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Mechanistic studies on the DNA linking activity of Epstein–Barr nuclear antigen 1

Tina M. Avolio-Hunter and Lori Frappier*

Department of Medical Genetics and Microbiology, University of Toronto, 1 Kings College Circle, Toronto, Ontario, Canada

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ABSTRACT

The DNA replication, plasmid segregation and trans-activation functions of Epstein–Barr nuclear antigen 1 (EBNA1) require the binding of EBNA1 to specific DNA recognition sites in the two non-contiguous functional elements of the Epstein–Barr virus latent origin of replication, *oriP*. EBNA1 molecules bound to these elements interact with each other resulting in the formation of looped individual DNA molecules and multiply linked DNA molecules. We have developed a glycerol gradient sedimentation assay suitable for quantitative analysis of the DNA linking activity of EBNA1 and used it to investigate the contribution of EBNA1 residues to the linking interaction and the mechanism of the interaction. Using overlapping internal deletion mutants, we found that two regions of EBNA1 can cause DNA linking, amino acids 40–100 and 327–377, but that the stabilities of the linked complexes formed by the two regions differ dramatically; only complexes formed through the latter region are stable to glycerol gradient sedimentation analysis. Mechanistic studies using EBNA1 in combination with GAL4–EBNA1 fusion proteins showed that linking interactions mediated by residues 327–377 are homotypic. Our results also suggest that only the DNA-bound form of EBNA1 participates in the protein–protein interactions seen in DNA linking.

INTRODUCTION

Epstein–Barr nuclear antigen 1 (EBNA1) is a multifunctional protein that plays several important roles during latent infection of human host cells by Epstein–Barr virus (EBV). First, EBNA1 activates replication of the episomal viral genome once every cellular S phase (1–4). Second, EBNA1 governs segregation of the viral episomes during cell division so that the EBV genome is stably maintained (5,6). Third, EBNA1 transactivates expression of other EBV latent gene products (7–9). All three of these EBNA1 functions require a direct interaction with the viral latent origin of replication, *oriP*. *oriP* is comprised of two non-contiguous functional elements, the dyad symmetry element (DS) and the family of repeats (FR) (10). The DS contains four EBNA1 binding sites and likely the initiation site for DNA synthesis (11–14). The FR contains 20 EBNA1 binding sites and, when bound by EBNA1, plays an important role in activating DNA replication from the DS, activating expression of other EBV latent genes and governing stable segregation of the EBV genome (5–8,11,14). The interaction of EBNA1 with the FR element has also been observed to cause replication forks to pause at this element (12,15).

In efforts to understand the mechanisms by which EBNA1 fulfils its replication, transactivation and segregation functions, we and others have purified EBNA1 and studied the properties of the purified protein *in vitro*. Although no enzymatic activities have been found to be intrinsic to EBNA1, the assembly of EBNA1 on *oriP* has been found to involve several types of homotypic interactions and to induce changes in the origin DNA structure (16–23). One type of EBNA1–EBNA1 interaction involves interaction at a distance between EBNA1 complexes assembled on the FR and DS elements. When the interaction occurs between the FR and DS elements on the same *oriP* molecule, the intervening DNA forms a loop and thus this has been termed the 'DNA looping' interaction (17,22,24). A similar interaction also occurs between EBNA1 molecules bound to FR and DS elements on different DNA molecules, which causes multiple DNA molecules to be linked together through a centralized EBNA1 complex and thus this interaction has been termed 'DNA linking' (24–27).

A region of EBNA1 that mediates the DNA looping and linking interactions has been localized to a central arginine-rich region between amino acids 327 and 377 (the looping domain) (24,27). These sequences retain the ability to mediate interactions between DNA-bound proteins when transferred to the GAL4 DNA binding domain and approximately half of this region is sufficient for linking activity (26–28). DNA looping and linking interactions mediated by the EBNA1 looping domain have been shown to stabilize EBNA1 binding to the DS element (22,27) but the *in vivo* function of these interactions has not yet been determined.

In this paper we have used a glycerol gradient assay to address questions concerning the mechanism of the EBNA1 linking interaction and the EBNA1 residues required for linking activity in the context of the complete EBNA1 protein. This assay has advantages over the assays used previously to study EBNA1 linking activity in that the amount of DNA linked can be readily quantified, multiple DNA fragments can be studied in combination and the stability of the linked complexes can be assessed.

MATERIALS AND METHODS

Construction of EBNA1 internal deletion mutants

EBNA1 mutants lacking amino acids 41–376 (bEBNAΔ41–376), 325–376 (bEBNAΔ325–376) and 356–362 (bEBNAΔ356–362),

*To whom correspondence should be addressed. Tel: +1 416 946 3501; Fax: +1 416 978 6885; Email: lori.frappier@utoronto.ca

in addition to amino acids 101–323 of the Gly-Ala repeat region, were constructed using two rounds of PCR amplification. In the first round of PCR two fragments were generated from the p205 version of EBNA1 (2), extending from the N-terminus to the deletion site and from the C-terminus to the deletion site. Primers adjacent to the deletion site contained *Spe*I restriction sites at their 5′-ends in addition to EBNA1 sequences. The amplified N- and C-terminal fragments of EBNA1 were gel purified, cleaved with *Spe*I and ligated together. Ligation products were subjected to a second round of PCR amplification using primers that hybridize to the N- and C-termini of EBNA1. In this way only ligation products that were joined through the cohesive *Spe*I ends were amplified. In addition to EBNA1 sequences, the N-terminal EBNA1 primer contained an *Eco*RI site and the C-terminal primer contained a *Bam*HI site at the 5′-end. The PCR products were digested with *Eco*RI and *Bam*HI and ligated between the *Eco*RI and *Bam*HI sites of the baculovirus transfer vector pVL1392 (29). These recombinant constructs were sequenced to confirm the EBNA1 deletion.

Recombinant baculoviruses were generated by co-transfecting Sf9 insect cells with the transfer vector containing EBNA1 sequences and Baculogold baculovirus DNA (Pharmingen, San Diego, CA). Recombinant viruses were subjected to two rounds of plaque purification prior to use for protein production. The EBNA1 proteins expressed from the recombinant viruses contain only EBNA1 sequences (as shown in Fig. 2) with the exception of two amino acids (Thr-Ser) that result from the *Spe*I linker spanning the deletion site.

Protein purification

bEBNA1 and full-length EBNA1 (containing the Gly-Ala repeat) were expressed from baculoviruses and purified as previously described (24,30). bEBNAΔ356–362 was expressed from a baculovirus and purified from Sf9 cells as described for bEBNA1 (24). $EBNA_{351–641}$ and $EBNA_{452–641}$ were produced in *Escherichia coli* and purified as described in Frappier *et al.* (27) and Barwell *et al.* (31), respectively. The production in *E.coli* and purification of GAL4–$EBNA_{351–377}$ and GAL4–$EBNA_{452–474}$ fusion proteins is described in Laine and Frappier (28). For the purification of EBNA1 internal deletion mutants, Sf9 monolayer cultures in 20 150 cm^2 tissue culture plates were infected with recombinant baculovirus and harvested 48 h post-infection as previously described (16). The cells were washed with phosphate-buffered saline and resuspended in 20 mM Tris–HCl, pH 7.5, 135 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 1% Nonidet P40, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT). After 1 h on ice, the lysate was clarified by centrifugation at 12 000 *g* for 30 min at 4°C. NaCl was added to the supernatant to a final concentration of 300 mM, then the extract was loaded onto a 20 ml heparin–agarose (BioRad) column equilibrated with 20 mM HEPES, pH 7.5, 300 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF (column buffer). The heparin column was washed with four column volumes of the column buffer and EBNA1 mutants were eluted with column buffer containing 1 M NaCl. EBNA1-containing fractions were pooled, diluted to a final concentration of 270 mM NaCl and loaded onto either a 1 ml HPLC Mono Q column (for bEBNAΔ41–376; Perceptive Biosystems) or a 1 ml HPLC MonoS column (for bEBNA-Δ325–376; Perceptive Biosystems) equilibrated with column buffer containing 270 mM NaCl. Both columns were developed with a 15 ml linear gradient from 270 mM to 1 M NaCl at 1 ml/min. Fractions containing the EBNA1 proteins were aliquoted and stored at –70°C. EBNA1 proteins in these fractions were estimated by SDS–PAGE and Coomassie staining to be >80% pure.

Electron microscopy

Saturating quantities of bEBNA1 were assembled onto a 3 kb DNA molecule containing the FR element of *oriP* and protein–DNA complexes were prepared for electron microscopy as previously described (17).

DNA fragments for glycerol gradient assays

The DNA fragment containing the DS element of *oriP* (310 bp) was excised from the plasmid pGEMoriP (16) by digestion with *Eco*RV and *Hin*dIII. The DNA fragment containing the FR element of *oriP* (800 bp) was excised from pGEMoriP by digestion with *Eco*RI and *Nco*I. The DNA fragment (170 bp) containing five tandem GAL4 DNA binding sites was generated by *Hin*dIII and *Xba*I digestions of $pGal4_5EIBTATACAT$ (32). The above DNA fragments were separated by agarose gel electrophoresis, purified using Qiaex beads (Qiagen) and end-labeled by filling in the recessed 3′-ends with [α-^{32}P]dCTP using DNA polymerase Klenow fragment.

Electrophoretic mobility shift assay (EMSA)

Purified bEBNA1 or $EBNA_{452–641}$ (28–1800 fmol dimer) was titrated onto 20 fmol end-labeled 800 bp FR DNA fragment in a 20 µl reaction containing 50 ng herring sperm DNA, 20 mM Tris–HCl, pH 7.5, 300 mM NaCl, 10 µM $ZnCl_2$, 5 mM $MgCl_2$, 0.5 mM DTT. After 10 min at room temperature, bound and unbound FR DNA molecules were separated by electrophoresis on a 5% non-denaturing polyacrylamide gel and DNA bands were visualized by autoradiography.

Glycerol gradient assays

End-labeled DNA fragments (200 fmol) were incubated for 10 min at room temperature with excess EBNA1 or GAL4–EBNA1 proteins (18–20 pmol protein except where otherwise indicated) in a 20 µl reaction containing 0.5 µg herring sperm DNA, 20 mM Tris–HCl, pH 7.5, 10 µM $ZnCl_2$, 5 mM $MgCl_2$, 0.5 mM DTT and either 300 (for EBNA1 proteins) or 150 mM (for GAL4–EBNA1 fusion proteins) NaCl. The higher salt concentration used for EBNA1 proteins was necessary to prevent precipitation of the protein that was not bound to DNA and the lower salt concentration used for the GAL4–EBNA1 fusion proteins was necessary for the GAL4 DNA binding domain to bind to the GAL4 sites. When DNA fragments containing EBNA1 sites and GAL4 sites were combined in the same reaction, 200 fmol each DNA fragment was used and EBNA1 was incubated with the DNA in 300 mM NaCl. After 10 min at room temperature, the GAL4–EBNA1 fusion protein was added, the NaCl concentration was diluted to 150 mM NaCl and reactions were further incubated for 10 min at room temperature.

Reactions were layered onto 2 ml 10–30% glycerol gradients at 4°C containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 µM ZnCl , 5 mM $MgCl_2$, 0.5 mM DTT and subjected to centrifugation for 30 min at 166 000 *g* at 4°C. After centrifugation, 17 115 µl fractions were collected from the top of the gradient and the bottom of the centrifuge tube was rinsed with 115 µl 3% SDS to resolubilize DNA that had been pelleted to the bottom of the tube

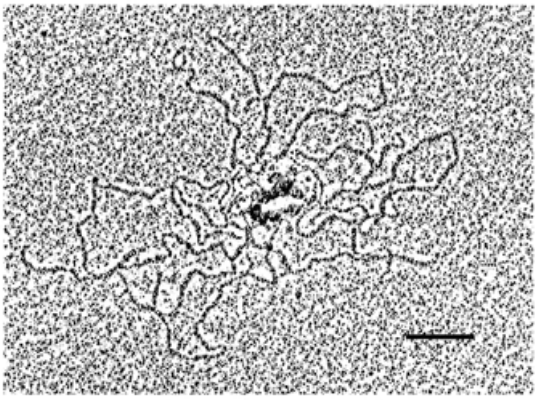

Figure 1. Electron micrograph of a linked complex. A linked complex generated by bEBNA1 binding to DNA containing the FR element is shown. Bar 0.1 µm.

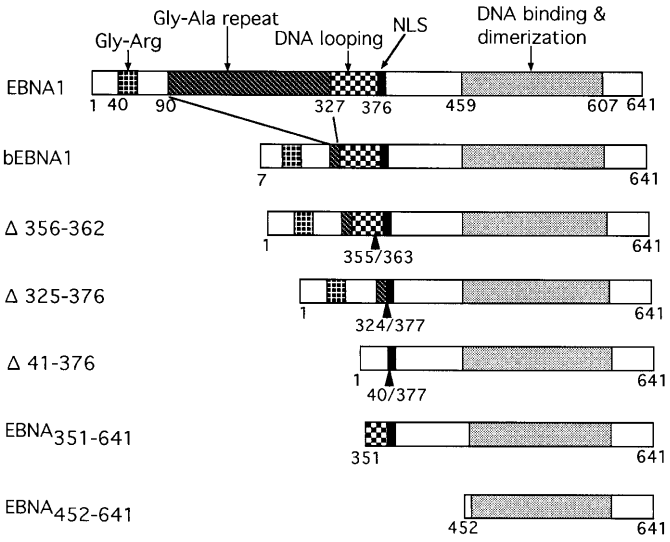

Figure 2. Schematic representation of the EBNA1 proteins used in this study. Amino acid numbers are indicated as are the Gly-Arg repeat, the Gly-Ala repeat, the DNA looping region, the nuclear localization sequence (NLS) and the DNA binding and dimerization domains. The position of the internal deletions are indicated by the arrowheads.

(fraction 18). Equal volumes of each fraction were subjected to electrophoresis on a 0.5× TBE 5% non-denaturing polyacrylamide gel. After electrophoresis, the gels were dried and the DNA fragments were visualized by autoradiography. For gradients that contained more than one DNA fragment, the amount of each DNA fragment in each fraction was quantified by PhosphorImager analysis (Molecular Dynamics) of the dried gels. For gradients which contained a single DNA fragment, the amount of DNA in each fraction was determined by counting a sample of each gradient fraction in the scintillation counter.

Ligation enhancement assays

Ligation enhancement assays were conducted as previously described using DNA templates generated by linearizing pGE-MoriP with *Mlu*I (24). In the resulting DNA fragment, the FR element is 280 bp from one end, the DS element is 710 bp from the other end and the two elements are separated by 3025 bp of plasmid DNA. This DNA template (45 fmol) was incubated with the indicated amounts of EBNA1 proteins in a 100 µl reaction containing 20 mM Tris–HCl, pH7.5, 300 mM NaCl, 20 mM $MgCl_2$, 2 mM ATP, 10 mM DTT, 2 mM spermidine. After 10 min at room temperature, the reaction was diluted 2-fold and one Weiss U T4 DNA ligase (Gibco BRL) was added, followed by a 5 min incubation at 15°C. The ligation reactions were stopped and the DNA was ethanol precipitated and analyzed by agarose gel electrophoresis as previously described (24).

RESULTS

The glycerol gradient assay

The interaction at a distance between DNA-bound EBNA1 molecules (resulting in DNA looping and linking) has previously been shown to occur by several methods but in none of these assays was the formation of linked DNA molecules readily quantifiable. We wished to develop an assay in which the DNA linking activity of EBNA1 was easier to quantify and that was more amenable to mechanistic studies. Previous electron microscopic (EM) studies have shown that EBNA1 binding to the FR or DS element of *oriP* causes the formation of large complexes in which multiple DNA molecules associate through EBNA1–EBNA1 interactions (22,24,27). An example of a linked complex formed by EBNA1 binding to DNA containing the FR element is shown in Figure 1. We explored whether glycerol gradient sedimentation could be used to separate these linked complexes from individually bound and unbound DNA molecules.

To this end, we incubated an end-labeled DNA fragment containing the FR element from *oriP* with either a biologically active version of EBNA1, containing both DNA binding and looping regions (bEBNA1; Fig. 2), or with a C-terminal fragment of EBNA1 that contains the DNA binding region but lacks the DNA looping domain ($EBNA_{452–641}$; Fig. 2). The binding reactions contained saturating quantities of the EBNA1 proteins (4-fold molar excess of EBNA1 to EBNA1 binding sites), as verified by EMSA (Fig. 3). Under these conditions >90% of DNA fragments were bound (as determined by quantification of the unshifted DNA molecules in the EMSA) and all 20 EBNA1 binding sites within each DNA fragment were filled. The filling of all 20 EBNA1 binding sites of the FR by saturating quantities of EBNA1 has been previously shown by us and others by DNase I footprinting (11; Frappier and O'Donnell, unpublished) and by determining the stoichiometry of EBNA1 dimers per *oriP* molecule (16). Protein–DNA complexes were layered onto glycerol gradients and subjected to ultracentrifugation. A very short centrifugation time (30 min) was used because it was our goal to separate the very large linked protein–DNA complexes from the individually bound DNA molecules. Fractions (1–17) were then collected from the top of the gradients and the bottom of the centrifuge tubes were rinsed with 3% SDS to resolubilize any very large DNA complexes (linked by EBNA1) that had sedimented against the bottom of the tube (fraction 18). The ^{32}P-labeled DNA fragment in each fraction was quantified by counting an aliquot in a scintillation counter. A second aliquot of each fraction was loaded (without altering the concentration of protein or buffer components) onto a non-denaturing polyacrylamide gel and, after electrophoresis, the positions of the labeled DNA fragments were detected by

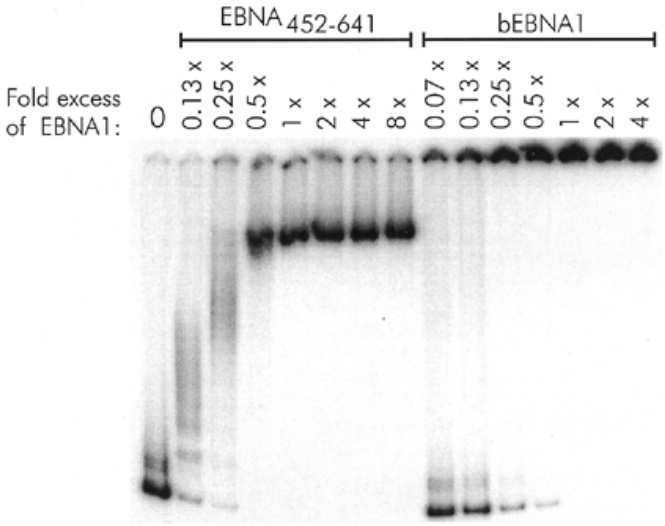

Figure 3. Electrophoretic mobility shift assay of bEBNA1 and $EBNA_{452–641}$ on the FR element. bEBNA1 and $EBNA_{452–641}$ were titrated onto the end-labeled FR DNA fragment and protein–DNA complexes were separated from unbound DNA by non-denaturing polyacrylamide gel electrophoresis. Linked bEBNA1–FR complexes are retained in the gel wells. The molar excesses of EBNA1 dimers over EBNA1 binding sites are indicated. The 4-fold molar excess of bEBNA1 and $EBNA_{452–641}$ corresponds to the amount used in the glycerol gradient analyses.

autoradiography. This EMSA was performed in order to verify that the DNA molecules that remained at the top of the gradient were still bound by EBNA1. We have previously shown that bEBNA1 and EBNA1 fragments containing the DNA binding domain form complexes with EBNA1 recognition sites that are extremely stable to dilution, high salt concentrations and competitor DNA (16; Cruickshank, Edwards and Frappier, unpublished) and, therefore, during the course of the glycerol gradient assay, the EBNA1–DNA complexes would not be expected to dissociate.

EMSA of the glycerol gradient fractions is shown in Figure 4. Under the centrifugation conditions used, the unbound FR fragments (Fig. 4A) and the FR DNA fragments bound by $EBNA_{452–641}$ (Fig. 4B) remained at the top of the gradient, with the latter complexes migrating further into the gradient than the unbound DNA. In contrast, incubation of the FR DNA with bEBNA1 resulted in the formation of much larger DNA complexes that sedimented completely to the bottom of the gradient tube (Fig. 4C). The electrophoretic mobility of the FR fragments at the top of the gradient in the $EBNA_{452–641}$ sample (Fig. 4B) is identical to the saturated FR–DNA complex, demonstrating, first, that EBNA1 remains stably assembled on the DNA during the assay and, second, that binding of EBNA1 to the DNA fragments was not sufficient to cause them to pellet to the bottom of the gradient. The FR fragments from the large protein–DNA complexes in fraction 18 did not co-migrate with the saturated DNA–protein complex because the SDS solution used to resolubilize the complexes stripped the protein from the DNA. In the experiments shown, 0.5 µg herring sperm DNA was included in the binding reaction; the same experiments were also conducted in the absence of competitor DNA with identical results.

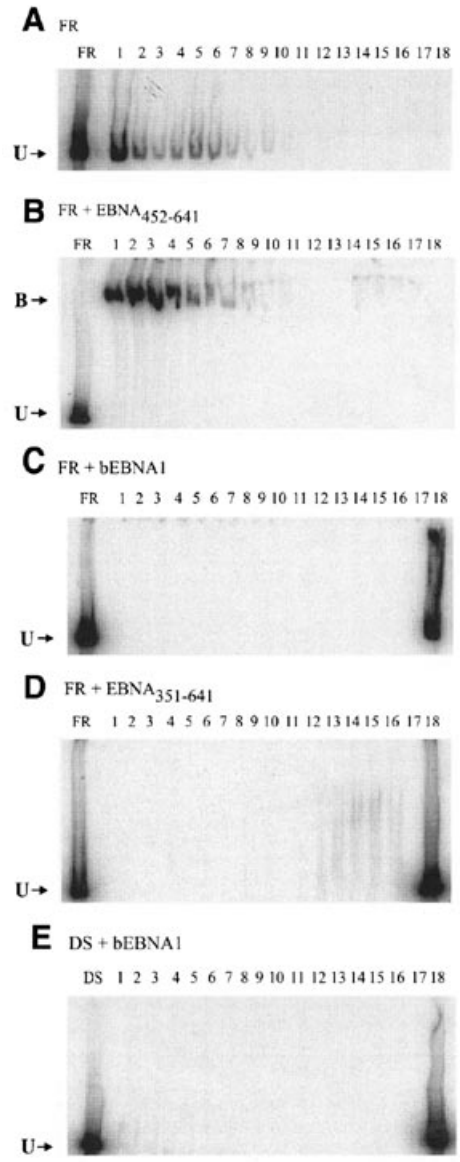

Figure 4. Glycerol gradient profiles. End-labeled FR (**A–D**) or DS (**E**) DNA fragments were assembled with an excess of the EBNA1 proteins indicated (B–E) then applied to the top of a 10–30% glycerol gradient or applied directly to the gradient without protein assembly (A). After centrifugation, samples were collected from the top (fraction 1) to the bottom (fraction 17) of the gradient and material pelleted on the bottom of the centrifuge tube was resolubilized in SDS (fraction 18). Equal volume samples from each fraction were analyzed by non-denaturing acrylamide gel electrophoresis along with an FR or DS DNA marker. Autoradiographs of the dried gels are shown and the positions of unbound (U) and bound (B) DNA molecules are indicated. Lane numbers refer to gradient fraction numbers.

The sedimentation of FR fragments to the bottom of the gradient by bEBNA1 but not by $EBNA_{452–641}$ is consistent with the results of previous assays that showed that sequences between amino acids 327 and 377 are important for DNA linking activity (24,27). To further verify that rapid sedimentation of the FR DNA was due to EBNA1 linking activity, we examined another EBNA1 truncation mutant that contains approximately half of the

DNA looping region but has been shown to retain full DNA linking activity ($EBNA_{351–641}$; Fig. 4D) (27). Consistent with previous results, $EBNA_{351–641}$ was found to mediate efficient linking of the FR DNA in the glycerol gradient assay. Like the FR, the DS element of *oriP* has been previously shown to form looped and linked complexes with EBNA1 (22,24). We analyzed the sedimentation of saturated EBNA1–DS complexes in the glycerol gradient and found that bEBNA1 linked the DS DNA with similar efficiency as the FR DNA (Fig. 4E). Thus the results of the glycerol gradient analysis are in excellent agreement with previous studies that measured the DNA looping and linking activity of EBNA1 and EBNA1 mutants.

From these studies we can conclude that the linked DNA complexes formed by EBNA1 are stable to glycerol gradient sedimentation and can be resolved from individually bound and unbound DNA molecules. The percentage of DNA molecules linked by EBNA1 could thus be quantified by determining the percentage of the ^{32}P-labeled DNA in fraction 18. Quantification of the DNA linking activities from Figure 4 showed that bEBNA1 linked 63% of the FR molecules to which it bound (Table 1; the percentage of FR molecules linked varied from 63 to 83 in multiple assays). The sum of the ^{32}P counts in each fraction indicated that recovery of the end-labeled DNA in the glycerol gradient assay was 90–100% (data not shown).

Table 1. EBNA1 amino acid requirements for DNA linking activity

Protein	DNA	DNA linked (%)[a]
0	FR	0.1
EBNA1 (+Gly-Ala)	FR	84.0
bEBNA1	FR	63.1
351–641	FR	80.1
452–641	FR	0.9
Δ41–376	FR	0.9
Δ325–376	FR	2.5
Δ356–362	FR	65.9
bEBNA1	DS	65.2
0	GAL4BS	0.3
$GAL4\text{-}EBNA_{452–474}$	GAL4BS	3.2
$GAL4\text{-}EBNA_{351–377}$	GAL4BS	69.2

[a]Percentage of labeled DNA in fraction 18 in Figures 4, 5 and 7.

EBNA1 amino acid requirements for DNA linking activity

All of the DNA looping/linking studies to date have been conducted on versions of EBNA1 lacking most of the Gly-Ala repeat region (amino acids 90–327). This region of EBNA1 is not required for the DNA replication, transactivation or DNA segregation functions of EBNA1 (2,33) but is important for evasion of the host immune response (34,35). Since wild-type EBNA1 contains this repeat region, we examined whether the Gly-Ala repeat affected the DNA linking activity of EBNA1. Figure 5B shows glycerol gradient sedimentation analysis of purified full-length EBNA1 on FR DNA fragments. Like bEBNA1, full-length EBNA1 linked the FR DNA efficiently (Table 1), indicating that the Gly-Ala repeat does not inhibit linking activity.

Using a series of N-terminal truncation mutants of bEBNA1, we showed previously that the EBNA1 arginine-rich region between amino acids 327 and 377 is required for DNA linking in the absence of the N-terminal portion of EBNA1 (24,27). However, we have not addressed the possibility that residues in the N-terminal portion of EBNA1 can also mediate DNA linking. In particular, we wished to test the possible involvement of the other arginine-rich region of EBNA1 which spans amino acids 40 to 53 (Gly-Arg repeat). To assess the possible contribution of this Gly-Arg repeat to DNA linking, we constructed internal deletion mutants of bEBNA1 that removed the previously defined DNA looping domain but left the N-terminal Gly-Arg repeat intact (bEBNAΔ325–376) or that removed both of these arginine-rich regions (bEBNAΔ41–376) (see Fig. 2). These proteins were produced from baculoviruses, purified and their DNA binding properties characterized. Saturated protein–DNA complexes were then assembled on FR DNA fragments and the complexes analyzed by glycerol gradient sedimentation. The removal of amino acids 325–376 was found to abolish the formation of linked DNA complexes, as did the larger 41–376 deletion (Fig. 5D and E and Table 1). The lack of linking activity was not due to dissociation of the proteins from the DNA; the FR DNA at the top of the gradient had an electrophoretic mobility consistent with a saturated EBNA1–FR complex (data not shown). The gradient sedimentation results therefore suggest that the Gly-Arg repeat or other N-terminal sequences cannot substitute for the internal looping domain in linking DNA.

Since the glycerol gradient sedimentation assay may not detect linked DNA complexes with fast dissociation rates, we next tested the possibility that N-terminal EBNA1 sequences mediated the formation of less stably linked DNA complexes that dissociated during centrifugation through the glycerol gradient. To this end, we employed the more rapid ligation enhancement assay, which can detect linked DNA complexes with faster off rates (24,27,28). In this assay EBNA1 proteins were assembled onto linear DNA templates that have the FR element close to one end and the DS element close to the other end, then ligase was added for a 5 min reaction. After removal of the proteins, the DNA products were separated by agarose gel electrophoresis. As shown in Figure 6, bEBNA1 induces the formation of DNA concatamers in this assay whereas $EBNA_{452–641}$ does not. These DNA concatamers reflect the linking of DNA molecules through the EBNA1 complexes bound near the DNA ends, which increases the efficiency with which the ends of the DNA molecules are ligated. When the assay was conducted with the EBNA1 internal deletion mutants, we found that bEBNA-Δ325–376, like bEBNA1, induced formation of DNA concatemers. In contrast, induction of DNA concatamers was not detected for bEBNAΔ41–376 even at much higher protein concentrations. The results indicate that EBNA1 sequences between amino acids 41 and 100 can link DNA (bEBNAΔ325–376 lacks amino acids 101–323 of the Gly-Ala repeat in addition to amino acids 325–376) but that these linked complexes are less stable than those that occur through the DNA looping domain (residues 325–376).

We also constructed and analyzed by glycerol gradient sedimentation the linking activity of a version of bEBNA1 containing a small deletion (Δ356–362) within the looping domain. As shown in Figure 5F, this mutation had no discernable effect on the DNA linking activity of bEBNA1 (see also Table 1).

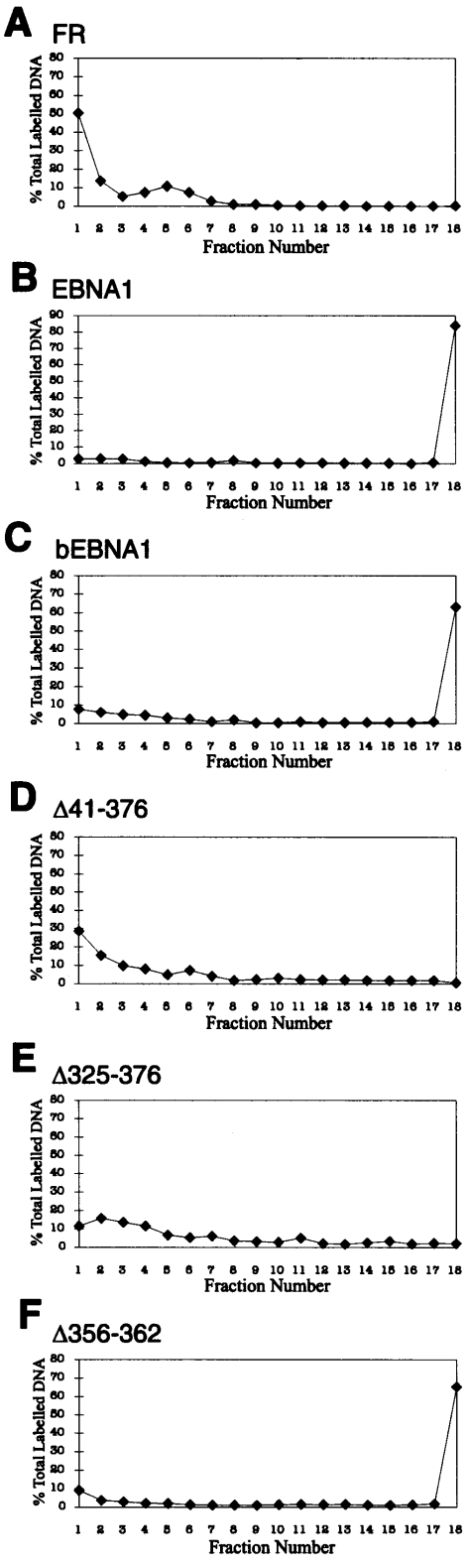

Figure 5. EBNA1 amino acid requirements for the formation of stably linked DNA complexes. End-labeled FR DNA fragments were left unassembled (**A**) or assembled with full-length EBNA1 (**B**), bEBNA1 (**C**), bEBNAΔ41–376 (**D**), bEBNAΔ325–376 (**E**) or bEBNAΔ356–362 (**F**) and then analyzed by glycerol gradient sedimentation and non-denaturing acrylamide gel electrophoresis as for Figure 4. The quantification of labeled DNA molecules in each glycerol gradient fraction is shown.

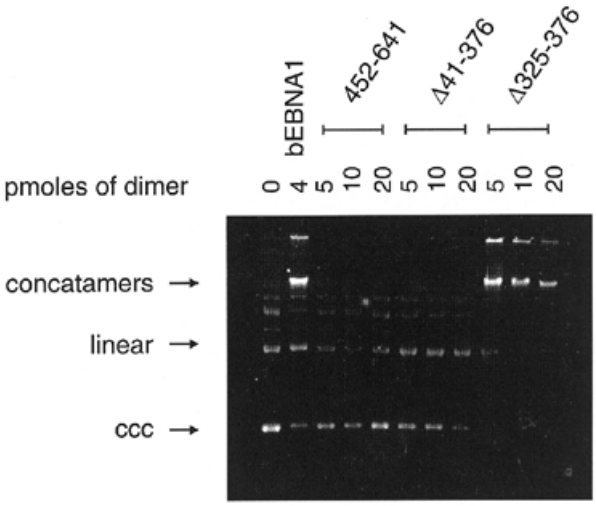

Figure 6. Ligation enhancement assay of EBNA1 mutants. bEBNA1, $EBNA_{452–641}$, bEBNAΔ41–376 and bEBNAΔ325–376 were titrated onto linear DNA templates containing the FR and DS elements. Ligase was then added to the protein–DNA complexes and, after a 5 min reaction, the protein was removed and the DNA products were separated by agarose gel electrophoresis in the presence of ethidium bromide. The position of the linear DNA substrate and covalently closed circular (CCC) and concatemeric DNA products are indicated. A ligation reaction performed in the absence of EBNA1 proteins is shown in the first lane (0).

This result is consistent with results from our previous studies that indicated that only approximately half of the looping domain sequence is required for linking activity (27,28).

We have previously shown by EM and ligation enhancement assays that amino acid sequences 320–355 and 351–377 from the EBNA1 looping domain retain DNA linking activity when transferred to the GAL4 DNA binding domain (28). To further assess the efficiency of formation and stability of the linked complexes generated by the GAL4–EBNA1 fusion proteins, we assembled saturating quantities of GAL4 fusion proteins containing the EBNA1 351–377 looping domain fragment or a negative control EBNA1 fragment (amino acids 452–474) onto DNA fragments containing five tandem GAL4 binding sites. These protein–DNA complexes were then analyzed by glycerol gradient sedimentation (Fig. 7). The fusion protein containing the looping domain sequences formed linked complexes with similar efficiency as bEBNA1 (Fig. 7C and Table 1), whereas the fusion protein containing EBNA1 sequences outside the looping domain ($GAL4–EBNA_{452–474}$) formed very few linked DNA complexes (Fig. 7B and Table 1). These results confirm that sequences from the looping region retain functionality when transferred to the GAL4 DNA binding domain and indicate that the linked DNA complexes formed by the fusion protein are similar in stability to those formed by EBNA1.

Mechanism of the DNA linking interaction

We next used the glycerol gradient sedimentation approach to ask several questions concerning the mechanism by which the EBNA1 looping domain mediates DNA linking. We first tested the possibility that the looping domain within bEBNA1 links DNA by directly interacting with DNA molecules *in trans*. We

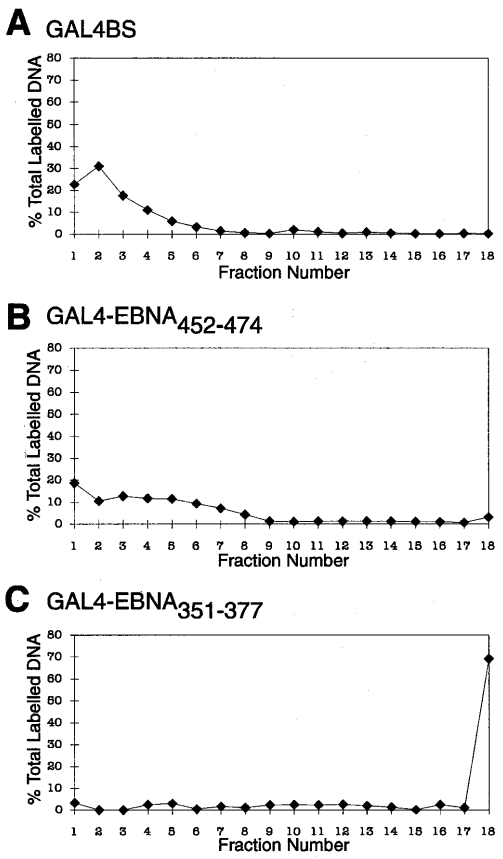

Figure 7. Glycerol gradient sedimentation analysis of GAL4–EBNA1 fusion proteins. A DNA fragment containing five tandem GAL4 sites was analyzed by glycerol gradient sedimentation either before (**A**) or after assembly with the indicated GAL4–EBNA1 fusion proteins (**B** and **C**).

incubated bEBNA1 with an equimolar mixture of two end-labeled DNA fragments of different lengths, one that contained the FR element and a second that lacked EBNA1 binding sites (GAL4BS), then analyzed the mixture by glycerol gradient sedimentation. The two DNA fragments from each gradient fraction were resolved using gel electrophoresis and quantified individually (Fig. 8A). The FR DNA fragments that were bound by bEBNA1 sedimented to the bottom of the tube but the DNA fragments that lacked EBNA1 binding sites were found at the top of the gradient. These results indicate that the looping domain of DNA-bound bEBNA1 preferentially associates with protein-bound DNA over naked DNA. In this experiment we excluded the herring sperm DNA normally added to the binding reactions, in order to facilitate detection of interactions with the labeled GAL4BS DNA.

We next asked whether the protein–protein association that results in DNA linking is homotypic or heterotypic, i.e. whether the looping domain is required on both DNA-bound proteins that interact to link the two DNA molecules. To address this question, we assembled, in one reaction an EBNA1 protein lacking the looping domain ($EBNA_{452–641}$) on FR or DS DNA fragments and a GAL4 fusion protein containing the EBNA1 looping domain ($GAL4–EBNA_{351–377}$) on a DNA fragment containing five tandem GAL4 binding sites (GAL4BS; saturating quantities of both proteins were used). Glycerol gradient sedimentation of

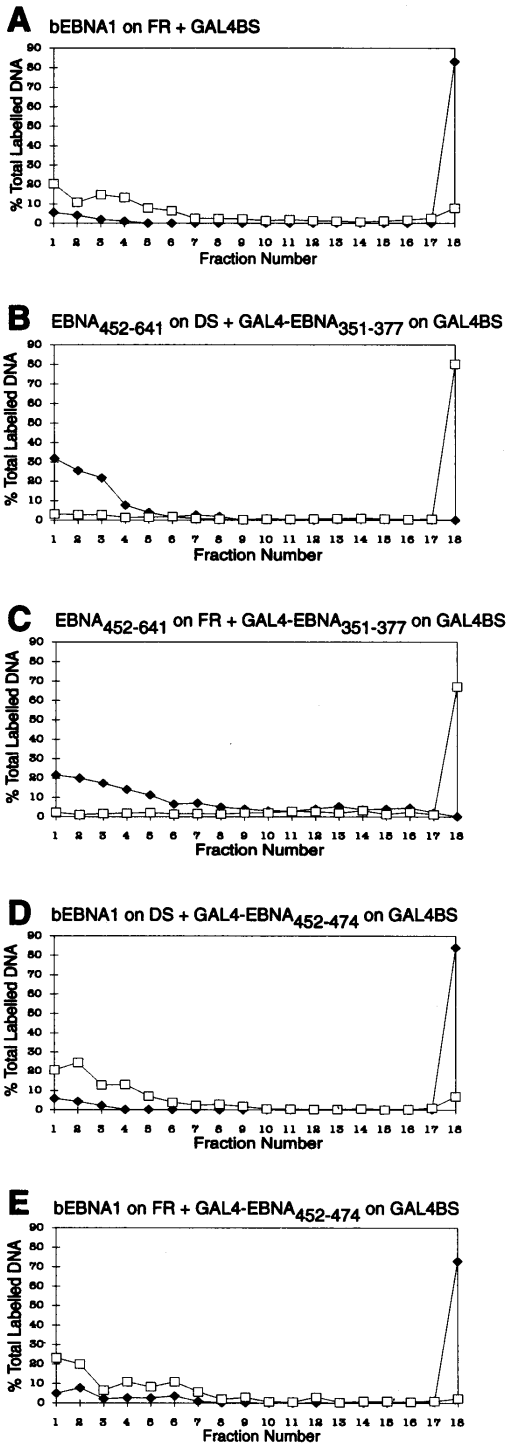

Figure 8. EBNA1-mediated DNA linking is a homotypic interaction. (**A**) bEBNA1 was assembled onto end-labeled FR DNA fragments in a reaction that also included end-labeled DNA fragments containing GAL4 binding sites (GAL4BS). (**B–E**) EBNA1 proteins that contained (bEBNA1) or lacked (EBNA452–641) the DNA looping domain were assembled onto end-labeled FR or DS DNA fragments and, in the same reaction, GAL4–EBNA1 fusion proteins that contained (GAL4–EBNA351–377) or lacked (GAL4–EBNA452–474) looping sequences were assembled onto end-labeled GAL4BS DNA fragments. The mixtures were analyzed by glycerol gradient sedimentation and non-denaturing acrylamide electrophoresis and, for each gradient fraction, the DNA fragments containing EBNA1 (closed diamonds) or GAL4 (open squares) binding sites were quantified separately.

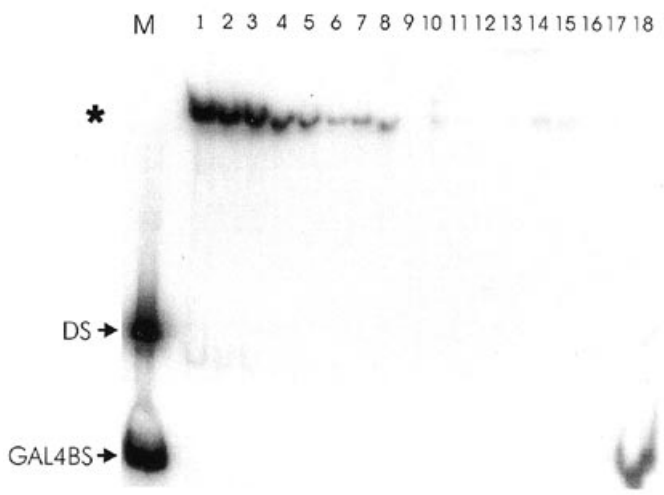

Figure 9. Electrophoretic mobility shift analysis of the glycerol gradient fractions of $EBNA_{452–641}$ on the DS with GAL4–$EBNA_{351–377}$ on GAL4BS. Non-denaturing polyacrylamide gel analysis of the glycerol gradient fractions corresponding to Figure 8B. A marker lane (M) shows the position of the unbound DS and GALBS DNA fragments. The position of the DS DNA fragment bound by $EBNA_{452–641}$ is also indicated (*). This shifted band was confirmed to contain the DS fragment (as opposed to the GAL4BS fragment) by incubating the fractions in SDS, to remove the bound protein, prior to electrophoretic analysis (data not shown).

the two DNA fragments was then analyzed. As shown in Figure 8B and C, GAL4–$EBNA_{351–377}$ linked the GAL4BS DNA fragments but the DNA fragments bound by $EBNA_{452–641}$ were not sequestered into these linked complexes. The EMSA of the gradient fractions that corresponds to Figure 8B is shown in Figure 9. The complementary experiment was also conducted in which the looping domain was present on the EBNA1 protein (bEBNA1) but not on the GAL4 fusion protein (GAL4–$EBNA_{452–474}$; Fig. 8D and E). In these cases the DNA fragments bound by bEBNA1 were linked together but were not found associated with the DNA fragments bound by the GAL4 fusion proteins. These results suggest that the looping domains are required on both partners in a linked complex.

We have previously shown that bEBNA1 and GAL4–$EBNA_{351–377}$ do not form large complexes stable to glycerol gradient sedimentation in the absence of DNA (16,28), indicating that DNA binding is a requirement for the protein–protein interactions that occur through the looping domain and lead to DNA linking. We have not, however, tested whether the looping domain of a bEBNA1 dimer in solution can interact with the looping domain of a DNA-bound EBNA1 molecule. If this were the case, then a large excess of EBNA1, over what is needed to fill the DNA binding sites, would be expected to inhibit linking by EBNA1-bound DNA molecules. To test this possibility we used the glycerol gradient sedimentation assay to compare the percentage of DS DNA molecules linked when the binding reactions contained saturating (6:1 molar ratio of EBNA1 dimers to EBNA1 binding sites) and super-saturating (25:1 and 125:1 molar ratio of EBNA1 dimers to EBNA1 binding sites) amounts of the EBNA1 molecules. As shown in Table 2, increasing the amount of EBNA1 from a 6- to a 125-fold excess did not inhibit DNA linking, indicating that soluble EBNA1 does not effectively compete for interactions with DNA-bound EBNA1.

Table 2. Effect of EBNA1 excess on DNA linking activity

Ratio of EBNA1 dimers to binding sites	DNA linked (%)
6:1	69.1
25:1	71.3
125:1	72.8

DISCUSSION

We have developed a glycerol gradient sedimentation assay for detection of linked DNA complexes generated by interactions between DNA-bound proteins and used this assay to analyze the DNA linking activity of EBNA1. This assay has an advantage over EM (22,24,27,28), ligation enhancement (24,27,28) and EMSA (26,36) used previously to study EBNA1 linking activity in that it is more quantitative and more amenable to mechanistic studies. Although EM can be used to determine the number of linked complexes formed, the number of DNA molecules sequestered into the linked complexes is difficult to determine by this method. For the ligation enhancements assays, quantification of the percentage of DNA molecules linked under different reaction conditions is complicated by the fact that several different ligated products are produced from linked DNA complexes and that the activity of the ligase used to detect the linked complexes is affected by changes in the reaction conditions. Measurements of linked DNA molecules by EMSA is difficult because it involves quantifiably distinguishing between low mobility DNA smears that result from protein–protein associations and discrete shifted bands generated by individually bound DNA molecules. This method also does not distinguish between linked DNA complexes formed in the binding reaction and the aggregation of protein–DNA complexes that occurs upon loading in the low salt, high pH conditions of the Tris/borate/EDTA electrophoresis buffer.

From our analysis of the DNA linking activity of EBNA1 by glycerol gradient sedimentation we can make the following conclusions. First, the linked DNA complexes formed by EBNA1 have slow dissociation rates and therefore are stable to glycerol gradient analysis. Second, sequences from the EBNA1 DNA looping region, between amino acids 327 and 377, are both necessary and sufficient to mediate interactions between DNA-bound proteins that lead to linked DNA complexes stable to glycerol gradient sedimentation. Third, DNA linking interactions mediated by the EBNA1 looping domain are homotypic interactions that preferentially occur between the looping domains of two DNA-bound proteins.

The finding that deletion of amino acids 325–376 from bEBNA1 disrupts the ability to form stably linked DNA complexes is in excellent agreement with our previous studies in which the DNA linking and looping activity of a series of N-terminal EBNA1 truncation mutants was analyzed by EM and ligation enhancement assay (24,27). In those studies, truncation mutants lacking most or all of the sequences between residues 327 and 376 were not observed to generate linked DNA complexes. The finding that deletion of amino acids 356–362 from bEBNA1 does not affect linking activity is in keeping with our previous studies using GAL4–EBNA1 fusion proteins, which showed that an EBNA1 fragment containing residues 320–355 was sufficient for linking activity in the absence of the remainder of the looping domain (28).

Using overlapping internal deletion mutants, we have also addressed the possible role of EBNA1 residues N-terminal of the looping domain in DNA linking. The results of ligation enhancement and glycerol gradient sedimentation assays indicate that sequences between amino acids 41 and 100 have some capacity to link DNA, but that this interaction is less stable than that formed through the DNA looping domain. The less stably linked DNA complexes may be mediated by the EBNA1 Gly-Arg repeat (amino acids 40–53), which is similar in sequence to the DNA looping domain, or by residues just C-terminal of this repeat (amino acids 54 and 89), as has been suggested by GAL4–EBNA1 fusion protein studies (26). Presently, the functional significance of the different stabilities of linked DNA complexes is not clear.

We have previously shown that, in the absence of DNA, dimers of EBNA1 or GAL4 fusion proteins that contain the EBNA1 looping domain do not interact to form complexes stable to glycerol gradient analysis (16,28). In addition, in both glycerol gradient assays and EM (17,24), soluble EBNA1 does not appear to disrupt interactions between DNA-bound bEBNA1 dimers. These results suggest that only DNA-bound forms of EBNA1 (or GAL4 fusion proteins) can interact through the DNA looping domain. The apparent requirement for DNA binding may simply reflect a requirement for multimerization of EBNA1 in order to detect EBNA1–EBNA1 interactions *in trans*. Alternatively, the DNA binding requirement might indicate that the looping domain must contact DNA before it is active in mediating protein interactions, possibly due to a change in structure of the looping region induced by the DNA interaction. The latter possibility is supported by the finding that peptides from the EBNA1 looping domain have a low but measurable affinity for DNA (our unpublished data). The looping domain could contact DNA flanking the EBNA1 recognition sequence or, since the EBNA1 DNA binding domain interacts with only one face of the DNA helix (23), could interact with the opposite face of the EBNA1 recognition sequence. The susceptibility of the EBNA1 looping domain to proteolysis suggests that it is unstructured in solution (Bochkareva, Edwards and Frappier, unpublished) and, therefore, we postulate that association of the looping domain with DNA is required for this region to adopt the structure involved in protein–protein interactions.

Ultimately we would like to determine the functional significance of the EBNA1–EBNA1 interactions that result in DNA looping and DNA linking. To this end we have been testing the activity of the EBNA1 internal deletion mutants characterized in this paper in transient DNA replication, long-term plasmid maintenance and transcription transactivation assays. Interestingly, we have found that deletion of amino acids 325–376, which disrupts formation of stably linked DNA complexes, has little effect on the ability of EBNA1 to support the transient replication of *oriP* plasmids but has a profound effect on the ability of EBNA1 to maintain these plasmids in long-term culture and to transactivate the expression of reporter genes (Shire, Ceccarelli, Avolio-Hunter and Frappier, in preparation; Ceccarelli and Frappier, in preparation). Thus the looping domain is biologically important and mediates the segregation and transactivation functions of EBNA1. The interpretation of these results, however, is complicated by the fact that the 325–376 deletion also disrupts the interaction of EBNA1 with at least one cellular factor of unknown function (Shire, Ceccarelli, Avolio-Hunter and Frappier, in preparation). Thus further studies are necessary to determine whether the functional importance of the looping domain is due to its ability to generate looped or linked EBNA1–DNA complexes or due to specific associations with cellular factors.

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