

Separation of the DNA replication and transactivation activities of EBNA1, the origin binding protein of Epstein-Barr virus

Research Article

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Summary

During latent infection of human B-lymphocytes, Epstein-Barr virus (EBV) genomes are stably maintained as DNA episomes that replicate once per cellular S phase. The replication and segregation of the EBV episomes requires the latent origin of replication, *oriP*, and one viral protein, Epstein-Barr Nuclear Antigen 1 (EBNA1). EBNA1 also activates the transcription of other latent viral genes and some cellular genes. EBNA1 fulfills all of its functions by directly interacting with EBV sequences, but the functional role of EBNA1 residues outside of the DNA binding domain is not well understood. We have explored the contribution of EBNA1 regions to transactivation activity. Our results indicate that the C-terminal acidic tail of EBNA1, that was previously implicated in transactivation, is not required for this function. Rather, the transactivation activity resides primarily in an internal arginine-rich region (amino acids 325-376) that was previously shown to mediate interactions at a distance between DNA-bound EBNA1 molecules as well as interactions with at least two cellular factors. An EBNA1 mutant, lacking amino acids 325-376, supports the transient replication of *oriP* plasmids at wild type levels but is severely impaired for transcriptional enhancement. Therefore, our results indicate that the replication and transactivation functions of EBNA1 can be separated.

I. Introduction

Epstein-Barr virus (EBV) is a human herpes virus that establishes a latent infection in peripheral B-lymphocytes, inducing them to proliferate (reviewed in Kieff, 1996; Rickinson and Kieff, 1996). During latency, only a fraction of the viral genes are expressed and infectious virions are not produced. Multiple copies of the 172 Kb viral genome persist as double-stranded circular DNA episomes within the cell nucleus. The viral DNA replicates once per cellular S phase, in concert with the host cell chromosomes, and segregates efficiently to the daughter cells, so that a constant copy number of EBV genome per cell is maintained (Adams, 1987; Yates and Guan, 1991; Yates et al., 1985).

The latent origin of replication, *oriP*, was identified as an 1800 bp fragment of the EBV genome that supported the replication and stable maintenance of plasmids in EBV-infected cells (Sugden et al., 1985; Yates et al., 1984). Subsequently it was shown that only one viral protein, Epstein-Barr Nuclear Antigen 1 (EBNA1), was required for the replication and stable maintenance of *oriP* plasmids in dividing cells (Lupton and Levine, 1985; Yates et al., 1985). *OriP* contains two essential elements, the family of repeats (FR) and the dyad symmetry (DS) element, which contain 20 and 4 EBNA1 binding sites, respectively (Rawlins et al., 1985). Bidirectional replication of *oriP* containing plasmids initiates at or near the DS element (Gahn and Schildkraut, 1989; Niller et al., 1995) but, in most cell lines, additional EBNA1 sites, either tandem DS

elements or the FR element is required for efficient replication (Harrison et al., 1994; Reisman et al., 1985; Wysokenski and Yates, 1989). Recombinant plasmids with more than one DS element are not amplified (Kirchmaier and Sugden, 1995), suggesting that *oriP* replication is regulated in a similar manner as human chromosomal origins.

The FR element of *oriP* activates replication and is located approximately 1 Kb from the DS element. The intervening sequences do not appear to be important for *oriP* function since large deletions and insertions in these sequences do not affect the replication activity of *oriP* (Reisman et al., 1985). The FR also governs the stable segregation of *oriP* plasmids to daughter cells (Chittenden et al., 1989; Reisman et al., 1985). Plasmid segregation requires 8 of the 20 EBNA1 binding sites and can occur in the absence of a DS element (Chittenden et al., 1989; Middleton and Sugden, 1994). The FR performs a third function as a transcriptional enhancer of viral latency promoters (Gahn and Sugden, 1995; Langle-Rouault et al., 1998; Pugeilli et al., 1996; Reisman and Sugden, 1986; Sugden and Warren, 1989). Transactivation assays with reporter gene constructs have shown that only 6-7 of the FR EBNA1 binding sites are required for enhancer activity and that the FR activates transcription when positioned upstream or downstream of a promoter (Reisman and Sugden, 1986; Wysokenski and Yates, 1989).

The three functions associated with *oriP*, namely DNA replication, DNA segregation and transactivation, require EBNA1. EBNA1 binds as a dimer to each of its recognition sites in the FR and DS (Ambinder et al., 1991; Frappier and O'Donnell, 1991a; Rawlins et al., 1985) and occupies *oriP* throughout all or most of the cell cycle (Hsieh et al., 1993). The mechanism by which EBNA1 activates replication from the DS element is not yet clear, but likely involves changes in the DNA structure of the DS and the recruitment of cellular factors. EBNA1 binds cooperatively to the multiple sites within the DS element (Harrison et al., 1994; Summers et al., 1996) causing localized distortion of the DNA (Bochkarev et al., 1996; Frappier and O'Donnell, 1992; Hearing et al., 1992; Hsieh et al., 1993; Summers et al., 1997). Transient replication of *oriP* plasmids requires two EBNA1 recognition sites separated by 3 bp suggesting that specific contact between the neighbouring EBNA1 dimers is important for initiation (Harrison et al., 1994). EBNA1 does not appear to possess any enzymatic activities (Frappier and O'Donnell, 1991a; Middleton and Sugden, 1992) suggesting that recruitment of cellular factors to *oriP* DNA through EBNA1 interactions is important for origin activation. DNA-bound EBNA1 has been shown to interact with replication protein A (RPA), the human replicative single-strand DNA binding protein, and this interaction may be important for the initiation of DNA replication (Zhang et al., 1998).

The mechanism by which EBNA1 mediates the segregation of EBV episomes and *oriP* plasmids is thought to involve "piggybacking" on the host chromosomes. This

hypothesis stems from the observation that the EBV genome, *oriP* plasmids and EBNA1 all localize to the host metaphase chromosomes (Delecluse et al., 1993; Harris et al., 1985; Petti et al., 1990; Simpson et al., 1996). Finally, EBNA1 can act as both an enhancer and repressor of transcription. Enhanced expression of EBV latent promoters and reporter gene constructs occurs upon EBNA1 interaction with the FR element (Gahn and Schildkraut, 1989; Reisman and Sugden, 1986). Repression occurs when EBNA1 binds to the two recognition sites present in the *Bam* HI-Q region of the EBV genome (Rawlins et al., 1985; Sample et al., 1992). The latter interaction negatively regulates the expression of EBNA1 from the Qp promoter (Sample et al., 1992).

EBNA1 consists of 641 amino acids and is shown schematically in **Figure 1**. A number of functional domains of EBNA1 have been identified but the contribution of many EBNA1 regions is still unknown. The large internal repeat of glycine and alanine residues (amino acids 101-325) is not required for EBNA1 replication, transactivation or segregation functions (Yates and Camiolo, 1988; Yates et al., 1985), but enables EBNA1 to evade cytotoxic T-lymphocyte responses (Levitskaya et al., 1995). The adjacent region of EBNA1 (325-376) is a glycine and arginine-rich domain, termed the looping domain, that mediates homotypic interactions at a distance between FR- and DS-bound EBNA1 molecules resulting in looped or linked DNA complexes (Avolio-Hunter and Frappier, 1998; Frappier et al., 1994; Goldsmith et al., 1993; Laine and Frappier, 1995; Mackey et al., 1995; Mackey and Sugden, 1997) and heterotypic interactions with some cellular proteins (Shire et al., submitted; Wang et al., 1997). This domain of EBNA1 has also been suggested to bind RNA (Snudden et al., 1994). The looping domain is followed by a sequence of basic amino acids (379-386) that functions as a nuclear localization signal for EBNA1 (Ambinder et al., 1991). The DNA binding and dimerization domains of EBNA1 colocalize to amino acids 459-607 (Ambinder et al., 1991; Summers et al., 1996) and high resolution structures of these EBNA1 domains have been determined (Bochkarev et al., 1996; Bochkarev et al., 1995). The extreme C-terminus of EBNA1 contains an aspartate and glutamate-rich region termed the acidic tail. The acidic tail was suggested to be a transactivation domain (Ambinder et al., 1991), but assignment of a functional role for this region has not been conclusive (Kirchmaier and Sugden, 1997; Polvino-Bodnar et al., 1988; Polvino-Bodnar and Schaffer, 1992; Yates and Camiolo, 1988).

Plasmids that contain *oriP* and express EBNA1 provide useful gene delivery vectors for gene therapy strategies due to their stable maintenance in extrachromosomal form in human cells (Franken et al., 1996; Judde et al., 1996; Robertson et al., 1996). However, a potential drawback to introducing EBNA1 into mammalian cells was revealed by the induction of B-cell neoplasia in EBNA1 transgenic mice (Wilson et al., 1996) and the induction of

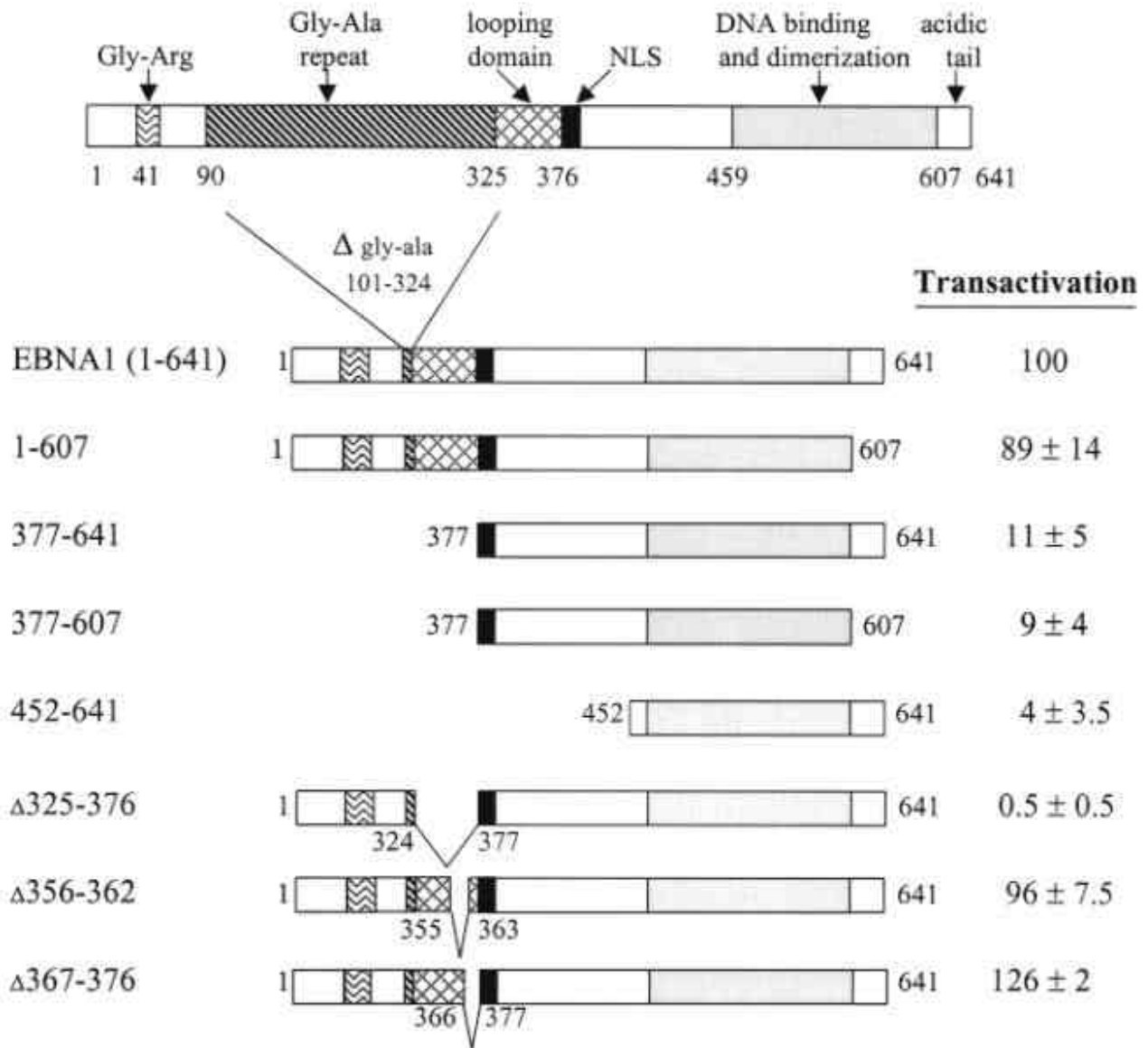


Figure 1. EBNA1 truncation and internal deletion mutants. EBNA1 amino acids present in each mutant are shown. The position of the DNA binding and dimerization domain, looping domain, nuclear localization signal (NLS) and other features of the EBNA1 polypeptide are shown. Residues 101-324 of the glycine-alanine repeat are not present in any of the EBNA1 proteins used in this study. The average transactivation activity of each mutant relative to wild type EBNA1 (\pm standard deviation) determined in this study are also shown.

recombinase activating genes, Rag1 and Rag2, in EBNA1-expressing human peripheral B-lymphocytes (Srinivas and Sixbey, 1995). In order for the EBNA1-oriP system to be useful for human gene therapy, the transactivation activity of EBNA1 needs to be more carefully defined and disabled.

To identify the transactivation domain, we have tested a series of truncated and internally deleted EBNA1 proteins for the ability to activate transcription of a reporter gene in human cells. Our results indicate that the arginine-rich

looping domain (amino acids 325-376) is critical for the transactivation activity but not the replication activity of EBNA1, and that the C-terminal acidic tail does not contribute to transactivation.

II. Results

In order to determine the regions of EBNA1 that contribute to transactivation functions, we constructed a

series of EBNA1 truncation and internal deletion mutants (**Figure 1**). Mutations were designed to specifically target two regions of EBNA1, the acidic tail and the looping domain, while maintaining the integrity of the DNA binding and dimerization domains. The transactivation activity of each EBNA1 protein in human cells was determined using chloramphenicol acetyl transferase (CAT) reporter assays. C33A cells were transfected with a plasmid expressing EBNA1 or EBNA1 mutants from the cytomegalovirus (CMV) promoter and a second plasmid (pFRTKCAT) containing the CAT reporter gene under control of the *oriP* FR element (Reisman et al., 1985). 24 hours post transfection, lysates were prepared and CAT assays were performed using equal amounts of total protein. The percentage of acetylated chloramphenicol was monitored and plotted relative to the reaction time in order to determine the acetylation rate for each mutant (**Figure 2**). Expression of all mutant proteins was confirmed by Western blotting (data not shown) using antisera directed against the DNA binding domain (kindly provided by Jaap Middeldorp). We first compared the transactivation activity of EBNA1 truncation mutants that lacked the C-terminal acidic tail (1-607), the N-terminal 376 amino acids including the looping domain (377-641), or both (377-607) with that of wild type EBNA1. As shown in **Figures 2 and 3**, removal of the acidic tail had no significant effect on the transactivation activity of EBNA1, while removal of the N-terminal 376 amino acids severely reduced transactivation. The small amount of transactivation activity observed with 377-641 was similar to that of the DNA binding and dimerization domain (452-641) and was not significantly decreased by the removal of the acidic tail. Transactivation results from multiple experiments are summarized in **Figure 1**. Our results indicate that the acidic tail is not a transactivation domain and that residues between 1-376 mediate transactivation by EBNA1.

A. Transactivation is mediated by the looping domain

The glycine and arginine-rich looping domain of EBNA1 is located within the N-terminal residues important for transactivation. This domain has been shown to mediate interactions between DNA-bound EBNA1 molecules (Avolio-Hunter and Frappier, 1998; Frappier et al., 1994; Goldsmith et al., 1993; Laine and Frappier, 1995; Mackey et al., 1995;), as well as with some cellular factors (Shire et al., 1998; Wang et al., 1997). To investigate the contribution of the looping domain to transactivation, we constructed internal deletions that lacked all (325-376) or part (356-362 and 367-376) of the looping domain and tested their ability to activate the FR-CAT reporter construct. Comparison of the acetylation rates with wild type EBNA1 (**Figure 2**) indicated that the removal of the fifty amino acids (325-376) comprising the looping domain resulted in a loss of transactivation activity. All EBNA1 mutants were expressed at similar levels as determined by Western blot of transfected cell

extracts (data not shown). Data from multiple experiments, summarized in **Figures 1 and 3**, showed that less than 1% of wild type transactivation activity was observed for the 325-376 looping domain mutant. Small deletions within the looping domain (356-362 and 367-376) resulted in levels of CAT activity comparable to wild type EBNA1 (**Figures 2 and 3**). These results are consistent with our previous findings that small deletions in the looping domain do not abrogate the protein-protein interactions mediated by this region (Avolio-Hunter and Frappier, 1998; Laine and Frappier, 1995; Shire et al., 1998).

B. The looping domain is dispensable for transient replication of *oriP* plasmids

Previous findings have suggested that the EBNA1 domains that contribute to replication and transactivation overlap since EBNA1 mutants that are deficient in only one of these two activities have not been isolated (Polvino-Bodnar and Schaffer, 1992; Yates and Camiolo, 1988). Therefore, we were interested in determining whether the 325-376 mutant that is defective for transactivation was able to support the transient replication of *oriP* plasmids in human cells. To this end, C33A cells were transfected with a plasmid containing both the EBNA1 expression cassette and *oriP*. 72 hours post-transfection, the plasmids were isolated, linearized and digested with Dpn I to remove plasmids that had not undergone replication in the human cells. A Southern blot of the recovered and replicated plasmid DNA is shown in Figure 4. Comparison of the amount of replicated plasmid DNA recovered with 325-376 and wild type EBNA1 indicated that 325-376 supports the transient replication of *oriP* plasmids. The average replication activity of 325-376 observed in four different experiments was 95% (\pm 45%) of the wild type replicative activity. The fact that 325-376 is functional for replication indicates that its lack of transactivation activity is not due to complete misfolding of the protein or due to an inability to enter the nucleus. The identification of an EBNA1 mutant that is defective for transactivation but active for replication suggests that these functions of EBNA1 are not entirely overlapping.

III. Discussion

We have identified a region of EBNA1 that is necessary for transactivation but not required for replication. This region maps to the EBNA1 looping domain (amino acids 325-376) which has previously been shown to mediate protein-protein interactions (Avolio-Hunter and Frappier, 1998; Frappier et al., 1994; Goldsmith et al., 1993; Laine and Frappier, 1995; Mackey et al., 1995; Shire et al., 1998; Wang et al., 1997). The EBNA1 mutant lacking the looping domain demonstrated less than 1% of the wild type transactivation activity but was functional in transient replication assays, indicating that the protein was properly folded, present in the nucleus, and capable of interacting

Figure 2. Activation of a CAT reporter gene by EBNA1 mutants. C33A cells were transfected with a plasmid (pFRTKCAT) containing the CAT reporter gene under control of the *oriP* FR element and a plasmid expressing EBNA1 or EBNA1 mutants. Equal amounts of protein from cell lysates were tested for CAT activity and aliquots were removed at 5, 20 and 60 minute time intervals. Acetylated and unacetylated chloramphenicol was separated by thin layer chromatography and quantified by phosphorimager analysis (Molecular Dynamics).

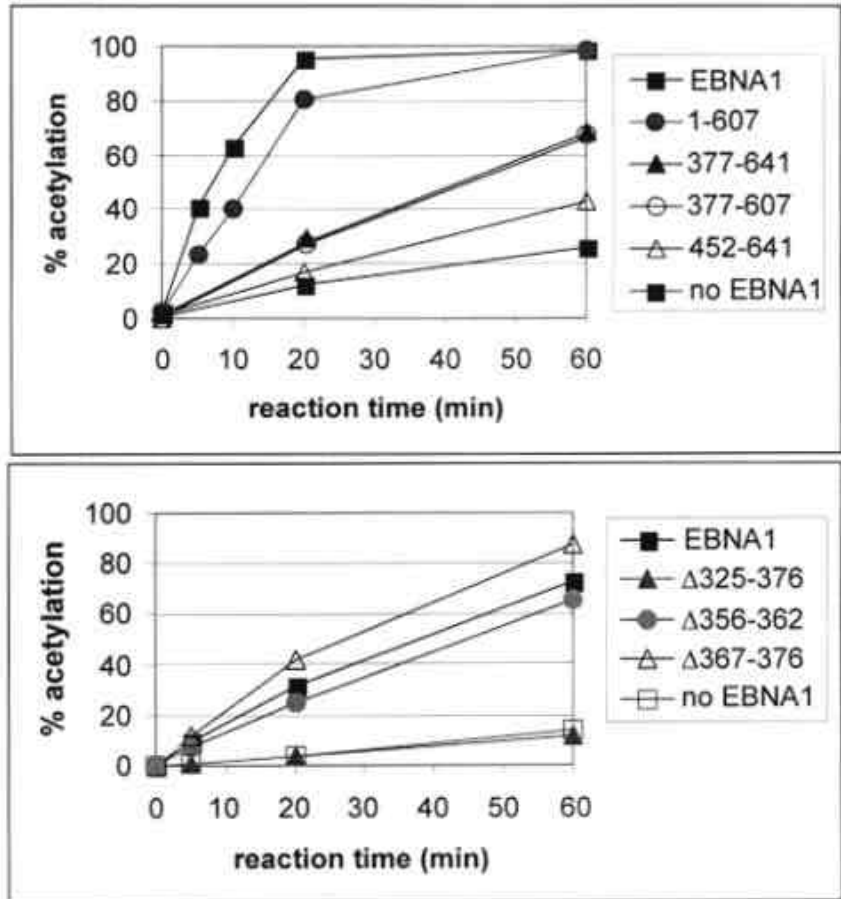


Figure 3. Relative transactivation ability for EBNA1 and EBNA1 mutants. Transactivation rates for EBNA1 mutants were determined for each experiment and expressed as a percentage of wild type EBNA1 activity. The results displayed for each EBNA1 mutant represent the average of multiple experiments (error bars, \pm standard deviation).

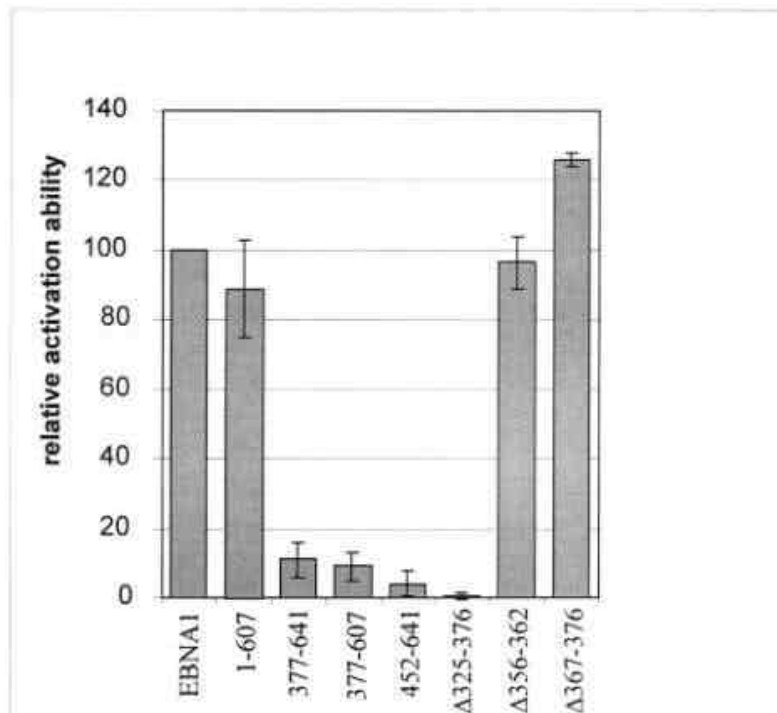




Figure 4. Transient replication of *oriP* plasmids in human cells expressing EBNA1 or 325-376. C33A cells were transfected with *oriP* plasmids expressing EBNA1, 325-376 or no EBNA1 (pc3*oriP*). Plasmids were isolated 72 hours post-transfection and linearized with *Xho* I. 90% of each sample was further digested with *Dpn* I to remove unreplicated plasmid DNA (*Xho* I/ *Dpn* I). The products were separated on a 1% agarose gel and visualized by Southern blotting. Replicated plasmids were quantified by phosphorimager analysis (Molecular Dynamics).

with *oriP*. Our finding that the looping domain is not required for the EBNA1 replication function is consistent with the results of Kim et al. (1997) who showed that a similar EBNA1 mutant (328-374) supported transient

replication. EBNA1 mutants containing small deletions within the looping domain (356-362 and D367-376) exhibited wild type levels of transactivation (this study), were functional for *oriP* plasmid replication (Shire et al., submitted) and mediated both homotypic and heterotypic protein-protein interactions (Avolio-Hunter and Frappier, 1998; Shire et al., submitted). The looping domain consists of six imperfect repeats of an eight amino acid sequence (Laine and Frappier, 1995) and the tolerance of this domain to small deletions suggests a degree of functional redundancy within this region.

We have also shown that the acidic tail of EBNA1 is neither required nor sufficient for transactivation. This finding is in contrast to a previous study by Ambinder et al. (1991) that suggested the acidic tail was important for transactivation, but is in agreement with the results of Yates and Camiolo (1988), Polvino-Bodnar and Schaffer (1992) and Kirchmaier and Sugden (1997). The latter study demonstrated that an EBNA1 fragment containing the DNA binding domain and acidic tail functions as a dominant negative inhibitor of the transactivation and replication activities of wild type EBNA1 (Kirchmaier and Sugden, 1997).

The looping domain of EBNA1 has a propensity to mediate both homotypic and heterotypic protein-protein interactions. Homotypic interactions occur between EBNA1 molecules bound to the FR or DS elements of *oriP*, resulting in the formation of looped (when interactions occur within a single *oriP* molecule) or linked (when interactions occur between different *oriP* molecules) DNA molecules (Avolio-Hunter and Frappier, 1998; Frappier et al., 1994; Frappier and O'Donnell, 1991b; Goldsmith et al., 1993; Laine and Frappier, 1995; Mackey et al., 1995; Middleton and Sugden, 1992; Su et al., 1991). Similar homotypic interactions mediated by the EBNA1 looping domain are observed when this domain is fused to the DNA binding domain of GAL4 (Laine and Frappier, 1995; Mackey et al., 1995).

The looping domain of EBNA1 has also been shown to mediate heterotypic interactions with two cellular proteins (Wang et al., 1997; Shire et al., submitted). Wang et al. (1997) showed that the P32/TAP protein, previously shown to interact with a wide variety of proteins, interacts with residues 40-60 and 325-376 of EBNA1. Based on the findings that the latter EBNA1 region is important for transactivation and that a fragment of P32/TAP activates transcription when fused to the GAL4 DNA binding domain, it has been suggested that the P32/TAP interaction may be important for EBNA1-mediated transactivation. A second cellular protein that interacts with residues 325-376 of EBNA1 was recently identified and called EBP2 (Shire et al., submitted). Functional assays with EBNA1 mutants show a correlation between the ability to bind EBP2 and the ability to mediate *oriP* plasmid segregation and transactivation. Therefore the EBNA1-EBP2 interaction

may be important for either or both of these EBNA1 functions.

One of the reasons for defining regions within EBNA1 that mediate specific functions is to design *oriP*-based vectors suitable for use in gene therapy. Plasmids that contain *oriP* and express EBNA1 are useful because they are replicated and stably maintained in human cells (Franken et al., 1996; Judde et al., 1996; Robertson et al., 1996). However, a negative aspect of this system stems from the observation that EBNA1 transactivates the expression of some cellular genes (Srinivas and Sixbey, 1995). The EBNA1-*oriP* system would be more useful for human gene therapy if a mutant of EBNA1 was utilized that was inactive for transactivation but active for DNA replication and segregation functions. While we successfully identified an EBNA1 mutant that lacked transactivation activity and replicated *oriP* plasmids, the mutant was not able to maintain the plasmids in long-term culture (Shire et al., submitted). A better understanding of how the EBNA1 looping domain and interacting cellular factors contribute to transactivation and segregation may facilitate the development of a safe EBV-based vehicle for the stable delivery of therapeutic genes into human cells.

IV. Materials and Methods

A. Cell culture

C33A cells (human papillomavirus negative cervical carcinoma) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine and penicillin/streptomycin.

B. Mammalian expression plasmids

The plasmids used for mammalian transfections were derived from pcDNA3 (Invitrogen, Carlsbad Ca.). The plasmid pcDNA3 was digested with *Hind* III, treated with mung bean nuclease to remove the 5' extensions and digested with *Bam* HI. DNA fragments encoding EBNA1 lacking the Gly-Ala repeat region (amino acids 101-324) or EBNA1 mutants additionally lacking amino acids 608-641 (EBNA 1-607), 1-376 (EBNA 377-641), both 1-376 and 608-641 (EBNA 377-607), or 1-451 (EBNA 452-641) were generated by PCR amplification from p205 (Yates et al., 1985) using an N-terminal primer containing an *Nde* I site and a C-terminal primer containing a *Bam* HI site. These DNA fragments were digested with *Nde* I, filled in with the Klenow fragment of DNA polymerase I, then digested with *Bam* HI. An EBNA1 mutant lacking amino acids 325-376 (325-376) was PCR amplified from pVLE 325-376 (Avolio-Hunter and Frappier, 1998) using an N-terminal primer containing an *Nco* I site and a C-terminal primer containing a *Bam* HI site. The DNA fragment was digested with *Nco* I, filled in with Klenow, then digested with *Bam* HI. DNA fragments containing the EBNA1 mutants lacking amino acids 356-362 (356-362) and 367-376 (367-376) were excised by digesting pVLE 356-362 and pVLE 367-376 (Laine and Frappier, 1995; Shire et al., 1998) with *Eco* RI, filling in the 5' overhang with Klenow then digesting with *Bam* HI. DNA fragments encoding EBNA1 or the EBNA1 mutants described above were ligated into

pcDNA3. Plasmids used for transient replication experiments were modified by the addition of EBV *oriP* DNA sequences. A DNA fragment encoding *oriP* was excised from pGEMoriP (Frappier and O'Donnell, 1991b) by digestion with *Bam* HI and *Rsa* I and inserted between the *Bgl* II and *Nru* I sites of pcDNA3 containing EBNA1 or EBNA1 mutants to generate pc3oriP-EBNA1.

C. Transcription enhancement assays

C33A cells were seeded in 60 mm dishes at a density of 1×10^6 cells/dish and grown for 24 hours prior to transfection by the calcium phosphate/DNA coprecipitation method. Five micrograms of pcDNA3 plasmids encoding EBNA1 or EBNA1 mutants were combined with 2 μ g of pFRTKCAT and 2.5 μ g of herring sperm DNA in 0.25 ml of 0.25 M CaCl_2 , then added dropwise to 0.25 ml of 2x HBS pH 6.95 (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4) with vortexing. After a 30 minute incubation at room temperature, the precipitate was added dropwise to the cells and incubated for 12-16 hours at 37°C. The cells were then washed twice with PBS, supplemented with fresh medium and incubated for 24 hours at 37°C. After the cells were harvested, a small portion of the sample was set aside for protein analysis while the remaining cells were lysed by three rounds of freezing and thawing. The supernatant was tested for CAT reporter expression levels. CAT assays contained 50 μ g of protein extract, 0.25 M Tris-HCl pH 7.5, 0.25 mM acetyl CoA and 3-6 pmol of C^{14} -chloramphenicol (NEN) in a 150 μ l reaction volume. The reactions were incubated at 37°C and 50 μ l aliquots were removed at various time points. Acetylated and unacetylated products were separated on thin layer chromatography plates (Whatman) in a chloroform/methanol (95:5) mixture and quantified by phosphorimager analysis using ImageQuant software (Molecular Dynamics).

D. Transient replication assays

C33A cells were plated in 10 cm dishes at 2.5×10^6 cells/dish and grown 24 hours prior to transfection. Transfections were performed as described for transcription assays except that 10 μ g of pc3oriP-EBNA1 plasmid DNA and 10 μ g of herring sperm DNA was used and the reaction volumes were doubled. Following removal of the DNA precipitate, cells were washed in PBS, split into 150 mm dishes and grown for 72 hours. Cells from each plate were collected and lysed in 700 μ l of 10 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.6% SDS. High molecular weight DNA was precipitated by the addition of NaCl to 0.83 M and incubated overnight at 4°C. Low molecular weight DNA in the supernatant was extracted with phenol:chloroform (1:1), ethanol precipitated and resuspended in TE pH 8.0. Half of each sample was linearized with *Xho* I and 90% of the linearized samples was subsequently digested with *Dpn* I (4 units) for 2 hours at 37°C. DNA fragments from the restriction digests were separated on a 1% agarose gel, transferred to Gene Screen Plus (NEN Research Products) and probed with pc3oriP that had been labeled with P^{32} -dCTP by random primer extension. Radiolabelled bands were visualized by autoradiography and quantified by phosphorimager analysis using ImageQuant software (Molecular Dynamics).

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