and linking is readily detected. Because the concentration of binding sites at oriP is approximately 500-fold higher, linking likely occurs in vivo.

Interactions between linking domains are mediated by specific sequences or structures of the linking domains. It is a distinct possibility that specific interactions between the linking domains of EBNA1 and other proteins also occur. Twenty amino acids of EBNA1 are sufficient to inhibit specifically linking of DNA by EBNA1 (GAL4(1–94)&372–391, Fig. 2). Smaller molecules may also be effective inhibitors of linking domain interactions. EBV is associated with many diseases including several malignancies. In all of these diseases EBNA1 is expressed in infected cells and sometimes only EBNA1 is expressed. Therefore, small molecules which inhibit EBNA1’s functions, such as DNA linking, could be clinically useful.

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REFERENCES
1. Adams, A. (1987) J. Virol. 61, 1743–1746
2. Yates, J. L., and Guan, N. (1991) J. Virol. 65, 483–488
3. Sugden, B., and Warren, N. (1988) Mol. Biol. Med. 5, 84–94
4. Kirchmaier, A. L., and Sugden, B. (1990) J. Virol. 64, 1280–1283
5. Reisman, D., Yates, J., and Sugden, B. (1985) Mol. Cell. Biol. 5, 1822–1832
6. Lupton, S., and Levine, A. J. (1985) Mol. Cell. Biol. 5, 2533–2542
7. Yates, J., Warren, N., Reisman, D., and Sugden, B. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 3806–3810
8. Ambinder, R. F., Shah, W. A., Rawlins, D. R., Hayward, G. S., and Hayward, S. D. (1996) J. Virol. 70, 2369–2379
9. Ambinder, R. F., Mullen, M., Chang, Y.-N., Hayward, G. S., and Hayward, S. D. (1991) J. Virol. 65, 1466–1478
10. Inoue, N., Harada, S., Homma, T., Kitamura, T., and Yanagi, K. (1991) Virology 182, 84–93
11. Chen, M.-R., Midldorff, J. M., and Hayward, S. D. (1993) J. Virol. 67, 4875–4885
12. Bochkareva, A., Barwell, J. A., Puetzner, R. A., Bochkareva, E., Fрапплиер, L., and Edwards, A. M. (1990) Cell 84, 791–800
13. Rawlins, D. R., Mulman, G., Hayward, S. D., and Hayward, G. S. (1985) Cell 42, 859–868
14. Jones, C. H., Hayward, D., and Rawlins, D. R. (1989) J. Virol. 63, 101–110
15. Iser, R., Bankier, A. T., Biggin, M. D., Deninger, P. L., Farrell, P. J., Gibson, T. J., Hatfull, G., Hudson, G. S., Satchwell, S. C., Seguin, T., Tuffnell, P. S., and Barrett, B. G. (1984) Nature 310, 207–211
16. Sugden, B., and Warren, N. (1989) J. Virol. 63, 2644–2649
17. Gahn, T. A., and Sugden, B. (1995) J. Virol. 69, 2633–2636
18. Puglielli, M. T., Desai, N., and Speck, S. H. (1997) J. Virol. 71, 129–128
19. Yates, J. L., and Camiloi, S. M. (1988) Cancer Cells 6, 197–205
20. Polvino-Bodnar, M., and Schaffer, P. A. (1992) Virology 187, 591–603
21. Su, W., Middleton, T., Sugden, B., and Echols, H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10870–10874
22. Frappilier, L., and O'Donnell, M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10875–10879
23. Frappilier, L., and O'Donnell, M. (1991) J. Biol. Chem. 266, 7819–7826
24. Middleton, T., and Sugden, B. (1990) J. Virol. 64, 489–495
25. Kirchmaier, A. L., and Sugden, B. (1997) J. Virol. 71, 1766–1775
26. Goldsmith, K., Bendell, L., and Frappilier, L. (1995) J. Virol. 67, 3418–3426
27. Frappilier, L., Goldsmith, K., and Bendell, L. (1994) J. Biol. Chem. 269, 1057–1062
28. Mackey, D., Middleton, T., and Sugden, B. (1995) J. Virol. 69, 6199–6208
29. Snudden, D. K., Hearing, J., Smith, P. R., Grassier, F. A., and Griffin, B. E. (1994) EMBO J. 13, 4840–4847
30. Middleton, T., and Sugden, B. (1994) J. Virol. 68, 4067–4071
31. Lillie, J. W., and Green, M. R. (1989) Nature 338, 39–44
32. Sambrook, J., Fritsch, E. F., and Maniatis (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
33. Miller, G., and Lipman, M. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 190–194
34. Laine, A., and Frappilier, L. (1995) J. Biol. Chem. 270, 30914–30918
35. Sternus, L., Middleton, T., and Sugden, B. (1990) J. Virol. 64, 2407–2410
36. Haieh, D. J., Camiolo, S. M., and Yates, J. L. (1993) EMBO J. 12, 4933–4944