A FUNCTIONAL ANALYSIS OF THE EBNA1 ORIGIN BINDING PROTEIN OF EPSTEIN-BARR VIRUS

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By

DEREK FRANK JOSEPH CECCARELLI, B.Sc.(Hons.)

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AUTHOR: Derek F.J. Ceccarelli, B.Sc. (McMaster University)

SUPERVISOR: Dr. Lori D. Frappier, Associate Professor

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ABSTRACT

Epstein-Barr nuclear antigen 1 (EBNA1) is the only viral protein required for the replication and stable maintenance of the Epstein-Barr virus genomes in latently infected human B lymphocytes. The latent origin of replication, *oriP*, contains multiple EBNA1 binding sites within two essential DNA elements, the dyad symmetry element (DS) and the family of repeats (FR). In addition to an essential role in DNA replication and segregation, EBNA1 activates the transcription of other latent viral genes. EBNA1 fulfills these functions by directly interacting with EBV sequences, however the functional role of EBNA1 residues outside of the DNA binding and dimerization domain is not well understood. A functional analysis of EBNA1 was performed in order to determine the contributions of four EBNA1 sequence motifs, the acidic tail, the glycine and arginine-rich looping domain, the WF motif and the proline loop, to DNA replication, plasmid maintenance and transactivation in human cells. The results show that the arginine-rich looping domain is important for transactivation and DNA segregation but not for DNA replication. Thus, the replication and transactivation functions of EBNA1 are separable as are the replication and segregation functions. Contrary to previous reports, the acidic tail does not appear to contribute to any of the activities examined in this thesis; thus, EBNA1 is not an acidic activator of transcription. The proline loop and WF motif are located within the DNA binding and dimerization domain of EBNA1 but were not previously observed to make DNA contacts. While *in vitro* DNA binding studies with EBNA1 proline loop and WF mutants identified a

contribution of both motifs to DNA binding, functional assays showed that the WF motif was not required for the EBNA1 functions tested. A functional contribution of the proline loop was also not evident but these results were hampered by the low expression level of the proline loop mutant proteins.

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This thesis is dedicated to my father, Frank Theodore Ceccarelli

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LIST OF ABBREVIATIONS

ACS	ARS consensus sequence
Ad	adenovirus
APC	anaphase promoting complex
ARS	autonomously replicating sequence
ATP	adenosine triphosphate
BCRF1	BamHI C rightward frame 1
BZLF1	BamHI Z leftward frame 1
BL	Burkitts lymphoma
BPV	bovine papillomavirus
BSA	bovine serum albumin
CD	circular dichroism
CAT	chloramphenicol acetyltransferase
CDC	cell division cycle
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CHO	chinese hamster ovary cells
CMV	cytomegalovirus
dATP	deoxyadenosine triphosphate
DHFR	dihydrofolate reductase
DME	Dulbecco's modified eagles media
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA

DTT	dithiothreitol
DS	dyad symmetry element
E. coli	*Escherichia coli*
EBER	Epstein-Barr expressed RNA
EBNA	Epstein-Barr nuclear antigen
EBP2	EBNA-binding protein 2
EBV	Epstein-Barr virus
ECF	enhanced chemifluorescence
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift analysis
FR	family of repeats
HBS	hepes-buffered saline
HEPES	n-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HLA	human leukocyte antigen
IPTG	isopropylthio-β-D-galactoside
kbp	kilobase pair
HPLC	high pressure liquid chromatography
LANA	latency-associated nuclear antigen
LB	Luria Bertani broth
LCL	lymphoblastoid cell line
LMP	latent membrane protein
MALDI-TOF	matrix-assisted laser-desorption ionization time-of-flight

MME	minichromosome maintenance element
mRNA	messenger RNA
NC	nitrocellulose
NLS	nuclear localization signal
NP40	nonidet P-40
NPC	nasopharyngeal carcinoma
NTP	nucleoside triphosphate(s)
OBP	origin binding protein
OD	optical density
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
ORC	origin recognition complex
oriLyt	EBV lytic origin of replication
oriP	EBV latent origin of replication
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PL	proline loop
PMSF	phenylmethylsulphonylfluoride
PVDF	polyvinylidene fluoride
RFC	replication factor C
RNA	ribonucleic acid
RMS	root mean square

RPA	replication protein A
SCC	sister chromatid cohesion
SDS	sodium dodecyl sulphate
SMC	structural maintenance of chromosomes
SV40	simian virus 40
TAE	tris, acetic acid, ethylenediaminetetraacetic acid
TBE	tris, boric acid, ethylenediaminetetraacetic acid
TBP	TATA element binding protein
TLC	thin layer chromatography
TPA	tetradecanoylphorbol-13-acetate
Tris	tris(hydroxymethyl)aminoethane
VZV	varicella zoster virus
WF	tryptophan and phenylalanine motif
ZRE	Zta responsive element

1.0 INTRODUCTION

1.1 Epstein-Barr Virus

Epstein-Barr virus (EBV) is a herpes virus found within human populations worldwide. Lytic replication of EBV within epithelial cells of the oropharynx generates viral particles that are shed in saliva and transmitted easily between individuals. Infection of B lymphocyte cells results in viral latency with expression of six Epstein-Barr nuclear antigens (EBNAs) and three latent membrane proteins (LMPs) that induce continuous proliferation of the cell (reviewed in Kieff, 1996). Epstein-Barr nuclear antigen 1 (EBNA1) is essential for maintaining the latent infection, governing the replication and the segregation of EBV genomes in the nucleus of proliferating cells.

1.1.1 Clinical manifestations of EBV infection

EBV has been associated with a number of clinical syndromes. Infection with EBV during infancy is usually asymptomatic, while adolescents are susceptible to develop self-limiting infectious mononucleosis (Henle et al., 1968). EBV was originally identified in cell lines derived from patients with Burkitt's lymphoma (BL), a B-cell lymphoma endemic in equatorial Africa (Epstein et al., 1964). Cultured B lymphocytes from peripheral blood yielded transformed and immortal lymphoblastoid cell lines (LCLs) that were EBV-positive and expressed the latent viral antigens (reviewed in Kieff, 1996). *In vitro* studies revealed a potent growth transformation of EBV infected B lymphocytes and suggested an etiological role for the latent virus in BL. EBV DNA and the latent antigens were subsequently identified in AIDS-related immunoblastic lymphomas, posttransplant lymphomas and Reed-Sternberg cells of Hodgkin's disease

(reviewed in Rickinson and Kieff, 1996). EBV has also been implicated in several carcinomas, and the latent viral genome has been detected in tumour-derived cells of gastric, thymic, salivary gland and nasopharyngeal carcinomas (Rickinson and Kieff, 1996). While the majority of the human population is latently infected with EBV, the factor(s) that predispose specific individuals to develop an EBV-associated malignancy are not known. Investigation of the EBV latent gene products will contribute to an understanding of the oncogenic potential of the virus.

1.1.2 Viral structure

Electron microscopy and antigenic studies have classified EBV as a lymphocryptovirus of the gammaherpes class (Epstein et al., 1965). Virion particles consist of a glycoprotein-studded envelope surrounding a tegument and a nucleocapsid core. The EBV genome was the first herpesvirus genome to be cloned (Dumbaugh et al., 1980) and completely sequenced (Baer et al., 1984; Hatfull et al., 1988). The viral genome is linear, 172 kb in length, and contains numerous repeated DNA sequence elements. Two major EBV subtypes, identified as EBV-1 and EBV-2, differ within the sequence of some latent gene products and in their ability to transform B lymphocytes (Dumbaugh et al., 1980; Sample et al., 1990).

The comparisons amongst EBV and other herpesvirus genes indicate that EBV has typical lytic functions and unique latent functions. A large number of EBV lytic gene products are homologous to proteins identified in other herpesviruses such as varicella zoster virus (VZV) (Davison and Taylor, 1987). Other EBV lytic genes have sequence and functional similarity to cellular gene products, including the amino acid sequence of BCRF1 which is 84 % homologous to human interleukin-10 (Moore et al., 1990). In

contrast, the EBV-encoded latent genes have no detectable homology with those of other herpes viruses except among the closely related gammaherpes papio, pan and samarai viruses (Karlin et al., 1994).

1.1.3 Lytic replication of EBV

Lytic replication of EBV is detectable in epithelial cells derived from patients, however lytic infection of tissue culture cells is inefficient and produces only low titers of virus (Sixbey et al., 1983; Sixbey et al., 1984). Therefore, most studies on the lytic replication of EBV have relied upon induction of latently infected B lymphocytes to produce virus. Lytic replication has been induced in cultured cells with tetradecanoylphorbol-13-acetate (TPA), n-butyrate or by crosslinking of cell surface immunoglobulin molecules (Luka, 1988). Expression of the EBV immediate early gene BZLF1 also induces the switch from latent to lytic replication. Zta, the product of the BZLF1 gene, is a transactivator of early EBV lytic gene expression and induces virus production (Rooney et al., 1989). Zta binds as a dimer to several Zta responsive elements (ZREs) within two EBV early lytic promoters and the N-terminal transactivation domain of Zta stabilizes preinitiation complexes with the basal transcription machinery (Lieberman et al., 1990; Lieberman and Berk, 1991). The transactivation domain also mediates the interaction of Zta with several EBV proteins required for lytic replication (Gao et al., 1998).

Lytic replication of the EBV genome occurs during S phase and requires several viral gene products including the transactivator Zta, a DNA polymerase (BALF1), a primase and helicase complex (BSLF1 and BBLF4), a single-stranded DNA binding protein (BALF2), and a processivity factor (BMRF1) (Fixman et al., 1992). The lytic

origin of replication, *oriLyt*, is distinct from the latent origin of replication, *oriP*, that will be described in greater detail in section 1.1.4.3. *OriLyt* is bound by Zta, followed by assembly of the EBV helicase-primase and the polymerase-associated factors into a lytic replication complex (Gao et al., 1998). Multiple copies of the viral genome are generated as concatamers, cleaved, packaged into virions and released from the dying cell (Cho and Tran, 1993; Hammerschmidt and Sugden, 1988).

1.1.4 EBV latent infection

EBV is able to infect and immortalize B lymphocytes *in vivo* and *in vitro*. EBV specifically recognizes the CD21 surface antigen of B lymphocytes, a complement receptor (Fingeroth et al., 1984; Nemerow et al., 1985). The EBV glycoprotein gp350 contains a region with sequence homology to the complement component C3d, a natural ligand of CD21 (Nemerow et al., 1987). The interaction of CD21 and gp350 is sufficient for attachment of EBV to the B lymphocyte surface and is a classic example of molecular mimicry by a pathogen.

Infection by the bound virus appears to require additional interactions since recombinant EBV lacking the minor glycoprotein gp42 (BZLF2), binds but fails to penetrate B lymphocytes (Wang and Hutt-Fletcher, 1998). The cell surface human leukocyte antigen (HLA) Class II protein, HLA-DR, binds gp42 and serves as a cofactor for infection of B cells (Li et al., 1997; Spriggs et al., 1996). These findings suggest a combination of virus-cell interactions are necessary for infection and may account for the narrow host cell range of EBV.

The process by which EBV becomes internalized in cytoplasmic vesicles, fuses with the vesicle membranes, and releases its genome into the cytoplasm for transport to

the nucleus is poorly understood (Kasamatsu and Nakanishi, 1998). Upon entry into the nucleus, the linear EBV genome circularizes via the terminal repeats and a pattern of latent gene expression initiates (Figure 1) (Alfieri et al., 1991; Hurley and Thorley-Lawson, 1988). Within 12 hours post-infection, EBV nuclear proteins EBNA-LP and EBNA-2 are detectable due to the high constitutive activity of the latent viral promoter, Wp. Following this initial period of protein expression, promoter usage switches to Cp and long transcripts are generated that become differentially spliced to encode EBNA3A, 3B, 3C and EBNA1. The most abundant mRNAs are produced from a bidirectional promoter that transcribes the LMP2A, 2B and LMP1 genes. By 32 hours, all EBNAs and LMPs are expressed and small RNA polymerase III transcripts, called EBER1 and EBER2, begin to accumulate. These nonpolyadenylated, nuclear RNAs become the most abundant EBV RNAs in the cell although their function in the nucleus remains unclear. During progression of the latent infection, EBNA1 binds to *oriP* and enables the replication and maintenance of the EBV genomes within an expanding pool of proliferating B lymphocytes.

1.1.4.1 Latent Infection Proteins

Only a fraction of the EBV gene products are expressed during latency. Six nuclear proteins (EBNA-LP,1,2,3A,3B,3C) and three membrane bound proteins (LMP1,2A,2B) play a role in inducing cellular proliferation and maintaining the latent infection in B lymphocytes (reviewed in Kieff, 1996). An additional EBV protein, BARF1, is expressed in latently infected epithelial cells (Hayes et al., 1999).

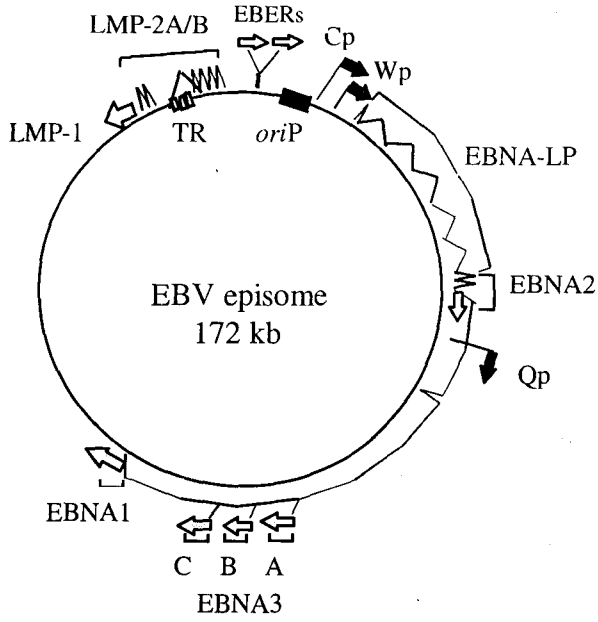


Figure 1. Schematic of the EBV genome (adapted from Kieff, 1996). The latent membrane proteins LMP-1, LMP-2A and LMP-2B are expressed from a bidirectional promoter near the terminal repeats (TR). The position of EBERs and the latent promoters Cp, Wp and Qp are shown along with the location of exons within a single primary transcript that encodes Epstein-Barr nuclear antigens EBNA-LP, EBNA2, EBNA3A, EBNA3B, EBNA3C and EBNA1. The position of the latent origin of replication, *oriP*, is also shown.

EBNA-LP is encoded within the leader region of each EBNA mRNA and is translated when splicing generates an in-frame initiation codon. This protein stimulates EBNA2- mediated transcriptional activation and is essential for growth transformation of B lymphocytes (Harada and Kieff, 1997; Mannick et al., 1991; Sinclair et al., 1994).

EBNA1 is required to establish and maintain EBV latent infection and is the only viral protein that associates with chromosomes during mitosis (Lee et al., 1999; Petti et al., 1990). EBNA1 binds to specific recognition sites located within the latent viral origin of replication, *oriP*, as well as upstream of the EBNA1 Qp promoter (Rawlins et al., 1985). Binding of EBNA1 to *oriP* sequences mediates the replication and segregation of the EBV episome within the nucleus of proliferating cells (Yates et al., 1985), and activates transcription at viral and heterologous promoters (Reisman and Sugden, 1986; Sugden and Warren, 1989). EBNA1 bound to sequences near the Qp promoter represses EBNA1 expression from Qp (Sample et al., 1992). Since EBNA1 has no detectable enzymatic activity, it is thought that EBNA1 fulfills its functions by mediating interactions with cellular proteins. A review of the functions and characteristics of EBNA1 are presented in section 1.1.5.

EBNA2 is essential for B lymphocyte transformation and functions as a transcriptional activator of cellular and viral latent genes (Sung et al., 1991; Wang et al., 1990). EBNA2 is an acidic protein that associates with the large subunit of the single stranded DNA binding protein, RPA, and the basal transcription components TFIIB and TAF40 (Tong et al., 1995). EBNA2 does not interact directly with DNA, but rather, associates with the DNA binding protein RBPJ kappa (Jκ) (Ling et al., 1993; Yalamanchili et al., 1994). Jκ is a transcriptional repressor with DNA recognition sites

that map to EBNA2 responsive elements within the EBV genome (Dou et al., 1994; Grossman et al., 1994; Waltzer et al., 1994). The interaction of EBNA2 with DNA-bound Jκ converts a transcriptional repressor signal into an activation signal and is important for EBNA2 responsiveness (Henkel et al., 1994; Hseih and Hayward, 1995; Waltzer et al., 1995). EBNA2 also interacts with Pu.1, a B lymphocyte-specific factor that binds directly to additional sites within the EBNA2 responsive elements (Johannsen et al., 1995). EBNA2 activated transcription of cellular genes, including CD23 (Wang et al., 1987), CD21 (Wang et al., 1990), and c-fgr (Knutson, 1990), may be facilitated by Jκ, PU.1 or other as yet unidentified factors.

EBNA3A,3B and 3C are large, related proteins that localize to intranuclear regions excluding the nucleolus (Petti et al., 1990). Recombinant EBV, generated by introducing specific mutations in each of the EBNA3 genes, revealed that EBNA3A and EBNA3C are essential for B lymphocyte growth transformation and that EBNA3B is dispensable (Tomkinson and Kieff, 1992; Tomkinson et al., 1993). EBNA3A and EBNA3C contain sequence motifs common to many transcription factors yet function as potent repressors of transcription from the EBV Cp and LMP latent promoters (Bain et al., 1996; Le Roux et al., 1994; Radkov et al., 1997). A physical interaction between EBNA3C and Jκ prevents the association of EBNA2-Jκ with DNA and is thought to be responsible for the EBNA3C mediated repression of EBNA2 activation (Robertson et al., 1996; Waltzer et al., 1996; Zhao et al., 1996).

LMP1 is a membrane protein with six transmembrane domains that is essential for B lymphocyte growth transformation. LMP1 aggregates within the plasma membrane and the C-terminal cytoplasmic domain associates with cellular proteins that are

mediators of the TNFR growth signalling pathways that activate NFκB, induce constitutive growth and inhibit apoptosis (Devergne et al., 1998; Eliopoulos et al., 1999; Fries et al., 1996; Mosialos et al., 1995). The upregulation of adhesion and coactivation molecules by LMP1 mimics the function of activated CD40 (Kilger et al., 1998). Thus, LMP1 appears to be a constitutively activated signalling receptor molecule (Gires et al., 1999).

LMP2A and LMP2B are both predicted to contain 12 transmembrane regions and differ only in an amino-terminal cytoplasmic region of the proteins. The N-terminal domain of LMP2A is a substrate for the *src* family of tyrosine kinases (Longnecker et al., 1991) and is specifically targetted by *lyn* kinase (Burkhardt et al., 1992). LMP2A blocks a switch from latent to lytic infection in B lymphocytes by disrupting the tyrosine kinase-mediated calcium signalling pathways (Miller et al., 1994). Since LMP2B lacks the N-terminal domain, LMP2B may modulate the effects of LMP2A.

1.1.4.2 Types of Latency

During latent infection of primary B lymphocytes, EBV expresses the six EBNA proteins, three latent membrane proteins and two small nonpolyadenylated RNAs. This pattern of viral gene expression is termed latency III and is evident in cells transformed into lymphoblastoid cell lines (LCL) that proliferate in a manner indistinguishable from mitogen or antigen stimulation (Hurley and Thorley-Lawson, 1988; Sugden and Mark, 1977). By contrast, EBV-positive tumor cells express only a subset of the latent genes and are designated as either latency I or II. Nasopharyngeal carcinoma (NPC) cells display latency II characteristics, expressing only EBNA1, the LMPs and BARF1 (Brooks et al., 1992; Fahraeus et al., 1988; Hayes et al., 1999; Kerr et al., 1992; Young et

al., 1988). BL cells display latency I and restrict detectable viral protein expression to EBNA1 (Gregory et al., 1990; Rowe et al., 1987). Latency I cells are not recognized by EBV-specific cytotoxic T-lymphocytes due to a unique immune evasion strategy of EBNA1 and the repression of all other viral antigens (Rooney et al., 1985; Levitskaya et al., 1995). In latency I and II, the Cp and Wp promoters are silent with EBNA1 autoregulating its own transcription from the Qp promoter (Sample et al., 1992; Schaefer et al., 1991; Schaefer et al., 1995). The expression of EBNA1 in all forms of latency highlights the essential role of this protein for maintenance of the viral genome and persistence of the infection in spite of continual immune surveillance against other viral gene products.

1.1.4.3 The Latent Origin of Replication

The EBV genome replicates once per cell cycle and is maintained as a supercoiled episome in the nucleus of infected cells (Adams, 1987; Lindahl et al., 1976). Electron microscopy studies of viral episomes purified from infected B lymphocytes revealed replication forks indicative of a bidirectional origin of replication (Gussander and Adams, 1984). Screening of the viral genome for *cis*-acting elements that permit plasmid replication identified *oriP*, the latent origin of replication (Sugden et al., 1985; Yates et al., 1984). The only viral *trans*-acting factor required for autonomous replication of *oriP* plasmids in human cells is the Epstein-Barr nuclear antigen 1 (EBNA1) protein (Lupton and Levine, 1985; Yates et al., 1985). In the presence of EBNA1, *oriP* plasmids replicate once per cell cycle and are maintained at a stable copy number in dividing cells (Yates et al., 1984; Yates and Guan, 1991). Thus, *oriP* appears to contain all the *cis*-acting requirements for both replication and segregation of the plasmid during cell division.

Analysis of the EBV genome, by two-dimensional gel techniques, detected replication bubbles at *oriP* (Gahn and Schildkraut, 1989). *OriP* may not be the only origin used for latent replication by the virus however, since initiation sites were also mapped in four different cell lines to a broad region upstream of *oriP* (Little and Schildkraut, 1995). While alternate initiation sites may exist, *oriP* remains the only genetically defined sequence shown to support plasmid replication.

The latent origin of replication, *oriP*, has been localized to a 2 kbp fragment of the EBV genome shown in Figure 2. *OriP* consists of two essential elements separated by 960 bp of DNA, the family of repeats (FR) and the dyad symmetry element (DS), which contain 20 and 4 EBNA1 binding sites respectively (Rawlins et al., 1985; Reisman et al., 1985). Each EBNA1 recognition site consists of an 18 bp imperfect palindromic sequence which is homologous to a consensus sequence 5' GG(A/G)TAGCATATGCTA(C/T)CC 3' (Ambinder et al., 1990; Rawlins et al., 1985).

Two *in vivo* assays have been used to examine *oriP* function. Following the introduction of *oriP* plasmids into mammalian cells, DNA replication and plasmid maintenance activities are determined from the analysis of plasmid DNA recovered from cells after various lengths of time in culture. The replication of o*riP* plasmids is determined by the resistance of the recovered DNA to digestion by the methylation-sensitive restriction enzyme Dpn I. The methylation of GATC sequences in DNA initially propagated in *E. coli* is not maintained upon

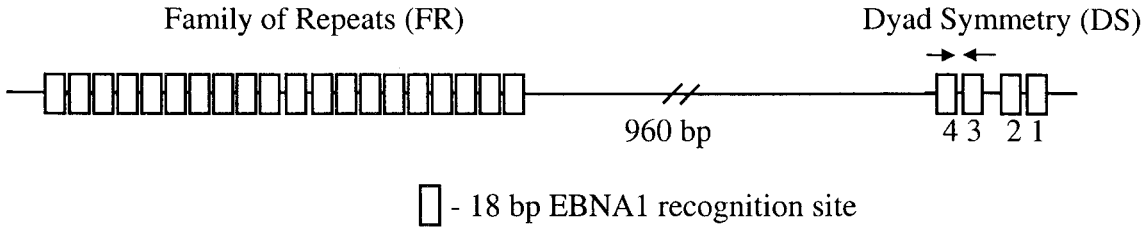


Figure 2. The latent origin of replication, *oriP*. The family of repeats contains 20 EBNA1 binding sites and the dyad symmetry element consists of two pairs of adjacent sites numbered site 1 to site 4 from right to left. Sites 3 and 4 of the DS are contained within a 65 bp sequence of dyad symmetry as illustrated by arrows.

replication of the DNA in mammalian cells. Thus, plasmids that replicate in mammalian cells are rendered resistant to Dpn I while those that are unable to replicate are sensitive to Dpn I digestion. Plasmid maintenance assays examine the stability of *oriP* plasmids in mammalian cells over extended periods of time in culture (Sugden et al., 1985; Yates et al., 1984). The plasmid contains a selectable marker and renders the transfected cell resistant to a drug that eliminates untransfected cells. The amount of plasmid DNA isolated from an equal number of drug-resistant cells is determined and the copy number per cell is calculated. Unstable plasmids are lost or not maintained in extracellular form due to defects in either DNA segregation, DNA replication or both processes. The replication and plasmid maintenance assays have allowed the function of *oriP* elements and EBNA1 to be examined.

The rearrangement and/or removal of EBNA1 binding sites has provided insight into the function of the *oriP* elements. When present together on a plasmid, the FR and DS support plasmid maintenance even when the orientation of the elements is altered (Lupton and Levine, 1985; Reisman et al., 1985). Sequences between the FR and DS were shown to be nonessential as this region can be removed or extra sequences added (up to 2400 bp) without any effect on long term plasmid retention (Reisman et al., 1985). Although neither the FR nor the DS can support DNA replication individually, the FR can be replaced with multiple DS elements and the modified *oriP* plasmid is still able to replicate (Reisman et al., 1985; Wysokenski and Yates, 1989). However, the DS element cannot be functionally replaced by the FR, suggesting that the DS is the origin of replication and that additional EBNA1 sites are required to efficiently activate replication at *oriP* (Wysokenski and Yates, 1989). The contention that the DS is the origin of

replication is also supported by two-dimensional gel electrophoresis analysis of replicating *oriP* plasmids. In these studies, replication was observed to initiate at or near the DS element (Gahn and Schildkraut, 1989; Platt et al., 1993). The four EBNA1 binding sites of the DS element are arranged as two pairs of adjacent sites (see Figure 2). Point mutations revealed that one pair of adjacent sites is sufficient for plasmid replication but replication was disrupted if 5 or 10 base pairs were inserted between the remaining two sites (Harrison et al., 1994). Therefore, the spacing and arrangement of EBNA1 binding sites within the DS element is important for the replication of *oriP* plasmids.

The FR element of *oriP* appears to have several functions. First, as mentioned above, the FR may activate DNA replication from the DS. Second, the FR governs the partitioning of *oriP* plasmids in dividing cells. While multiple copies of the DS allow replication in the absence of the FR element, *oriP* plasmids require the FR element for their efficient maintainence in cells (Wysokenski and Yates, 1989). The entire FR element is not essential for plasmid partitioning since the number of EBNA1 binding sites within the FR could be reduced from 20 to 7 without any significant loss in plasmid replication or maintenance activity (Chittenden et al., 1989; Wysokenski and Yates, 1989). The rate of *oriP* plasmid loss has been measured in a number of cell lines and varies between 2-6 % per generation (Kirchmaier and Sugden, 1995; Sugden and Warren, 1988; Yates et al., 1984). The FR also mediates the retention of plasmid DNA in the absence of a functional origin of replication. While plasmids lacking the DS element are retained, the copy number decreases as the unreplicated plasmids are diluted within a population of dividing cells (Middleton and Sugden, 1994). Additional evidence for the

maintenance activity of the FR was provided by a study demonstrating that plasmids containing the FR and human DNA sequences were maintained in cells that express EBNA1, whereas the same plasmids lacking the FR element were unable to persist (Krysan et al., 1989).

The mechanism by which the EBV episomes and *oriP* plasmids segregate is thought to involve the attachment of *oriP* sequences to the host cell metaphase chromosomes. This hypothesis is supported by fluorescence *in situ* hybridization studies that observed EBV genomes and *oriP*-containing DNA randomly decorating metaphase chromosomes (Delecluse et al., 1993; Harris et al., 1985; Simpson et al., 1996). The interaction between *oriP* and metaphase chromosomes is thought to be mediated by EBNA1 because EBNA1 also associates with condensed cellular chromatin during mitosis (Grogan et al., 1983; Marechal et al., 1999; Petti et al., 1990). Although the nature of the interaction between EBNA1 and the host chromosomes is not known, the identification of cellular proteins that may mediate these interactions would increase our understanding of DNA segregation.

A third role for the FR is that of a transcriptional enhancer element. In the presence of EBNA1, the FR element enhances transcription when positioned either upstream or downstream of a promoter and functions efficiently with as few as 7 of the 20 EBNA1 binding sites (Reisman and Sugden, 1986; Wysokenski and Yates, 1989). In addition to studies using artificial reporter constructs, the FR enhances transcription in an EBNA1-dependent manner from the Cp, Wp and LMP latent EBV promoters (Gahn and Sugden, 1995; Pugeilli et al., 1996; Sugden and Warren, 1989). Transcriptional activation occurs in cells lines that are not permissive for *oriP*-mediated plasmid replication,

demonstrating that the FR enhancer activity is not dependent on DNA replication (Reisman and Sugden, 1986; Wysokenski and Yates, 1989). Investigations in other viral systems have revealed that transcriptional enhancers contribute to the activation of replication from SV40 and polyoma virus origins (DePamphilis, 1988). The transcriptional enhancer element from SV40, however, cannot substitute for the FR element to activate replication from the DS, suggesting that transcriptional activation is not sufficient to activate *oriP* replication (Reisman and Sugden, 1986).

Finally, the FR element has been reported to be a pause or termination site for DNA replication forks (Gahn and Schildkraut, 1989). Analysis of DNA replication intermediates by two-dimensional gel electrophoresis revealed a pattern consistent with the existence of a replication fork barrier at the FR element and unidirectional replication of *oriP* plasmids (Gahn and Schildkraut, 1989). The replication fork, initiating at the DS element in both plasmids and the EBV episome, proceeds towards the FR and is paused at that site while a second replication fork moving in the opposite direction progresses around the DNA molecule and terminates at the FR element (Gahn and Schildkraut, 1989). Replication fork arrest on a DNA template *in vitro* requires at least 6 of the 20 EBNA1 binding sites and the presence of EBNA1 (Dhar and Schildkraut, 1991). EBNA1 binds to sites in the FR element and the pausing of replication forks at the FR element is thought to be due to the high affinity binding of multiple EBNA1 dimers to this region (Ermakova et al., 1996; Frappier and O'Donnell, 1991; Jones et al., 1989). The FR does not completely block replication fork movement *in vivo*, however, since a plasmid containing the DS element flanked by two FR elements was maintained only slightly less efficiently than the wild-type *oriP* plasmid (Kirchmaier and Sugden, 1995). While the

functional significance of replication fork arrest at the FR element is not known, this process might play a role in the regulation of replication from *oriP*.

1.1.5 Epstein-Barr nuclear antigen 1 (EBNA1)

EBNA1 is essential for three functions associated with *oriP*, namely DNA replication, DNA segregation and the enhancement of transcription. Each recognition site in the FR and DS is bound by an EBNA1 dimer (Ambinder et al., 1991; Frappier and O'Donnell, 1991a; Rawlins et al., 1985) and these sites are occupied throughout most or all of the cell cycle (Hsieh et al., 1993; Niller et al., 1995). Recognition sites of the DS differ in their binding affinity for EBNA1, with sites one and four having higher affinity than sites two and three (see Figure 2) (Ambinder et al., 1990; Summers et al., 1996). EBNA1 displays cooperative binding to adjacent sites such that the binding of EBNA1 to site two is stimulated in the presence of site one and the four sites of the DS fill simultaneously upon titration of EBNA1 (Harrison et al., 1994; Summers et al., 1996).

1.1.5.1 EBNA1 Replication Mechanism

The mechanism by which EBNA1 activates replication from the DS element has not been elucidated, but is likely to involve changes in the DNA structure of the DS and recruitment of cellular factors to *oriP*. Four types of structural alterations in *oriP* DNA have been shown to be associated with EBNA1 binding to the DS and FR elements. First, a single thymine residue in sites one and four of the DS element becomes sensitive to oxidation by potassium permanganate upon binding of EBNA1 both in *vitro* and in *vivo* (Frappier and O'Donnell, 1992; Hearing et al., 1992; Hsieh et al., 1993). Since permanganate sensitivity occurs at distorted or melted duplex DNA, the appearance of sensitive thymine residues suggested that EBNA1 binding to the DS induces a distortion

at sites one and four. The interaction of EBNA1 with site one alone also displayed permanganate reactivity and it was shown that the inversion of an adenine and thymine base pair differentiates a reactive and unreactive EBNA1 recognition site (Summers et al., 1997). The structures of permanganate-reactive and -unreactive DNA recognition sites in complex with EBNA1 were determined by x-ray crystallography and subtle differences were noted between the two structures (Bochkarev et al., 1996; Bochkarev et al., 1998). Specifically, in the permanganate-sensitive DNA, the interstrand hydrogen bonds were lengthened at three consecutive base pairs spanning the permanganate-reactive base pair. These observations suggest that the distortion detected upon EBNA1 binding may involve localized breathing of the DNA strands but not complete melting of the origin.

A second structural alteration is predicted to occur upon the cooperative assembly of EBNA1 on adjacent sites of the DS element. The structure of EBNA1 bound to a single DNA site allowed the cooperative assembly of EBNA1 to adjacent sites to be modelled (Bochkarev et al., 1996). Steric hinderance was observed between two EBNA1/DNA complexes separated by 3 bp of B-form DNA. The model predicted that further unbending and unwinding of the DNA, in addition to the distortion observed at individual sites, would be required to alleviate the steric clash.

The third and fourth structural distortions of *oriP* that occur upon EBNA1 binding were revealed by electron microscopy of EBNA1-DNA complexes. DS DNA molécules in which both ends appeared to exit the roughly spherical EBNA1 complex on the same side suggested the folding or wrapping of DNA around the EBNA1 complex (Frappier and O'Donnell, 1991b). In addition, EBNA1 complexes bound to the FR and DS

elements of *oriP* were observed to interact, generating a DNA loop between the two elements (Frappier and O'Donnell, 1991b; Su et al., 1991). This DNA looping interaction stabilizes the association of EBNA1 on the DS element and is thought to be an important step in the activation of replication from *oriP* (Frappier et al., 1994; Su et al., 1991).

Several lines of evidence suggest that EBNA1 activates replication by recruitment of cellular factors to *oriP*. First, the assembly of EBNA1 on *oriP* is required for origin activation despite the fact that EBNA1 does not appear to possess any enzymatic activities. This suggests that cellular initiation factors may be recruited to *oriP* through interactions with EBNA1 (Middleton and Sugden, 1992). Second, origin binding proteins in all other viral systems act as assembly sites for replication proteins (Hassell and Brinton, 1996). Third, DNA-bound EBNA1 interacts with replication protein A (RPA), the human single stranded binding protein, suggesting that this interaction may be important for the initiation of DNA replication (Zhang et al., 1998). Fourth, the replication of *oriP* DNA displays species specificity; primate cells are permissive for EBV replication, while rodent cells are not (Yates et al., 1985). Such species specificity is likely due to specific protein-protein interactions. Finally, EBNA1 is bound to *oriP* throughout most or all of the cell cycle (Hsieh et al., 1993; Niller et al., 1995). Therefore the signal that activates DNA replication is not EBNA1 binding to DNA, but likely, the association of a cellular protein with the EBNA1-DNA complex.

1.1.5.2 EBNA1 Segregation Mechanism

The mechanism by which EBNA1 mediates the segregation of EBV episomes and *oriP* plasmids is thought to involve an attachment or association with the cellular chromosomes. Fluorescence *in situ* hybridization and immunofluorescence studies have

revealed that the EBV genome, *oriP* plasmids and EBNA1 all localize to the host metaphase chromosomes (Grogan et al., 1983; Harris et al., 1985; Petti et al., 1990; Simpson et al., 1996). Recognition of *oriP* sequences and nuclear localization of EBNA1 is not sufficient to mediate plasmid maintenance, indicating that other unidentified functions of EBNA1 are required (Middleton and Sugden, 1994). Immunofluorescence studies with green fluorescent protein-EBNA1 fusion constructs have identified at least three chromosomal binding fragments of EBNA1 outside of the DNA binding domain (Marechal et al., 1999). The mechanism by which EBNA1 attaches to metaphase chromosomes is still unclear but is thought to involve an interaction with cellular proteins rather than a direct interaction with chromosomal DNA. A recently identified human protein, EBNA1 binding protein (EBP2), appears to be a good candidate for this function. EBNA1 and EBP2 colocalize on metaphase chromosomes and the association of these proteins on mitotic chromatin depends on a glycine-arginine rich region of EBNA1 (Shire et al., 1999; Wu et al., submitted). Further studies are needed to establish whether the chromosome and EBP2 binding domains of EBNA1 are necessary for the maintenance of *oriP* plasmids in replicating cells.

1.1.5.3 EBNA1-Mediated Activation of Transcription

EBNA1 acts as both an enhancer and a repressor of transcription, however the mechanism of action and the responsible domains of EBNA1 are unknown. Transcription from the EBV latent promoters and reporter gene constructs linked to the FR element is enhanced in EBNA1-expressing cells (Gahn and Sugden, 1995; Reisman and Sugden, 1986; Sugden and Warren, 1989). During latency III, EBNA1 enhances its own expression, as well as that of other EBV latent genes, through activation of the viral

BamHI-C and -W promoters (Pugeilli et al., 1996; Sugden and Warren, 1989). EBNA1-mediated transcriptional activation in cell lines that are not permissive for EBV latent replication suggested that the mechanism for the enhancement of transcription is more general than the species-specific nature of *oriP* replication (Wysokenski and Yates, 1989). A functional chimeric transactivation protein, constructed by fusing the EBNA1 DNA binding domain with the transactivation domain of the estrogen receptor, failed to activate *oriP* replication (Middleton and Sugden, 1992). These results determined that a transactivation function is not sufficient for origin activation.

During latency I and II, EBNA1 is expressed from the BamHI-Q promoter (Qp) (Nonkwelo et al., 1996; Schaefer et al., 1995). Qp is activated by cellular factors while EBNA1 negatively regulates its own expression by binding two recognition sites immediately upstream of the promoter (Sample et al., 1992; Schaefer et al., 1991). The mechanism of EBNA1-mediated repression is not understood but competitive interference with transcriptional activation proteins for binding sites at adjacent promoter sequences has been suggested (Nonkwelo et al., 1997).

1.1.5.4 Other Functions of EBNA1

In addition to its role in the replication, segregation and transactivation of the EBV genome, EBNA1 may play a role in the immortalization of host cells. Transgenic mice that express EBNA1 within B cells develop fatal lymphomas whereas littermate control animals have no pathological phenotypes (Wilson et al., 1996). Additionally, EBNA1 is the only EBV protein expressed in tumour cell lines derived from Burkitts lymphoma patients (Gregory et al., 1990; Rowe et al., 1987). These results suggest that EBNA1 may be considered a viral oncogene that contributes to the establishment,

progression and/or maintenance of B cell lymphomas.

EBNA1 also serves a role in the evasion of host immune responses (Rickenson et al., 1996). Type I latency cells, which express EBNA1 alone, escape recognition by cytotoxic T lymphocytes (Steven et al., 1996). This immune escape occurs because EBNA1 is not processed by the proteosome and, as a result, EBNA1 epitopes are not presented on the cell surface (Levitskaya et al., 1995; Levitskaya et al., 1997).

1.1.5.5 EBNA1 Protein Domains

The EBNA1 gene, from the EBV strain B95-8, encodes a protein of 641 amino acids (Baer et al., 1984) that is shown schematically in Figure 3. Although a number of functional domains have been identified, many regions of the EBNA1 protein remain uncharacterized. The N-terminal region of EBNA1 contains a glycine and arginine-rich region of unknown function. A large internal region of EBNA1, comprised of repeated glycine and alanine residues (amino acids 90-324), is not required for the replication, transactivation or segregation functions of EBNA1 (Yates and Camiolo, 1988; Yates et al., 1985). The Gly-Ala repeat is responsible for the ability of EBNA1 to evade cytotoxic T-lymphocyte responses (Levitskaya et al., 1995; Levitskaya et al., 1997). The adjacent region of EBNA1 (amino acids 325-376) is a glycine and arginine rich domain, termed the looping domain, that mediates interactions between the FR and DS-bound EBNA1 molecules and results 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). EBNA1 has also been suggested to bind RNA through the RGG (Arg-Gly-Gly) motifs found within the looping domain and more N-terminal sequences (Snudden et al., 1994) but the functional significance of RNA binding

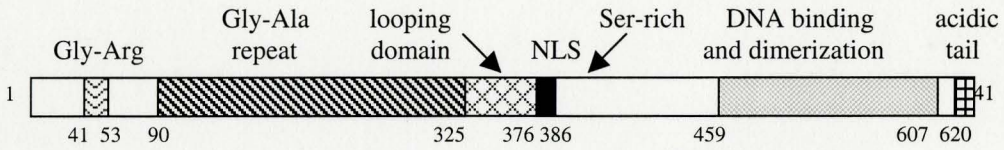


Figure 3 Schematic representation of the EBNA1protein. Amino acid numbers are indicated for the Gly-Arg rich region, the Gly-Ala repeat, the looping domain, the nuclear localization signal (NLS), the Ser-rich region, the DNA binding and dimerization domain and the acidic tail.

(if any) is unknown.

The looping domain is followed by a sequence of basic residues (amino acids 379-386) that function as a nuclear localization signal for EBNA1 (Ambinder et al., 1991) and a serine-rich region (amino acids 383 to 393) that is the predominant region of phosphorylation. EBNA1 was shown to be phosphorylated only at serine residues in both EBV-infected B lymphocytes and when expressed from a recombinant baculovirus in insect cells (Frappier and O'Donnell, 1991; Hearing and Levine, 1985). It is not yet known whether the phosphorylation is important for any EBNA1 function. The C-terminal region of EBNA1 (amino acids 620-641) contains 13 aspartate and glutamate residues and has been termed the acidic tail. This domain was suggested to be required for transactivation (Ambinder et al., 1991), and plasmid maintenance (Yates and Camiolo, 1988), however, the assignment of a functional role for this region has not been conclusive (Kirchmaier and Sugden, 1997; Polvino-Bodnar and Schaffer, 1992; Yates and Camiolo, 1988).

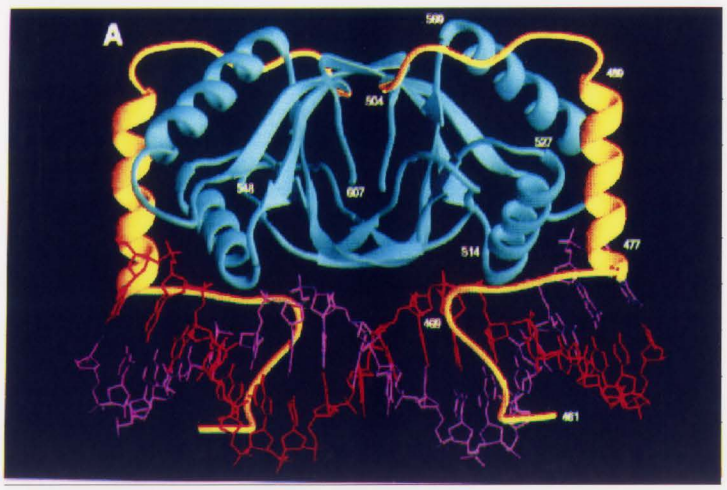
1.1.5.5.1 The DNA binding and dimerization domain of EBNA1

EBNA1 residues responsible for the DNA binding and dimerization of the protein have been shown to exist between amino acids 459 to 607 (Ambinder et al., 1991; Shah et al., 1992; Summers et al., 1996). Biochemical approaches defined the boundaries of the DNA binding domain within a larger C-terminal fragment of EBNA1 that bound *oriP* DNA in an equivalent manner to the full length protein (Ambinder et al., 1991; Rawlins et al., 1985). Gel filtration and glycerol gradient sedimentation analyses of purified EBNA1, determined that the protein exists as a dimer in solution (Frappier and O'Donnell, 1991a). Electrophoretic mobility shift assays, performed with *in vitro*

translated polypeptides of different sizes, determined that EBNA1 also bound DNA as a dimer (Ambinder et al., 1991). Limited proteolysis identified a smaller fragment of EBNA1 with increased protease resistance upon DNA binding (Shah et al., 1992). Internal deletions within this region of EBNA1 perturbed dimerization and abolished DNA binding suggesting that these two activities co-exist within a discrete domain (Shah et al., 1992). Residues important for dimerization were identified between amino acids 501 and 532 and between 554 and 598 (Chen et al., 1993). Dimerization is essential for DNA binding, however, mutations outside of this region also implicated amino acids 459 to 487 as a DNA recognition motif (Chen et al., 1993).

Crystal structures of the EBNA1 DNA binding and dimerization domain, free and in association with two different 18 bp EBNA1 binding sites (Figure 4), have been determined (Bochkarev et al., 1996; Bochkarev et al., 1995; Bochkarev et al., 1998). The structure revealed that each EBNA1 monomer contains four β-strands, two α-helices and a loop, between amino acids 504 and 604, that are collectively termed the core domain (Figure 4A, in blue shading) (Bochkarev et al., 1995). This region corresponds to the dimerization domain defined by Chen et al. (1993), and displays structural homology to the DNA binding and dimerization domain of the E2 protein from bovine papillomavirus (Figure 4b) (Bochkarev et al., 1995; Hegde et al., 1992). In both proteins, the four β-strands from each monomer comprise an 8 stranded antiparallel β-barrel dimer with a hydrophobic interface. The EBNA1 and E2 core domain structures are almost identical in structure (rms deviation 0.908Å) without any apparent sequence conservation (Bochkarev et al., 1995). Sequence specific DNA contacts by E2 dimers are mediated by two helices (termed the recognition helices) that have structural counterparts in EBNA1

A



B

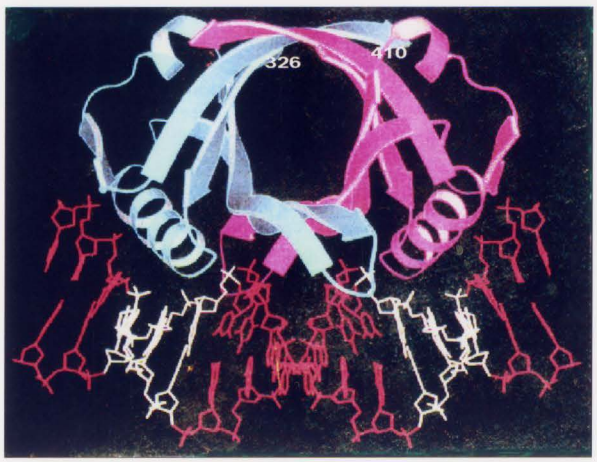


Figure 4 Structure of the DNA binding domains of EBNA1 and the papillomavirus E2 proteins bound to DNA. (A) An EBNA1 dimer bound to its 18 base pair binding site. The core domain (amino acids 504 to 604) is shown in blue and the flanking domain (amino acids 459 to 503) is shown in yellow (adapted from Bochkarev et al, 1996). (B) A dimer of the E2 protein bound to its 16 base pair binding site (adapted from Hegde et al, 1992). The two monomers of the DNA binding domain are shown in blue and magenta and are structurally homologous to the core domain of EBNA1

that will also be referred to as the recognition helices (Hegde et al., 1992). Although the crystal structure showed no base contacts between the recognition helices of EBNA1 and the DNA, point mutations in these helices severely impair DNA binding without affecting protein folding or stability (Cruickshank et al., submitted). Thus, the results to date suggest that the EBNA1 recognition helices make transient DNA contacts that are required for the loading of the flanking domain on the DNA (Cruickshank et al., submitted).

The primary difference in the structure of the EBNA1 core domain and the E2 DNA binding domain lies in the loop that connects β-strands 2 and 3. This loop in EBNA1 is 9 amino acids longer that in E2 and, unlike the sequence in E2, is very proline-rich and referred to as the proline loop (Bochkarev et al., 1995). Protease sensitivity in this region of the EBNA1 core domain suggested that the proline loop may be exposed and flexible in DNA-bound EBNA1 (Shah et al., 1992). The crystallographic data determined that the proline loop is in an extended conformation and solvent-accessible in both free and DNA-bound EBNA1 structures (Bochkarev et al., 1996; Bochkarev et al., 1995). The proline loop is conserved in the EBNA1-homologue from two related lymphocryptoviruses, which possess either three or all five prolines in a loop of the same length, suggesting that this region is important for EBNA1 function (Blake et al., 1999; Yates et al., 1996). Proline rich sequences have been found in other proteins, including the transcription factors CTF/NF-1 and OCT-2, and are known to mediate protein-protein interactions (Kim and Roeder, 1994; Tanaka et al., 1994). For these reasons, the proline loop is a region of the protein that is thought to mediate associations between EBNA1 and cellular proteins.

In addition to the core domain, amino acids between residues 459 and 503 have also been implicated in DNA binding. The crystal structure of EBNA1 bound to DNA revealed that this region is comprised of an α-helix and an extended peptide chain collectively referred to as the flanking domain (Figure 4a, in yellow shading). All of the base contacts observed in the co-crystal structure were mediated by residues in the flanking domain (Bochkarev et al., 1996; Bochkarev et al., 1998). Specifically, residues Lys461, Gly463, Arg469 and Lys477 of the flanking domain make a total of seven direct and two water-mediated hydrogen bonds with bases of the DNA recognition site. Residues 461 to 469 form an extended peptide that is inserted within the DNA minor groove. EBNA1 mutants lacking this region display a 20-fold weaker association with sites in the DS element and fail to generate the permanganate sensitivity of two EBNA1 binding sites in the DS element (Bochkarev et al., 1998; Summers et al., 1996; Summers et al., 1997). Within the extended chain, the two aromatic side chains of Trp464 and Phe465 (WF motif) are positioned in a region of the DNA minor groove that is widened by 2-3Å (Bochkarev et al., 1996). These residues are also conserved in EBNA1 homologues of the related rhesus and baboon lymphocryptoviruses (Blake et al., 1999; Yates et al., 1996). The WF motif does not appear to be required to induce permanganate-sensitivity in susceptible EBNA1 binding sites and a role for the DNA distortion caused by these residues remains to be determined (Summers et al., 1997).

1.1.5.6 EBNA1-Interacting Proteins

Attempts have been made to identify cellular proteins that interact with and contribute to the activities described for EBNA1. The yeast two-hybrid screen has been effectively used to identify interacting partners of numerous proteins and to date, yeast

two-hybrid screens of human cell libraries conducted in four different laboratories have yielded three EBNA1 interacting proteins. First, a protein of the nuclear import complex, called Rch1/karyopherin α2/importin α/hsrp 1α, was identified in two separate screens and shown to interact with the nuclear localization signal of EBNA1 (Fischer et al., 1997; Kim et al., 1997). The association of Rch1 and EBNA1 is thought to mediate the transport of EBNA1 into the nucleus but has not been shown to directly contribute to the functions of EBNA1. Second, an EBNA1-interacting protein, p32/TAP, was identified in all of the two-hybrid screens conducted in different laboratories (Aiyar et al., 1998; Kim et al., 1997; Shire et al., 1999; Wang et al., 1997). P32/TAP interacts with two separate glycine/arginine-rich domains within the N-terminus of EBNA1 and the results suggested that P32/TAP may contribute to the transactivation activity of EBNA1 (Wang et al., 1997). P32/TAP has been found to interact with diverse factors, such as nuclear splicing factor SF2, the human immunodeficiency virus REV protein and extracellular components including hyaluronic acid and the complement component C1Q (Chen et al., 1998; Ghebrehiwet et al., 1994; Luo et al., 1994; Mayeda et al., 1992). Therefore the function of p32/TAP is unclear as is the significance of its interaction with EBNA1.

A nucleolar protein of unknown function, previously called p40 and renamed EBNA1 binding protein 2 (EBP2), was recently identified from a yeast two-hybrid screen of an EBV-transformed B lymphocyte library (Chatterjee et al., 1987; Shire et al., 1999). EBP2 interacts with the glycine and arginine-rich looping domain between amino acids 325-376 of both free and DNA-bound EBNA1. The EBNA1-EBP2 interaction is not mediated by nucleic acids and occurs in both yeast and insect cell environments (Shire et al., 1999). EBP2 is conserved in eukaryotes and functional characterization of the *S.*

cerevisiae homologue suggests that this protein functions in the processing of rRNA transcripts (Huber et al., submitted). Interestingly, recent immunofluorescence studies conducted in our lab on human EBP2, show that this protein colocalizes with EBNA1 on the condensed cellular chromosomes during mitosis (Wu et al., submitted). Thus EBP2 might mediate the segregation function of EBNA1.

The small number of EBNA1-interacting proteins identified in the two-hybrid screens may be due to the cell-cycle dependent and/or transient nature of protein interactions at *oriP*. In addition to screening approaches, a biochemical approach was used to demonstrate the interaction of purified EBNA1 with a known replication protein, RPA (Zhang et al., 1998). The interaction of RPA and EBNA1 was observed by surface plasmon resonance both in solution and when EBNA1 was bound to the DS element (Zhang et al., 1998). RPA is a heterotrimer that binds to single-stranded DNA and interacts with a number of proteins involved in initiating DNA replication including DNA polymerase-α-primase, SV40 large T antigen and papillomavirus E1 (Dornreiter et al., 1992; Han et al., 1999). EBNA1 binds to the large subunit of RPA through either the C-terminal acidic tail or undefined sequences in the internal region of EBNA1 (Weissart, Frappier and Fanning, unpublished). Although the functional significance of the EBNA1-RPA interaction has not been demonstrated, the association of RPA with DNA-bound EBNA1 may facilitate assembly of replication factors at the origin.

1.2 DNA Replication in Eukaryotes

The replication of eukaryotic DNA is a highly regulated event of the cell cycle. In spite of the complexity of this fundamental process, common mechanisms observed among prokaryotic, eukaryotic and viral systems have provided a general model for DNA

replication (Kornberg and Baker, 1992). The initiation of DNA synthesis occurs at specific sites within the genome that assemble multi-protein complexes prior to S phase. Origin binding proteins (OBPs) recognize sequences within the replication origin and recruit additional factors required for DNA replication through both protein-protein and protein-DNA interactions. DNA strand separation at the origin results from this protein assembly and, during the replication process, the DNA is further melted by a helicase that moves with the replication fork. DNA synthesis involves a primase that synthesizes short RNA primers on both the leading and lagging strands and one or more DNA polymerases along with their associated factors. In eukaryotic chromosomes, replication forks migrate bidirectionally from the origin until termination occurs at the chromosome ends or between opposing forks from adjacent origins. The entire genome is duplicated with high fidelity and sister chromatids remain associated until the DNA is segregated to daughter cells at mitosis.

Much of our understanding of the DNA replication process and replication proteins in eukaryotic cells has been obtained through the investigation of systems, namely yeast and viral, that are amenable to genetic and/or biochemical approaches. The following section will briefly review what is known about replication origins and proteins identified in SV40 and papillomavirus, yeast and multicellular eukaryotes.

1.2.1 Replication of eukaryotic viruses

Most information about eukaryotic replication proteins and their mechanisms of action has come from studying the replication of viral DNA that utilizes the host cell replication machinery. The SV40, polyoma, papilloma, and Epstein-Barr viruses replicate in mammalian cells and depend heavily on the cellular replication proteins. A summary

of the replication of these viruses is outlined in the following sections.

1.2.1.1 Viral Replication Origins

Origins of DNA replication have been characterized in the genomes of several DNA viruses that replicate in mammalian cells. A comparison of the origins of SV40, polyomavirus, papillomavirus, and adenovirus led to the conclusion that viral origins contain modular DNA elements comprising core and auxiliary functions (DePamphilis, 1993). Core origin is the minimal *cis*-acting sequence required to initiate DNA replication under all conditions and contains an origin recognition element, a DNA unwinding element and an A/T-rich element. The origin recognition element is a DNA recognition site for origin binding proteins that assemble the replication machinery. DNA unwinding elements are easily unwound sequences with a critical spatial orientation to the origin recognition element, while A/T-rich sequences are easily distorted and may facilitate either protein binding to the origin recognition element or melting of the DNA unwinding element.

Auxiliary components consist of enhancer elements that facilitate origin firing and timing during S phase but are not required under all conditions (DePamphilis, 1988). Enhancer elements are comprised of transcription factor binding sites suggesting that transcriptional activator proteins play a role in enhancing DNA replication (He et al., 1993; Heintz, 1992). Several mechanisms have been proposed to explain how transcription elements may stimulate origin activity. First, enhancer-mediated transcription through the origin of mitochondrial DNA generates an RNA primer that is used for replication of mitochondrial DNA (Clayton, 1991). Second, transcriptional elements may promote origin melting. The removal of auxiliary sites adjacent to the

SV40 origin does not affect the binding of large T-antigen to origin sequences but greatly reduces origin unwinding activity and subsequent replication (Gutierrez et al., 1990). Third, transcription factors bound to enhancer elements may mediate the assembly of pre-replication complexes by recruiting or stabilizing the binding of replication proteins to the origin. For example, Adenovirus (Ad) type-2 replication is enhanced by cellular transcription factors NF1 and NF3 that facilitate the formation of a pre-initiation complex with the Ad-terminal protein and Ad-polymerase at the viral origin (Hay, 1996). Finally, transcription factors can prevent chromatin-mediated repression by modifying or displacing nucleosomes at origin sequences. For example, stimulation of SV40 origin activity by NF1 was only observed when the DNA was organized into a repressive chromatin structure (Cheng and Kelly, 1989).

1.2.1.2 Papovavirus Replication

SV40 and polyoma virus are related papovaviruses that multiply in primate and rodent cells respectively. The replication of SV40 and papillomaviral DNA requires only one viral protein, large T-antigen, and relies on cellular factors that are assembled at the viral origin (reviewed in Brush et al., 1995; Hassell and Brinton, 1996). A cell-free system for the replication of SV40 DNA was developed using primate cell extracts (Li and Kelly, 1984). Through fractionation of the extracts, this *in vitro* replication assay led to the purification of several proteins that were subsequently shown to be essential for both SV40 and cellular DNA replication (Hurwitz et al., 1990; Kelly, 1988; Stillman, 1989). Polyoma large T-antigen is thought to require additional factors for the stimulation of DNA replication although those proteins remain to be identified (Hassell and Brinton, 1996; Melendy et al., 1995). The cell-free SV40 replication system has

proven to be invaluable for the identification and characterization of cellular replication factors and has facilitated the investigation of the molecular mechanisms of SV40 DNA replication. A brief summary of the events involved in the initiation of DNA synthesis follows.

The initiation of DNA synthesis begins with the ATP-dependent assembly of large T-antigen hexamers on the two recognition sites in the origin. This induces structural distortion and DNA melting within an adjacent A/T-rich sequence and the DNA unwinding element (Boroweic et al., 1990). The DNA is then progressively unwound by the helicase activity of large T-antigen (Dean et al., 1992). Cellular proteins that interact with T-antigen and assemble at the replication fork have been purified and allowed the reconstitution of SV40 DNA replication with purified proteins (Waga et al., 1994). RPA is recruited to the origin through direct interactions with T-antigen and stabilizes regions of single-stranded DNA that are required for the primase activity of polymerase-α-primase (Dornreiter et al., 1992; Melendy and Stillman, 1993). Polymerase-α-primase initiates the leading DNA strand synthesis and reinitiates each Okazaki fragment on the lagging strand by the synthesis of an RNA primer that is partially extended by the DNA polymerase activity (Bullock et al., 1991; Tsurimoto and Stillman, 1991). A clamp loader complex, RFC (replication factor C), recognizes the primer-template junction on the leading strand and at each Okazaki fragment, assembling a trimeric ring of PCNA (proliferating cell nuclear antigen) around the DNA (Lee et al., 1991; Tsurimoto, 1998; Tsurimoto and Stillman, 1991). PCNA associates with DNA polymerase-δ, establishing a processive polymerase complex that replicates the leading DNA strand in a continuous manner and the lagging strand by the discontinuous

synthesis of each Okazaki fragment (Waga and Stillman, 1994). Ahead of the replication fork, the parental DNA strands are unwound by the helicase activity of large T-antigen. The positive supercoiling that accumulates ahead of the progressing replication fork is relieved by a topoisomerase activity. The RNA primers within the newly synthesized DNA strand are removed by FEN-1 and RNase HI nuclease activities and DNA gaps are repaired and ligated together to complete DNA replication (Waga et al., 1994).

1.2.1.3 Papillomavirus Replication

Papillomavirus constitutes a large family of related DNA viruses that transform epithelial cells and replicate at low copy numbers without lysing the host cell (Howley, 1996). The replication of papillomavirus DNA is under copy number control and the regulation of initiation events at a single origin is not limited to once per cell cycle (Gilbert and Cohen, 1987; Ravnan et al., 1992). The mechanism of DNA replication requires two viral proteins, E1 and E2, in addition to a small *cis*-acting region of the genome (Stenlund, 1996). The viral origin of replication comprises an E1 binding site flanked by A/T rich sequences and two E2 binding sites (Ustav et al., 1993). Auxilliary sequences within an upstream regulatory region are required for stable replication and contain an additional ten E2 binding sites (Piirsoo et al., 1996). E1 is an initiator protein that binds the origin in a multimeric form and possesses ATPase and helicase activities that are absolutely required for papillomavirus replication (Seo et al., 1993; Yang et al., 1993). E2 is a transcriptional enhancer that interacts with E1 and is specifically required for replication of the viral DNA *in vivo* (Frattini and Laimins, 1994; Mohr et al., 1991). A careful analysis of the E1 and E2 DNA binding requirements for replication has determined that E2 facilitates the formation of a multimeric E1 protein complex at the

origin (Sanders and Stenlund, 1998; Sedman and Stenlund, 1995). Cellular proteins including polymerase-α-primase and RPA interact with E1, suggesting that assembly of a replication complex occurs at the origin (Han et al., 1999; Park et al., 1994). Unlike SV40 large T antigen, E1 does not appear to be able to melt the DNA at the replication origin, suggesting that cellular factors are required for this purpose (Sedman and Stenlund, 1998).

1.2.2 DNA replication in *Saccharomyces cerevisiae*

The amenability of the budding yeast *S. cerevisiae* to genetic and biochemical approaches has made it a valuable eukaryote for studying DNA replication. Numerous origins of DNA synthesis are required to replicate large genomes that consist of multiple chromosomes. Both the DNA sites that serve as replication origins, called autonomously replicating sequences (ARS elements), and a multiple protein complex that assembles at ARS elements have been identified (Bell and Stillman, 1992; Newlon, 1988).

1.2.2.1 ARS Elements

Eukaryotic origins of DNA replication have been best defined in the budding yeast *S. cerevisiae* (Newlon, 1996; Newlon, 1988). A genetic screen of yeast chromosomes for replication origins identified an autonomously replicating sequence, called ARS1, that permits high efficiency transformation and extrachromosomal maintenance of plasmid DNA (Stinchcomb et al., 1979). Two-dimensional gel analysis of ARS1-containing plasmids isolated from S phase cells determined that a replication bubble, indicative of an origin of bidirectional replication, co-localizes with ARS1 (Brewer and Fangman, 1987; Huberman et al., 1987; Newlon et al., 1993). A replication bubble was also detected at the chromosomal position of ARS1 indicating that this DNA

element is a functional origin (Huberman et al., 1988). An additional technique that identifies the transition from discontinuous to continuous DNA synthesis, called replication initiation point mapping, confirmed that DNA replication initiated at a single site within ARS1 (Bielinsky and Gerbi, 1999; Gerbi and Bielinsky, 1997).

Comparison of several identified ARS elements indicated that the essential components of an ARS include an 11 bp consensus sequence (ACS) and less conserved DNA elements collectively called the B domain (Broach et al., 1983; Marahrens and Stillman, 1992). The ACS is the only highly conserved DNA sequence in the ARS element and point mutations in the ACS either abolish or reduce ARS activity (Van Houten and Newlon, 1990). The B domain consists of modular elements 3' to the T-rich strand of the ACS that are interchangeable among ARS elements (Rao et al., 1994; Theis and Newlon, 1994). ARS1 contains three B elements that comprise a DNA unwinding element, a recognition site for the TATA-binding protein and a binding site for the transcription factor ABF1 (ARS binding factor 1) (Lue and Kornberg, 1993; Natale et al., 1992; Umek, 1988). Mutations within the individual B elements reduced ARS activity and combinations of mutations demonstrated that the B elements were collectively essential for ARS function (Marahrens and Stillman, 1994). The spacing and orientation of ACS and B elements is critical and suggests multiple interactions between DNA elements and proteins at the origin are required for origin activation.

The activation of ARS elements as functional replication origins is influenced by chromosomal context and subject to temporal regulation within S phase (Friedman et al., 1996; Newlon, 1996). Adjacent ARS elements appear to interfere with each other, as initiation was observed to occur equally at either one of two closely spaced ARS

elements but was not observed to occur from both of these origins in the same DNA molecule (Brewer and Fangman, 1993). Several observations indicate that the time at which an ARS element initiates replication is influenced by its chromosomal context. A proximity to telomeres has been observed to delay origin firing. For example, the timing of origin activation was delayed until late S phase when a DNA fragment containing an early replicating ARS element was placed near a telomeric repeat and, conversely, occurred early in S phase when a late firing ARS element was placed in a plasmid (Ferguson et al., 1991; Ferguson and Fangman, 1992). However, at least two origins maintain a late firing pattern when located on plasmids, indicating that telomeres are not the only determinant of late activation (Friedman et al., 1996). Replication timing also appears to be influenced by the transcriptional status of the chromosomal locus; ARS elements in transcriptionally active loci tend to replicate earlier than those in loci that are not transcribed (Simon and Cedar, 1996). For example, unexpressed HML and HMR loci are located in late-replicating sub-telomeric regions, however these elements replicate early upon translocation to the transcriptionally active MAT locus (Reynolds et al., 1989). Colocalization of early replication sites and transcriptionally active chromatin may be a result of structural organization within the nucleus or alternatively, transcription factors may facilitate the assembly or stability of replication complexes (Heintz, 1996). Thus, the activity of ARSs appears to be regulated by a combination of *cis*-acting elements that mediate regional effects and *trans*-acting factors that function directly at the origin.

1.2.2.2 The Origin Recognition Complex of *S. cerevisiae*

A complex of six proteins, called the origin recognition complex (ORC), protects

the ARS consensus sequence and part of the B domain from nuclease digestion (Bell and Stillman, 1992). ORC binds ARS elements in an ATP-dependent manner but does not melt the origin DNA or possess helicase activity (Bell and Stillman, 1992). Mutations in the ARS consensus sequence that reduce ORC binding *in vitro* also reduce ARS function *in vivo* suggesting that the ORC-ARS interaction is functionally important (Bell and Stillman, 1992; Rowley et al., 1995). Yeast strains containing temperature sensitive mutations in ORC subunits lose chromosomes and arrest at the G1-S transition point at the non-permissive temperature (Bell et al., 1993; Foss et al., 1993). ORC binds to the origin throughout the cell cycle demonstrating that, although ORC may be necessary for DNA replication, it is not sufficient for origin activation (Aparicio et al., 1997; Diffley and Cocker, 1992). Monitoring the ARS1 binding site throughout the cell cycle revealed an extension of the ORC footprint upon exit from mitosis that remained until the onset of S phase (Diffley et al., 1994). The presence of proteins, in addition to ORC, could account for the extended footprint and suggests that a pre-replication complex is assembled with ORC prior to the initiation of DNA replication (Diffley et al., 1994).

1.2.2.3 Replication Initiation Proteins

Genetic approaches in yeast were initially used to identify proteins involved in the replication of DNA. *S. cerevisiae* mutants were generated that do not progress through a complete cell cycle or are defective in minichromosome maintenance, and were designated cell division cycle (CDC) or minichromsome maintenance (MCM) mutants, respectively (Hartwell, 1976; Maine et al., 1984). A subset of CDC genes encode replication proteins and of these, homologues have also been identified and characterized in the fission yeast *Schizosaccharomyces pombe* (Dutta and Bell, 1997; Newlon, 1996;

Nurse et al., 1976). A screen for yeast proteins that interact with ORC and are involved in the initiation of DNA synthesis identified cdc6 (Liang et al., 1995). Cdc6 mutants failed to maintain ARS-containing plasmids, however, the defect was rescued by providing multiple ARS elements on the plasmid suggesting a role for cdc6 in the initiation of DNA replication (Hogan and Koshland, 1992; Liang et al., 1995). Expression of cdc6p during G1 is required for the formation of a pre-replicative complex at ARS elements and, following entry into S phase, the protein is targeted for proteolysis by the ubiquitin-conjugation pathway (Piatti et al., 1996; Sanchez et al., 1999). In the absence of cdc6, cells fail to replicate DNA and the footprint at ARS elements resembles that of purified ORC (Cocker et al., 1996; Piatti et al., 1995).

The function of cdc6 appears to be in the loading of a complex of MCM (min-chromosome maintenance) proteins onto the ORC-bound ARS element (Donovan et al., 1997; Liang and Stillman, 1997; Tanaka et al., 1997). MCM2 through 6 are related, abundant proteins and homologues have been detected in all eukaryotes from yeast to humans (Tye, 1999). Following entry into S phase, MCM proteins are phosphorylated and are released from the origin (Lei et al., 1997). After leaving the origins, it has been suggested that MCMs may be exported to the cytoplasm, (Liang and Stillman, 1997; Tanaka et al., 1997; Young and Tye, 1997) or they may move along the DNA with components of the replication fork (Aparicio et al., 1997). Each MCM subunit has ATPase motifs also found in enzymes that unwind DNA (Koonin, 1993; Lei et al., 1996). Weak helicase activity has been detected with a purified sub-complex of human MCMs (MCM 4,6, and 7) and has led to considerable interest in this family of proteins (Dutta and Bell, 1997; Ishimi, 1997). At present, it is still unclear whether or not MCM

complexes are responsible for origin melting and/or DNA unwinding at replication forks.

In addition to ORC, cdc6 and MCMs, several other proteins including cdc45, S-phase cyclin-dependent kinases (CDKs) and the cdc7-dbf4 kinase complex are required to render the origin competent to fire. Assembly of pre-replication complexes, including ORC, cdc6 and MCMs, at origins occurs in periods of low CDK activity (during G1) and further assembly is inhibited upon entry into S phase (Detweiler and Li, 1998; Tanaka et al., 1997). The appearance of B-type cyclins and CDK activity at the G1/S transition blocks the formation of new pre-replication complexes, in addition to activating replication at exisiting origin complexes (Schwob et al., 1994). Cyclin B/cdc28 associates with and phosphorylates cdc6 *in vitro* (Elsasser et al., 1996) and, given the inhibitory effect of CDK activity on cdc6 function in yeast (Piatti et al., 1996), CDK may phosphorylate cdc6 *in vivo* and inhibit the re-establishment of pre-replication complexes within the same S phase (Jallepalli and Kelly, 1997).

A second kinase activity that peaks at G1/S and is essential for DNA replication is encoded by cdc7 (Jackson et al., 1993). The regulatory subunit of cdc7, a protein called dbf4, associates with origins suggesting that the kinase is localized to origins to promote initiation of replication (Dowell et al., 1994). Temperature-sensitive mutants of cdc7 fail to activate late origins when shifted to the non-permissive temperature after release from an S phase block (Bousset and Diffley, 1998). Transient expression of cdc7 at the beginning of S phase activates early but not late-firing origins (Donaldson et al., 1998). The extended S phase, consistent with replication from a reduced number of origins, suggests that cdc7-dbf4 functions to directly activate each origin. Genetic and biochemical studies suggested that MCMs are likely candidates for cdc7-mediated

phosphorylation (Hardy et al., 1997; Lei et al., 1997) and that this modification might be required for recruitment of RPA to the origin (Tanaka and Nasmyth, 1998).

Cdc45 is another protein required to initiate DNA replication (Zou et al., 1997). Association of cdc45 with the pre-initiation complex requires CDK activity suggesting that the protein functions just prior to initiation of DNA replication (Owens et al., 1997; Zou and Stillman, 1998). Interaction of cdc45 with MCMs has been detected and, like MCMs, cdc45 has been reported to move with the replication fork (Aparicio et al., 1997).

1.2.3 DNA replication in metazoans

As progress is made in our understanding of metazoan DNA replication, the proteins and mechanisms being investigated are, for the most part, similar to those already described for *S. cerevisiae*. An emerging theme, therefore, is the conserved nature of DNA replication factors among eukaryotes. Despite these similarities with yeast, additional complexities exist in the replication of a large genome within the developmental framework of a multicellular organism. Rapid S phase and nuclear division during early embryogenesis require different mechanisms to those of the normal cell cycle, including the use of additional sites of replication and unsynchronization of origin firing to complete DNA synthesis in a shorter period of time (Carminati and Orr-Weaver, 1996). Replication origins in metazoans are poorly defined and the assembly of ORC, cdc6 and MCMs at these sites remains uncharacterized.

1.2.3.1 Metazoan Origins of Replication

Origins in metazoan cells have been difficult to identify due to the complexity of the genomes and limitations in available assays. However, some similarities with ARS elements and viral origins have emerged from the analysis of multiple replication sites in

the chromosomes of flies, frogs and mammals (reviewed in DePamphilis, 1999). For instance, DNA synthesis does not initiate at random sequences but requires regions that are larger than the well defined viral and yeast origins. The efficiency of origin usage may be dictated by nuclear organization and chromatin structure that is subject to regulation in response to cellular differentiation during development. As a result, many potential origins may not fire in a given cell cycle. The DNA sequences of origins that fire once per S phase in multi-cellular organisms remain poorly defined and this has limited the progress in understanding replication in these systems.

The most studied mammalian origin of replication is that of the hamster dihydrofolate reductase (DHFR) locus. A number of origin mapping strategies have been applied to the single copy DHFR locus of Chinese hamster ovary (CHO) cells and amplified locus of CHOC400 cells (reviewed in DePamphilis, 1999). Multiple initiation events were observed throughout the 55 kb DHFR locus, however, two origins of bidirectional replication were preferentially labelled at the beginning of S phase (Dijkwel and Hamlin, 1995; Dijkwel et al., 1994; Leu and Hamlin, 1989). One origin, *ori*-β, contains A/T-rich and bent DNA sequences that are bound by multiple protein factors, including the transcription factors AP-1 and OCT-1 (Heintz, 1996). Two sites within ori-β are recognized by RIP-60, a protein that mediates the formation of a DNA loop and may contribute to origin melting or destabilization (Mastrangelo et al., 1993). Despite the recent progress, further investigations of DHFR origin sequences remain hindered without a plasmid-based assay and the ability to investigate individual *cis*-acting elements.

Xenopus egg extracts support DNA replication and provide a cell-free system for

the investigation of replication under cell-cycle control (DePamphilis, 1999). DNA templates are completely duplicated following assembly of decondensed chromatin and a nuclear membrane from activated egg extract components (Blow and Laskey, 1986). Initiation events in egg extracts occur randomly along naked DNA but initiate specifically at the DHFR locus within intact late G1-phase CHO nuclei extract (Gilbert et al., 1995; Wu and Gilbert, 1996). This finding suggests that origin selection is dictated by pre-existing nuclear structure and proteins that are activated by factors in the *Xenopus* egg extract. The commitment to origin-specific replication, termed the origin decision point, is sensitive to a protein kinase inhibitor suggesting that phosphorylation of nuclear or egg extract proteins is required to initiate DNA synthesis at origin pre-replication complexes (Wu and Gilbert, 1997).

1.2.3.2 Metazoan Replication Initiation Proteins

Homologues of ORC, cdc6, and MCM proteins have been identified in humans, frogs, flies and rodents (Gavin et al., 1995) and likely have analogous functions as in yeast. The assembly of these initiation factors on DNA has not been investigated since well defined metazoan origins remain elusive. However, a number of co-immunoprecipitation, co-purification and yeast two-hybrid studies have demonstrated interactions between ORC, cdc6 and MCM subunits (reviewed in Dutta and Bell, 1997). The importance of ORC, cdc6 and MCMs in the *Xenopus* egg replication system is evidenced by the fact that immunodepletion of these factors from the egg extracts results in a loss of replication activity that can be restored by addition of the recombinant proteins (Coleman et al., 1996; Madine et al., 1995; Romanowski et al., 1996)

1.2.4 EBV latent replication as a model system

Like SV40, EBV relies almost entirely on the host cell for replication and thus provides a useful system for studying mammalian DNA replication. Unlike SV40, the EBV origin binding protein, EBNA1, lacks origin melting and helicase activities (Frappier, 1991a). Therefore, EBV must use cellular proteins for these activities and investigation of the mechanism of EBNA1 function should identify replication factors in addition to those used by SV40. Regulatory factors might also be identified using EBV as a model system because, unlike the replication of other viral genomes, replication from the EBV latent origin, *oriP*, is regulated to once per cell cycle.

1.3 DNA Segregation in Eukaryotes

Following the completion of S phase, duplicated cellular chromosomes remain associated as sister chromatids and begin to condense prior to the onset of mitosis (reviewed in Nasmyth, 1999). Mitosis consists of several distinct stages culminating in the separation of sister chromatids and their segregation to opposite poles of the dividing cell. Condensed chromatids become associated with microtubule arrays and are positioned at the cellular midpoint between two spindle poles during prophase and metaphase. A series of molecular events triggers the separation of sister chromatids during anaphase and their transport to opposite spindle poles. A new nuclear envelope forms around the daughter chromosomes and cellular division is completed during telophase and cytokinesis respectively. While mitotic chromosomes were first visualized by microscopy over 100 years ago, the factors required for cohesion, condensation and segregation of sister chromatids have only recently been investigated at the molecular level. Genetic and biochemical analysis of yeast mutants, coupled with cell-free studies

using *Xenopus* egg extracts, have identified several proteins involved in the cohesion and condensation of sister chromatids that are required for proper DNA segregation (reviewed in Nasmyth, 1999). The conserved nature of these factors suggests that a similar mechanism of chromosomal segregation may exist in all eukaryotes.

1.3.1 Chromosome cohesion and condensation

Sister chromatids remain tightly associated following DNA replication and become condensed at the onset of mitosis. A family of four structurally related proteins, called structural maintenance of chromosome (SMC) proteins, that mediate chromosome cohesion and condensation have been identified in *S. cerevisiae* (reviewed in Hirano, 1999; Koshland and Strunnikov, 1996). SMC proteins each contain an N-terminal nucleotide binding motif, two central coiled-coil motifs and a conserved Asp-Ala rich region (DA-box) that are essential for their activity (Strunnikov et al., 1995). SMC1 is essential for viability during mitosis, and loss of function causes defects in nuclear division resulting in chromsomal loss (Strunnikov et al., 1993). A specific role for SMC1 in chromosome segregation was implicated when a genetic screen identified SMC1 mutants that demonstrated a premature separation of sister chromatids. SMC1 was identified along with SMC3, SCC1 (sister chromatid cohesion), SCC3 and Trf4p as members of a cohesion complex that binds to chromsomes during S phase (Castano et al., 1996; Guacci et al., 1997; Michaelis et al., 1997). Cohesion between sister chromatids is established following DNA replication but is lost during the metaphase-anaphase transition. The dissociation of SCC1 from chromatids depends on the anaphase promoting complex (APC), a multi-protein complex that mediates the destruction of proteins that act as inhibitors of anaphase (Michaelis et al., 1997). The APC-mediated

degradation of a protein called Pds1, releases a second factor, called Eps1, from its tight association and results in the displacement of SCC1 from chromosomes (Ciosk et al., 1998). Thus, the loss of sister chromatid cohesion at the onset of anaphase is triggered by APC and allows chromosomes to segregate to opposite poles of the dividing cell (Zachariae and Nasmyth, 1999).

The condensation of chromosomes at the onset of mitosis is thought to reduce the incidence of chromosome damage or loss from tangled DNA strands pulled to opposite poles of the dividing nucleus. Protein complexes that mediate chromosome condensation have been isolated from *Xenopus* egg extracts (Hirano and Mitchison, 1994). XCAP-E and XCAP-C are heterodimeric components of a condensin complex with homology to the SMC2 and SMC4 proteins of *S. cerevisiae* (Hirano et al., 1997). The condensins have ATPase activity in cell-free extracts and can introduce superhelical tension into DNA (Kimura and Hirano, 1997). The regulation of condensin activity and characterization of additional proteins associated with condensed chromatin remains to be determined.

1.3.2 Centromeres

The segregation of replicated chromosomes to daughter cells with high fidelity requires a chromosomal region termed the centromere. Centromeric DNA in *S. cerevisiae* is comprised of three sequence elements within a nuclease-resistant chromatin structure (reviewed in Hegemann and Fleig, 1993). Genetic and biochemical interactions have been detected among a number of yeast proteins and centromeric DNA elements that form the kinetochore (Pluta et al., 1995; Meluh and Koshland, 1997). The kinetochore associates with the microtubule apparatus and delays anaphase until all chromosomes are attached to the mitotic spindle (reviewed in Hyman and Sorger, 1995).

Centromeric elements in higher eukaryotes consist of large blocks of repetitive DNA sequences and remain poorly defined. In spite of the size and complexity of the kinetochore complex at the constriction point of sister chromatids, a number of kinetochore proteins have been identified and the centromeric proteins CENP-A and CENP-C possess sequence similarity to yeast kinetochore components (reviewed in Maney et al., 1999). Characterization of these kinetochore components has only recently been undertaken and numerous factors that are important for the regulation of chromosome segregation likely remain to be identified.

1.3.3 Viral genome segregation mechanisms

To be maintained at a constant copy number, the low copy papillomavirus and latent Epstein-Barr virus genomes must be efficiently partitioned to the daughter cells at each cell division. Integration of viral DNA into a host chromosome would fulfill the requirement, however this is rarely observed. Both EBV and papillomavirus genomes are maintained as multicopy plasmids in the nucleus of infected cells, and therefore each virus must have a segregation mechanism. Both viruses appear to have adopted a similar segregation mechanism that involves attachment to the host cell chromosomes.

For the bovine papillomavirus genome, efficient partitioning involves a minichromosome maintenance element (MME) as well as two viral proteins, E1 and E2 (Piirsoo et al., 1996). The MME is located within the upstream regulatory region that contains ten E2 recognition sites (Piirsoo et al., 1996). The BPV genome and MME-containing plasmid DNA are closely associated with mitotic chromatin in cell lines that express E1 and E2 (Ilves et al., 1999; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). E2 is a transcriptional activator and also activates papillomavirus

replication by aiding in the assembly of the E1 origin binding protein on the origin (see section 1.2.1.3). E2 is phosphorylated at multiple serine residues and mutations that prevent this phosphorylation disrupt plasmid maintenance despite normal transcription and replication of the viral genome (Lehman et al., 1997). Colonies of cells expressing E2 phosphorylation mutants display sectoring of viral DNA that indicates an inability to maintain the episome (Lehman and Botchan, 1998). Deletion of E2 binding sites within the MME, or mutations within E2 disrupt the association with mitotic chromosomes and result in loss of plasmid maintenance (Ilves et al., 1999; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). Therefore, the interaction of E2 and the viral episome with mitotic chromosomes appears to be the mechanism for BPV plasmid maintenance (Calos, 1998). The cellular factors that mediate the interaction of MME-bound E2 with the mitotic chromosomes is unknown.

There are many similarities between the segregation of papillomavirus and Epstein-Barr virus genomes in proliferating cells. As discussed above and in section 1.1.5.2, both viral partitioning systems involve *cis*-acting elements of the genome with multiple binding sites for the essential origin binding proteins. Additionally, both the viral genomes and the origin binding proteins associate with cellular mitotic chromosomes. Therefore, EBNA1 and *oriP* plasmids provide another system to study the mechanisms of plasmid partitioning in the absence of a centromere (Calos, 1998).

Recently, the genome of the Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) and the latency associated nuclear antigen (LANA) have been shown to colocalize on host metaphase chromsomes (Ballestas et al., 1999). A *cis*-acting region of the viral genome and the LANA protein are sufficient for the maintenance of plasmid

DNA in dividing lymphocytes (Ballestas et al., 1999). This finding provides a third example of viral persistence mediated through the attachment of viral episomes to a component of the host cell chromosomes.

1.4 Activation of Transcription

The production of messenger RNA (mRNA) requires the formation of multiprotein complexes at specific promoter sites in an analogous fashion to pre-replication complex formation at origins. The assembly and activation of the transcriptional machinery is subject to multiple levels of regulation and recent progress in understanding the molecular mechanisms that underlie these processes may provide insight into the mechanisms of EBNA1-activated transcription.

1.4.1 Promoters and the basal transcription machinery

The production of mRNA by RNA polymerase II requires the formation of a multi-protein complex, termed the preinitiation complex, on the promoter element (reviewed in Zawal and Reinberg, 1995). Most promoters are identified by a TATA motif that is specifically recognized by the TATA binding protein (TBP) of the basal transcription factor complex TFIID. The general transcription factors, TFIIA and TFIIB, bind directly to TBP and increase the stability of TBP on the TATA box. The TBP complex, which includes several TBP-associated proteins called TAFs, is recognized by the RNA polymerase II holoenzyme, thereby establishing a preinitiation complex at the promoter. Although the preinitiation complex is competent for the initiation of transcription, several rate-limiting steps in the assembly of this complex limit the level of basal transcription in cells.

1.4.2 Activators of transcription

Several mechanisms for transcriptional enhancement have been proposed and different strategies may synergize to activate transcription. A transcriptional activator binds enhancer elements and stimulates one or more rate-limiting steps in the assembly of an active preinitiation complex at the promoter. Although different transcriptional activators are required for distinct classes of promoters and cellular conditions, the underlying mechanisms of activation are similar. Promoter sequences in the nucleus are thought to be poorly accessible because genes are packaged into chromatin. Also, the levels of RNA polymerase II holoenzyme are limiting and thus, competition for promoter assembly requires active mechanisms to recruit and assemble the preinitiation complex onto an accessible promoter site (Zawal and Reinberg, 1995).

Several classes of transcriptional activators have been described (Mitchell and Tjian, 1989). First, activators that bind directly to enhancer elements, such as GAL4, are composed of a DNA binding and an activation region found within separate domains of the protein. The DNA binding domain recognizes sites upstream of the promoter and imparts gene specificity, while the activation domain mediates protein-protein interactions with components of the transcription machinery and facilitates the assembly or stability of the protein complex on the DNA. Activation domains of this class of activator proteins have been categorized on the basis of their amino acid composition into acidic, proline-rich and glutamine-rich categories (Mitchell and Tjian, 1989). Transactivation domains that are rich in acidic residues have been identified in a number of activator proteins including VP16, P53 and GAL4. Acidic transactivator proteins promote the initiation and elongation steps of transcription through interactions with

TFIIB, TFIID and TFIIH (Blau et al., 1996; Lin et al., 1991; Stringer et al., 1990; Xiao et al., 1994). The activation domain of CTF-1 is proline-rich and facilitates the TFIIB assembly step during transcriptional activation (Kim and Roeder, 1994). SP1 is rich in glutamine residues and has been demonstrated to mediate interactions with the TATA-binding protein (Emili et al., 1994). In summary, while transactivation domains may be diverse in composition, the ability to mediate protein-protein interactions and facilitate the assembly of pre-initiation complexes appears to be a common mechanism of action.

The second class of activator proteins, called coactivators, do not bind DNA directly but must associate with specific DNA-bound proteins to mediate their function. The molecular targets of coactivators vary widely but include (i) proteins that facilitate promoter assembly, such as the general transcription factors; (ii) proteins of the RNA polymerase II holoenzyme that facilitate transcription initiation and promoter clearance; and (iii) elongation factors that facilitate read-through of paused or arrested polymerase complexes on the template (reviewed in Greenblatt, 1997).

A third class of activator proteins recruit or activate chromatin remodelling factors that clear or modify nucleosomes (reviewed in Kadonaga, 1998). These multisubunit complexes contain subunits that bind enhancer elements and others that catalyze the acetylation or deacetylation of histones. Neutralization of the charge in the basic regions of histones has been proposed to reduce histone-DNA affinity and lead to increased access of transcription factors to chromatin. In summary, the collective rôle of all transcriptional activators is to increase the efficiency of assembly and function of the transcriptional machinery.

1.4.3 Transactivation by EBNA1

EBNA1 falls into the DNA-binding category of transcriptional activator proteins. The mechanism by which FR-bound EBNA1 activates transcription is unknown. Residues of EBNA1 in addition to the DNA binding and dimerization domain are required for the activation of transcription (Middleton and Sugden, 1994). Interactions with known components of the basal transcription machinery have not been detected and the transactivation domain of EBNA1 has not been well defined.

1.5 Rationale of this Thesis

The purpose of the research conducted in this thesis was to identify regions of the EBNA1 protein that are required for the replication and maintenance of *oriP* plasmid DNA and the activation of transcription. The approach undertaken involved the generation of EBNA1 mutants that targetted four regions of the protein, namely the acidic tail, the proline loop, the WF motif and the looping domain. EBNA1 mutant proteins were expressed and examined for their functional activity in human cells. It was hoped that this information would lead to an increased understanding of the mechanisms by which EBNA1 governs replication, segregation and transcriptional activation and ultimately would facilitate the identification of human cellular proteins that are involved in the EBNA1-mediated functions at *oriP*.

2.0 MATERIALS AND METHODS

2.1 Construction of EBNA1 Mutants

EBNA1 mutants, in which amino acids 545-549 (PL1) and 541-553 (PL2) of the proline loop were substituted with Gly-Ala-Ser-Gly, were constructed using two rounds of polymerase chain reaction (PCR) amplification. All EBNA1 constructs described in this thesis were generated from an EBNA1 template (plasmid p205) that does not encode amino acids 101-323 of the non-essential glycine-alanine repeat (Yates et al., 1985). The first round of PCR from p205 amplified DNA fragments of the EBNA1 gene that extend from the N-terminus (N-terminal primer) to the deletion site (PL1-C or PL2-C) and from the deletion site (PL1-N or PL2-N) to the C-terminus (C-terminal primer) at amino acid 641. Primers adjacent to the deletion site on each fragment contained Nhe I restriction sites at their 5' ends. The primers used were as follows:

N-terminal 5' CGGAATTCATATGTCTGACGAGGGGCCA 3'

PL1-C 5' TCCGCTAGCCACCGGCCATTCCAAAGGGGAGACG 3'

PL1-N 5' GGTGCTAGCGGACAACCTGGCCCGCTAAGGGAG 3'

PL2-C 5' TCCGCTAGCACCGGGGAGACGACTCAATGGTGT 3'

PL2-N 5' GGTGCTAGCGGACTAAGGGAGTCCATTGTCTGT 3'

C-terminal 5' CCTCCAGGATCCTCACTCCTGCCCTTCCTCACC 3'

The N- and C-terminal fragments of EBNA1 were purified from agarose gels using a Qiaex kit (Qiagen) according to the manufacturer's instructions. Each DNA fragment was resuspended in TE (10 mM Tris-HCl pH 8, 1 mM EDTA), digested with 20 units of

Nhe I (New England Biolabs; NEB) and ligated together in 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 μg/ml BSA using 800 units of T4 DNA ligase (NEB) overnight at 15 °C. Ligation products of the appropriate length were purified from an agarose gel and subjected to a second round of PCR using the primers that anneal to the N- and C-termini of $EBNA_{1\text{-}641}$. In addition to EBNA1 sequences, the N-terminal primer contained an Nde I site and the C-terminal primer contained a Bam HI site at the 5' end. PCR products were gel purified then digested with 20 units of Nde I and Bam HI. The DNA fragments were purified from the reaction using a Qiaex kit and 80 fmol was ligated with 20 fmol of a pET15b vector (Novagen) that had been digested with Nde I and Bam HI. The ligation products were used to transform 100 µl of competant DH5α *E. coli* cells. Transformants were screened for positive clones by restriction digest analysis of small scale DNA preparations (Sambrook et al., 1989). Each clone was confirmed by sequencing performed by the central facility of the Institute for Molecular Biology and Biotechnology (MOBIX, McMaster University).

The DNA binding and dimerization domain and the acidic tail (amino acids 452-641) of $EBNA_{1\text{-}641PL1}$ and $EBNA_{1\text{-}641PL2}$ were amplified by PCR to generate $EBNA_{452\text{-}641PL1}$ and $EBNA_{452\text{-}641PL2}$. The N-terminal primer contained a Nde I restriction site and the C-terminal primer contained a Bam HI site allowing the PCR products to be digested and ligated between the Nde I and Bam HI sites of pET15b. The primers used to amplify amino acids 452-641 were the C-terminal primer listed above and the 452-N primer as follows:

452 -N 5' CGTCGACATATGGGTCAGGGTGATGGAGGC 3'

The resulting constructs express the DNA binding and dimerization domain of EBNA1 in bacteria as an N-terminal fusion to a hexa-histidine tag and a thrombin protease site.

2.1.1 Generation of mammalian expression constructs

The plasmids used for the transfection of human cells were derived from pcDNA3 (Invitrogen). DNA fragments encoding the full length protein ($EBNA_{1\text{-}641}$), or EBNA1 mutants lacking amino acids 608-641 ($EBNA_{1\text{-}607}$), or 1-451 ($EBNA_{452\text{-}641}$) were generated by PCR amplification from p205 using an N-terminal primer containing an Nde I site and a C-terminal primer encoding a Bam HI site. DNA fragments encoding EBNA1 mutants lacking amino acids 1-376 ($EBNA_{377\text{-}641}$) or both 1-376 and 608-641 ($EBNA_{377\text{-}607}$) were amplified using an N-terminal primer containing an Nco I site and a C-terminal primer containing a Bam HI site. The primers that hybridize at amino acids 377 or 607 are shown below; all other primers were listed above.

377-N 5' CTGGATCCATGGGAGAAAAGAGGCCCAGG 3'

607-C 5' CGTGCAGGATCCTCAAGGCAAATCTACTCCATC 3'

After PCR, the DNA fragments were digested with Nde I or Nco I and the 5' overhangs were filled in using the Klenow fragment of DNA polymerase I (NEB) in a reaction containing 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 7.5 mM DTT and 33 μM of each dNTP for 15 minutes at 25 °C. The DNA fragments were then digested with Bam HI. DNA fragments encoding the EBNA1 proline loop mutants, $EBNA_{1\text{-}641PL1}$ and $EBNA_{1\text{-}641PL2}$, were generated by digesting pET15b $EBNA_{1\text{-}641PL1}$ and pET15b $EBNA_{1\text{-}641PL2}$ with Nde I, filling in the 5' overhang with Klenow (as described above), then digesting with Bam HI. DNA fragments encoding EBNA1 mutants lacking amino acids 356-362

($EBNA_{\Delta 356-362}$) and 367-376 ($EBNA_{\Delta 367-376}$) were generated by digesting pVLEΔ356-362 (Laine and Frappier, 1995) and pVLEΔ367-376 (Shire et al., 1999) with Eco RI, filling in the 5' overhang with Klenow, then digesting with Bam HI.

The mammalian expression plasmid pcDNA3 was digested with Hind III, filled in with the Klenow fragment of DNA polymerase I and digested with Bam HI. DNA fragments encoding EBNA1 or EBNA1 mutants described above were ligated into pcDNA3 and the recombinant plasmids were sequenced. The resulting plasmids express EBNA1 or EBNA1 mutant proteins from the CMV promoter when transfected into mammalian cells. The pcDNA3-EBNA1 plasmids were further modified by the addition of EBV *oriP* DNA sequences. A DNA fragment encoding *oriP* was excised from pGEMoriP (Frappier and O'Donnell, 1991) by digestion with Bam HI and Rsa I and inserted between the Bgl II and Nru I sites of pcDNA3 to generate pc3oriP. This procedure was repeated for each plasmid encoding EBNA1 or an EBNA1 mutant. The plasmids encoding $EBNA_{\Delta 325-376}$ and $EBNA_{\Delta 41-376}$ (called pc3oriP-$EBNA_{\Delta 325-376}$ and pc3oriP-$EBNA_{\Delta 41-376}$) were generated by Tina Avolio-Hunter as described in Shire et al (1999). $EBNA_{1-641WF}$ contains a double point mutation of Phe464 and Trp465 in which both amino acids were substituted with alanines. The plasmid encoding $EBNA_{1-641WF}$ (called pc3oriP-$EBNA_{1-641WF}$) was generated by Kathy Shire. $EBNA_{1-641WF}$ was constructed using two rounds of PCR amplification. In the first PCR round, two DNA fragments of the EBNA1 gene from p205 were generated, extending from amino acids 1 to 463 and from amino acids 464 to 641. The internal primers used were as follows, where the WF-N primer encoded the double point mutation:

WF-C 5' CCCTCCTTTTTTGCGCCT 3'

WF-N 5' GCGGCGGGAAAGCATCGTGGTCAA 3'

The N- and C-terminal fragments of EBNA1 were purified from an agarose gel, ligated together and subjected to a second round of PCR using the EBNA1 N-terminal and C-terminal primers. The PCR products were gel purified, digested with Bam HI (at the C-terminal end), and ligated into pc3oriP as described for the other EBNA1 constructs.

2.2 Expression of EBNA1 Proline Loop Mutants in *E. coli*

pET15b constructs encoding $EBNA_{452\text{-}641PL1}$ and $EBNA_{452\text{-}641PL2}$ were used to transform competent BL21(DE3) *E. coli* (Studier et al., 1990). The cells were plated on LB-agar plates (10 g/L bactotryptone, 5g/L yeast extract, 10 g/L NaCl, 15 g/L agar) containing 100 μg/ml ampicillin and incubated overnight at 37 °C. A single colony was used to inoculate a 50 ml liquid LB culture containing 100 μg/ml ampicillin. The culture was grown in a orbital shaker at 37 °C to an optical density (OD) at 600 nm of 0.60 and used to innoculate a 2 L culture of LB containing 100 μg/ml ampicillin that was similarly grown to an OD of 0.60. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 1 mM. Following a 3 hour incubation at 37 °C, the bacterial cells were harvested by centrifugation at 5000 rpm in a GSA rotor (Sorvall) for 15 minutes at 4 °C, rinsed in 10 mls of 20 mM Tris-HCl pH 7.5, 10 % sucrose, then frozen at -70 °C.

2.2.1 Purification of EBNA1 proline loop mutants

Frozen bacterial cells were thawed on ice in 20 ml of lysis buffer (20 mM Tris-HCl pH 8, 500 mM NaCl, 10 % glycerol, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA). The cells were lysed by three 30 second rounds of sonication (Vibracell) on ice and the lysate was clarified by centrifugation at 25 000 rpm in a SW28 rotor (Beckman) for 30 min. The soluble fraction was passed through a 25 ml DE52 (Whatman) column equilibrated with 50 mM HEPES (N-2-hydroxyethylpiperazine- N'-2-ethanesulfonic acid) pH 7.5, 350 mM NaCl, 1 mM DTT and 10 % glycerol. The DE52 flow through was diluted to a final NaCl concentration of 200 mM with buffer A (50 mM HEPES pH 7.5, 1 mM DTT, 1mM PMSF, 1 mM benzamadine, 10 % glycerol) and applied to a 10 ml heparin-agarose column (Bio-Rad) equilibrated with buffer A plus 200 mM NaCl. The column was washed with four column volumes of buffer A at 1 ml/min and the protein was eluted with a 75 ml salt gradient from 200 mM to 1 M NaCl in buffer A. The fractions containing EBNA1 protein, as determined by polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie staining, were pooled and dialyzed overnight in buffer B (50 mM HEPES pH 7.5, 750 mM NaCl, 10 % glycerol). The EBNA1 protein was then loaded onto a 5 ml HPLC metal-chelating column (PerSeptive Biosystems) that had been charged with nickel and equilibrated in buffer B plus 5 mM imidazole. The column was washed with five column volumes of buffer B plus 5 mM imidazole, then with five column volumes of buffer B containing 50 mM imidazole. EBNA1 proteins were eluted at 1 ml/min with buffer B containing 300 mM imidazole and DTT was immediately added to the eluates to a final concentration of 10 mM. The fractions containing EBNA1 were pooled and dialyzed in

buffer B plus 1 mM DTT. The histidine tag was then removed from the EBNA1 protein by incubation with 1 μg of thrombin per mg of protein for 2 hours at 4 °C in the presence of 2.5 mM $CaCl_2$. The removal of the histidine tag was confirmed by the increased mobility of the protein on SDS-PAGE. The digested protein was diluted with buffer A to a final NaCl concentration of 200 mM and loaded onto a 1 ml HPLC Mono S column (Perseptive Biosystems) equilibrated with buffer A plus 200 mM NaCl. The EBNA1 protein was eluted at 0.5 ml/min with buffer A containing 1 M NaCl and was determined to be approximately 90 % pure when examined by SDS PAGE and Coomassie staining. EBNA1-containing fractions were aliquoted and stored at -70 °C.

2.3 DNA Binding Assays

2.3.1 Preparation of oligonucleotides and DNA fragments

Site 1 and site 2 of the DS element were generated from complementary oligonucleotides synthesized by MOBIX (McMaster University). They are as follows:

Site 1 oligomers 5'CGGGAAGCATATGCTACCCG 3'

5' CGGGTAGCATATGCTTCCCG3'

Site 2 oligomers 5' GGATAGTATATACTACTACCC 3'

5' GGGTAGTAGTATATACTATCC 3'

Each oligonucleotide pellet was dissolved in TE to a concentration of 50 μM. For end-labelling reactions, 5 pmol of one oligo from each pair was labelled by a 30 minute incubation at 37 °C with 10 units of polynucleotide kinase (NEB) and 30 μCi of γ^{32}P-ATP in a 20 μl reaction containing 70 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT.

The reaction was terminated by the addition of EDTA to a final concentration of 25 mM. For quantification purposes, a 0.25 μl aliquot of the reaction was spotted on DE-81 paper and the remainder of the DNA was purified from unincorporated nucleotides by passage through a G-25 Sephadex spin-column (Bohringer-Mannheim). A 0.25 μl aliquot of the purified DNA was spotted in duplicate on DE-81 paper. One of the duplicate DE-81 papers, along with the sample spotted before the spin column, was washed three times for 5 minutes each in 300 mM ammonium formate, 10 mM sodium pyrophosphate, rinsed in H_2O, then 100 % ethanol and finally dried. This procedure removes unincorporated ^{32}P-ATP from the papers leaving the oligonucleotides bound. The amount of radioactivity bound to the washed and unwashed papers was quantified by liquid scintillation counting (Beckman) and the specific activity of the labelled oligonucleotide was determined from these numbers. To generate the double-stranded site 1 or site 2, the radiolabelled oligonucleotide (400 fmol) and the unlabelled complementary oligonucleotide (800 fmol) were incubated together at 94 °C for 5 minutes, then cooled to room temperature at a rate of 1 °C per minute in a PCR machine (MJ Research).

DNA fragments containing the FR, DS or both sites 1 and 2 of the DS were generated from plasmids, purified and quantitated by absorbance at 260 nm. A 999 bp DNA fragment containing the FR element was excised from pGEMoriP by digestion with Eco RI and Mlu I. A 178 bp DNA fragment containing the DS element was PCR amplified from pGEMdyad using the following primers:

Dyad up 5' GCTCTAGAGCAGAAGGTTCGTCCAACCAA 3'

Dyad down 5' CCCAAGCTTGGGTATAGTGTCACCTAAATCG 3'

A 180 bp DNA fragment containing sites 1 and 2 was generated from pGEMsite1/2 (Summers et al., 1996) by digestion with Xba I. Each DNA fragment was end-labelled by filling 3' recessed ends with α-^{32}P-dCTP. Five pmol of the DNA fragment was incubated for 30 minutes at 37 °C with 5 units of the Klenow fragment of DNA polymerase I and 30 μCi of α^{32}P-dCTP in a 20 μl reaction containing 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 7.5 mM DTT, 33 μM dATP, 33 μM dGTP, 33 μM dTTP. The labelled DNA was purified from unincorporated nucleotides by passage through a G-25 Sephadex column and quantified as described above.

2.3.2 Electrophoretic mobility shift assays (EMSAs)

Electrophoretic mobility shift assays (EMSA) were performed using $EBNA_{452-641}$ that was purified by Jennifer Cruickshank as described in Barwell et al (1995), $EBNA_{452-641WF}$ that was purified by Angela Flemming as described in Summers et al (1997), and $EBNA_{452-641PL1}$ and $EBNA_{452-641PL2}$ that were purified as described in section 2.2.1. Protein-DNA binding reactions were performed using 10 fmol radiolabelled target DNA (site 1, site 2, site 1/site 2, DS or FR) in 10 mM HEPES pH 7.5, 5 mM $MgCl_2$, 300 mM NaCl, and 1 μg sheared herring sperm DNA. Protein concentrations were determined by absorbance at 280 nm and proteins were titrated into a final reaction volume of 20 μl. Following a 20 minute incubation at room temperature, 4 μl of 30 % glycerol was added and the sample was loaded onto a native 5 % or 12 % polyacrylamide gel and run in 0.5 x TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA pH 8). After electrophoresis, gels were dried onto Whatman paper and labelled DNA was visualized by autoradiography on Kodak XAR-5 film and quantified by phosphorimager analysis using

ImageQuant software (Molecular Dynamics). The dissociation coefficients for $EBNA_{452-641}$, $EBNA_{452-641PL1}$, $EBNA_{452-641PL2}$ and $EBNA_{452-641WF}$ bound to site 1, site 2 and site 1/site 2 were determined as the amount of EBNA1 protein required to bind 50 % of the DNA in the reaction, using the equation:

$$Kd = \frac{[EBNA_{free}][DNA_{free}]}{[DNA_{bound}]}$$

According to this equation, when 50 % of the DNA is bound, $Kd = [EBNA_{free}]$. The moles of $EBNA_{free}$ was calculated by subtracting the moles of $EBNA_{bound}$ (equal to moles of DNA_{bound} for one EBNA1 binding site and 2 x moles DNA_{bound} for two sites) from the total amount of EBNA1 added to the reaction. The amount of EBNA1 protein required to shift 50 % of the FR and DS elements was determined by phosphorimager analysis but an actual dissociation coefficient was not calculated since this would require assumptions about the actual number of sites bound.

2.4 Circular Dichroism

Circular dichroism (CD) spectroscopy was used to compare the secondary structure of $EBNA_{452-641}$, $EBNA_{452-641PL1}$, $EBNA_{452-641PL2}$, and $EBNA_{452-641WF}$. A 10 μM solution of each protein was brought to a final volume of 200 μl with PBS and scanned in a 0.1 cm cuvette using an Aviv 62A DS circular dichroism spectrometer. Samples were scanned in 1 nm steps from 300 to 200 nm at 25 °C with a 1 second averaging time. The average elipticity values of 5 scans conducted on each protein was subtracted from that of a buffer only scan and plotted.

2.4.1 Protein stability studies

The stability of $EBNA_{452-641}$, $EBNA_{452-641PL1}$, $EBNA_{452-641PL2}$, and $EBNA_{452-641WF}$ was examined using the Aviv 62A DS circular dichroism spectrometer. A 25 μM solution of each protein was brought to a 160 μl volume in 300 mM NaCl, 10 mM HEPES, pH 7.5, and 5 mM $MgCl_2$ for each scan. Protein unfolding was monitored upon the addition of unfolding buffer (7.2 M GuHCl, 300 mM NaCl, 10 mM HEPES pH 7.5, 5 mM $MgCl_2$) to a final GuHCl concentration of 5.0 to 6.6 M and to a final protein concentration of 2 μM in a 2 ml volume. Protein unfolding was monitored by scanning the sample at 222 nm for 3600 seconds at 25 °C. The raw data was fit to the equation, y=1-exp(-kt) using Excel (Microsoft Corp.), where t is the time in seconds, y is the fraction of the protein folded and k is the unfolding rate constant.

2.5 Functional Assays

2.5.1 Cell culture

C33A cells, a human HPV negative cervical carcinoma (obtained from Dr. P. Whyte) were maintained in Dulbecco modified Eagle's medium (DME) containing 10 % fetal bovine serum, 0.03 % glutamine supplemented with penicillin and streptomycin at 37 °C in a humidified 5 % CO_2 atmosphere.

2.5.2 Cesium chloride purification of plasmid DNA

The plasmid DNA that was used to transfect C33A cells was purified from cesium chloride gradients. A DH5α strain of *E. coli* containing the plasmid of interest was grown in 500 mls of medium (LB and 100 μg/ml ampicillin) overnight at 37 °C. The bacteria were pelleted by centrifugation and resuspended in 10 mls of P1 buffer (50 mM Tris-HCl

pH 8, 10 mM EDTA). Following the addition of 10 mls of lysis buffer (0.2 M NaOH, 1 % SDS) and a 5 minute incubation at room temperature, 10 mls of P3 (2.55 M KOAc pH 4.8) was added with vigorous mixing. The cellular debris was removed by centrifugation for 30 minutes at 14 000 rpm in a SS34 rotor (Sorvall) and the supernatant was transferred to a 50 ml conical tube. Nucleic acids were precipitated from the supernatent by the addition of 18.5 mls of isopropanol and pelleted by centrifugation at 3000 rpm in a GS-6KR centrifuge (Beckman) for 30 minutes. The DNA pellet, 10 g of CsCl and 2 mg of ethidium bromide were dissolved in 9.5 mls of TE and transferred to a 13 ml polyallomer seal cap tube (Beckman). Following centrifugation at 55 000 rpm for 16 hours in a VTi 65.1 rotor (Beckman), the band containing supercoiled plasmid DNA was removed by a syringe and 18.5 gauge needle. The DNA solution was transferred to a 15 ml conical tube and the ethidium bromide was removed by several washes with CsCl-saturated isopropanol. The DNA was diluted with two volumes of ddH_2O and precipitated by the addition of six volumes of absolute ethanol. The pelleted DNA was resuspended in TE and quantified by absorbance at 260 nm. The purity of the supercoiled DNA was confirmed by agarose gel electrophoresis.

2.5.3 Transcription enhancement assays

C33A cells were transfected by the calcium phosphate coprecipitation method (Graham and van der Eb, 1973). Cells were plated in 60 mm dishes at a density of 1×10^6 cells/dish and grown for 24 hours prior to transfection with cesium chloride purified plasmid DNA. Five micrograms of pcDNA3 plasmids encoding EBNA1 or EBNA1 mutants were combined with 2 μg of pFR-tkCAT (obtained from Dr. B. Sugden, University of Wisconson at Madison) and 2.5 μg of herring sperm DNA in 0.25 ml of

0.25 M $CaCl_2$. A β-galactosidase reporter plasmid (pCH110, Pharmacia) was included as an internal control for transfection efficiency (see section 2.5.3.1). The DNA/$CaCl_2$ solution was added dropwise to 0.25 ml of 2 x HBS pH 6.95 (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4) with vortexing. After 30 minutes at room temperature, the solution was added dropwise to cells in 4 ml of medium and the cells were incubated with the precipitate for 12-16 hours at 37 °C. The cells were then washed in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4), given fresh medium and incubated for 24 hours at 37 °C . Half of the cells harvested were frozen at -70 °C for analysis of β-galactosidase activity and for Western blots of EBNA1, while the rest of the cells were lysed by three rounds of freezing and thawing. Lysates were clarified by centrifugation at 14 000 rpm in a microfuge and the supernatant was used in CAT assays (Ausubel et al., 1991). The protein concentration of the supernatant was determined by the Bio-Rad protein assay, and 50 μg of protein was used in a 150 μl reaction containing 0.25 M Tris-HCl pH 7.5, 0.25 mM acetyl CoA and 3 pmol of C^{14} chloramphenicol (NEN). The reactions were incubated at 37 °C, and at various time points, 50 μl aliquots were removed and vortexed with 300 μl of ethyl acetate. The C^{14} chloramphenicol extracted into ethyl acetate was dried overnight in a fumehood, resuspended in 20 μl of ethyl acetate and spotted onto a cellulose thin layer chromatography plate (Whatman). The plates were developed in a chloroform/methanol (95:5 % vol) mixture and air dried. The CAT assay products were visualized by phosphorimager analysis of the dried plates and quantified using ImageQuant software (Molecular Dynamics).

2.5.3.1 β-Galactosidase Assays

C33A cell extracts were prepared for Western blot analysis (described in section 2.6.1) and assayed for β-galactosidase activity. A 20 μl aliquot of cell extract was incubated with 100 mM sodium phosphate pH 7.5, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β -mercaptoethanol and 80 nM o-nitrophenyl-β-D-galactopyranoside (ONPG) for 2 hours at 37 °C. Following the addition of 200 μl of 1 M sodium carbonate and 50 μl of isopropanol, the absorbance of the solution at 420 nm and 550 nm was measured in a spectrophotometer (Pharmacia). The β-galactosidase activity was determined and normalized according to the protein concentration in each sample, as described in Sambrook et al (Sambrook et al., 1989). Transfections with a greater than two-fold difference in β-galactosidase activity among samples were excluded from functional analysis.

2.5.4 Transient replication assays

C33A cells were plated in 10 cm dishes at 2.5 x 10^6 cells/dish and grown 24 hours prior to transfection. Ten micrograms of pc3oriP encoding EBNA1 or EBNA1 mutants was combined with 10 μg of herring sperm DNA. The transfection was performed as described for transcription enhancement assays (section 2.5.3) except that the volume of the $CaCl_2$/DNA and HBS solutions was doubled to 0.5 ml. Following the removal of DNA precipitate, cells were washed in PBS, split into 140 mm dishes and grown for 72 hours. Approximately 5 x 10^6 cells from each plate were collected and lysed in 700 μl of 10 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0, 0.6 % SDS. The remaining cells were pelleted and frozen at -70 °C for analysis of EBNA1 protein expression by Western blot

(section 2.6.1). High molecular weight DNA was precipitated by the addition of NaCl to 0.83 M and incubation overnight at 4 °C (Hirt, 1967). Low molecular weight DNA in the supernatent was extracted with phenol:chloroform (1:1), ethanol precipitated and resuspended in TE pH 8. Half of each sample was linearized with Xho I and 9/10 of the linearized samples were further digested with Dpn I (4 units) for 2 hours at 37°C. DNA fragments from the restriction digests were separated on a 0.9 % agarose gel, transferred to Gene Screen Plus (NEN) and probed with pc3oriP $EBNA_{1\text{-}641}$ that had been labelled with α^{32}P-dCTP by random primer extension (section 2.5.4.1). Radiolabelled bands were visualized by autoradiography and quantified by phosphoimager analysis using ImageQuant software (Molecular Dynamics).

2.5.4.1 Random Primer Labelling of DNA

A uniformly labelled probe for Southern blots was prepared by random primer labelling of pc3oriP-$EBNA_{1\text{-}641}$. The plasmid was linearized and 300 ng was denatured with 1 μg of hexanucleotides (Pharmacia) at 94 °C for 5 minutes and then rapidly cooled on ice. The denatured plasmid and primers were incubated for 2 hours at room temperature with 50 μCi of α^{32}P-dCTP and 5 units of the Klenow fragment of DNA polymerase I in a reaction containing 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 7.5 mM DTT, 50 μM dATP, 50 μM dGTP, 50 μM dTTP. The radiolabelled probe was separated from unincorporated nucleotides on a G-25 Sephadex spin column and quantified by liquid scintillation counting.

2.5.4.2 Southern Blots

DNA isolated from C33A cells, that were harvested after transient replication and plasmid maintenance assays, was resuspended in 20 µl of TE and separated on a 0.9 % agarose gel in TAE (90 mM Tris pH 8, acetic acid, EDTA) overnight at 2 V/cm. The gel was incubated in 0.25 M HCl for 10 minutes with gentle agitation, rinsed in dH_2O and incubated in denaturation buffer (0.4 N NaOH, 0.6 M NaCl) for 30 minutes. The agarose gel was placed upon a nylon membrane (Genescreen Plus; NEN Inc.) and a vacuum blotter apparatus (Pharmacia) was used to transfer the DNA from the gel to the membrane according to the manufacturers instructions. Denaturation buffer was continuously added to the gel for 1 hour at 50 mbars of vacuum. Following the denaturation step, 20 x SSC (3 M NaCl, 0.3 M Na_3Citrate pH 7) was continuously added to the gel for 30 minutes after which time the membrane was removed, briefly rinsed in 2 x SSC (0.3 M NaCl, 30 mM Na_3Citrate pH 7) and dried. The membrane was wetted in 2 x SSC and added to 10 ml of prehybridization buffer (50 % formamide, 0.5 % SDS, 0.1 % ficoll, 0.1 % polyvinyl pyrrolidone, 0.1 % BSA, 6 x SSC, 100 µg/ml herring sperm DNA) in a hybridization oven (InterScience) for 2 hours at 42 °C. A random primer labelled DNA probe ($5x10^6$ cpm) was added to the hybridization bottle and incubated overnight at 42 °C. The membrane was washed twice in 2 x SSC, 1 % SDS at room temperature for 10 minutes, twice at 65 °C for 30 minutes and once at room temperature in high stringency wash buffer (0.2 x SSC, 0.1 % SDS) for 30 minutes.

2.5.5 Plasmid maintenance assays

C33A cells were plated in 10 cm dishes at 2.5 x 10^6 cells/dish and grown 24 hours prior to transfection. One microgram of pc3oriP encoding EBNA1 or EBNA1 mutants was combined with 19 μg of herring sperm DNA and the transfection was performed as described in section 2.5.4. Following removal of the DNA precipitate, cells were washed in PBS, split into 140 mm dishes and grown in medium containing 400 µg/ml G418 (Gibco BRL). Following 2 weeks of culture, $5x10^6$ cells from each plate were harvested and lysed for DNA purification (Hirt, 1967) while the remaining cells were pelleted and frozen at -70 °C. Low molecular weight DNA was isolated as described in section 2.5.4, digested with Xho I and Dpn I, separated by agarose gel electrophoresis, Southern blotted and probed with ^{32}P-labelled pc3oriP-EBNA$_{1\text{-}641}$. Radiolabelled DNA bands were visualized by autoradiography and quantified by phosphoimager analysis using ImageQuant software (Molecular Dynamics).

2.6 Quantification of EBNA1 Protein Expressed in Functional Assays

2.6.1 Western blot analysis

C33A cells from transcription enhancement, transient replication and plasmid maintenance assays were harvested for analysis of EBNA1 protein expression following each experiment. The frozen cell pellet was suspended in 100 µl of Western lysis buffer (20 mM Tris-HCl pH 8, 500 mM NaCl, 0.1 % Triton, 0.5 mM EDTA, 1 mM PMSF, 1 mM benzamadine), sonicated for 6 seconds and, following a 30 minute incubation on ice, the cellular debris was pelleted during a 30 minute spin at 14 000 rpm in a microfuge. The supernatent was recovered and the protein concentration was determined by the Bio-

Rad protein assay. Protein samples (30 μg) were run on a 12 % SDS-polyacrylamide gel with prestained low molecular weight SDS-PAGE standards (BioRad) as size markers. The proteins were transferred to a nitrocellulose (NC) filter (MilliPore) or a polyvinylidene fluoride (PVDF) membrane (Gelman Science) using a wet transfer apparatus (BioRad) containing 25 mM Tris, 190 mM glycine and 20 % methanol according to manufacturers instructions. The NC and PVDF blots were blocked in milk buffer (5 % powdered non-fat Carnation milk in PBS) for a minimum of one hour at room temperature. This and all subsequent steps were done with gentle agitation on a platform shaker (Hoefer). The blots were incubated with the primary antibody in milk buffer at room temperature for one hour. For all EBNA1 proteins, the rabbit polyclonal antibody K67 (from Dr. Jaap Middledorp) was used at a dilution of 1:5000. The blots were washed four times in milk buffer and incubated with the secondary antibody at a dilution of 1:5000 in milk buffer; a goat anti-rabbit monoclonal antibody conjugated to peroxidase for NC blots or goat-anti-rabbit monoclonal antibody conjugated to alkaline phosphatase for PVDF blots (both from Kirkegaard and Perry Laboratories). The blots were incubated at room temperature for one hour, then washed four times in PBS for five minutes each time. Equal volumes of two developing buffers for enhanced chemiluminescence (ECL; by NEN Inc.) were mixed together and incubated with the NC blot for 1 minute at room temperature. The NC was exposed to Kodak XAR film for various time intervals until the signal from the protein samples was clearly visible. The reagent for enhanced chemifluorescence (ECF, Amersham Inc.) was incubated with the PVDF blot for 5 minutes according to the manufacturers instructions. A Storm 860

scanner and ImageQuant software (Molecular Dynamics) were used to quantify the relative intensity of fluorescence from each protein signal.

2.6.2 EMSAs with C33A extracts expressing EBNA1

C33A cell extracts that were prepared for Western blot analysis (described above) were used in protein-DNA binding reactions. Increasing amounts of protein extract were incubated in a reaction containing 10 fmol of radiolabelled site 1 as described in section 2.3.2. The amount of radiolabelled DNA bound per μg of cellular protein was calculated and used to compare the relative binding activity of each EBNA1 protein.

3.0 RESULTS

3.1 EBNA1 Mutants

To gain an understanding of the mechanisms by which EBNA1 functions in DNA replication, DNA segregation and transcriptional activation, the functional contributions of four motifs within the protein were examined. Mutations in EBNA1 were designed to specifically target the acidic tail, the looping domain, the WF motif and the proline loop. These regions of the EBNA1 protein were of particular interest for the following reasons. The acidic tail has been suggested to be important for DNA segregation and the activation of transcription, although the experiments that demonstrated these findings were not conclusive (Yates and Camiolo, 1988; Ambinder, 1991). I wanted to conclusively determine what contribution, if any, the acidic tail made to these processes. The looping domain mediates protein-protein interactions between DNA-bound EBNA1 molecules and also interacts with some cellular factors. To understand the possible importance of these protein interactions, it was necessary to test the functional contributions of the looping domain. The WF motif is composed of two aromatic amino acids (Trp464 and Phe465) within the DNA binding and dimerization domain. The WF motif is in an extended chain that sits in the minor groove of the DNA and the peculiar conformation of this motif appears to push apart the DNA strands of the EBNA1 recognition site (Figure 5). The WF side chains appeared to contribute to the widening of the minor groove observed at this point and thus were proposed to be important for the DNA melting step of DNA replication (Bochkarev et al., 1996). The proline loop is within the core DNA binding and dimerization domain. Its extended conformation on the

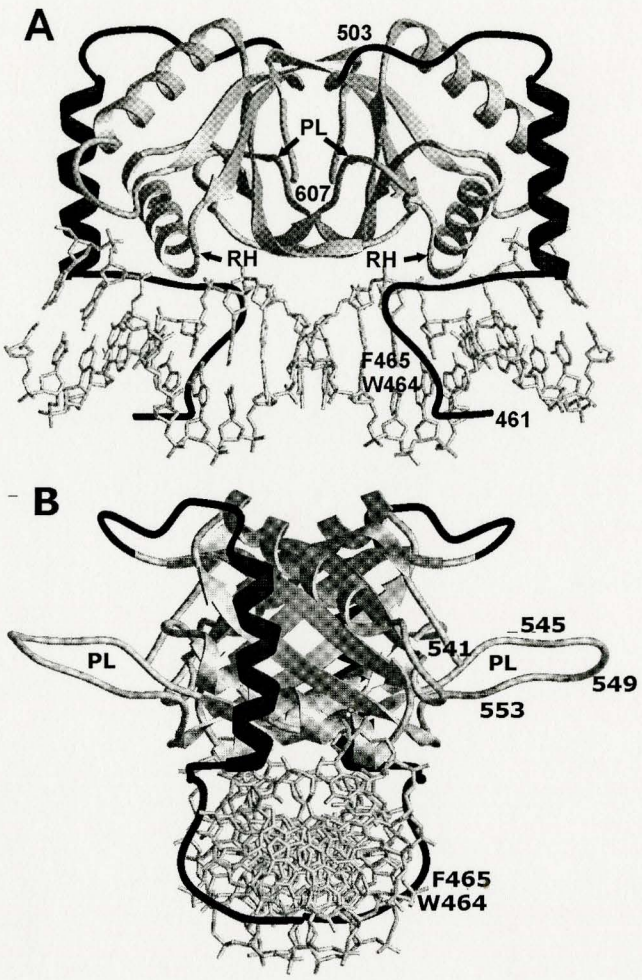


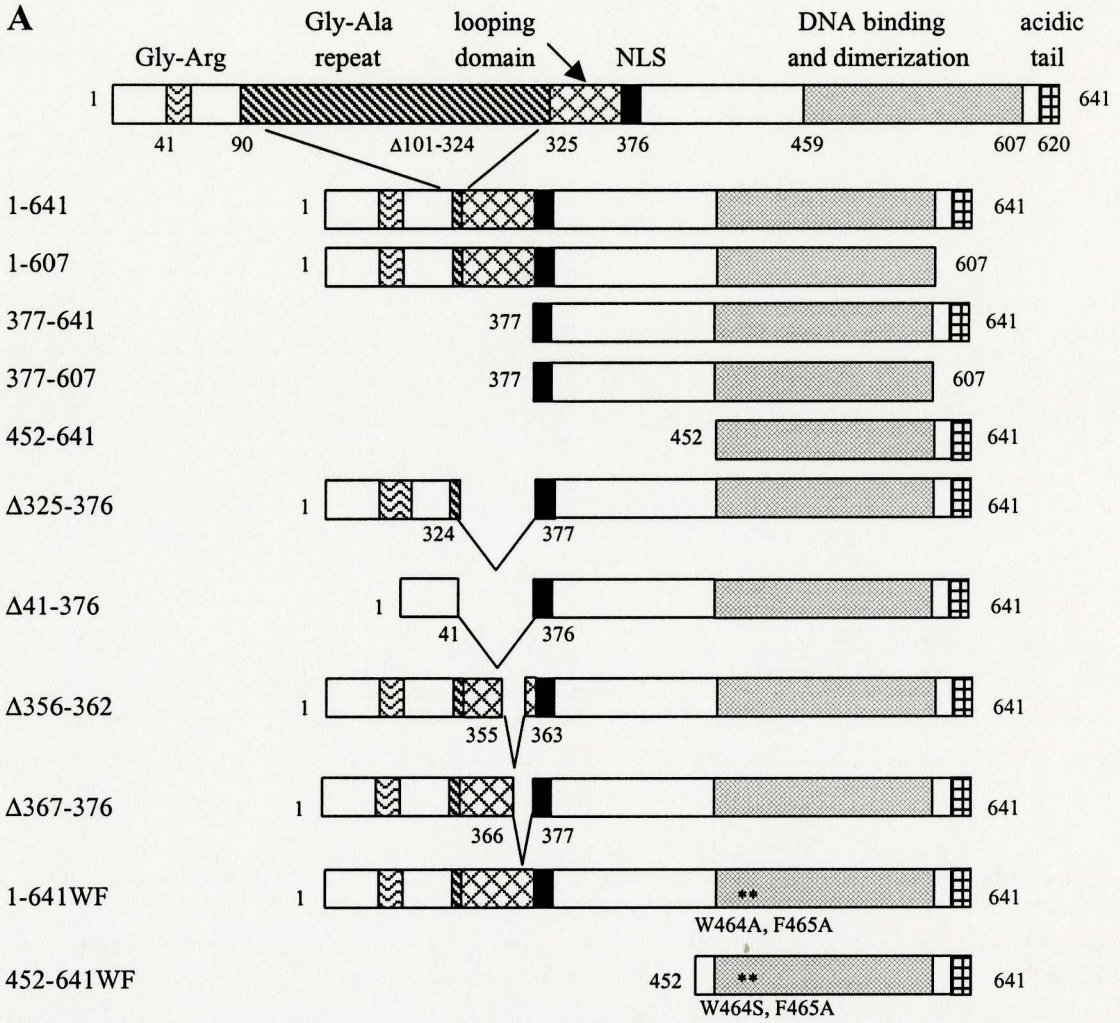
Figure 5 Structure of the EBNA1 DNA binding and dimerization domain bound to DNA. The core domain is in light shading and the flanking domain is in dark shading. (A) A view perpendicular to the DNA axis (adapted from Summers et al, 1997). The positions of the proline loop (PL), the recognition helix (RH) and the WF motif, tryptophan (W464) and phenylalanine (F465) within the DNA minor groove, are shown. (B) A view perpendicular to the DNA axis (adapted from Edwards et al, 1998). The approximate position of residues substituted in the proline loop mutations (PL1 545 to 549; PL2. 541 to 553) are shown.

surface of the protein (see Figure 5b) suggested that it might be involved in mediating protein-protein interactions (Bochkarev et al., 1996). The EBNA1 mutants were expressed in human cells and their functional activity was compared to that of wildtype EBNA1. The version of EBNA1 used as wildtype in these studies is one that lacks most of the Gly-Ala repeat (Figure 6a) and has been previously shown to be functional for replication, segregation and transactivation (Yates and Camiolo, 1988; Yates et al., 1985).

EBNA1 must bind to the recognition sites within *oriP* in order to mediate its activities in the cell. Since the WF motif and the proline loop are within the previously defined DNA binding and dimerization domain (Bochkarev et al., 1996; Summers et al., 1996), the effect of mutations within these regions on the DNA binding affinity of EBNA1 was first examined.

3.1.1 Purification of the EBNA1 DNA binding domain mutants

Two mutations of the EBNA1 proline loop, shown in Figure 6b, were designed to replace part (PL1) or all (PL2) of the proline rich sequence with a flexible linker. The EBNA1 mutants were constructed by a two-step PCR strategy that substituted five (PL1) or thirteen residues (PL2) of the proline loop with Gly-Ala-Ser-Gly and the DNA fragments were cloned into a pET expression vector. The PL2 mutation decreased the size of the loop from 22 to 13 amino acids. This size reduction was not expected to disrupt the DNA binding domain since the structurally homologous region in the E2 protein of papillomavirus has a 13 amino acid loop at the same position (Bochkarev et al., 1995; Hegde et al., 1992). $EBNA_{1\text{-}641}$ is not expressed at detectable levels in bacteria, however a truncated protein containing the DNA binding and dimerization domain and



B

Wild type	539 540 541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 Leu Pro Phe Gly Met Ala Pro Gly Pro Gly Pro Gln Pro Gly Pro Leu Arg Glu
PL1	539 540 541 542 543 544 550 551 552 553 554 555 556 Leu Pro Phe Gly Met Ala Gly Ala Ser Gly Gln Pro Gly Pro Leu Arg Glu
PL2	539 540 554 555 556 Leu Pro Gly Ala Ser Gly Leu Arg Glu

Figure 6. EBNA1 mutants used in this study (A) The position of the DNA binding and dimerization domain, looping domain, nuclear localization signal (NLS) and other features of the EBNA1 protein are shown. Residues 101-324 of the glycine-alanine repeat are not present in the constructs. (B) Amino acids of the proline loop, that were substituted in PL1 and PL2 by glycine-alanine-serine-glycine, are shown.

the acidic tail ($EBNA_{452-641}$) has been expressed at high levels in *E. coli*, purified and shown to be functional for DNA binding (Barwell et al., 1995). Therefore, to assess the effects of the PL1 and PL2 mutations on DNA binding, these mutations were generated in the context of $EBNA_{452-641}$. $EBNA_{452-641PL1}$ and $EBNA_{452-641PL2}$ were amplified by PCR and cloned into pET15b, generating fusion proteins with N-terminal hexa-histidine tags separated from the EBNA1 protein by a thrombin cleavage site. His-tagged $EBNA_{452-641PL1}$ and $EBNA_{452-641PL2}$ were produced in *E. coli* and purified on heparin and nickel columns as described in section 2.2.1. The His-tags were removed from the protein by thrombin digestion and the EBNA1 proteins were further purified and concentrated on a Poros S column. Purified $EBNA_{452-641PL1}$ and $EBNA_{452-641PL2}$ were estimated to be greater than 90 % pure by SDS-PAGE and Coomassie-blue staining (Figure 7).

A double point mutation of the EBNA1 WF motif, that substituted Phe464 and Trp465 with serine and alanine respectively, was generated by Angela Flemming. The $EBNA_{452-641WF}$ protein was purified by Angela Flemming on heparin and nickel columns and concentrated on a Porous S column following removal of the histidine tag (Summers et al., 1997).

3.1.2 Site-specific DNA binding by the PL and WF mutants

The DNA binding affinity of purified $EBNA_{452-641}$, $EBNA_{452-641PL1}$, $EBNA_{452-641PL2}$ and $EBNA_{452-641WF}$ for recognition sites of the DS element was determined by electrophoretic mobility shift asssays (EMSAs). For these assays, EBNA1 proteins were titrated into binding reactions containing a radiolabelled EBNA1 recognition site which corresponds to site 1 of the DS element. Following non-denaturing polyacrylamide gel electrophoresis, bound and unbound DNA fragments were quantified by phosphorimager

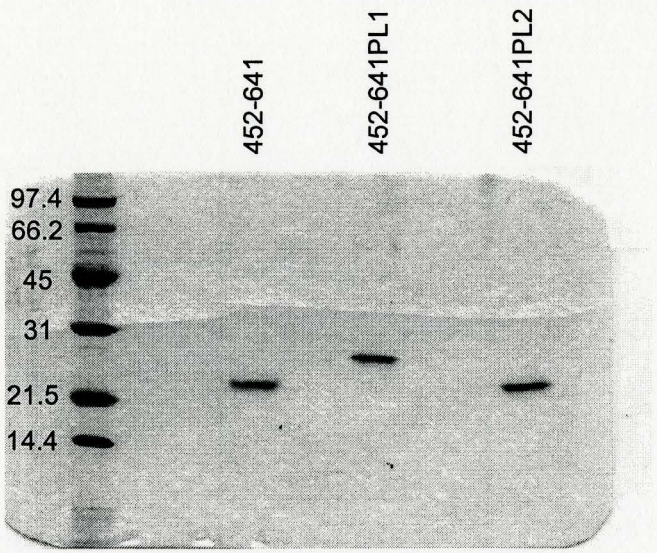


Figure 7 SDS-polyacrylamide gel of purified EBNA1 proteins. EBNA1 amino acids 452-641 containing the wild-type sequences, or PL1 or PL2 mutations are shown. Proteins (300 ng of each) were separated on a 12 % SDS-polyacrylamide gel and stained with Coomassie blue. The molecular weight of protein size markers (Bio-Rad) is shown. $EBNA_{452\text{-}641PL1}$ has a carboxy-terminal extension of 19 amino acids that accounts for the larger size of the protein.

analysis (Figure 8a). The amount of EBNA1 protein required to bind 50 % of the DNA molecules was determined and a dissociation constant for site 1 was calculated (Table 1). $EBNA_{452\text{-}641}$ and $EBNA_{452\text{-}641PL1}$ bound to site 1 with similar affinities of 8 and 11 nM, while $EBNA_{452\text{-}641PL2}$ and $EBNA_{452\text{-}641WF}$ exhibited a 14- and 17-fold decrease in affinity, respectively, as compared to $EBNA_{452\text{-}641}$ (Figure 8a and Table 1). Therefore, the PL1 mutation had no significant effect on DNA binding affinity, while the PL2 and WF mutations reduced DNA affinity by a similar extent.

EMSAs were also performed using the FR and DS elements of *oriP* to assess the relative efficiencies of EBNA1 binding to the intact DNA elements (Figure 9). The amount of EBNA1 protein required to bind 50 % of each DNA fragment was determined and reported in Table 1. Dissociation constants were not calculated since this requires assumptions about the number of sites bound which could not be confirmed. The amounts of $EBNA_{452\text{-}641PL2}$ protein required to bind 50 % of the DS and FR were 18- and 12-fold higher, respectively, than with $EBNA_{452\text{-}641}$ and are comparable to the difference in the Kd values of these proteins for site 1 (Table 1).

The EBNA1 DNA binding and dimerization domain has previously been shown to assemble cooperatively on the four sites of the DS element (Summers, 1996; Harrison, 1994). The cooperative interaction between adjacent EBNA1 dimers on the DS is thought to be important for origin activation. Our lab has previously shown that cooperative interactions of EBNA1 can be detected by EMSAs by comparing the dissociation constants for EBNA1 assembly on site 2 alone versus site 2 in the context of a DS fragment containing both site 1 and site 2 (site 1/ site 2). Cooperative binding of the EBNA1 DNA binding and dimerization region was reflected in the fact that the binding

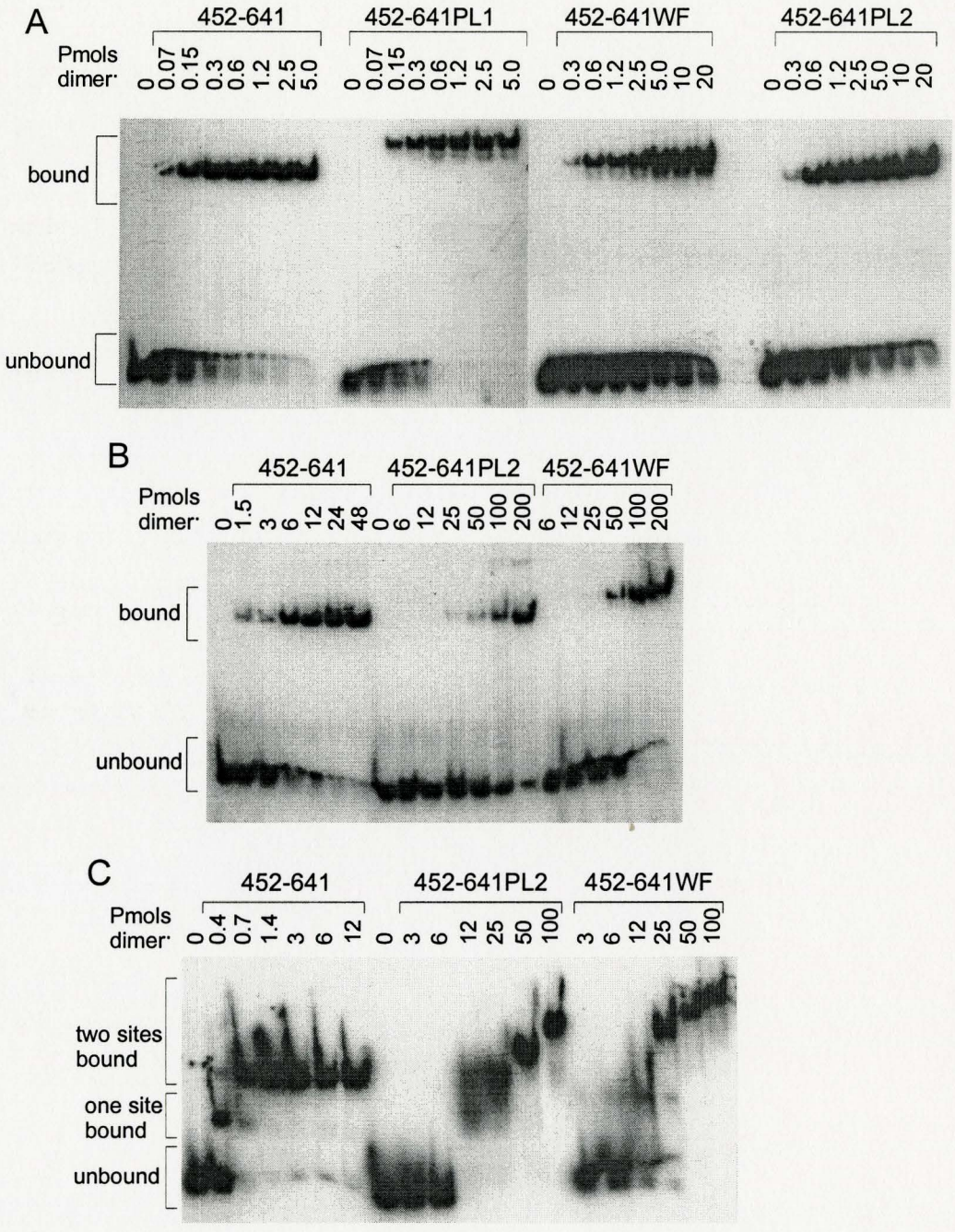


Figure 8. EMSAs of EBNA1 proline loop and WF mutants on sites 1 and 2. Purified $EBNA_{452-641}$, $EBNA_{452-641PL1}$, $EBNA_{452-641PL2}$ and $EBNA_{452-641WF}$ proteins were titrated into a reaction with radiolabelled DNA containing either site 1 (A), site 2 (B) or site 1 and site 2 (C) of the DS element. Bound and unbound DNA fragments were separated on a 12 % polyacrylamide gel and visualized by autoradiography.

Table 1. Dissociation constants for EBNA1 on *oriP* binding sites

EBNA1 fragment	Kd (nM)			concentration required to bind 50 % of the DNA (nM)	
	site 1	site 2	site 1/ site 2*	DS element	FR element
$EBNA_{452-641}$	8	75	20	7	2
$EBNA_{452-641PL1}$	11	nd†	nd	nd	2
$EBNA_{452-641PL2}$	116	2574	553	125	25
$EBNA_{452-641WF}$	141	1832	482	125	nd

* the values were calculated for binding to the second site
† not determined

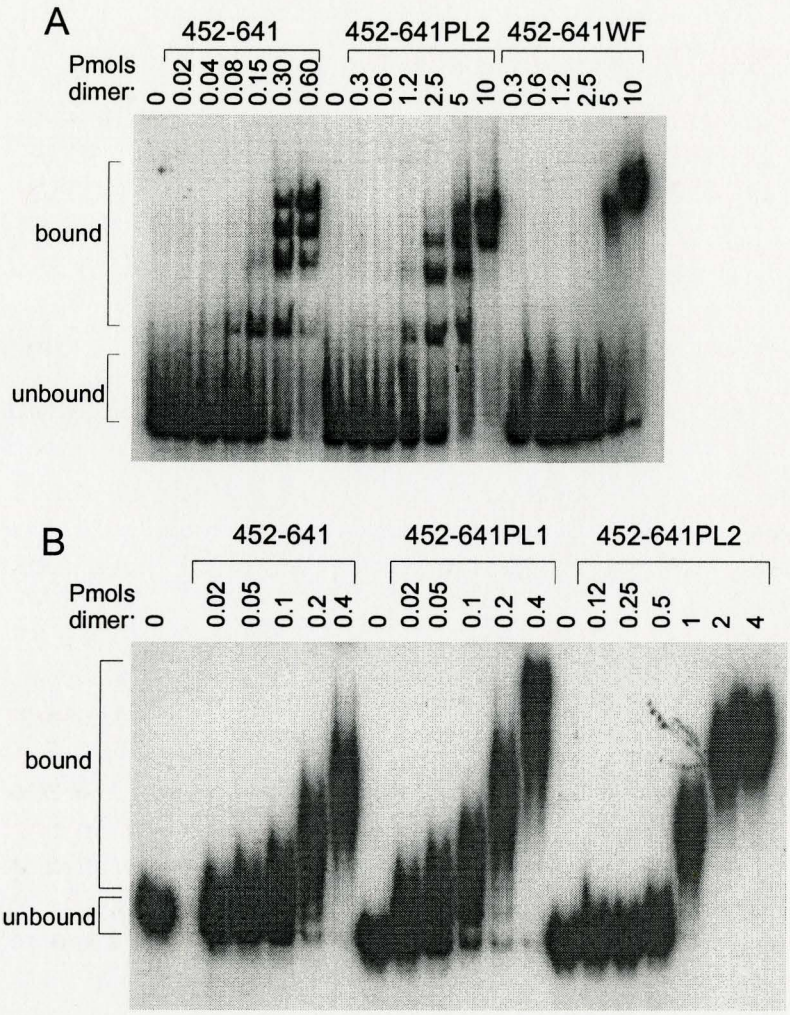


Figure 9 EMSAs of the EBNA1 proline loop and WF mutants on the *oriP* DS and FR elements. Purified $EBNA_{452-641}$, $EBNA_{452-641PL1}$, $EBNA_{452-641WF}$, and $EBNA_{452-641PL2}$ were titrated into a reaction with radiolabelled DNA containing either the DS element (A) or the FR element (B). Bound and unbound DNA fragments were separated on a 5 % polyacrylamide gel and visualized by autoradiography.

to site 2 was stimulated when site 1 was present (Summers, 1996).

The cooperative assembly of $EBNA_{452-641}$, $EBNA_{452-641PL2}$ and $EBNA_{452-641WF}$ was assessed by performing EMSAs on DNA fragments containing site 2 alone (Figure 8b) or containing site 1 and site 2 separated by the same 3 bp spacing found in the DS element (Figure 8c). On site 2 DNA fragments, $EBNA_{452-641}$ demonstrated a 9-fold weaker affinity for site 2 than for site 1 and similar findings have been reported for smaller truncations of the EBNA1 DNA binding and dimerization domain (Summers et al., 1996). Similarly, the affinities of $EBNA_{452-641PL2}$ and $EBNA_{452-641WF}$ for site 2 were 22- and 13-fold lower than for site 1 (Table 1). Within the site 1/ site 2 DNA fragment, $EBNA_{452-641}$, $EBNA_{452-641PL2}$ and $EBNA_{452-641WF}$ bound both DNA sites efficiently. The dissociation constant for binding site 1/ site 2 was determined from the second shifted complex which should represent the binding to site 2. The values for each EBNA1 protein were 4- to 5-fold lower than for site 2 alone (Table 1) indicating that the proline loop and WF mutations did not disrupt the cooperative binding of DNA by EBNA1. Therefore, although the PL2 and WF mutants have reduced binding affinities for EBNA1 recognition sites, both proteins are able to assemble cooperatively on the DS when protein concentrations are sufficient.

3.2 Protein Stability of Proline Loop Mutants

It was possible that the decreased DNA binding activity of $EBNA_{452-641PL2}$ and $EBNA_{452-641WF}$ was due to improper folding or reduced stability of these proteins. To determine if mutation of the proline loop and WF motif disrupted the folding or stability of the EBNA1 DNA binding and dimerization domain, circular dichroism (CD) was used to examine the secondary structure of the purified proteins. CD spectra reflect the

conformation of a polypeptide chain and are sensitive to changes in the secondary structure of the protein. Significant differences between the spectral patterns of the wildtype and mutant proteins would suggest changes in the overall protein conformation. A 10 μM solution of $EBNA_{452-641}$, $EBNA_{452-641PL1}$, $EBNA_{452-641PL2}$ and $EBNA_{452-641WF}$, in phosphate-buffered saline, was scanned from 300 to 200 nm using a circular dichroism spectrometer (Aviv). All four proteins had similar spectra with elliptical minima at 208 and 222 nm in keeping with their helical content (Figure 10). These results indicated that the proline loop and WF mutations did not disrupt the overall secondary structure of EBNA1 under native conditions.

The stability of the wildtype and mutant proteins were compared by measuring their unfolding rates in a denaturant. EBNA1 proteins were diluted in a solution containing various concentrations of guanidine hydrochloride and the ellipticity was monitored by CD at 222 nm. The change in ellipticity at 222 nm over time indicates the unfolding rate as helical structures of the protein denature. $EBNA_{452-641}$ was very stable, requiring a 28 minute incubation in 6.6 M guanidine for half of the protein to unfold (Table 2). The WF mutation had no effect on protein stability since $EBNA_{452-641WF}$ required 32 minutes for 50 % of the protein to unfold under the same conditions. Both $EBNA_{452-641PL1}$ and $EBNA_{452-641PL2}$ unfolded rapidly in 6.6 M guanidine but demonstrated increased stability in 6 M guanidine and remained stable for approximately two hours in 5 M guanidine. Although $EBNA_{452-641PL1}$ and $EBNA_{452-641PL2}$ are less stable than $EBNA_{452-641}$, these proteins are still considerably more resistant to unfolding than single domain proteins such as barstar, ribonuclease H1, T4 lysozyme and lambda repressor (Hanagan et al., 1998; Ramachandran and Udgaonkar, 1996; Roberge et al., 1998).

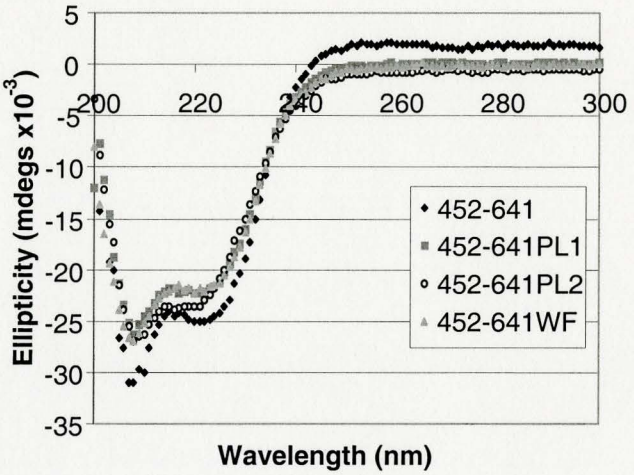


Figure 10. Analysis of EBNA1 proline loop and WF mutants by circular dichroism. CD spectra of $EBNA_{452\text{-}641}$, $EBNA_{452\text{-}641PL1}$, $EBNA_{452\text{-}641PL2}$ and $EBNA_{452\text{-}641WF}$ were determined by scanning a 10 μM solution of each protein from 300 nm to 200 nm. All proteins exhibit the helical minima at 208 and 222 nm that is indicative of their helical content.

Table 2. Denaturation rates of EBNA1 proline loop and WF proteins

Protein	[GuHCl] (M)	k^{-s} ¶	$T_{1/2}$ (min) ‡
452-641	6.6	4.1×10^{-4}	28
452-641 PL1	6.6	3.0×10^{-3}	4
	6.0	1.3×10^{-3}	9
	5.0	9.6×10^{-5}	120
452-641 PL2	6.6	9.5×10^{-3}	1
	6.0	3.4×10^{-3}	3.5
	5.0	1.2×10^{-4}	100
452-641 WF	6.6	3.6×10^{-4}	32

¶ unfolding rate constant

‡ time required for half of protein to unfold

Therefore, the modest reduction in protein stability caused by the proline loop mutations is not likely to be a factor under the no-guanidine conditions of the DNA binding or functionality assays.

3.3 DNA Replication Activity of EBNA1 Mutants

The mechanism by which EBNA1 activates replication from the DS element has not been elucidated, but is thought to involve multiple changes in the DNA structure of the DS and the recruitment of cellular factors to the origin. In order to investigate the EBNA1 motifs that distort DNA and are thought to be involved in protein-protein interactions, the looping domain, WF motif, proline loop and the acidic tail were mutated in full length EBNA1. The mutant proteins were examined for their ability to replicate *oriP* plasmids in human cells. For these studies, plasmids were constructed that contained *oriP*, a neomycin selectable marker and expressed EBNA1 or the EBNA1 mutants from a CMV promoter (Figure 11a). These plasmids were used to transfect C33A cells by the calcium phosphate coprecipitation method (Graham and van der Eb, 1973). To perform transient replication assays, C33A cells were harvested 72 hours post-transfection. Plasmid DNA was isolated from approximately 5 x 10^6 cells, linearized by digestion with Xho I, and 9/10 of the sample was treated with Dpn I to digest unreplicated DNA. The plasmid pc3oriPEBNA$_{1\text{-}641}$ was radiolabelled and used as a probe for Southern blots of the linear and Dpn I treated DNA. Plasmid DNA was visualized by phosphorimager analysis and quantified using ImageQuant software (Molecular Dynamics). The linearized DNA samples served as a control for the efficiency of plasmid DNA recovered (data not shown). The replication activity of each EBNA1 mutant was determined by comparing the amount of Dpn I-resistant plasmid DNA to that obtained for EBNA$_{1\text{-}641}$ in

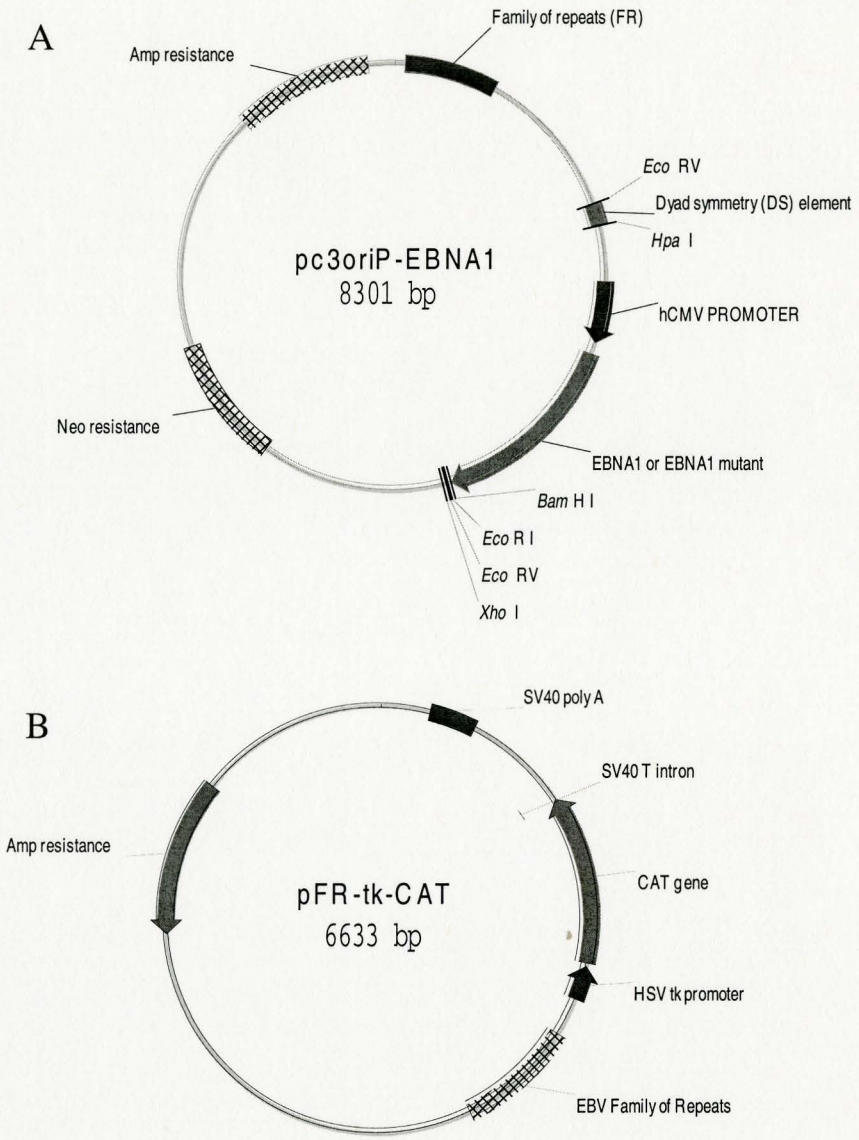


Figure 11 Plasmids used to transfect C33A cells. (A) EBNA1 coding sequences were inserted between the Hind III (destroyed) and Bam HI sites of pcDNA3 (Invitrogen) and the plasmid was further modified by the addition of *oriP* between the Bgl II and Nru I sites (both destroyed) to generate pc3oriPE. (B) A plasmid containing the FR element of *oriP* positioned 200 bp upstream of a herpes simplex virus thymidine kinase (HSV-tk) promoter and the chloramphenicol acetyltransferase (CAT) gene was a gift of Dr Bill Sugden (University of Wisconsin, Madison).

the same experiment. A small amount of Dpn I-resistant DNA was sometimes recovered from pc3oriP transfected cells that do not express EBNA1 (on average 4 % of wild type EBNA1), however, this signal was always lower than the amount detected with any of the EBNA1 mutants and was deemed to be background activity.

The expression of the EBNA1 proteins in transfected cells was confirmed by Western blot and, for the truncation mutants, by determining the site 1 binding activity of the lysate. For the latter assays, increasing amounts of lysate, from pc3oriPE-transfected C33A cells harvested 24 hours post-transfection, was incubated with radiolabelled site 1 in the presence of competitor DNA. Each EBNA1 protein expressed in C33A cells bound site 1 (Figure 12 and data not shown), while no shifted DNA was observed with 20 μg of protein from C33A cells that were tranfected with an empty vector (pc3oriP). The position of the shifted DNA complex corresponded to the size difference of the EBNA1 proteins. $EBNA_{1\text{-}641}$ and $EBNA_{1\text{-}607}$ retain the looping domain and, as a result, these proteins form linked complexes with multiple DNA molecules that are too large to enter the acrylamide gel. The results of the EMSAs indicated that the EBNA1 proteins were expressed in C33A cells and were functional for DNA binding.

3.3.1 The acidic tail and DNA replication

In order to determine if the acidic tail is required for DNA replication, an EBNA1 mutant that lacks this region, $EBNA_{1\text{-}607}$, was examined. $EBNA_{1\text{-}607}$ was found to support DNA replication although the efficiency was somewhat less (58% on average) than that of $EBNA_{1\text{-}641}$ (Figure 13a and Table 3). The lower replication level could be due to reduced expression levels of $EBNA_{1\text{-}607}$ as suggested by an EMSA of transfected cellular lysates (Figure 12). This apparent decrease in replication efficiency is unlikely to be

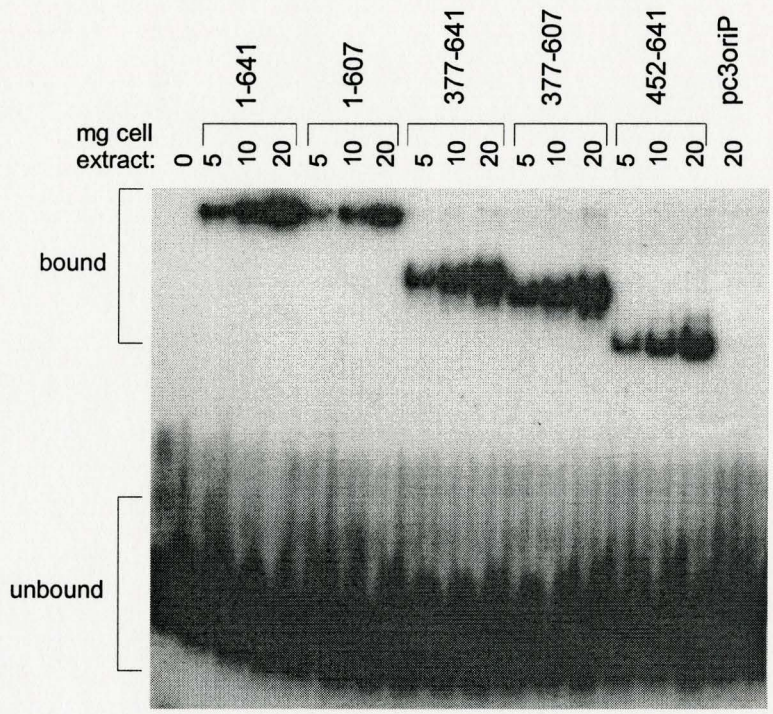


Figure 12. EMSAs with EBNA1 proteins expressed in C33A cells. Protein extracts were prepared from C33A cells that were transfected with pc3oriPE encoding $EBNA_{1\text{-}641}$, $EBNA_{1\text{-}607}$, $EBNA_{377\text{-}641}$, $EBNA_{377\text{-}607}$, $EBNA_{452\text{-}641}$ or no EBNA1 (pc3oriP). An increasing amount of protein was titrated into a reaction containing radiolabelled site 1 The bound and unbound DNA fragments were separated on a 12 % polyacrylamide gel and visualized by autoradiography.

significant as $EBNA_{1-607}$ was subsequently shown to be fully active for plasmid maintenance activity(Section 3.4.1). A second EBNA1 mutant, $EBNA_{377-641}$, that retains the acidic tail but lacks N-terminal sequences, supported a low level of replication that was, on average, 34% the efficiency of wild-type EBNA1 (Figure 13a and Table 3). This suggests that residues between amino acids 1 and 366 are important for DNA replication. Therefore, data obtained from at least four transfections for each mutant indicates that the acidic tail of EBNA1 is neither necessary nor sufficient, when combined with the DNA binding and dimerization domain, for DNA replication (Table 3).

3.3.2 The looping domain and DNA replication

The looping domain is an Arg-rich sequence that lies between amino acids 325 and 376 and, therefore, was part of the N-terminal sequences deleted in $EBNA_{377-641}$. Since $EBNA_{377-641}$ has only weak replication activity, a role for the looping domain in DNA replication was examined. Removal of the 50 amino acid looping domain did not appreciably affect the replication efficiency of EBNA1 as $EBNA_{\Delta 325-376}$ was found to replicate with 95 % of the efficiency, on average, of wildtype EBNA1 (Figure 13b and Table 3). This mutant was previously shown to lack the ability to mediate the EBNA1-EBNA1 interactions that result in the linking of multiple DNA molecules together (Avolio-Hunter and Frappier, 1998). These results indicate that the looping domain is not required for replication and suggest that amino acids 1 to 100 are important for replication (since residues 101-324 of the Gly-Ala repeat were not present in any of the EBNA1 constructs). A larger deletion, $EBNA_{\Delta 41-376}$, was also examined that removes a second Arg-rich region in addition to the looping domain. $EBNA_{\Delta 41-376}$ retained

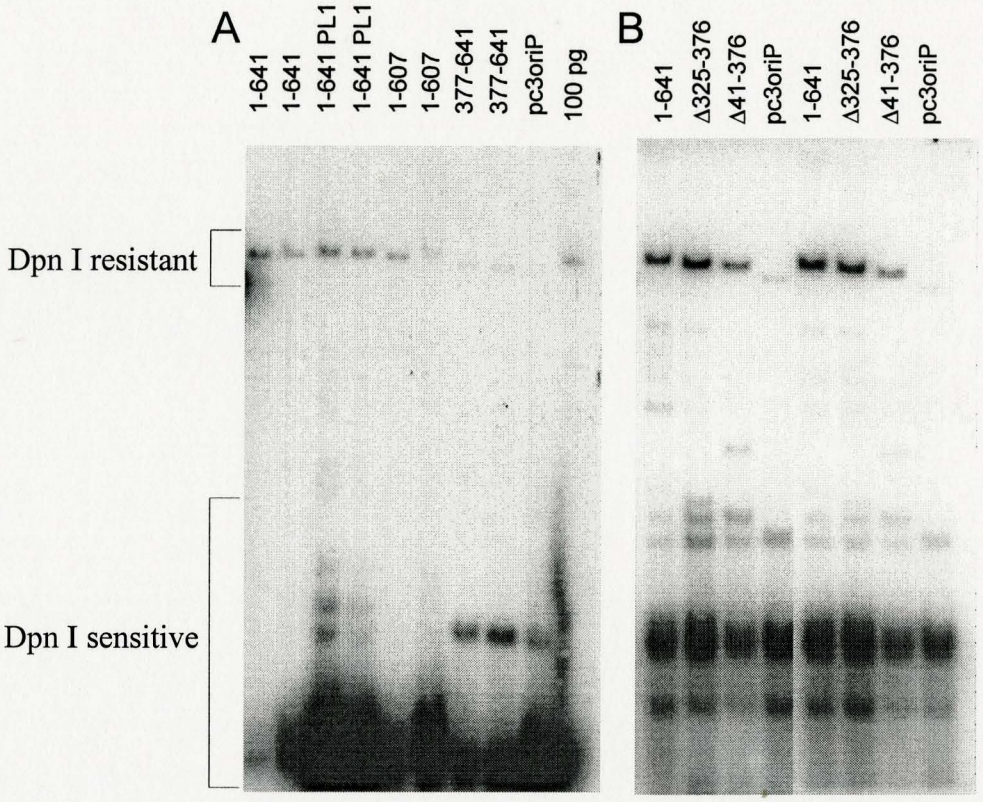


Figure 13 Transient replication assay of EBNA1 mutants. *Ori*P plasmids encoding $EBNA_{1\text{-}641}$, $EBNA_{1\text{-}641PL1}$, $EBNA_{1\text{-}607}$, $EBNA_{377\text{-}641}$, $EBNA_{\Delta325\text{-}376}$, $EBNA_{\Delta41\text{-}376}$ or no EBNA (pc3*ori*P) were used to transfect C33A cells. Low molecular weight DNA was purified from 5 x 10^6 cells three days post-transfection, linearized with Xho I and digested with Dpn I. DNA fragments were separated on a 0.9 % TAE agarose gel, Southern blotted and visualized by autoradiography. The replication activity of each mutant was determined by comparing the amount of Dpn I resistant DNA to that obtained for wildtype $EBNA_{1\text{-}641}$

Table 3. Transient replication activity of EBNA1 mutants

Protein	Individual Experiments*									Average§
	A	B	C	D	E	F	G	H	I	
1-641	100	100	100	100	100	100	100	100	100	100 n = 9
1-607							24 73	71 63		58 ± 23 n = 4
377-641						32 10	78	25 24		34 ± 26 n = 5
Δ325-376		168 83	92 37							95 ± 54 n = 4
Δ41-376		55 26	49 57							47 ± 14 n = 4
1-641PL1	80 85					14 7	24 23	143 89		58 ± 48 n = 8
1-641PL2							4.1 4.2		23 19 14	13 ± 8 n = 5
1-641WF					86 71		68 92		154 220 153	120 ± 57 n = 7
pc3oriP	9	10	4	9.5	0	0	0	3	5	4.2 ± 4 n = 9

* The transient replication activity was determined from the amount of Dpn I-resistant *oriP* plasmid DNA recovered three days post-transfection and expressed as a percentage of $EBNA_{1-641}$ activity. Where two values are shown, duplicate samples of the mutant were assayed.

§ The average activity of each EBNA1 mutant as a percentage of $EBNA_{1-641}$ activity followed by the standard deviation and number of experiments (n).

replication activity, although the efficiency of replication was reduced to 47 % of wild-type EBNA1 (Figure 13b and Table 3). The reduced replication of $EBNA_{\Delta 41\text{-}376}$ relative to $EBNA_{\Delta 325\text{-}376}$ might indicate that the sequences between amino acids 41 and 100 contribute to replication efficiency or that the Δ41-376 deletion disrupts the structure of an N-terminal domain that is important for replication.

3.3.3 The WF motif and DNA replication

The WF motif was thought to play a role in DNA replication because positioning of these residues in the DNA minor groove is such that they could contribute to DNA melting (Figure 5). An EBNA1 mutant with the WF motif substituted by two alanine residues was constructed and the effect of this mutation on the ability of EBNA1 to replicate *oriP* plasmids was examined. This mutation reduced DNA binding affinity (Table 1) but $EBNA_{1\text{-}641WF}$ was found to support DNA replication with an activity that was, on average, 120 % the efficiency of wildtype EBNA1 (Figure 14a and Table 3). Therefore, the WF motif does not appear to contribute to DNA replication. These results also indicate that the reduced DNA binding affinity associated with the WF mutation is not sufficient to prevent DNA binding in this assay.

3.3.4 The proline loop and DNA replication

I next investigated the role of the EBNA1 proline loop in DNA replication. As shown above (Section 3.1.2), the small proline loop mutation (PL1) had no significant effect on DNA binding activity while the large proline loop mutation (PL2) decreased binding to *oriP* (Table 1). The DNA binding ability of $EBNA_{452\text{-}641PL2}$ was, however, similar to that of the WF mutant, a protein that retained wildtype DNA replication

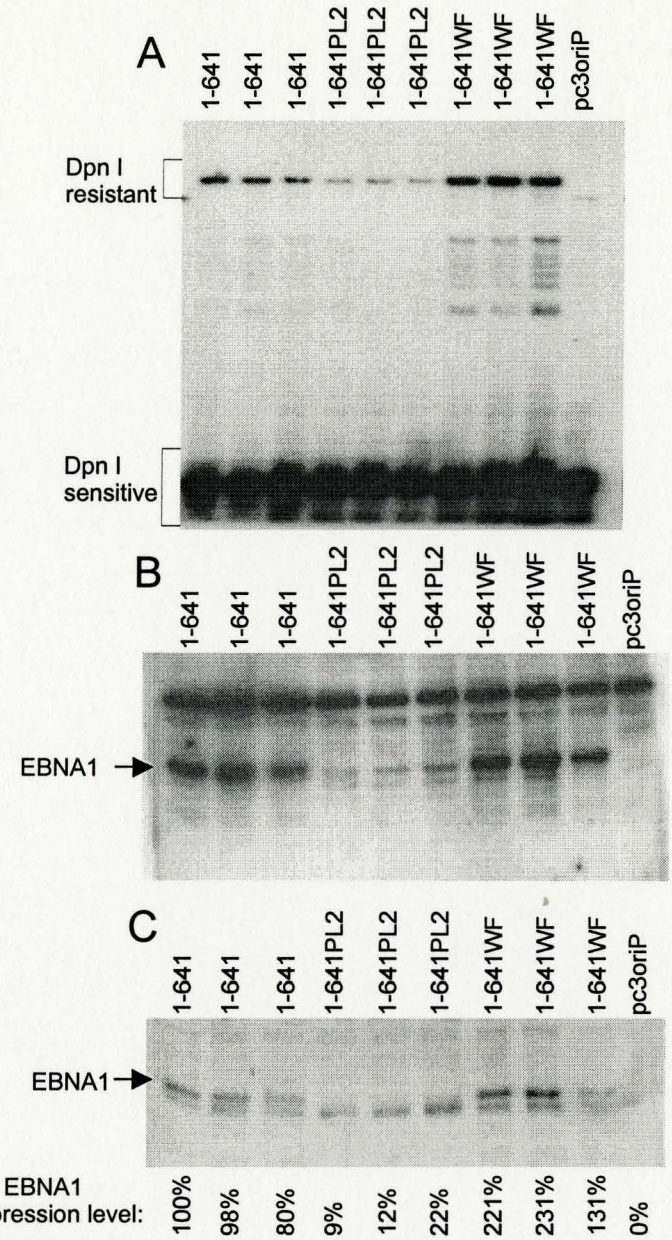


Figure 14. Transient replication assay of EBNA PL2 and WF mutants. *OriP* plasmids encoding $EBNA_{1\text{-}641}$, $EBNA_{1\text{-}641PL2}$ and $EBNA_{1\text{-}641WF}$ or no EBNA1 (pc3oriP) were used to transfect C33A cells. Following three days of cell growth, plasmid DNA was isolated, linearized and treated with Dpn I. (A) The digested DNA was separated on a 0.9 % gel, Southern blotted and visualized by autoradiography. The Dpn I-resistant DNA was quantified by phosphorimager analysis. (B and C) Aliquots of cell lysates containing 30 μg of protein were analyzed for EBNA1 expression by Western blot using ECL (B) or ECF (C) detection systems. The expression level of each mutant, expressed as a percentage of the $EBNA_{1\text{-}641}$ level, is shown below panel C.

activity. Therefore, the effects of the proline loop mutations on the ability of EBNA1 to mediate the replication of *oriP* plasmids was examined. $EBNA_{1\text{-}641PL1}$ displayed replication activity that varied considerably from one experiment to the next and, on average, was 58 % of $EBNA_{1\text{-}641}$ (Figure 13a and Table 3). The replication activity of $EBNA_{1\text{-}641PL2}$ was, on average, 13 % of wildtype EBNA1 and was consistently less than that of $EBNA_{1\text{-}641PL1}$ (Figure 14a and Table 3). In order to determine if the apparent lack of activity might be due to the failure of $EBNA_{1\text{-}641PL2}$ to bind DNA, we compared the expression level of $EBNA_{1\text{-}641PL2}$ to that of $EBNA_{1\text{-}641WF}$. Since $EBNA_{1\text{-}641WF}$ is active for replication and has the same DNA binding affinity as $EBNA_{1\text{-}641PL2}$, we reasoned that, if $EBNA_{1\text{-}641PL2}$ is expressed at levels that are equal to or above that of $EBNA_{1\text{-}641WF}$, the $EBNA_{1\text{-}641PL2}$ concentration should be sufficient to bind *oriP*.

The levels of protein expression were compared by Western blot analysis on aliquots of transfected cells from the transient replication assays. Enhanced chemiluminescence (ECL) and enhanced chemifluorescence (ECF) Western blots of whole cell lysates were performed using a rabbit polyclonal antisera raised against the DNA binding and dimerization domain of EBNA1 (provided by Dr. J. Middledorp). The ECL Western blot, in Figure 14b, suggested that levels of $EBNA_{1\text{-}641PL2}$ protein were lower than that of $EBNA_{1\text{-}641}$ and $EBNA_{1\text{-}641WF}$. However, since ECL does not give quantitative results, the Western blots were repeated using the quantitative ECF procedure. ECF Western blot analysis showed that the levels of $EBNA_{1\text{-}641PL2}$ protein in the transfected cells were 5-10 fold lower than $EBNA_{1\text{-}641}$ and 10-25 fold lower than $EBNA_{1\text{-}641WF}$ (Figure 14c). The low expression level of $EBNA_{1\text{-}641PL2}$, coupled with its DNA binding defect, raises the distinct possibility that the lack of activity associated with

the PL2 mutation is due to insufficient DNA binding by $EBNA_{1\text{-}641PL2}$.

3.4 DNA Segregation Activity of EBNA1 Mutants

The maintenance of *oriP* plasmids in dividing cells requires DNA replication and an active segregation mechanism. To gain an understanding of the contributions of the four EBNA1 motifs to plasmid maintenance, EBNA1 mutants were examined for their ability to maintain *oriP* plasmids during a two week period of cell culture. C33A cells were transfected with pc3oriPE plasmids and grown in the presence of 400 μg/ml G418 for two weeks. Colonies of C33A cells appeared on the plates during this period of time and $5x10^6$ cells were harvested from each plate. Plasmid DNA was isolated and prepared for Southern blotting in the same manner as for the transient replication assays. Plasmids were linearized with Xho I and digested with Dpn I to eliminate unreplicated DNA that occasionally adheres to the tissue culture plates. The Dpn I resistant plasmid DNA from each sample was visualized by autoradiography of Southern blots and quantified by phosphorimager analysis using ImageQuant software (Molecular Dynamics). A 100 pg sample of pc3oriPE and/or pc3oriP was included on the Southern blot in order to allow the amount of plasmid DNA recovered from the transfected cells to be quantified. The average plasmid copy number per cell was determined and compared to the value obtained for wild type EBNA1.

The plasmid maintenance assay results represent a combination of the DNA replication and segregation activities. EBNA1 mutants shown to be defective in transient replication should also be defective for plasmid maintenance. For EBNA1 mutants with wildtype replication activity, a defect in plasmid miantenance should represent a segregation defect.

3.4.1 The acidic tail and plasmid maintenance

To investigate a role for the acidic tail in plasmid maintenance, an EBNA1 mutant that lacked the acidic tail, $EBNA_{1\text{-}607}$, was tested for its ability to maintain *oriP* plasmids in C33A cells. $EBNA_{1\text{-}607}$ consistently maintained *oriP* plasmids at a level equivalent to wild type EBNA1 indicating that the acidic tail is not required for replication or segregation (Figure 15,16a and Table 4). This finding is in contrast with results reported by Yates and Camiolo, that suggested the acidic tail was important for plasmid maintenance (Yates and Camiolo, 1988). My results also indicate that the small decrease in replication efficiency observed for $EBNA_{1\text{-}607}$ in transient assays was not functionally significant.

Plasmid maintenance activity of $EBNA_{377\text{-}641}$ was also examined. This mutant was unable to maintain *oriP* plasmids, indicating that the acidic tail is not sufficient for plasmid maintenance when combined with the DNA binding and dimerization domain (Figure 15, 17a and Table 4). Since $EBNA_{377\text{-}641}$ was shown to be impaired for replication in transient assays, the defect in plasmid maintenance may be due to replication alone or to a combination of replication and segregation defects.

3.4.2 The looping domain and plasmid maintenance

The looping domain mediates the interaction between DNA-bound EBNA1 molecules and may also mediate interactions with host cellular proteins. In order to determine if such looping domain-mediated interactions are required for plasmid maintenance, EBNA1 mutants with deletions in the looping domain were examined for the ability to maintain *oriP* plasmids in C33A cells. $EBNA_{377\text{-}641}$ lacks the entire N-terminus, including the looping domain, and failed to maintain *oriP* plasmids (Table 4).

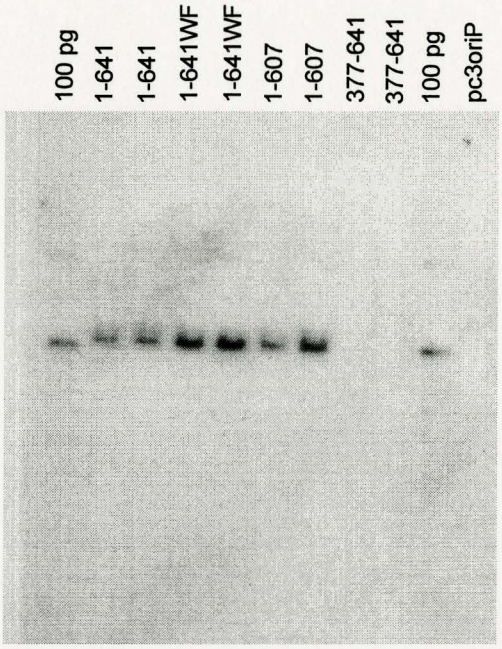


Figure 15. Plasmid maintenance assay of EBNA1 WF and truncation mutants. *Ori*P plasmids encoding $EBNA_{1\text{-}641}$, $EBNA_{1\text{-}641WF}$, $EBNA_{1\text{-}607}$, $EBNA_{377\text{-}641}$ or no EBNA1 (pc3oriP) were used to transfect C33A cells. Following 14 days of culture in the presence of G418, plasmid DNA was purified from 5 x 10^6 cells, linearized and treated with Dpn I. The digested DNA was separated on a 0.9 % agarose gel, Southern blotted and visualized by autoradiography. Plasmid DNA was quantified by phosporimager analysis and the average copy number per cell was determined by comparing the amount of plasmid recovered to a 100 pg marker of pc3oriP-$EBNA_{1\text{-}641}$ The plasmid maintenance activity of each mutant was determined by comparing the amount of plasmid DNA recovered with that of wildtype EBNA1

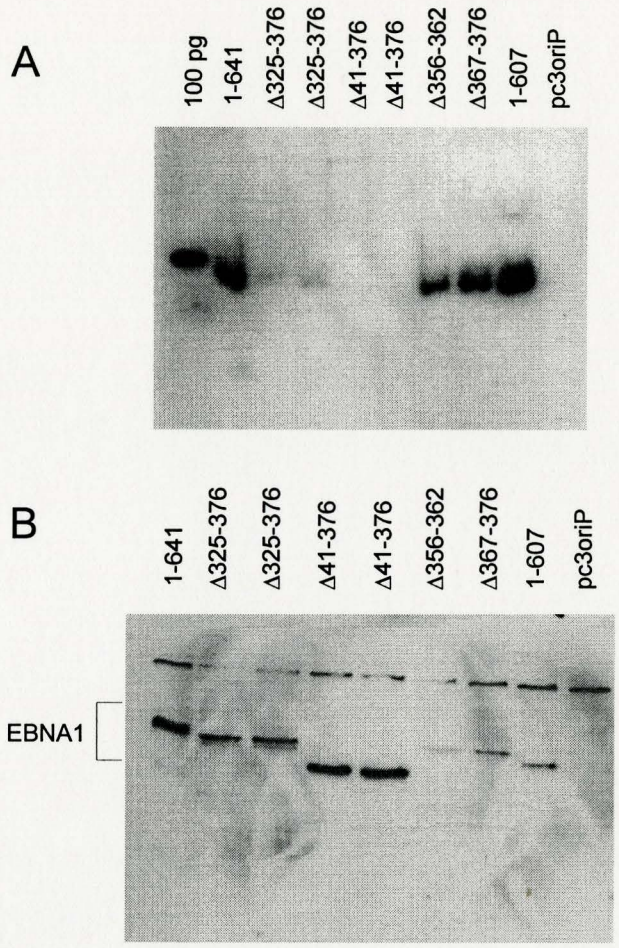


Figure 16. Plasmid maintenance assay of EBNA1 looping domain and acidic tail mutants. *OriP* plasmids encoding $EBNA_{1\text{-}641}$, $EBNA_{\Delta 325\text{-}376}$, $EBNA_{\Delta 41\text{-}376}$, $EBNA_{\Delta 356\text{-}362}$, $EBNA_{\Delta 367\text{-}376}$, $EBNA_{1\text{-}607}$ or no EBNA1 (pc3oriP) were used to transfect C33A cells. Low molecular weight DNA was prepared from 5 x 10^6 G418-resistant cells after two weeks in culture, linearized and treated with Dpn I. (A) Southern blot of the digested DNA The plasmid maintenance activity of each mutant was determined by comparing the average copy number of plasmid DNA recovered per cell with that of $EBNA_{1\text{-}641}$ (B) Expression of EBNA1 protein was detected by an enhanced chemiluminescence (ECL) Western blot performed on 30 μg of protein from G418-resistant cell lysates.

Table 4. Plasmid maintenance activity of EBNA1 mutants

Protein	Individual Experiments*									Average§
	A	B	C	D	E	F	G	H	I	
1-641	100 (1.5)	100 (2.2)	100 (5.0)	100 (2.0)	100 (2.4)	100 (2.7)	100 (1.0)	100 (4.5)	100 (2.0)	100 n = 9
1-607				105 (2.1)	63 (1.5)	152 (4.1)			118, 231 (2.3) (4.5)	134 ± 63 n = 5
377-641								0, 0 (0) (0)	0, 0 (0) (0)	0 ± 0 n = 4
Δ325-376			0 (0)			1.9, 3 (0.05) (0.1)				1.6 ± 1.5 n = 3
Δ41-376			0 (0)			0, 0 (0) (0)				0 ± 0 n = 3
Δ356-362	67 (1.0)	105 (2.3)				42 (1.1)				71 ± 32 n = 3
Δ367-376	120 (1.8)	105 (2.3)				68 (1.8)				98 ± 27 n = 3
1-641PL1				145 (2.9)	145 (3.5)		87, 197 (0.9) (2.0)	200, 180 (9.0) (8.2)		159 ± 43 n = 6
1-641PL2				0 (0)	0 (0)			0, 0 (0) (0)		0 ± 0 n = 4
1-641WF	113 (1.7)	50 (1.1)							251, 277 (4.9) (5.4)	173 ± 109 n = 4
Pc3oriP	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 ± 0 n = 9

* The plasmid maintenance activity was determined from the amount of Dpn I-resistant *oriP* plasmid DNA recovered from 5 x 10^6 cells after two weeks under selection and expressed as a percentage of $EBNA_{1-641}$ activity. The average number of Dpn I-resistant *oriP* plasmids recovered per cell is in parentheses. Where two values are shown, duplicate samples of the mutant were assayed.

§ The average activity of each EBNA1 mutant is a percentage of $EBNA_{1-641}$ activity followed by the standard deviation and number of experiments (n)

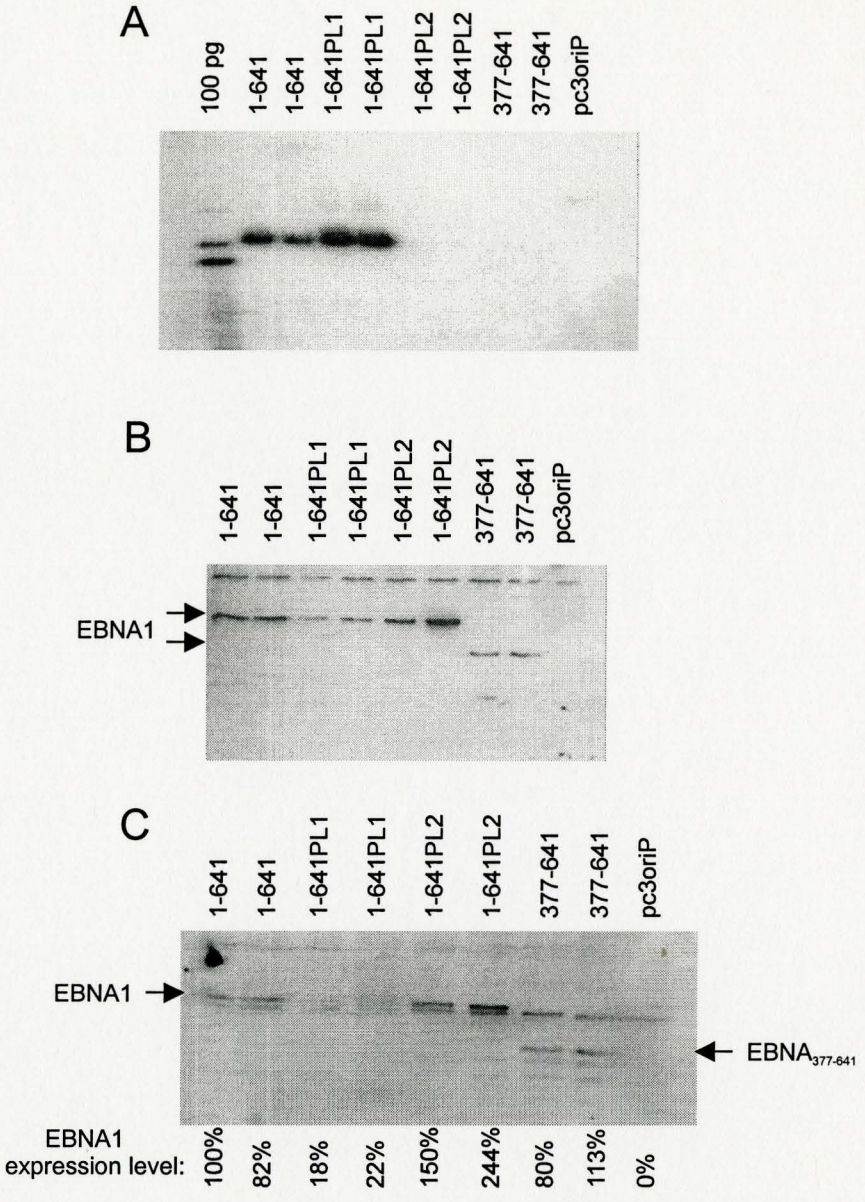


Figure 17 Plasmid maintenance assay of EBNA1 proline loop mutants and $EBNA_{377\text{-}641}$. *OriP* plasmids encoding $EBNA_{1\text{-}641}$, $EBNA_{1\text{-}641PL1}$, $EBNA_{1\text{-}641PL2}$, $EBNA_{377\text{-}641}$ or no EBNA1 (pc3oriP) were used to transfect C33A cells. Following 14 days of selection, DNA was purified from 5 x 10^6 cells, linearized and treated with Dpn I. (A) Southern blot of the digested DNA. Lane 1 contains 100 pg each of linearized pc3oriP and linearized pc3oriP-$EBNA_{1\text{-}641}$ (B and C) A lysate was prepared from the harvested cells and Western blots for EBNA1 expression were performed on 30 μg of protein. Antibodies were visualized by ECL (B) and ECF (C). The expression level of each mutant, expressed as a percentage of the $EBNA_{1\text{-}641}$ level, is shown below panel C.

(Table 3). The cumulative effect of inefficient replication over a two week period in culture could explain the inability to detect plasmid DNA in C33A cells and therefore, the contribution of the N-terminal sequences to segregation could not be assessed using this mutant.

More definitive results on the contribution of N-terminal sequences to EBNA1 segregation function were obtained using internal deletion mutants encompassing the looping domain. $EBNA_{\Delta 325\text{-}376}$ was shown in transient assays to be completely active for replication, but this mutant failed to maintain *oriP* plasmids following a two week period of cell culture (Figure 16a). The lack of plasmid maintenance activity is therefore likely due to a defect in segregation. A similar plasmid maintenance defect was observed for the Δ41-376 mutant. Both $EBNA_{\Delta 325\text{-}376}$ and $EBNA_{\Delta 41\text{-}376}$ were detected by ECL Western blots (Figure 16b) and the DNA replication activity of the proteins indicates that they were expressed, properly folded and able to bind *oriP* DNA.

Small deletions within the looping domain were also examined to determine if the entire looping domain was required for plasmid maintenance activity. $EBNA_{\Delta 356\text{-}362}$ and $EBNA_{\Delta 367\text{-}376}$ each contained a deletion that removed one of six repeated sequence elements within this region (Laine and Frappier, 1995). Both proteins were able to maintain *oriP* plasmids at levels close to wildtype EBNA1, despite the apparent lower protein expression levels as observed by Western blot (Figure 16a,b). Therefore, these small deletions do not disrupt the replication or segregation activity of EBNA1. These results indicate either that amino acids 325-355 are required for plasmid maintenance or that the repetitive sequences of the looping domain have redundant activities.

3.4.3 The WF motif and plasmid maintenance

The effect of the WF mutation on the ability of EBNA1 to maintain *oriP* plasmids in dividing cells was examined. $EBNA_{1\text{-}641WF}$ expressing C33A cells efficiently maintained *oriP* plasmids at levels that were, on average, 173 % of wildtype (Figure 15 and Table 4). Therefore, the WF motif is not required for DNA replication or segregation. It is not clear at this time whether the apparent increase in plasmid maintenance activity observed for $EBNA_{1\text{-}641WF}$ reflects a bonafide stimulation of replication and/or segregation activities.

3.4.4 The proline loop and plasmid maintenance

To investigate the possible role of the proline loop in plasmid maintenance, $EBNA_{1\text{-}641PL1}$ and $EBNA_{1\text{-}641PL2}$ were examined for their ability to maintain *oriP* plasmids in C33A cells. The PL1 mutation did not result in a loss of maintenance activity since the average plasmid copy number per cell was slightly higher than for $EBNA_{1\text{-}641}$ (Figure 17a and Table 4). This result confirms the earlier finding that $EBNA_{1\text{-}641PL1}$ is functional for DNA replication and it also indicates that the PL1 mutation does not affect the segregation activity of EBNA1. In contrast, $EBNA_{1\text{-}641PL2}$ was not able to maintain plasmid DNA in the transfected cells (Figure 17a and Table 4). The loss of activity could be due to inefficient DNA replication and/or inefficient segregation which might stem from the reduced DNA binding ability of this mutant.

Expression of $EBNA_{1\text{-}641PL2}$ in transfected cells was examined by ECL and ECF Western blots (Figure 17b,c). The relative levels of $EBNA_{1\text{-}641PL2}$ protein were higher than $EBNA_{1\text{-}641}$ after two weeks of culture, even in the absence of detectable plasmid DNA (Figure 17c). These results suggest that after two weeks in selective culture,

$EBNA_{1\text{-}641PL2}$ expression was driven from plasmid sequences that had integrated at a chromosomal locus. Such integration would be expected if the plasmid could not replicate. Although expression of $EBNA_{1\text{-}641PL2}$ after integration was higher than that of the wild type protein, expression of this protein from the plasmid three days post-transfection was relatively low (see Figure 14c). This low expression level, combined with the reduced DNA binding activity caused by the PL2 mutation, may be responsible for the lack of plasmid maintenance activity observed. Therefore, whether or not the PL2 mutation directly affects replication or segregation could not be determined.

3.5 Transactivation Activity of EBNA1 Mutants

In addition to playing an essential role in the replication and maintenance of *oriP* plasmids, EBNA1 mediates the activation of transcription. Transcriptional activation by EBNA1 occurs when EBNA1 binds the FR element, a transcriptional enhancer within the viral *oriP* sequences (Reisman and Sugden, 1986). EBNA1 regions in addition to the DNA binding and dimerization domain are required for transactivation activity (Polvino-Bodnar and Schaffer, 1992; Yates and Camiolo, 1988), but the transactivation domain has not been definitively mapped. Previous findings have suggested that the EBNA1 domains that contribute to replication and transactivation overlap since mutants that are deficient in only one of these two activities have not been isolated (Polvino-Bodnar and Schaffer, 1992; Yates and Camiolo, 1988).

In order to investigate the contribution of the acidic tail, looping domain, proline loop and WF motif to transactivation, a reporter plasmid and the pc3oriPE plasmids were used to co-transfect C33A cells. The reporter plasmid (pFR-tk-CAT) contains the FR element 200 bp upstream of the herpesvirus thymidine kinase (HSV-TK) promoter and

the chloramphenicol acetyltransferase (CAT) gene (Figure 11b; obtained from Dr. Bill Sugden, University of Wisconsin at Madison). C33A cells were harvested 24 hours post-transfection and 50 μg of protein from a clarified lysate was assayed for CAT activity. Aliquots taken after various reaction times were spotted onto cellulose thin layer chromatography (TLC) plates and the amount of acetylated and nonacetylated chloramphenicol was determined by phosphorimager analysis of the developed TLC plates. The percent of acetylated chloramphenicol versus time was plotted and the activity of each EBNA1 mutant was determined by comparing the acetylation rate with the value obtained for $EBNA_{1\text{-}641}$. For each transfection experiment, a negative control consisting of a plasmid (pc3oriP) that does not express EBNA1 was included to determine the background level of CAT activity within the C33A cells. The amount of background activity was subtracted from each sample before the values obtained for EBNA1 and EBNA1 mutants were compared.

3.5.1 The acidic tail and transactivation

In order to determine if the acidic tail of EBNA1 functions as a transactivation domain, the ability of several EBNA1 truncation mutants to activate expression of the CAT gene was determined. First, the transactivation activity of $EBNA_{1\text{-}607}$ was compared to wildtype EBNA1. As shown in Figure 18a and Table 5, removal of the acidic tail had no significant effect on the transactivation activity of EBNA1. To determine if the acidic tail might be a transactivation domain that is redundant in the context of full length EBNA1, the transactivation activity was examined using two N-terminal truncation mutants ($EBNA_{377\text{-}641}$ and $EBNA_{452\text{-}641}$) that contained the DNA binding domain and the acidic tail. $EBNA_{377\text{-}641}$ and $EBNA_{452\text{-}641}$ had transactivation activity only 4 % and 13 %

of that of wildtype EBNA1, respectively (Figure 18a and Table 5). The small amount of activity observed with $EBNA_{377-641}$ was not significantly reduced by the removal of the acidic tail in $EBNA_{377-607}$ (Figure 18a and Table 5). These results indicate that the acidic tail is neither required nor sufficient for transactivation and that the transactivation domain of EBNA1 is located between amino acids 1 to 376.

3.5.2 The looping domain and transactivation

The looping domain of EBNA1 is located within the N-terminal residues that the above results suggest are important for transactivation. To investigate the contribution of the looping domain to transactivation, internal deletion mutants that lacked all (Δ41-376 and Δ325-376) or part (Δ356-362 and Δ367-376) of the looping domain were tested for their ability to activate transcription of the FR-CAT reporter construct. Comparison of the acetylation rates with wildtype EBNA1 indicated that the removal of 50 amino acids comprising the looping domain (Δ325-376) resulted in a severe loss of transactivation activity (Figure 18b). Data from multiple experiments, summarized in Table 5, showed that on average 2.5 % of wild-type transactivation activity was observed for the Δ325-376 looping domain mutant. Similar results were obtained with the larger Δ41-376 deletion mutant (Table 5). The loss of transactivation activity was not due to insufficient protein expression as Western blot analysis showed that $EBNA_{\Delta 325-376}$ was expressed at levels as high or higher than the wildtype protein (Figure 18b). Also the fact that $EBNA_{\Delta 325-376}$ was active in replication (Table 3) and has wildtype DNA binding activity *in vitro* (Avolio-Hunter and Frappier, 1998) suggests that this protein is not defective in binding to the FR element. Therefore, the results indicate that the transactivation domain

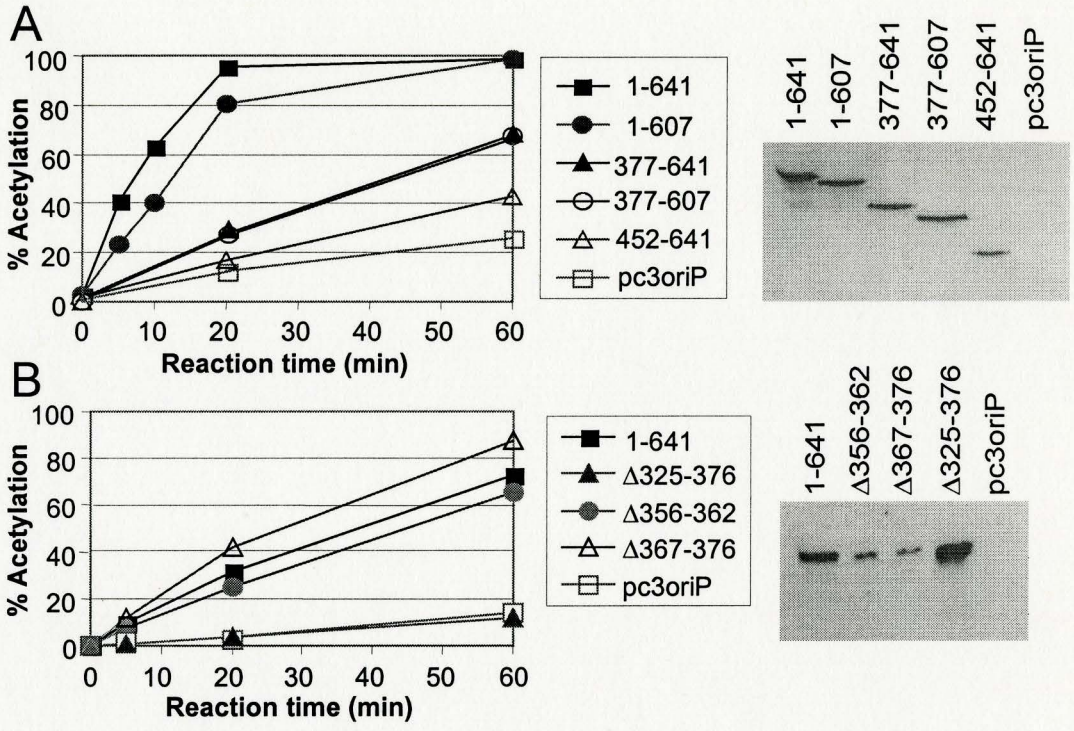


Figure 18. Transactivation activity of EBNA1 truncation and looping domain mutants. A CAT reporter construct (pFR-tkCAT) and pc3oriP encoding $EBNA_{1-641}$ or EBNA1 mutants were used to transfect C33A cells. Following 24 hours in culture, a cellular lysate was prepared and 50 μg of protein was assayed for CAT activity. The percent of acetylated chloramphenicol at various time points was plotted. ECL Western blots for EBNA1 expression (right panel) were performed on 30 μg of cellular protein and visualized by enhanced chemiluminescence

Table 5. Transactivation activity of EBNA1 mutants

Protein	Individual Experiments*													Average§
	A	B	C	D	E	F	G	H	I	J	K	L	M	
1-641	100	100	100	100	100	100	100	100	100	100	100	100	100	100 n = 13
1-607	85	113	69	78	100									89 ± 14 n = 5
377-641	16	7	4	19	21									13 ± 7 n = 5
377-607	17	5	6	9										9 ± 4 n = 4
452-641	7	2	0		18									4 ± 3.5 n = 4
Δ325-376				0	8	2	0							2.5 ± 0.8 n = 4
Δ41-376				1	6				4 14					6 ± 5 n = 4
Δ356-362						104	89	45 67	115 128					91 ± 31 n = 6
Δ367-376				96	118			113 108		141 139				125 ± 13 n = 6
1-641PL1				96	118					22 23	122 64	45 52	58 59	66 ± 35 n = 10
1-641PL2								21 16	23 18	11 26	40 26	12 14	25 26	21 ± 8 n = 12
1-641WF				75	130							79 69	94 91	90 ± 22 n = 6

* The transactivation activity was determined from the rate of chloramphenicol acetylation 24 hours post-transfection and expressed as a percentage of $EBNA_{1-641}$ activity. Where two values are shown, the mutant was transfected in duplicate.

§ The average activity of each EBNA1 mutant is a percentage of $EBNA_{1-641}$ activity followed by the standard deviation and number of experiments (n).

of EBNA1 is between residues 325 and 376.

To further define the sequence contributions to transactivation, the effect of small deletions within the looping domain (Δ356-362 and Δ367-376) on transactivation was also examined. $EBNA_{\Delta 356\text{-}362}$ and $EBNA_{\Delta 367\text{-}376}$ activated CAT expression at levels comparable to wildtype EBNA1 (Figure 18b and Table 5). These results suggest either that transactivation is mediated by residues 325-355 or that the repetitive sequences of the looping domain make redundant contributions to transactivation. The latter interpretation is consistent with 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).

3.5.3 The WF motif and transactivation

Although the WF motif was not expected to directly contribute to an interaction of EBNA1 with the cellular transcription machinery, the effect of the DNA binding defect caused by the WF mutation on the transcriptional activation of a CAT gene was examined. $EBNA_{1\text{-}641WF}$ demonstrated, on average, 90 % of wild-type EBNA1 tranasactivation activity (Figure 19a and Table 5). Therefore, the WF motif is not required for the activation of transcription from the FR element and the decreased DNA binding affinity associated with the WF mutation is not sufficient to disrupt binding to the FR *in vivo*.

3.5.4 The proline loop and transactivation

Proline-rich sequences have been shown to activate transcription in at least one protein (Kim and Roeder, 1994). In order to determine if the proline loop of EBNA1 contributes to transactivation activity, the proline loop mutants were tested for their

ability to transactivate the CAT reporter gene. $EBNA_{1\text{-}641PL1}$ gave variable results but, on average, had 66 % of wild-type activity (Figure 19a and Table 5). $EBNA_{1\text{-}641PL2}$ consistently transactivated CAT expression at lower levels than $EBNA_{1\text{-}641}$ and, on average, had 21 % of wildtype activity (Table 5). To determine if the apparent lack of $EBNA_{1\text{-}641PL2}$ activity might be due to low protein expression, ECL and ECF Western blots were performed (Figure 19b,c). Quantitative ECF Western blots showed that $EBNA_{1\text{-}641PL1}$ and $EBNA_{1\text{-}641PL2}$ were expressed at levels that were approximately 3-fold lower than $EBNA_{1\text{-}641}$ and $EBNA_{1\text{-}641WF}$ (Figure 19c). The results suggest that low protein expression, coupled with the DNA binding defect of $EBNA_{1\text{-}641PL2}$ protein, might be responsible for the lack of activity associated with the PL2 mutation. An attempt was made to increase the level of $EBNA_{1\text{-}641PL2}$ protein in the cell by doubling the amount of expression construct transfected, however, this approach did not result in an increase in the level of protein expressed (data not shown).

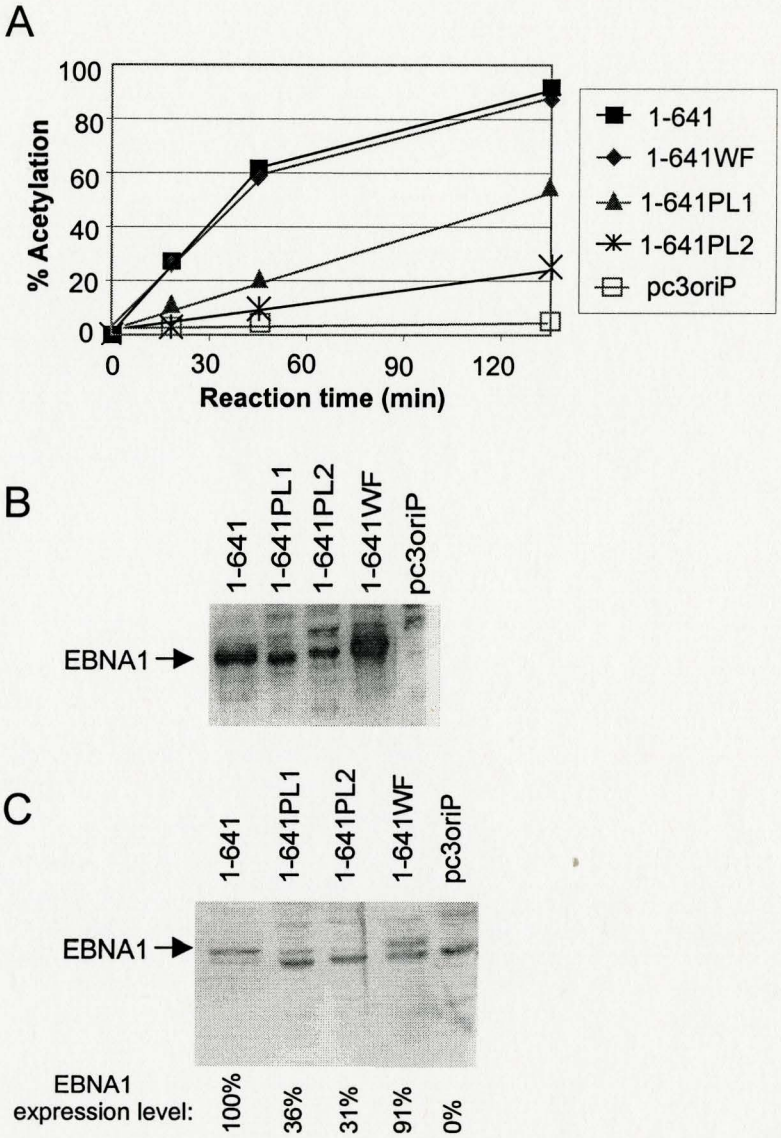


Figure 19 Transactivation assay of the EBNA1 proline loop and WF mutants. *OriP* plasmids encoding $EBNA_{1\text{-}641}$, $EBNA_{1\text{-}641PL1}$, $EBNA_{1\text{-}641PL2}$, $EBNA_{1\text{-}641WF}$ or no EBNA1 (pc3oriP) were used to transfect C33A cells. Following 24 hours in culture, a cellular lysate was prepared and 50 μg of protein was assayed for CAT activity The percent of acetylated chloramphenicol at various time points was plotted (A). Western blots for EBNA1 expression were performed on 30 μg of cellular protein and visualized by ECL (B) and ECF (C). The expression level of each mutant, expressed as a percentage of $EBNA_{1\text{-}641}$ level, is shown below panel C.

4.0 DISCUSSION

In efforts to understand the mechanisms by which EBNA1 activates transcription and governs the replication and segregation of *oriP* episomes, a functional analysis of the EBNA1 protein was undertaken. A series of mutations and deletions within four regions of EBNA1 were examined in this thesis and a summary of the results is shown in Table 6. Three different functional outcomes were observed among the various EBNA1 mutants. First, no significant effect on EBNA1 function was observed upon removal of the acidic tail region or mutation of the WF motif. A role for the acidic tail remains undefined while the WF motif contributes to the DNA binding affinity of EBNA1. Second, the arginine and glycine-rich looping domain was not required for the replication of *oriP* plasmids but removal of this EBNA1 region resulted in a loss of plasmid maintenance and transcription activation functions. Third, replacement of the entire proline loop compromised all three EBNA1 functions that were examined but also reduced the DNA binding affinity and expression level of EBNA1. The functional significance and mechanistic implications of each region of the protein examined in this thesis will be discussed individually in the sections that follow.

4.1 The Acidic Tail is not Required for EBNA1 Function

The acidic tail comprises the C-terminal 21 amino acids of EBNA1. Previous studies have reported that this region is not required for the replication of *oriP* plasmids in primate or human cells (Polvino-Bodnar and Schaffer, 1992; Yates and Camiolo, 1988). Residues 608 to 619 that link the DNA binding domain and acidic tail have not been previously examined for a contribution to EBNA1 function. I have shown that

$EBNA_{1\text{-}607}$ retains the ability to replicate *oriP* plasmids in human cells (Table 6). Therefore, the region of EBNA1 between residues 608 and 641, including the acidic tail, is not required for DNA replication and suggests that interactions between EBNA1 and cellular proteins that initiate DNA replication at *oriP* can occur in its absence.

In addition to the ability to replicate DNA, $EBNA_{1\text{-}607}$ also maintained *oriP* plasmids in a population of human cells under selection, indicating that the acidic tail was not required for the segregation function of EBNA1 (Table 6). This finding is in contrast to results reported by Yates and Camiolo (1988) that suggested the acidic tail was important for plasmid maintenance activity. The experiments were performed under similar conditions, and therefore, the discrepancy may be due to differences in the position of the EBNA1 C-terminal truncation. The EBNA1 acidic tail mutant used by Yates and Camiolo extended to amino acid 619 whereas the mutant I studied was truncated at amino acid 607. Biochemical studies subsequent to the functional analysis of Yates and Camiolo have shown that the DNA binding and dimerization domain of EBNA1 exists between amino acids 459 to 607 (Ambinder et al., 1991) and this finding influenced our selection of the C-terminal truncation site used in this study. The presence of amino acids 608 to 619 has no effect on the DNA binding affinity of EBNA1 (Summers et al., 1996) but may have inhibited the plasmid maintenance function of EBNA1 by interfering with protein-protein interactions required for DNA segregation. Alternatively, the exposure of residues 608 to 619, caused by the removal of the acidic tail, may have destabilized the protein or decreased the expression level of EBNA1 in the cell.

Table 6. Activity summary of EBNA1 mutants

EBNA1 Protein	Transactivation *	Transient Replication*	Plasmid Maintenance*
1-641	100 (n=13)	100 (n=9)	100 (n=9)
1-607	89 ± 14 (n=5)	58 ± 23 (n=4)	134 ± 63 (n=5)
377-641	13 ± 7 (n=5)	34 ± 26 (n=5)	0 ± 0 (n=4)
377-607	9 ± 4 (n=4)	nd†	nd
452-641	4 ± 3.5 (n=4)	nd	nd
Δ325-376	2.5 ± 0.8 (n=4)	95 ± 54 (n=4)	1.6 ± 1.5 (n=3)
Δ41-376	6 ± 5 (n=4)	47 ± 14 (n=4)	0 ± 0 (n=3)
Δ356-362	91 ± 31 (n=6)	nd	71 ± 32 (n=3)
Δ367-376	125 ± 13 (n=6)	nd	98 ± 27 (n=3)
1-641PL1	66 ± 35 (n=10)	58 ± 48 (n=8)	159 ± 43 (n=6)
1-641PL2	21 ± 8 (n=12)	13 ± 8 (n=5)	0 ± 0 (n=4)
1-641WF	90 ± 22 (n=6)	120 ± 57 (n=7)	173 ± 109 (n=4)
no EBNA1 (pc3oriP)	0•	4.2 ± 4 (n=9)	0 ± 0 (n=9)

* The activity of each protein is listed as a percentage of the value obtained for $EBNA_{1-641}$ followed by the standard deviation and number of experiments (n).
† not determined
• transactivation activity obtained without EBNA1 was subtracted from the values obtained for each EBNA1 mutant.

Many transcription factors contain acidic transactivation domains, and the acidic tail was suggested to be important for this function of EBNA1 (Ambinder et al., 1991). The activation of transcription by EBNA1, however, was not significantly compromised by the removal of residues 608 to 641 (Table 6). Additionally, the acidic tail was not able to restore transcriptional enhancer activity upon removal of the N-terminal region of EBNA1 (compare $EBNA_{377\text{-}641}$ and $EBNA_{377\text{-}607}$ in Table 6). Therefore, the acidic tail does not constitute an acidic transactivation domain since this region was neither necessary nor sufficient for the activation of gene expression. This finding is in agreement with three additional studies that reported similar results with deletions that focussed on the C-terminal 21 residues of the acidic tail (Kirchmaier and Sugden, 1997; Polvino-Bodnar and Schaffer, 1992; Yates and Camiolo, 1988).

Despite the lack of apparent function, three lines of evidence suggest that the acidic tail is at least partially exposed and thus capable of mediating interactions with cellular proteins. First, limited protease treatment of the EBNA1 DNA binding domain (amino acids 450-641) generated a smaller protease-resistant fragment that remained DNA-bound and mapped between amino acids 459 and 617 (Shah et al., 1992). The susceptibility of the acidic tail to protease cleavage is consistent with the exposed and potentially flexible nature of this region. Second, the EBNA1-DNA crystal structure revealed that Pro607, the C-terminal residue in the EBNA1 fragment, is exposed and forms a hydrogen bond with Gly542 of the proline loop. The proximity of the EBNA1 DNA binding domain C-terminal residue and the proline loop suggests that the acidic tail is positioned along the exterior surface of the DNA binding domain. Third, the

hydrophilic nature of the acidic tail suggests that it would be on the outer surface of the protein as opposed to the hydrophobic interior of the dimerization domain.

The conservation of the acidic tail C-terminus among EBNA1 homologues of related lymphocryptoviruses (Blake et al., 1999; Yates et al., 1996), suggests that this region of the protein contributes an important function that was not examined in this thesis. Possible roles for the acidic tail are as follows: First, there is reason to believe that EBNA1 plays a direct role in cellular immortalization by EBV and the acidic tail might contribute to this process. Second, EBNA1 expression results in increased levels of some cellular gene products such as CD25, RAG1 and RAG2 (Kube et al., 1999; Srinivas and Sixbey, 1995). Although the mechanism of cellular gene activation is not known, it could involve the acidic tail. Third, the acidic tail may contribute to EBNA1's ability to avoid proteosomal processing. This lack of processing is important because it enables cells expressing EBNA1 to evade cytotoxic T-lymphocyte responses (Rickenson et al., 1996). Fourth, the acidic tail may increase the solubility and/or expression level of EBNA1 within human cells as has been observed with the EBNA1 proteins expressed in *E. coli*. Fifth, EBNA1 negatively regulates transcription from the Qp promoter (Sample et al., 1992) and the acidic tail may play a role in this process.

4.2 The Functional Contribution of the Looping Domain

4.2.1 The looping domain is a transcriptional activation domain

The results in this thesis demonstrated that the looping domain of EBNA1 is required for the activation of transcription from the FR element of *oriP* (Figure 20). Removal of amino acids 325-376 reduced transcriptional activation to background levels (see $EBNA_{\Delta 325\text{-}376}$ in Table 6). The importance of the looping domain and associated

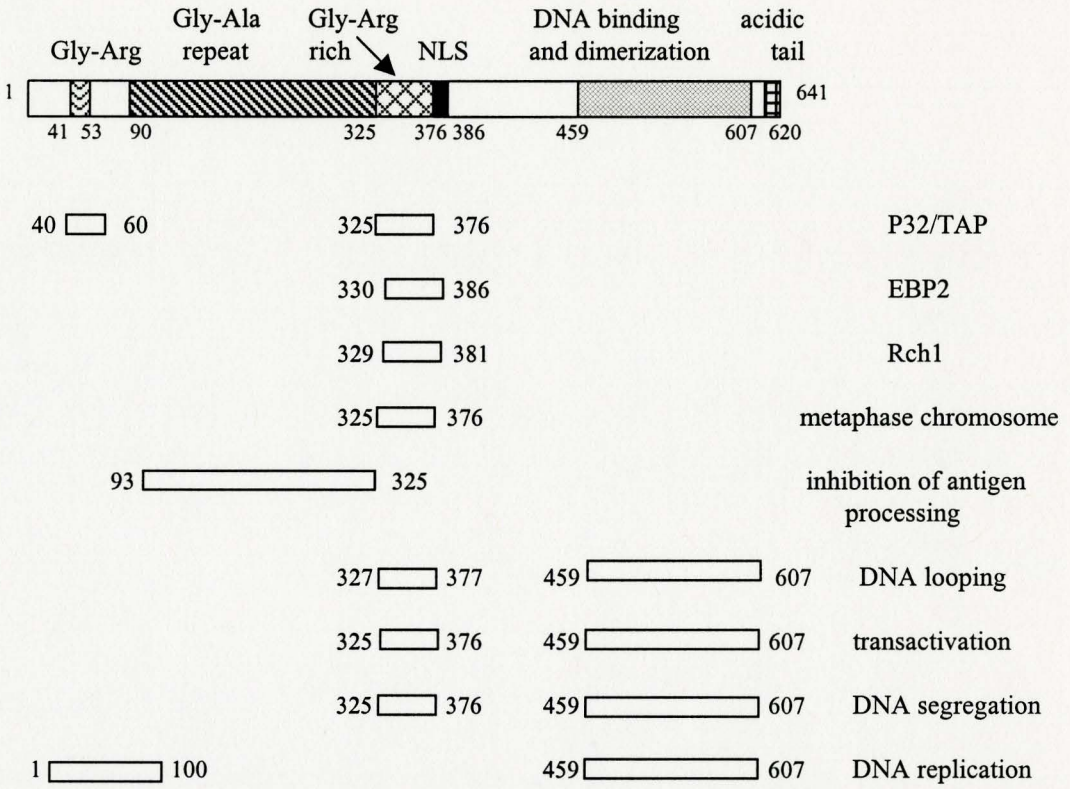


Figure 20. Summary of known protein interactions and functional domains of EBNA1 Amino acid numbers are indicated for the regions of EBNA1 required to mediate interactions with P32/TAP (Chen et al., 1997), EBP2 (Shire et al., 1999) and Rch1 (Kim et al., 1997). Association with metaphase chromosomes requires amino acids 328 to 376 (Marachel et al., 1999; Wu et al., submitted), inhibition of EBNA1 processing requires amino acids 83 to 325 (Levitskaya et al., 1995) and DNA looping requires residues 327 to 377 in addition to the DNA binding domain of EBNA1 (Avolio-Hunter and Frappier, 1998; Mackey and Sugden, 1995). EBNA1 functional activities (transactivation, DNA segregation and DNA replication) all require the DNA binding and dimerization domain in addition to the regions defined in this thesis.

looping activity to the activation of transcription has also been investigated by Mackey and Sugden (Mackey and Sugden, 1999). They reported that transcriptional activation occurs in the absence of residues 325 to 376 upon duplication of an N-terminal region of EBNA1 (amino acids 41 to 89) that possesses weak looping activity (Mackey and Sugden, 1999). Amino acids 41 to 89, however, are not functionally equivalent to the looping domain since their deletion from EBNA1 that contains the looping domain reduced transcriptional activation to 60 % of wildtype levels (Mackey and Sugden, 1999). Furthermore, no significant difference in transcriptional activation was observed in the presence or absence of residues 41 to 89 (compare $EBNA_{\Delta 325\text{-}376}$ and $EBNA_{\Delta 41\text{-}376}$ in Table 6). Therefore, the looping domain is the predominant transactivation domain within EBNA1.

Since it has been shown previously that the deletion of amino acids 325 to 376 does not disrupt the DNA binding ability of EBNA1 (Avolio-Hunter and Frappier, 1998), the looping domain is thought to mediate protein-protein interactions thereby tethering cellular proteins to the DNA. The looping domain may function by mediating interactions between FR-bound EBNA1 molecules and components of the basal transcription machinery or with cellular proteins that facilitate the remodeling of chromatin at the promoter. Indeed, several studies have demonstrated that the looping domain mediates 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; Mackey and Sugden, 1997) as well as interactions with at least two cellular factors, EBP2 and P32/TAP (Shire et al., 1999; Wang et al., 1997). The interactions with EBP2 and P32/TAP are not likely to be responsible for the transcriptional activity of EBNA1.

EBP2 is a nucleolar protein and evidence suggests that the interaction with EBNA1 is important for DNA segregation (see section 4.4.2). Although a published report suggests that P32/TAP binds to EBNA1 through the looping domain (Figure 20), our lab has shown that P32/TAP binds to the $EBNA_{\Delta 325\text{-}376}$ mutant that does not activate transcription (Wang et al., 1997; Holowaty and Frappier, unpublished) and therefore, the ability to bind P32/TAP does not correlate with the transcriptional activation function of EBNA1.

Although the EBNA1 looping domain is required for transcriptional activation, it is not known if the looping domain is sufficient for function when combined with the DNA binding domain of EBNA1 or a heterologous DNA binding domain. I attempted to address this issue by fusing the looping domain to the GAL4 DNA binding and dimerization domain and measuring the activation of transcription of a reporter gene under control of the GAL4 binding sites. Activation of transcription was not detected, however, I was unable to detect expression of the fusion proteins and thus, the results were inconclusive.

Part of the transactivation activity associated with EBNA1 may be due to an increased nuclear uptake of FR-containing plasmids (Langle-Rouault et al., 1998). The increased uptake may involve the interaction of EBNA1 with the nuclear import factor Rch1/importin-α. EBNA1 has been shown to directly interact with Rch1 (Figure 20) and the binding was reduced upon removal of the looping domain (Kim et al., 1997). This might suggest that the looping domain is required for the nuclear uptake of *oriP* plasmids, however two lines of evidence indicate that this is not the case. First, the looping domain deletion supports DNA replication activity and therefore must enter the

nucleus. Second, recent microscopy studies in our lab have shown that the Δ325-376 mutant is nuclear.

Studies suggest that acidic transcriptional activator proteins recruit histone acetyltransferases as a mechanism to remodel nucleosomes and activate transcription (Wang et al., 2000). Recent results in our lab suggest that purified EBNA1 is able to destabilize nucleosomes that are positioned on multiple EBNA1 binding sites *in vitro* (Avolio-Hunter and Frappier, unpublished) and that the EBNA1 looping domain interacts with at least one chromatin remodelling factor (Holowaty and Frappier, unpublished). Further investigations will determine if nucleosome destabilization and interactions with nucleosome remodelling factors are required for the transcriptional activation function of EBNA1.

4.2.2 The looping domain is not required for DNA replication

Although residues 325-376 of EBNA1 play a critical role in the activation of transcription, I have shown that deletion of the looping domain had no significant effect on the transient replication activity of EBNA1 (see $EBNA_{\Delta 325\text{-}376}$ in Table 6). The finding that the looping domain is not required for DNA replication is consistent with the results of Kim et al (1997), who showed that a similar EBNA1 mutant lacking amino acids 328 to 374 supported transient replication. Therefore the transactivation and replication activities of EBNA1 are separable and likely occur by different mechanisms. I have found that larger deletions that removed additional residues N-terminal to the looping domain reduced the replication efficiency of EBNA1 ($EBNA_{\Delta 41\text{-}376}$ and $EBNA_{377\text{-}641}$ in Table 6). The Gly-Ala repeat between 101 and 324 was not present in any of the EBNA1

constructs used in this thesis, thus, residues between amino acids 1 to 100 contribute to DNA replication (Figure 20).

The DNA looping interaction that occurs between EBNA1 molecules on the FR and DS elements has been shown to stabilize EBNA1 binding to the DS element and therefore, was thought to be important for the activation of replication from the DS (Frappier et al., 1994; Su et al., 1991). Our lab has shown that $EBNA_{\Delta 325\text{-}376}$ has little or no DNA looping activity *in vitro* (Avolio-Hunter and Frappier, 1998), and yet this mutant is active for replication. This suggests that DNA looping activity is not required for the replication of *oriP* plasmids under the experimental conditions. Alternatively, it is possible that the DNA looping interaction is important for replication from the DS but, under *in vivo* conditions, looping can be mediated by EBNA1 residues outside of amino acids 325 to 376. Indeed, amino acids 41 to 89 possess some limited capacity to link DNA (Avolio-Hunter and Frappier, 1998; Mackey and Sugden, 1997).

Mackey and Sugden reported that looping activity is important for DNA replication since the removal of either residues 41 to 89 or the looping domain reduced *oriP* replication to less than 25 % of wildtype EBNA1 (Mackey and Sugden, 1999). The looping domain mutation investigated by Mackey and Sugden, however also deleted residues 1 to 40 of EBNA1. Therefore, while their findings suggest that both regions of EBNA1 that possess looping activity contribute to replication, the results in this thesis suggest that residues 325 to 376 are not required for this EBNA1 function. Furthermore, the experimental conditions used by Mackey and Sugden differ from the methods described in this thesis and may contribute to the difference between the findings. In their DNA replication assays, two plasmids were used to transfect human cells; one contained

oriP and was monitored for DNA replication while the other plasmid expressed EBNA1 protein but lacked a functional origin and segregation element. Thus, the EBNA1 expression plasmid is not replicated or maintained in the cell by an active partitioning system and will become diluted among the dividing cells. The resulting decrease in EBNA1 protein over the four day period in culture might have affected the replication efficiency determined by Mackey and Sugden (1999).

4.2.3 The looping domain is required for plasmid maintenance

Although fully active for DNA replication, the looping domain mutant is unable to maintain *oriP* plasmids in long-term culture, indicating that this mutant is defective in DNA segregation (see $EBNA_{\Delta 325\text{-}376}$ in Table 6). This finding may explain the results of Mackey and Sugden (1999) who reported that the looping domain was important for DNA replication. In their transient replication experiments, *oriP* plasmids were recovered from human cells four days post-transfection. With increasing lengths of time in culture, the plasmid maintenance defect of the looping domain mutant would contribute to the reduction in the number of *oriP* plasmids recovered from cells. As a result, the contributions of the looping domain to replication is complicated by the effects on plasmid segregation.

EBNA1 is thought to govern the segregation of *oriP* plasmids by mediating their attachment to a component of the host chromosomes. In keeping with this model, EBNA1 looping domain sequences have been shown to be important for the attachment of EBNA1 to mitotic chromosomes (see Figure 20) (Marechal et al., 1999; Wu, Ceccarelli and Frappier, submitted). Immunofluorescence staining of cell lines expressing EBNA1 and $EBNA_{\Delta 325\text{-}376}$ revealed that both proteins were nuclear and

therefore the defect in plasmid maintenance of $EBNA_{\Delta 325\text{-}376}$ was not due to failure to enter the nucleus (Wu, Ceccarelli and Frappier, submitted). Unlike wildtype EBNA1, however, $EBNA_{\Delta 325\text{-}376}$ did not associate with condensed host chromosomes in mitosis. This observation provided further support for the EBV segregation model and for an essential role for the looping domain in this function of EBNA1.

Recent studies suggest that the component of the host chromosome to which EBNA1 attaches is the cellular factor EBP2 (Shire et al., 1999). EBP2 binds to the EBNA1 looping domain sequences and appears to colocalize with EBNA1 on mitotic chromosomes (Shire et al., 1999; Wu, Ceccarelli and Frappier, submitted). The binding of EBP2 to chromosomal DNA does not require EBNA1, suggesting that EBP2 may mediate the noncovalent attachment of EBNA1 to the mitotic chromosomes (Shire et al., 1999; Wu, Ceccarelli and Frappier, submitted).

The looping domain consists of six imperfect repeats of an eight amino acid sequence (Laine and Frappier, 1995). Two EBNA1 mutants, with small deletions that each remove a single repeat region of the looping domain (Δ356-362 and Δ367-376), retain plasmid maintenance activity. $EBNA_{\Delta 356\text{-}362}$ and $EBNA_{\Delta 367\text{-}376}$ interacted with EBP2 and the immunofluorescence staining pattern of both proteins on condensed chromosomes was indistinguishable from wildtype EBNA1 (Shire et al., 1999; Wu, Ceccarelli and Frappier, submitted). These results suggest that either the repetitive arginine and glycine-rich sequence of the looping domain contains redundant function, or that amino acids 325 to 355 are specifically required for the plasmid maintenance activity. Further mutations within this region, including the examination of an EBNA1

mutant lacking amino acids 325 to 355, would increase our understanding of the requirements for functional activity of the looping domain.

4.3 The WF Motif Contributes to the DNA Binding Affinity of EBNA1

The WF motif is within the DNA binding and dimerization domain of EBNA1 and consists of two aromatic amino acids that are conserved in the EBNA1 homologues of related lymphocryptoviruses (Blake et al., 1999; Yates et al., 1996). The phenylalanine and tryptophan residues are positioned in the minor groove of EBNA1-bound DNA and the aromatic rings appear to push the backbones of both DNA strands apart since the minor groove is widened by 2-3 Å at this region (Bochkarev et al., 1996). Although the WF motif is not required to induce permanganate sensitivity in susceptible EBNA1 binding sites, it was thought that these residues might promote origin melting and therefore be important for the initiation of replication from *oriP* (Summers et al., 1997). However, I found that mutation of W464 and F465 to alanine residues ($EBNA_{1\text{-}641WF}$), had no effect on transcriptional activation, DNA replication or the maintenance of *oriP* plasmids. Therefore, any distortion of the minor groove generated by the WF motif does not appear to be required for origin melting.

An unexpected finding of the WF mutation was the effect on DNA binding affinity. Mutation of the WF residues within the context of the EBNA1 DNA binding and dimerization domain reduced the affinity for a single EBNA1 recognition site 17-fold relative to the wildtype EBNA1 domain. The reduction in DNA affinity was not due to the unfolding or decreased stability of the protein since the CD spectra of $EBNA_{452\text{-}641WF}$ were indistinguishable from those of $EBNA_{452\text{-}641}$ under native and denaturing conditions (Figure 10 and Table 2).

The EBNA-DNA co-crystal structure, when combined with biochemical DNA binding studies using EBNA1 point mutants, suggests that EBNA1 binds DNA by a two-step mechanism (Cruickshank et al., submitted). Residues of the recognition helices make the first DNA contacts and position EBNA1 so that residues 461 to 469 of the flanking domain can be loaded into the minor groove of the DNA, enabling amino acids 461, 463 and 469 to make a total of five sequence-specific contacts. The WF sequence falls within the minor groove extended chain and, according to the co-crystal structure, these residues do not make hydrogen bonds with the DNA. The apparent DNA binding affinity of the WF mutant was similar to that of an EBNA1 truncation mutant ($EBNA_{468\text{-}607}$) lacking most of the minor groove extended chain (Bochkarev et al., 1996; Summers et al., 1996). My results, therefore, suggest that the WF motif plays a role in orienting the extended chain residues within the DNA minor groove, thereby enabling the side chains to form the base contacts. Kinetic binding studies that examine the association and dissociation rates of $EBNA_{452\text{-}641WF}$ would be useful to further investigate the role of W464 and F465 in the DNA binding process. A co-crystal structure of $EBNA_{459\text{-}607}$ with the WF mutation would show if this mutation disrupts base contacts in the minor groove and if the WF motif is responsible for widening of the minor groove.

4.4 The Proline Loop Contributes to DNA Binding

The proline loop of EBNA1 extends outward from the DNA binding and dimerization domain and does not directly contact DNA (Figure 5). Both the exposed positioning of the loop and its proline-rich sequence, which resemble proline-rich transcriptional activation domains of proteins such as hBRM (the human homologue of the yeast SNF2/SWI2 gene product) and human I-rel (Bours et al., 1994; Muchardt and

Yaniv, 1993), suggested that it might mediate interactions with other proteins and contribute to EBNA1 function. I have found that the DNA binding affinity of EBNA1 was not affected by the replacement of residues 545 to 549 with a flexible linker (PL1), but was affected by replacement of sequences 541 to 553 with the same four amino acid linker (PL2) (Table 1). The decreased length of the loop was not expected to disrupt the structure of the entire DNA binding domain, as the loop length in the PL2 mutant is identical to that of the structurally homologous loop of the E2 papillomavirus protein.

CD analyses of the proline loop mutants showed that the mutants were folded (Figure 9). However circular dichroism spectra are not sensitive to subtle changes in the tertiary structure and small differences in the EBNA1 DNA binding domain conformation may only be detected by a high resolution structure. Therefore, the possibility remains that some subtle conformational changes were caused by the proline loop mutations. I also examined the stability of the PL1 and PL2 mutant proteins under denaturing conditions. These experiments were performed to determine whether the mutations destabilized the dimerization of EBNA1. The structure of the EBNA1 dimerization interface is such that dissociation of the dimer must be accompanied by protein unfolding (Mok et al., 1996a; Mok et al., 1996b) and, therefore, the stability of the protein in denaturant reflects the tendency for the monomers to dissociate. The stability of the EBNA1 proline loop mutants in guanidine, although somewhat less than for wildtype EBNA1, was very high as compared to that of other proteins under similar conditions. The EBNA1 PL mutants required 5 to 6 M guanidine to unfold (Table 2), while the single domain proteins barstar, xylanase and lambda terminase unfold between 1 and 3 M guanidine (Hanagan et al., 1998; Ramachandran and Udgaonkar, 1996;

Roberge et al., 1998). Thus, the unfolding and disruption of the PL1 and PL2 dimers is unlikely to be a factor under the zero guanidine conditions of the DNA binding and functional assays.

In the EBNA1-DNA co-crystal structure, proline loop residues were not observed to directly contact the DNA (Bochkarev et al., 1996). This observation is consistent with the wildtype DNA binding affinity of the PL1 mutant (Table 1). The PL2 mutations, however, removed amino acids 541 to 553 and reduced the affinity for individual EBNA1 binding sites 14-fold. This suggests that interactions mediated by these residues are important for the high affinity binding to DNA. Four residues adjacent or within the proline loop mediate interactions that might play a role in DNA binding (Bochkarev et al., 1996). Arg538 is positioned just N-terminal to the proline loop and makes an electrostatic interaction with the phosphodiester backbone of DNA. Glu556 is C-terminal to the proline loop and interacts with a residue of the flanking domain from the other monomer (Arg469) that makes two sequence-specific interactions with the DNA minor groove. The Glu556-Arg469 interaction appears to be important for DNA binding because a point mutation of Glu556 to alanine reduces DNA binding affinity approximately 400-fold (Shire, Flemming and Frappier, unpublished data). Although Arg538 and Glu556 are not removed in the PL2 mutant, it is possible that the change in the length and sequence of the proline loop introduced in this mutant affected the positioning of these neighbouring residues.

Gly542 and Pro553 of the proline loop, which are deleted in the PL2 mutant but not in the PL1 mutant, form hydrogen bonds with residues Arg532 and Leu533 that are adjacent to the EBNA1 recognition helices. Recent mutational analyses of the recognition

helices indicate that these helices play an important role in DNA binding by contacting the major groove (Cruickshank et al., submitted). It is possible therefore that the interaction between the proline loop and residues 532 and 533 are important for the proper positioning of the recognition helices in the major groove and accounts for the decreased DNA binding activity of the PL2 mutant. The EBNA1 crystal structure also showed that Gly542 of the proline loop forms a hydrogen bond with Pro607, which is located in a flexible extended chain that joins the DNA binding domain to the C-terminal acidic tail (Bochkarev et al., 1996). Disruption of this interaction by the PL2 mutation might alter the positioning of the acidic tail relative to the DNA binding domain. This could result in decreased DNA binding affinity by increasing steric hinderance or other interference by the acidic tail or by the residues joining the DNA binding domain to the acidic tail.

4.4.1 A functional role for the proline loop remains undetermined

$EBNA_{1\text{-}641PL1}$ was found to replicate DNA and maintain plasmids at or above wildtype levels and to activate transcription at levels close to wildtype EBNA1 (Table 6). Therefore, the proline-rich sequence at the tip of the proline loop is not required for DNA replication, segregation or transactivation activities of EBNA1. However, the PL1 mutant still contained some of the proline-rich sequences of the loop which could be sufficient to mediate functional interactions with cellular proteins. The PL2 mutation, which removes all of the proline loop sequence, was therefore constructed. $EBNA_{1\text{-}641PL2}$ did not support plasmid maintenance and exhibited only low levels of transient replication and transactivation activities (Table 6). The reduced DNA binding affinity of the PL2 mutant protein (see Table 1) and defect of each EBNA1 function suggested that the PL2 mutant

might not be bound to the *oriP* sequences *in vivo*. The DNA binding affinity of the PL2 mutant was found to be approximately the same as the WF mutant which was fully active for all three EBNA1 functions. Therefore, if the PL2 mutant protein was expressed at similar or higher levels as the WF mutant, we would expect that PL2 should be bound to *oriP* in the functional assays. Quantitative Western blots revealed that, during the period of time cells were cultured for transactivation and transient replication assays, $EBNA_{1\text{-}641PL2}$ was expressed at only 4 to 34 % of the level of $EBNA_{1\text{-}641WF}$. This finding suggests that the compromised functionality of $EBNA_{1\text{-}641PL2}$ might be due to a failure to bind the recognition sites in *oriP*. However, since I did not directly determine whether the *oriP* sequences were bound by the PL2 mutant *in vivo*, it is still possible that the PL2 mutant was bound to *oriP* and that the PL2 mutation disrupts functional protein interactions or cellular localization.

Both $EBNA_{1\text{-}641PL1}$ and $EBNA_{1\text{-}641PL2}$ were expressed at levels, on average, 33 % and 23 % of wildtype EBNA1 respectively. Since all EBNA1 constructs were transcribed from the same CMV promoter, variations in protein expression were not thought to be due to differences in the level of mRNA transcripts although this possibility has not been tested directly. The EBNA1 PL1 and PL2 mutant proteins might, however, have a decreased solubility (only soluble cell fractions were examined in Western blots) or decreased half-life in the cell due to an enhanced sensitivity to proteases and/or ubiquitination-mediated degradation. The half-life of EBNA1 mutants could be examined by performing pulse-chase labeling of EBNA1 in transfected cells.

Elevated levels of $EBNA_{1\text{-}641PL2}$ protein were detected in cells from the plasmid maintenance assays and could be due to an effect of cellular growth under selective

conditions. Since *oriP* plasmids were not recovered in extrachromosomal form from cells expressing $EBNA_{1\text{-}641PL2}$, plasmid sequences including the EBNA1 open reading frame and the neomycin-resistance gene must have integrated into a chromosomal locus. The integration event may have increased transcription from the CMV promoter (relative to that in the plasmid) due to the action of adjacent chromosomal transcriptional enhancer elements or to other aspects of chromosomal context.

4.5 Future Directions

While the looping domain is the major transcriptional activation domain within EBNA1, it remains to be determined if residues 325 to 376 are sufficient for this function. N-terminal truncations could be examined in order to determine if sequences between residues 1 to 100 of EBNA1 contribute to transcriptional activation along with the looping domain. While the looping domain is distinct from the EBNA1 DNA binding domain, the ability to transfer the transcriptional enhancer function of the looping domain to a heterologous DNA binding domain has not been demonstrated. The EBNA1-GAL4 fusion constructs should, therefore, be re-examined for transcriptional activation function. EBNA1 looping domain sequences could also be fused directly to the EBNA1 DNA binding domain and tested for transcriptional activation.

To better understand the mechanism by which the looping domain contributes to transcriptional activation, interactions between the EBNA1 looping domain and components of the RNA polymerase II transcriptional machinery can be examined by biochemical approaches. Both EBNA1, $EBNA_{\Delta 325\text{-}376}$ and GAL4-EBNA1 looping domain fusion proteins could be useful to investigate specific interactions between the EBNA1 transactivation domain and basal transcription factors including TFIIB and

TFIID. Recently, a cellular chromatin remodeling factor, Nap1, that binds $EBNA_{1\text{-}641}$ and not $EBNA_{\Delta 325\text{-}376}$ was identified by affinity chromatography (Walter et al., 1995; Holowaty and Frappier, unpublished). This finding suggests that transcriptional activation by EBNA1 might involve chromatin remodeling. The functional significance of the EBNA1-Nap1 interaction could be examined by performing *in vitro* chromatin remodeling assays on *oriP* nucleosomes.

Further mutational analysis of the looping domain sequences is necessary to map the exact residues that are important for transactivation and segregation activities. Deletion mutants lacking all or part of the 325 to 355 region will be useful for determining whether these sequences are responsible for the transactivation and segregation activities or whether the repeated sequences of the looping domain have redundant functions. The results of these mutational analyses are also of interest as they will determine whether the amino acid requirements for transactivation and segregation are the same or different.

My studies have only examined how EBNA1 transactivates gene expression when bound to the viral FR element, but data suggests that EBNA1 can also activate the expression of cellular genes. The transcriptional activation of cellular genes in response to EBNA1 could be examined by the use of DNA microarray technology. mRNA could be isolated before and after induction of EBNA1 or EBNA1 mutant protein expression in human cells and labelled with fluorescent probes. The altered transcription pattern of cellular genes upon EBNA1 induction could then be monitored by hybridizing microarrays of human cDNAs with the labelled RNA. This approach would be useful for identifying cellular targets of EBNA1 transactivation as a second strategy to identify

transcriptional activation domains of EBNA1.

The mechanism by which EBNA1 activates the replication of *oriP* plasmids has not yet been elucidated but recent studies in our laboratory suggest that it might involve chromatin remodeling. A role for EBNA1 in chromatin remodelling at *oriP* is suggested by two lines of evidence. First, Tina Avolio-Hunter, in our laboratory, has shown that EBNA1 destabilizes nucleosomes formed on the DS element of *oriP*. Second, Melissa Holowaty found that EBNA1 binds to a chromatin remodeling factor previously shown to be required for the replication of adenovirus chromatin (Matsumoto et al., 1993; Holowaty and Frappier, unpublished).

By analogy to the role of the origin recognition complex in *S. cerevisiae*, EBNA1 might also recruit cellular initiation proteins, such as cdc6 and MCMs, to the origin. Screens for physical interaction with EBNA1 have failed to isolate cdc6 or MCM proteins but this does not rule out the possibility that weak or transient functional interactions occur in the human cell nucleus. Possible functional interactions between EBNA1, cdc6 and MCMs will be assessed in the laboratory by determining the effect of the purified proteins on the melting of *oriP* sequences, the first step in DNA replication.

Although the segregation defect associated with the looping domain mutant of EBNA1 was inferred from its inability to maintain *oriP* plasmids in human cells, a direct quantitative assay for DNA segregation is lacking. The long-term *oriP* plasmid maintenance assay is not an ideal way of measuring segregation because plasmid maintenance is also dependent on the replication function of EBNA1. Therefore, the segregation activity of EBNA1 mutants that are defective for DNA replication cannot be assessed. Methods to separate the replication and segregation of plasmids in mammalian

cells are currently being explored. One strategy involves the generation of a plasmid that is duplicated from a replication origin that is unrelated to *oriP* but depends on the EBV segregation system (i.e. the FR element and EBNA1) for efficient partitioning. Plasmids containing the papillomavirus minimal origin replicate in rodent cells that express the E1 and E2 papillomavirus proteins but are not maintained in long-term culture due to the lack of the segregation element. A plasmid containing the papillomavirus minimal origin, the EBV FR element and an EBNA1 expression cassette has been generated in our laboratory and is being tested for replication and long term maintenance in hamster cells that express E1 and E2. If successful, this approach would allow EBNA1 mutants to be tested for their ability to partition the FR-containing plasmid in the absence of any effects of the mutations on the replicative function of EBNA1

In order to determine if the acidic tail and proline loop contribute to EBNA1 functions that were not revealed in this thesis, mutants lacking these motifs could be used to examine the interactions of EBNA1 with cellular proteins. MALDI-TOF mass spectrometry has allowed the identification of several proteins in Hela cell lysates that are specifically retained on immobilized EBNA1-affinity columns. A comparison of the proteins that bind wildtype EBNA1 and/or EBNA1 mutants could identify cellular proteins that require the acidic tail or proline loop for the interaction. The disruption of a protein interaction would provide evidence that the mutated region of EBNA1 was important for the interaction with the cellular protein and may facilitate the assignment of a functional role for the acidic tail and the proline loop of EBNA1

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