Identification of Epstein-Barr Virus Nuclear Antigen 1 Protein Domains That Direct Interactions at a Distance between DNA-bound Proteins*

(Received for publication, September 1, 1995, and in revised form, October 18, 1995)

Alexandra Laine and Lori Frappier†

From the Institute for Molecular Biology and Biotechnology, Cancer Research Group, Department of Pathology, McMaster University, Hamilton, Ontario L8N 3S5, Canada

The EBNA1 protein of Epstein-Barr virus (EBV) binds to and activates DNA replication from the EBV latent origin of replication, oriP, via a direct interaction with the two noncontiguous subelements of oriP. The EBNA1 molecules bound to the oriP subelements interact efficiently with each other by a DNA looping mechanism. We have previously mapped a region of EBNA1 (termed the looping region) that is required to mediate the interaction of the EBNA1 molecules bound to the oriP subelements. We now demonstrate that two fragments of this region of EBNA1, which consist largely of an eight amino acid repeat, can mediate homotypic interactions when transferred to another DNA-binding protein. Protein interactions mediated by the EBNA1 looping region appear to be dependent on DNA binding since these interactions were detected between DNA-bound forms of the proteins only.

The interaction at a distance between DNA-bound proteins by a DNA looping mechanism has been reported to underlie a variety of biological processes including transcription, DNA replication, and site-specific DNA recombination (reviewed in Matthews (1992) and Schleif (1992)). Proteins that participate in this type of interaction include the λ and lac repressors (Kramer et al., 1987; Hochschild and Ptashne, 1988), the E2 protein of bovine papilloma virus (Knight et al., 1991), the Ultrabithorax homeodomain protein (Beachy et al., 1993), heat shock transcription factor 2 (Wyman et al., 1995), Mu A and B proteins (Adzuma and Mizuuchi, 1989), and the replication initiation proteins of plasmids R6K and P1 (Chattoraj et al., 1988; Mukherjee et al., 1988a, 1988b). Despite the apparent importance of this type of protein-protein interaction, the nature of the protein domains that mediate these interactions and the mechanism(s) by which they occur remain unclear. In order to address these issues, we have examined in detail the DNA looping interactions mediated by the EBNA1 protein of Epstein-Barr virus (EBV).1

During latent infection of human host cells, EBV genomes are maintained as double-stranded DNA episomes that replicate once every cell cycle (Adams, 1987; Yates and Guan, 1991). EBNA1, in conjunction with host replication factors, governs this replication through a direct interaction with the genetically defined latent origin of replication, oriP (Rawlins et al., 1985; Reisman et al., 1985). oriP consists of two subelements, the family of repeats (FR) and the dyad symmetry element (DS), that are separated by approximately 1 kb of DNA (Reisman et al., 1985). The DS element contains 4 EBNA1 binding sites and appears to be the actual origin of replication (Rawlins et al., 1985; Gahn and Schildkraut, 1989). The FR contains 20 EBNA1 binding sites and, when bound by EBNA1, activates DNA replication from the DS element, enhances transcription from viral promoters, and governs the stable segregation of the EBV episomes during cell division (Reisman et al., 1985; Reisman and Sugden, 1986; Krysan et al., 1989; Gahn and Sugden, 1995).

Examinations of the interaction of pure EBNA1 with oriP DNA sequences have revealed that the EBNA1 dimers that assemble on the FR and DS elements of oriP interact efficiently with each other bringing the two elements together. This interaction results in the generation of looped DNA molecules (when the interaction occurs between EBNA1 bound to FR and DS elements in the same DNA molecule) and in the cross-linking of multiple DNA molecules through EBNA1 (when the interaction occurs between EBNA1 bound to FR and DS elements on different DNA molecules) (Frappier and O'Donnell, 1991a; Su et al., 1991; Middleton and Sugden, 1992). Such interactions between EBNA1 dimers depend on EBNA1 binding to DNA and do not occur between EBNA1 dimers in solution (Frappier and O'Donnell, 1991b). Interactions between EBNA1 molecules bound to the FR and DS elements stabilize EBNA1 on the DS element and likely are an important part of the mechanism by which the FR activates DNA replication from the DS element (Su et al., 1991; Frappier et al., 1994). In order to better understand the mechanism by which these interactions occur, we have used mutational analyses to identify a region of EBNA1 (termed the DNA looping region) that is required to mediate the interaction between FR- and DS-bound EBNA1 molecules (Goldsmith et al., 1993; Frappier et al., 1994). This region is distinct from the DNA binding and dimerization domains of EBNA1 and therefore likely directs the homotypic protein interaction. We now demonstrate that the DNA looping region is composed of at least two independent domains that are sufficient to mediate homotypic protein interactions and that retain the ability to mediate these interactions when transferred to another DNA-binding protein.

MATERIALS AND METHODS

DNA—GAL4 DNA substrates for ligation enhancement assays contain two copies of five tandem 17-bp GAL4 binding sites positioned 50 and 780 bp away from each end of the DNA fragment, respectively, and separated by 3.9 kb of DNA. oriP DNA substrates have been described.
Previously (Goldsmith et al., 1993) and are similar in construction to the GAL4 substrates except that they contain the two orP subelements (containing 4 and 20 copies of the EBNA1 binding site) instead of GAL4 binding sites. Both of these substrates have cohesive ends. The GAL4 DNA substrates for electron microscopy contain two copies of five tandem 17-bp GAL4 binding sites positioned 0.7 and 3 kb away from each end of a DNA fragment, respectively, and separated by 1 kb of DNA.

Plasmids expressing GAL4EBNA351–377, GAL4EBNA320–355, and GAL4EBNA452–477 fusion proteins were constructed in two steps. Fragments of the EBNA1 gene encoding amino acids 351–450 or 320–450 were amplified by polymerase chain reaction and cloned between the NcoI and BamHI sites of pAS2 (a gift from Stephen Elledge; Harper et al., 1988a, 1988b). A fragment with the GAL4 and hemagglutinin gene fragments was amplified from the first codon of the GAL4 gene to the 377th (for the 351–450 template), the 355th (for the 320–450 template), or the 474th (for the 452–641 template) codon of EBNA1 by polymerase chain reaction and cloned into the BamHI site of pET15b (Novagen).

GAL4-EBNA1 Fusion Proteins—pET15b plasmids containing GAL4-EBNA1 fusion constructs were used to transform Escherichia coli strain BL21(DE3)pLySS (Studier et al., 1990). Transformants were grown at 37°C in 2 liters of Luria broth supplemented with 50 μg/ml ampicillin and 50 μg/ml chloramphenicol until the A600 was 0.6–0.8. Isopropyl-β-D-galactopyranoside was then added to 1 mM. The cells were harvested 3 h postinduction, resuspended in 40 ml of 50 mM Tris-HCl, pH 8.0, 0.1% Nonidet P-40, 10% sucrose, 1 mM EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulfonfluoride, and frozen at −70°C for at least 1 h. After the cells were thawed, NaCl and ZnCl2 were added to final concentrations of 200 mM NaCl and 10 mM ZnCl2, respectively, and the lysates were sonicated to reduce the viscosity of the solution. Lysates were clarified by centrifugation at 53,000 g for 30 min at 4°C then loaded onto a 25-ml heparin agarose column (Bio-Rad) equilibrated with buffer A (20 mM Hepes, pH 7.5, 10% glycerol, 10 mM ZnCl2, 2 mM phenylmethylsulfonfluoride) plus 200 mM NaCl. The column was developed with a linear gradient from 200 mM NaCl to 1 M NaCl in buffer A. The fractions containing the GAL4-EBNA1 fusion proteins were applied to a 5-ml HPLC metal-chelating column (PerSeptive Biosystems) charged with nickel and equilibrated in buffer B (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10 mM ZnCl2, 2 mM phenylmethylsulfonfluoride) plus 5 mM imidazole. The proteins were eluted with a linear gradient from 5 mM to 1 M imidazole in buffer B. Dithiothreitol and EDTA were added immediately to the eluted proteins to a final concentration of 1 mM each, and fractions containing the GAL4-EBNA1 fusion proteins were applied to a 3-ml HPLC heparin column or a 5-ml HPLC Poros S column (PerSeptive Biosystems) equilibrated with buffer A plus 200 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA. Proteins were eluted with buffer A containing 1 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA and stored at −70°C. 

GAL4-EBNA1 fusion proteins were identified by Western blotting using monoclonal antibodies against the hemagglutinin (HA) epitope (Boehringer Mannheim).

Ligation Enhancement Assays—GAL4 DNA substrates (45 fmol) were incubated with amounts of GAL4-EBNA1 fusion proteins sufficient to bind 50% of the DNA fragment in 100-μl reactions containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM MgCl2, 10 mM dithiothreitol, 2 μM spermidine, 2 μM ATP for 10 min at room temperature. Reactions were diluted 2-fold, and then 1 unit of T4 DNA ligase (Life Technologies Inc.) was added and incubated for 5 min at 15°C. Reactions were stopped by the addition of EDTA to 20 mM, and the DNA was precipitated with ethanol. Samples were resuspended in 1 mM Tris-HCl, 80 mM EDTA, 1% SDS and subjected to electrophoresis on an 0.8% agarose gel containing 0.5 μg/ml ethidium bromide. As a positive control, the above experiment was performed using oriP DNA substrates and an amount of EBNA1 (2 pmol as dimer) sufficient to bind 100% of the DNA molecules. The EBNA1 protein was that previously described by Frappier and O'Donnell (1991b).

Electron Microscopy—DNA fragments (10 fmol) containing GAL4 binding sites were incubated with amounts of GAL4-EBNA1 fusion proteins sufficient to bind 100% of the DNA fragments in 20 μl of a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM MgCl2, 0.5 mM dithiothreitol, 10 μM ZnCl2, for 10 min at room temperature. Samples were then diluted 6-fold in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM MgCl2, 2 mM spermidine, 10 μM ZnCl2, and spread, without fixation, on glow-charged carbon-coated copper grids (400-mesh; JEM Services). Grids were stained with 5% aqueous uranyl acetate, rotary-shadowed with tungsten, and observed using a JEM-1200 EXII electron microscope.

RESULTS 

EBNA1 Fragments Can Direct the Interaction between DNA-bound GAL4 Proteins—We have demonstrated previously that the interaction at a distance of DNA-bound EBNA1 molecules requires part (amino acids 351–377) of the internal basic region of EBNA1 (termed the looping region) between amino acids 327 and 377 (see Figs. 1; Frappier et al., 1994) that the amino-terminal amino acid numbers reported in Frappier et al. (1994) are off by 1, i.e. the 350–641 truncation mutant is really 351–641. In order to determine whether EBNA1 amino acids 351–377 are sufficient for this interaction, we fractionated this portion of EBNA1 to the GAL4 DNA binding domain (Fig. 1). Since the amino acid sequence of the EBNA1 351–377 fragment is very similar to that between amino acids 327 and 350, we also tested the ability of an EBNA1 fragment spanning this region (320–355) to support DNA looping interactions. A fusion protein containing an EBNA1 fragment (452–474) previously shown to be insufficient for DNA looping (Goldsmith et al., 1993) was also tested as a negative control (Fig. 1). The fusion proteins were overproduced in E. coli, purified to >75% purity, and assayed for DNA binding activity by electrophoretic mobility shift using DNA fragments containing 5 tandem GAL4 binding sites (data not shown). The amount of each protein required to bind 50% of the DNA molecules was then tested for the ability to mediate interactions between the GAL4 binding sites in a ligation enhancement assay (Fig. 2). In this assay, interactions between the proteins bound at their cognate DNA recognition sequences bring the cohesive ends of the DNA substrates close together, resulting in an increased ligation efficiency (Mukherjee et al., 1988a, 1988b; Miron et al., 1992). We have shown previously that EBNA1 induces the concatenation of oriP DNA molecules in this assay due to the ability of EBNA1 to mediate interactions between multiple DNA looping domains of EBNA1.
DNA fragments containing the oriP subelements (Goldsmith et al., 1993; Frappier et al., 1994; also see Fig. 2). When this assay was conducted with the GAL4-EBNA1 fusion proteins using DNA substrates containing 2 sets of 5 tandem copies of the GAL4 binding sites positioned close to each end of the DNA molecule, both the GAL4EBNA320–355 and the GAL4EBNA351–377 fusion proteins (where the subscript refers to the EBNA1 amino acids in the fusion protein) induced DNA concatenemes indistinguishable from those induced by EBNA1 (Fig. 2). No such interactions were observed with the fusion protein containing EBNA1 amino acids 452 to 474 (data not shown). Concatenemic DNA products were not induced by any of the GAL4-EBNA1 fusion proteins when the ligation enhancement assay was performed with DNA fragments lacking GAL4 binding sites (data not shown). These results indicate that EBNA1 fragments spanning amino acids 351 to 377 and 320 to 355 are sufficient to mediate protein-protein interactions when tethered to DNA through a DNA binding domain.

We also used electron microscopy to visualize the structures formed upon incubation of the GAL4 fusion proteins with DNA fragments containing 2 sets of 5 tandem copies of the GAL4 binding sites separated by 1 kb of intervening DNA (the same spacing as that for the oriP subelements). In keeping with the results of the ligation enhancements assays, GAL4EBNA320–355 and GAL4EBNA351–377, but not GAL4EBNA452–474, were observed to mediate interactions between multiple DNA molecules resulting in the formation of multiple complexes shown in Fig. 3 (see also Table 1). Multiple complexes were not observed when the fusion proteins were incubated with DNA fragments lacking GAL4 binding sites (data not shown). Although the majority of the DNA molecules that were bound by GAL4EBNA320–355 and GAL4EBNA351–377 were observed to be sequestered into these multiple complexes, the individual DNA molecules that were observed to have protein bound at both sets of GAL4 sites were in a looped configuration consistent with protein-mediated interactions between the two sets of sites. The frequency of multiple complexes and looped structures induced by GAL4EBNA320–355 and GAL4EBNA351–377 is almost identical with that observed previously for the EBNA351 truncation mutant bound to oriP DNA (Frappier et al., 1994).

**Protein Interactions Mediated by the EBNA1 Looping Region**

**Are Dependent upon DNA Binding**—In the context of the EBNA1 protein, the homotypic protein interactions mediated by the looping region of EBNA1 are dependent on DNA binding (Frappier and O'Donnell, 1991b). In order to determine whether the interactions mediated by the looping region of EBNA1 are DNA-dependent in the context of the GAL4 fusion proteins, we used glycerol gradient sedimentation to compare the native oligomerization state of GAL4-EBNA1 fusion proteins that do and do not mediate protein interactions on DNA (under protein and salt concentrations identical with those used for the ligation enhancement assay). The results in Fig. 4 show that both GAL4EBNA452–474 and GAL4EBNA351–377 migrate as discrete species with very similar sedimentation coefficients (3.2 and 3.7, respectively). Since the interaction of multiple GAL4EBNA351–377 molecules would result in complexes with much larger sedimentation coefficients, these results indicate that the interaction of multiple GAL4EBNA351–377 molecules, which occurs when this protein binds to DNA, does not occur in the absence of DNA. Therefore, interactions mediated by the EBNA1 looping region appear to be dependent on DNA binding.

**TABLE 1**

| GAL4-EBNA1 fusion proteins | Percent of observed structures |
|---------------------------|-------------------------------|
|                           | Multiple complex | Looped<sup>a</sup> | Other<sup>b</sup> |
| None                      | 0                 | 0.5               | 99.5               |
| 320–355                   | 78                | 3                 | 19                 |
| 351–377                   | 80                | 2                 | 18                 |
| 452–474                   | 0                 | 0                 | 100                |

<sup>a</sup> Individual DNA molecules in which the position of the loop is consistent with an interaction between the 2 sets of GAL4 binding sites.

<sup>b</sup> Other structures comprise individual nonlooped DNA molecules with protein bound at one or both (452–474 only) sets of GAL4 binding sites and unbound DNA molecules.

**Fig. 2. Detection of the interactions of DNA-bound GAL4EBNA320–355 and GAL4EBNA351–377 by ligation enhancement.** A DNA fragment containing two sets of five tandem copies of the GAL4 recognition sequence (GAL4) was incubated with amounts of GAL4-EBNA1 fusion proteins required to bind 50% of the DNA fragments or without protein (0). After the addition of ligase, samples were incubated for 5 min at 15°C prior to ethanol precipitation and agarose gel electrophoresis in 0.5 μg of ethidium bromide per ml. Positions of the linear DNA substrate (L) as well as covalently closed circular (CCC) and concatenemic (*) ligation products are indicated. The assay was also performed with a DNA fragment containing the oriP subelements (oriP) incubated with or without (0) EBNA1. The sizes of the largest concatenemeric products cannot be determined accurately from this gel system but are greater than 20 kb.

**Fig. 3. Multiple complexes induced by GAL4-EBNA1 fusion proteins.** Interactions between multiple DNA molecules were observed when GAL4EBNA320–355 (A) or GAL4EBNA320–355 (B) was incubated with a DNA fragment containing GAL4 binding sites and visualized by electron microscopy as described under “Materials and Methods.” Bar = 0.1 μm.
DISCUSSION

The results of both the ligation enhancement assay and the electron microscopy showed that both the 320–355 and 351–377 EBNA1 fragments constitute independent, transferable domains that are sufficient to mediate interactions at a distance between DNA-bound proteins. These two fragments of EBNA1 are highly conserved in sequence (53% identical according to the Lipman-Pearson protein alignment) and are composed largely of glycine and arginine residues (Fig. 5). Examination of these sequences reveals an eight amino acid repeat present in triplicate in both the 320–355 and 351–377 EBNA1 fragments (Fig. 5). Since both of these fragments are composed almost exclusively of these repeats, our results suggest that this repeated sequence can direct the interaction at a distance of DNA-bound EBNA1. Whether it is the actual sequence of the octamer repeat or the amino acid content of the repeat that is important for this interaction remains to be determined.

We have shown previously that an EBNA1 truncation mutant containing three octamer repeats (EBNA351) supports efficient interactions between the FR and DS elements and that the efficiency with which these interactions occur drops dramatically when two of the octamer repeats (amino acids 351–362) are deleted. Therefore, one copy of the octamer repeat sequence of the DNA looping region is insufficient to mediate stable interactions at a distance between DNA-bound EBNA1 molecules; two or three copies are required. Three copies of the octamer repeat also appear to be sufficient to confer complete DNA looping activity, since EBNA351 mediates interactions between FR and DS elements as efficiently as a larger replica-ADZUMA, K., AND MIZUUCHI, C. (1989) J. Mol. Biol. 215, 475–494.

Acknowledgments—We thank Klaus Shultes for assistance with the sample preparation for electron microscopy. We are also grateful to Drs. Aled Edwards, Jim Smiley, J ohn Hassell, and Peter Whyte for critical reading of the manuscript and to Aled Edwards and Jim Smiley for helpful comments throughout the course of this work.

Note Added in Proof—While this paper was under review, Mackey et al. (Mackey, D., Middleton, T., and Sugden, B. (1995) J. Virol. 69, 6199–6208) published data which also demonstrate that EBNA1 fragments with looping activity retain functionality when transferred to the GAL4 DNA binding domain.

REFERENCES

Adams, A. (1987) J. Virol. 61, 1743–1746.
Adzuma, K., and Mizuuchi, K. (1989) Cell 57, 41–47.
Ambinder, R. F., Muller, M., Chang, Y., Hayward, G. S., and Hayward, S. D. (1991) J. Virol. 65, 1466–1478.
Barwell, J., Bockharev, A., Pfuetzner, R., Tong, H., Yang, D., Frappier, L., and Edwards, A. (1995) J. Biol. Chem. 270, 20554–20559.
Beachy, P., Varkey, J., Young, K., von Kessler, D., Sun, B., and Ekker, S. (1993) Mol. Cell. Biol. 13, 6941–6956.
Bockharev, A., Barwell, J., Pfuetzner, R., Firey, W., Edwards, A., and Frappier, L. (1993) Cell 73, 39–46.
Burd, C., and Dreyfuss, G. (1994) Science 265, 615–621.
Chattoraj, D. K., Mason, R. J., and Wickner, S. H. (1988) Cell 52, 551–557.
Frappier, L., and O'Donnell, M. (1993a) Proc. Natl. Acad. Sci. U. S. A. 90, 10875–10879.
Frappier, L., and O'Donnell, M. (1995). J. Biol. Chem. 270, 20554–20559.
Goldsmith, K., and Bendell, L. (1994). J. Biol. Chem. 269, 1057–1062.
Kahn, T. A., and Schildkraut, C. L. (1989) Cell 58, 527–535.

2 A. Laine and L. Frappier, unpublished results.
Transferable DNA Looping Domains of EBNA1

Gahn, T., and Sugden, B. (1995) J. Virol. 69, 2633-2636
Goldsmith, K., Bendell, L., and Frappier, L. (1993) J. Virol. 67, 3418-3426
Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993) Cell 75, 805-816
Hochschild, A., and Ptashne, M. (1988) Nature 336, 353-357
Keegan, L., Gill, G., and Ptashne, M. (1986) Science 231, 699-704
Knight, J. D., Li, R., and Botchan, M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3204-3208
Kramer, H., Niemoller, M., Amouyal, M., Revet, B., von Wilden-Bergmann, B., and Muller-Hill, B. (1987) EMBO J. 6, 1481-1491
Krysan, P. J., Haase, S. B., and Calise, M. P. (1989) Mol. Cell. Biol. 9, 1026-1033
Matthews, K. (1992) Microbiol. Rev. 56, 123-136
Middleton, T., and Sugden, B. (1992) J. Virol. 66, 489-495
Miron, A., Mukherjee, S., and Bastia, D. (1992) EMBO J. 11, 1205-1216
Mukherjee, S., Erickson, H., and Bastia, D. (1988a) Cell 52, 375-383
Mukherjee, S., Erickson, H., and Bastia, D. (1988b) Proc. Natl. Acad. Sci. U. S. A. 85, 6287-6291
Rawlins, D. R., Milman, G., Hayward, S. D., and Hayward, G. S. (1985) Cell 42, 859-868
Reisman, D., and Sugden, B. (1986) Mol. Cell. Biol. 6, 3838-3846
Reisman, D., Yates, J., and Sugden, B. (1985) Mol. Cell. Biol. 5, 1822-1832
Schleif, R. (1992) Annu. Rev. Biochem. 61, 199-223
Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Methods Enzymol. 185, 60-89
Su, W., Middleton, T., Sugden, B., and Echols, H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10870-10874
Wyman, C., Gratkopp, E., Bustamante, C., and Nelson, H. C. M. (1995) EMBO J. 14, 117-123
Yates, J. L., and Guan, N. (1991) J. Virol. 65, 483-488
Identification of Epstein-Barr Virus Nuclear Antigen 1 Protein Domains That Direct Interactions at a Distance between DNA-bound Proteins
Alexandra Laine and Lori Frappier

J. Biol. Chem. 1995, 270:30914-30918.
doi: 10.1074/jbc.270.52.30914

Access the most updated version of this article at http://www.jbc.org/content/270/52/30914

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 21 of which can be accessed free at http://www.jbc.org/content/270/52/30914.full.html#ref-list-1