The role of the Epstein Barr Virus Nuclear Antigen-1 in the production of antibodies to dsDNA

by

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Abstract

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Previous studies have shown an association between the Epstein Barr Virus (EBV) and the development of the autoimmune disease, Systemic Lupus Erythematosus (SLE). However, it has not yet been proven that EBV plays a causative role in the etiology of SLE. In the present study, I demonstrate that mice injected with the major EBV nuclear protein, EBNA-1, can develop antibodies to double stranded DNA (dsDNA), which are the hallmark of SLE. To understand the basis for the anti-dsDNA response, I generated monoclonal antibodies (MAbs) to EBNA-1 from EBNA-1 injected mice. I made the novel observation that some of these MAbs cross-react with dsDNA. One of these MAbs, designated 3D4, was shown to bind to the glomeruli of mouse kidneys. This is a feature of the pathogenic anti-dsDNA antibodies in lupus, which can deposit in the kidney and cause renal damage (nephritis).

In an effort to map the epitope in EBNA-1 that elicits cross-reactivity to dsDNA, I generated several truncated fragments of the EBNA-1 protein and examined the binding of the cross-reactive MAbs to these fragments. All of the cross-reactive MAbs that I examined recognized an epitope that resides within amino acids 459 and 607 in the carboxyl region of EBNA-1. This 148 amino acid region is confined to the viral binding site (VBS) of EBNA-1 and contains a well-

defined secondary structure, although, it is not yet known whether the epitope is linear or conformational. We are currently trying to map this epitope further to define a smaller peptide that these MAbs recognize. Identification of a small epitope that serves as a peptide mimic for dsDNA may help in the design of diagnostic strategies for screening and therapeutic strategies for treating patients with SLE.

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Chapter 1: Introduction

1.1 Antibody Structure and Specificity

Antibodies, also referred to as immunoglobulins, are glycoproteins that bind antigens with high specificity and affinity and help the host to get rid of the antigen. Antibodies are produced and secreted by B cells. All antibodies are comprised of two identical heavy chains (HC) and two identical light chains (LC), which are bound together by disulphide bonds (Figure 1). There are five different types of H chains, μ,γ,α,δ,ε which determine the IgM, IgG, IgA, IgD and IgE class of antibody respectively. There are two different types of light chains namely-λ and κ. Each antibody uses only one type of light chain. The N terminal portion of both HC and LC form the variable region, which recognizes and binds the antigen. The C terminal portion forms the constant region, which is responsible for the effector functions of the antibody.

The gene segments that encode the HC locus are located on chromosome 12 in mice and chromosome 14 in humans. The H chain is comprised of three regions, variable (V), diversity (D) and joining (J) regions. There are multiple V, D and J segments and one segment of each region is randomly selected to rearrange. The heavy chain locus in humans has about 40 functional V_H, 25 D_H and $6J_H$ gene segments. The HC locus contains a series of C regions each of which correspond to a different isotype (1).

The H chain rearranges by creating a DJ recombination followed by a V to DJ joining. This process of VDJ recombination creates a DNA sequence encoding a complete H chain V region.

The primary mRNA transcript consists of a rearranged V DJ region. This transcript undergoes RNA splicing to juxtapose the VDJ region to the Cμ region (2). After successful rearrangement of the Ig HC gene segments, the cell commits to expression of a particular V region for its HC and excludes the HC rearrangement on the other allele. This process known as allelic exclusion ensures that each B cell makes HCs all of which contain a V region encoded by the same VDJ sequence.

This V, D, J recombination is guided by conserved non-coding DNA sequences, present at the site of recombination, known as Recombination Signal Sequences or RSS. RSS consists of a conserved heptamer followed by a non-conserved spacer region (which is either 12 or 23 bp) followed by a conserved nonamer region. The heptamer is always adjacent to the coding sequence. The recombination generally follows a 12/23 rule which means that a gene segment containing a 12 bp RSS typically joins a gene segment flanked by a 23 bp RSS.

Two important enzymes required for recombination are RAG1 and RAG2. RAG2 deficient mice lack mature B and T cells, as they are unable to undergo VDJ recombination (3). The RAG1 and RAG2 complex makes ds DNA breaks which facilitates the rest of the recombination process (4).

After the HC has successfully rearranged LC rearrangement occurs. The LC does not contain a D segment. The kappa V, J and C regions are present on chromosome 6 in mice and chromosome 2 in humans. The lambda LC locus is present on chromosome 16 in mice and chromosome 22 in humans (5, 6). A complete mRNA transcript is made by VJ recombination followed by splicing

out of the intervening region between the C region. In mice and humans the κ LC locus rearranges first. The κ locus in humans has 40 V segments followed by 5 J segments and only a single C gene whereas the λ locus consists of 30 functional V segments and 4 pairs of J and C gene segments.

After successful rearrangement of the LC on one of the alleles, resulting in a functional LC, the rearrangement on the other allele is inhibited by allelic exclusion. Hence, the cell commits to making LCs all of which contain a V region encoded by the same VJ sequence. This ensures that each B cell will produce antibodies of exactly the same specificity.

In order to protect the host from numerous pathogens, the immune system is capable of generating antibodies with different specificities. It is estimated that 10^{11} different specificities of Abs are present in the B cell repertoire (7). Ig diversity is generated by several mechanisms (8) (9). The presence of multiple V, D and J segments encoding the HC and multiple V and J segments encoding the LC contributes to the diversity (1). Random recombination of the V, D, J and the VJ segments also adds to the diversity (10). Also, imprecise joining of these VDJ segments and nucleotide additions and deletions at the joning regions expands the diversity further. At the protein level, random selection and pairing of different combinations of LC and HC provides another level of diversity. Finally, when B cells move to the periphery and participate in germinal center reactions they undergo somatic hypermutation and affinity maturation, which represents the final mechanism of antibody diversification (11).

The normal process of generating antibody diversity, sometimes can lead to the production of antibodies that have affinity to self antigens. The production of these auto-antibodies is generally down regulated by several mechanisms of tolerance which includes, clonal deletion, receptor editing and anergy (12-15).

1.2 B cell development

B cells are one of the two major types of lymphocytes. B cells express an immunoglobulin or antibody, also known as the B cell receptor (BCR) on their surface. This receptor has specificity for an antigen. When a B cell encounters a foreign antigen, it binds to the antigen via its BCR and if it also gets a signal from a T cell, then it differentiates into an antibody producing cell. The antibodies produced and secreted have the same specificity as the BCR.

B cell precursors originate in the bone marrow and complete most of their development there. The different stages of B cell development are- early pro-B, late pro-B, large pre-B, small pre-B, immature B cell and mature B cell.

The pro-B cell stage is the earliest stage of B cell development where immunoglobulin gene rearrangement begins. The heavy chain rearranges first (16). The rearrangement of D-J_H occurs at the early pro-B cell stage and V_H to DJ_H rearrangement marks the late Pro-B cell stage. When a productive HC rearrangement takes place, μ chains are expressed in the B cell in complex with a surrogate light chain. λ5 and V pre-B constitute the surrogate light chain (17). This complex known as the pre-B cell receptor is present mainly in the cytoplasm and to some extent on the cell surface also. Production of the pre B cell receptor signals successful HC gene rearrangement and enforces allelic exclusion (18).

This process stimulates the late pro-B cell to further differentiate to the large pre-B cell, which undergoes proliferation. At the small pre-B cell stage, the cell stops expressing surrogate light chain and undergoes V-JL for LC rearrangement (19). Once LC rearranges on one allele, the LC associates with the μ HC and if this is successful this complex inhibits the rearrangement of the other LC allele thereby enforcing allelic exclusion. LC recombination occurs first between Vκ and Jκ segments. If a productive rearrangement doesn`t occur on either κ allele, rearrangement occurs on λ LC allele (20).

When the light chain is successfully rearranged and pairs with the μ HC, it is expressed on the surface of the B cell and this marks the immature B cell stage of development. If the LC is not capable of pairing with the HC then the B cell dies.

Immature B cells that have no strong reactivity to self antigens are allowed to mature. The cells migrate to the periphery where they may encounter an antigen. However, if the B cell receptor exhibits reactivity to self, the development is halted and the cell undergoes regulation by clonal deletion, receptor editing, and anergy (12-15). If a lymphocyte escapes these processes, a response directed against self antigen can occur, resulting in a autoimmune diseases that could be either organ specific like diabetes and thyroiditis or systemic diseases like Rheumatoid Arthritis and SLE.

Immature B cells that have successfully passed the regulatory checkpoints in the bone marrow may undergo further maturation in the periphery. Alternative mRNA splicing produces a δ heavy

chain which leads to expression of IgD in addition to the IgM, on the cell surface, in the naïve mature B cell.

When a mature B cell comes in contact with a foreign antigen, it will bind that antigen and internalize it to the endosomal compartment. The antigen is then processed and presented by MHC Class II molecules to T helper cells. These B cells are then triggered by the T cell help, to proliferate and give rise to clones secreting the antibody of the same specificity. This process of clonal selection also known as Jerne`s hypothesis, leads to expansion and differentiation of the antigen specific B cell to either antibody secreting plasma cell or memory B cell (21).

1.3 Antigen Structure

A molecule recognized by an antibody is an antigen. Any biochemical molecule can act as an antigen such as protein, polysaccharides, nucleic acids and lipids. Amongst all these molecules proteins are most immunogenic. This is because of their large sizes and complexity they can contain multiple epitopes. Nucleic acids and polysaccharides are generally poor immunogens but they can become more immunogenic when coupled with the protein carriers. Lipids are rarely immunogenic however; immune responses to some glycoproteins and sphingolipids have been reported.

B cells recognize extracellular antigens. The part of the antigen that the antibody binds to is called antigenic determinant or an epitope. B cell epitopes are generally accessible and present on the surface of an antigen for example-terminal side chains of polysaccharides or hydrophilic amino acids on protein molecules. The B cell epitopes can be linear or conformational. Linear epitopes consist of sequential residues, while conformational epitopes are due to folding of molecules, which may bring non-sequential residues together. The size of the B cell epitope is approximately 5-7 amino acids (22).

It has been observed that antibodies with specificity to one epitope may bind to another epitope that is either sequentially or structurally similar to the first epitope. The epitope that mimics this response is known as a mimetope and the phenomenon is known as cross-reactivity. This will be discussed further in the following section.

1.4 Cross- reactive antibodies and molecular mimicry

The term molecular mimicry was coined by Fujinami and Oldstone. It is a phenomenon in which one antibody binds to two seemingly different antigens because of some similarity in linear sequence or some conformational determinant that is found in both antigens (23). Molecular mimicry can result in cross activation of autoreactive B cells by foreign pathogens. For instance, antibodies to microbial antigens such as glycolipid components of the cell wall of Mycobacterium tuberculosis, phosphorylcholine in the cell wall of Streptococcus pneumoniae or proteins in Burkholderia fungorum have been observed to cross-react with dsDNA (24-27).

Another example of cross-reactivity to self antigen is that antibodies to a Group A streptococcal antigen, cross-react with an antigen in cardiac muscle. This can result in the heart damage found in rheumatic fever (28).

Some examples of viruses that have been implicated in human autoimmune disease via molecular mimicry are Coxsackie virus and Rubella virus in Type-I diabetes. Antibodies to the Coxsackie B4 virus protein 2C have been found to cross react with the pancreatic islet autoantigen, glutamic acid decarboxylase and may play a role in the pathogenesis of insulin dependent diabetes mellitus (29). It was observed by Ou et al that antibodies to several Rubella virus determinants also cross react with the enzyme glutamic acid decarboxylase and these epitopes were also recognized at high frequencies by Type I diabetic patients (30). Another example of molecular mimicry by a virus is with HSV-1. HSV-1 leads to autoimmune stromal keratitis due to cross reactivity between a viral coat protein and corneal antigens (31).

Some other studies showed that antibodies to a variety of self proteins cross-react with double stranded DNA (dsDNA), such as antibodies to an extracellular matrix protein HP8, ribosomal P protein, elongation factor-2 (EF-2), and Sm D (32-35). Some studies have identified the peptide mimics or mimetopes of dsDNA such as DWEYSVWLSN and RLTSSLRYNP (36, 37).

It is unclear how these antigens act as molecular mimics of dsDNA. As previously mentioned, certain linear peptides or negatively charged peptides may also serve as molecular mimics for dsDNA However evidence from other studies suggest that conformational epitopes are also the targets of antibodies that cross-react with dsDNA and self proteins (38, 39).

1.5 SLE and Anti-dsDNA antibodies

SLE is an autoimmune disease characterized by the production of antibodies to double-stranded DNA (ds-DNA) and ribonucleoproteins (RNPs). These antibodies may deposit in the skin, joints, kidney, brain and other organs. The only two organs where these autoantibodies have been definitively liked to tissue damage are the kidneys and the brain (40, 41). SLE is 9 times more common in women than in men. It primarily affects women in their child bearing years (42). It is 3 times more common in African Americans, Hispanic and native American populations than Caucasians (43). The etiology of SLE is unknown, although several genetic and environmental causes have been suggested. It has been shown that there is a 25% increased risk of developing SLE among monozygotic twins as compared to approximately 2% in dizygotic twins (44). It has also been shown that inherited deficiencies of early components of the complement pathway like C1q, C2 or C4 also play an important role in development of lupus (45). Amongst the environmental factors, several viruses have been linked to SLE however; the strongest association has been made with the Epstein-Barr virus (EBV) (46-48).

Several laboratories identified the presence of antibodies to dsDNA in the sera of patients with lupus (49-52). The fact that these antibodies might lead to pathogenesis in lupus was initially demonstrated by a study in which anti-dsDNA antibodies were eluted from kidneys of patients with lupus nephritis (53).

It has been shown that active lupus is associated with the presence of IgG rather than IgM and IgA anti-dsDNA antibodies (54). Studies also indicate that the subclass of IgG anti ds-DNA antibodies may also be important in the disease. This can be due to their differing capacity to activate complement and engage Fc receptors (55).

The exact mechanism by which these anti ds-DNA antibodies can contribute to tissue inflammation and damage is not known. Some studies showed that both murine and human anti-dsDNA antibodies can penetrate living cells and interfere with the cell cycle progression and metabolism (56-58). Another possible mechanisms could be, the recruitment of inflammatory cells via the IgG Fc receptors and the activation of complement pathway (59). Recruitment of complement to the sites of immune complex deposition in the kidney can lead to inflammation and tissue damage which is the characteristic of lupus nephritis (60). However, it has also been shown that genetic deficiencies of the early components of the complement cascade can lead to a build up and lack of clearance of anti dsDNA immune complexes leading to lupus like disease (44) .

Sequence analysis of monoclonal anti-dsDNA in both mice and humans have a shown a high prevelance of arginine, asparagine and lysine residues in the CDR`s of these antibodies. The accumulation of these amino acids appear to be due to somatic hypermutation which helps to increase their antigen affinity and to promote the autoreactive B cell survival (61, 62).

1.6 Association of SLE with Epstein Barr Virus

Epidemiological studies have demonstrated a higher incidence of EBV infection and higher titers of antibodies to EBV in both young and adult lupus patients than in normal individuals. More recently James et al., observed seroconversion (IgG antibodies to EBV viral capsid antigen) in 99% of adolescent SLE patients compared to 70% of normal adolescents and 72% of adolescents with other rheumatic diseases (63-68). In addition, PCR analysis revealed the presence of EBV DNA in lymphocytes of 100% of SLE patients tested, compared to 72% of controls. In another study, IgA antibody against the EBV viral capsid protein was noted to be associated with recurrent SLE disease activity (flare) in adult patients in Taiwan (69). McClain et al. observed that antibodies to a major EBV nuclear antigen, Epstein Barr Virus Nuclear antigen-1 (EBNA-1), which is continuously expressed in latently infected B cells, arose in all of pediatric SLE patients tested compared to only 69% of healthy pediatric controls (70).

Although a direct causal relationship between EBV and SLE has not been found, independent case studies have reported the onset of lupus either concurrently or immediately following infection with EBV suggesting a possible association between EBV and SLE. Several studies have reported that antibodies to EBNA-1 obtained from sera from SLE patients recognize different epitopes on EBNA-1 than antibodies from sera of healthy individuals exposed to EBV suggesting that lupus patients have an altered immune response to EBNA-1. The dominant epitope recognized by normal sera is the gly-ala repeat located in the middle of EBNA-1,

whereas 30% of lupus sera predominantly recognize epitopes in the amino and carboxy terminal regions of EBNA-1 (Figure 2) (70-72).

1.7 Epstein Barr Virus

EBV was discovered in 1964 while studying electron micrographs of cells cultured from Burkitt`s lymphoma patients (73). It is a dsDNA γ-herpes virus that infects 90-95% of the adult population in the US (74). Viruses of the herpes family share a common structure. A single viral particle known as a virion consists of a glycoprotein envelope, a tegument layer containing viral proteins and mRNAs, and a protein capsid enclosing the linear dsDNA genome (75). EBV, also known as the human herpes virus (HHV4), primarily infects B lymphocytes by binding to the CD21 receptor on the surface of B cells through gp350, a major viral envelope glycoprotein. It also engages HLA class II molecules through another glycoprotein, gp42 as a co-receptor. EBV also infects epithelial cells through less efficient and yet undefined pathways (76, 77).

EBV can establish a lytic infection, which results in the production of infectious virions that bud off the host cells. Alternatively, it can remain latent within B cells allowing it to be dormant in the host for years. EBV DNA is carried as multiple extra chromosomal copies known as viral episome in the transformed B cells. Limited sets of viral latent proteins are constitutively expressed in all infected B cells. The latent proteins include six nuclear antigens (EBNA-1, 2A, 2B, 3B, 3C and LP) and three latent membrane proteins (LMPs 1, 2A and 2B). Additionally, small non polyadenylated RNAs, EBER1 and EBER2 are also detected in EBV infected B cells (78). Epstein Barr Virus nuclear antigen-1 (EBNA-1) binds to the Ori P of the EBV and controls the replication of the viral genome. It also helps in the maintenance of viral latency in the infected cells. EBNA-2 blocks B cell differentiation and promotes proliferation of EBV infected cells (79). Latent membrane proteins LMP1 and LMP2A provide the infected B cell with

survival and growth signals by mimicking the functions of CD40 and the B cell receptor (BCR) respectively. LMP2A interacts with Lyn kinase, through its immunoreceptor tyrosine based activation motifs (ITAMs) and hence mimics signaling via the BCR (80, 81). These EBV transformed B cells then migrate to the germinal centers and differentiate into the memory B cells. These latently infected memory B cells express very low levels of costimulatory molecules which enables them to persist but prevent them from being activated by T cells to secrete antibody (82).

Despite its significant transforming capacity, EBV causes tumors in only a few of the infected individuals (83). EBV has been linked to wide range of malignancies and various autoimmune diseases. Some diseases associated with EBV are infectious mononucleosis, Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma, Systemic Lupus Erythematosus and Rheumatoid Arthritis (73, 84-86). Two types of EBV have been identified; EBV 1 and EBV 2, based on differences in the DNA sequence of their latent genes. EBV 1 is more prevelant in western countries whereas both EBV 1 and EBV 2 are found in Africa and Papua New Guinea (87). This might be one of the reasons for the different types of diseases associated with EBV. In addition, differences in diet and other environmental factors like the presence of endemic diseases in a particular region might also account for differences in EBV manifestation in different geographical locations. The ability of EBV to infect and transform resting B cells is thought to be a major reason for this virus to cause Burkitt's lymphoma and Hodgkin's lymphoma whereas nasopharyngeal carcinoma is a cancer resulting from infection and transformation of epithelial cells in the pharynx. EBV has also been linked to autoimmune diseases like SLE and Rhuematoid Arthritis because of the presence of high titers of anti-EBV

antibodies, defective T cell responses and the presence of EBV DNA in the infected cells of these patients (83, 88).

1.8 Epstein Barr Virus Nuclear Antigen (EBNA-1)

EBNA-1 is a 69.7 KDa, DNA binding protein that controls the replication of the EBV virus within infected cells. It is also required to maintain viral latency. The protein sequence consists of three chromosomal DNA binding sites CBS1 (aa 72-84), CBS 2 (aa 328-365) & CBS 3 (aa 8-67). These sites allow EBNA-1 to interact with the host cell DNA. The other major domain in EBNA-1 is the Viral DNA binding/ dimerization site (aa 459-607) that allows EBNA-1 to interact with viral DNA. One other feature of this viral protein is a glycine-alanine repeat that spans from amino acids 90-328. The gly-ala repeat has been shown to interfere with antigen processing and MHC Class 1 presentation and to inhibit EBNA-1, mRNA translation (89, 90).

EBV DNA is very rarely integrated into the host cell genome and is mostly carried as an episome in latently infected cells (91). Hence, EBV requires a way of replicating viral DNA before mitosis and distributing episomes into progeny cells during cell division. EBNA-1 initiates replication by binding as dimers to the viral origin of replication OriP from its carboxyl end and via its chromosomal binding sites to the host cell chromosome (78). In addition to its role in viral replication, EBNA-1 binds to the EBV latency promoter and trans activates it. Thus, EBNA-1 also has a role in the regulation of expression of latency genes and in the maintenance of viral latency.

EBNA-1 is expressed in all EBV-associated tumors and EBV positive cells in healthy EBV carriers. EBNA-1 residues that mediate viral DNA binding and dimerization have been localized

to the C-terminal portion of the protein between amino acids 459-607. Crystal structure of this C-terminal portion reveals that this region is comprised of two closely associated domains, referred to as the core domain and the flanking domain. The core domain (amino acids 504-604) forms a dimerizing interface and also plays role in the sequence specific DNA interactions. The flanking domain (amino acids 461-503) also mediates base contacts with the DNA. Thus, both the core and flanking domains of EBNA-1 play direct roles in DNA recognition (92, 93).

1.9 Background Studies

Several laboratories have observed that antibodies to epitopes on EBNA-1 cross-react with epitopes on Sm, a ribonucleoprotein complex consisting of a core of polypeptides (B/B`, D, E, F, G). Sm B/B` and Sm D are specific targets of some SLE sera. A study by Sabbatini et al. demonstrated that antibodies to Sm D could be generated in mice immunized with a gly-arg rich peptide derived from the amino terminal end of EBNA-1 (94). Another study reported by James et al revealed that antibodies to Sm B/B` could be elicited in rabbits and mice following immunization with a proline rich peptide in the carboxy end of EBNA-1 (95). More recently, Poole et al showed that rabbits and mice injected with the proline rich peptide of EBNA-1, subsequently develop antibodies to U1 ribonucleoproteins, RNP A and RNP C as a consequence of epitope spreading (96).

Antibodies that recognize another peptide in the amino end of the EBNA-1 protein (amino acids 58-72) have more recently been shown to cross react with a peptide in the ribonucleoprotein, Ro (amino acids 169-180) that has no primary sequence homology to EBNA-1, although the peptides have similarly high isoelectric points (97). Following the immune response to each of these peptides, antibodies to additional intramolecular epitopes as well as other components of the spiceosomal complex and also to ds-DNA have been found to develop. These autoantibodies have been postulated to arise as a consequence of epitope spreading. Epitope spreading is a mechanism by which an immune response initiated by a particular autoantigen spreads to include responses directed against a different part of the same protein (intramolecular spreading) or a different protein in the complex (intermolecular spreading) (98). These studies suggest that

exposure to EBNA-1 following EBV infection, can lead to a cross reactive autoimmune response in some individuals which may play a role in SLE disease etiology. In addition to this, immunization of animals with cross-reactive peptides from Sm B/B', Sm D1 or Ro, have been shown to elicit antibodies that cross react with EBNA-1 (88, 99-101).

Previous studies in our laboratory demonstrated that BALB/c mice immunized with an expression vector (pcEF) expressing complete EBNA-1 protein or EBNA-1 lacking most of the gly-ala repeat (pcΔGA) developed antibodies to dsDNA and Sm in addition to EBNA-1. Mice injected with the empty expression vector (pcDNA3) had no response. The anti-EBNA-1 and anti-dsDNA response was found to be strain specific as DBA/2 mice were unable to produce an antibody response to ds-DNA and EBNA-1 (102). It was assumed that the antibodies to Sm arose because of cross-reactivity with EBNA-1 as previously reported, however, the basis for the anti-dsDNA response was unknown.

The present study was undertaken to examine whether the mice injected with rEBNA-1 protein elicited anti-dsDNA antibodies. I also wanted to understand the basis of this anti-dsDNA response. This work demonstrates that BALB/c mice injected with rEBNA-1 protein elicited an anti-dsDNA response and antibodies elicited in response to EBNA-1 may cross-react with dsDNA.

Chapter 2: Materials and Methods

2.1 Extraction, Purification and Characterization of rEBNA-1

The EBNA-1 baculovirus expression vector used in this study was a generous gift from Dr. Lori Frappier (McMaster University, Ontario, Canada). Recombinant EBNA-1 protein (rEBNA-1) expressed by a baculovirus expression vector was islolated from SF9 insect cells according to Lori Frappier (personal communication and modifications of Frappier and O'Donnell) (103). This vector encodes an EBNA-1 protein that has a deletion of most of the Gly-Ala repeat and has a 6 X His tag on the N-terminus, which allows for the protein's isolation on a Ni^{2+} affinity column (Figure 3B). SF9 cells were grown in serum-free insect cell culture medium (Sf-900 II SFM Invitrogen, Carlsbad, CA) at 27°C. Cells were resuspended at a concentration of $1x10^6$ cells per ml and 100 ml of cells (1.0×10^8 cells total) were infected with 500 µl of high titer ($5x10^7$ pfu) recombinant EBNA-1 baculovirus and grown in 500 ml Erlenmeyer flasks (Corning, Acton, MA) at 27°C in an air shaker for 60 hours. The cells were then harvested by centrifuging at 2000 rpm at 4°C for 10 minutes. The cell pellets were resuspended in 25 ml of a hypotonic buffer (20 mM HEPES pH 7.8, 1 mM $MgCl_2$) containing protease inhibitors (1 mM PMSF and 10 µM leupeptin) and allowed to swell on ice for 10 minutes. Cells were then dounced 20 times on ice and centrifuged at 4°C at 3000 rpm for 10 minutes. Supernatant was discarded. The pellet containing intact nuclei was resuspended in 25 ml of hypotonic buffer containing 2.7 ml of 5 M NaCl. After douncing on ice to open the nuclear envelope, the fraction was centrifuged at 18,000 rpm for 20 minutes and the supernatant containing rEBNA-1 protein was collected. Further purification of rEBNA-1 was performed employing a nickel agarose (Ni^{2+}-NTA) (QIAGEN, Valencia, CA) column according to modifications of Ceccarelli and Frappier (104). Ni^{2+}-NTA

agarose (1ml) was equilibrated in column buffer (0.2 M HEPES pH 7.8, 0.5 M NaCl, 10% glycerol) containing 5 mM imidazole, at room temperature. The nuclear extract was incubated with pre-equilibrated Ni^{2+}-NTA at room temperature for 2 hours, with rocking. After incubation, a column was packed with the nuclear extract/Ni^{2+}-NTA slurry. The column was washed slowly with column buffer containing 5 mM imidazole followed by column buffer containing 25 mM imidazole. Next, the rEBNA-1 protein was eluted with column buffer containing 300 mM imidazole. The protein was then concentrated and the buffer exchanged with PBS, 250 mM NaCl using an Amicon Centrifugal filter with a 10,000 molecular weight cut off (Millipore, Billerica, MA). The protein was then resolved by 12% SDS-PAGE followed by a Western blot and immunostaining with a monoclonal antibody to EBNA-1.

2.2 Injection of mice with rEBNA-1 protein

All animals were handled in strict accordance with good animal practice as defined by federal and state policies set forth by The Public Health Service Policy on the Humane Care and Use of Laboratory Animals (PHS 1986) and The USDA Animal Welfare Act (CFR 1985). All work done with animals in this study, was approved by The Institutional Animal Care and Use Committee (IACUC) at The City College of New York.

Twenty five, six week old, female BALB/c mice were used for injection studies. Five mice were injected intraperitoneally (ip) with 50 µg of rEBNA-1 protein in complete Freund's adjuvant (CFA) (Sigma, St Louis, MO) in a 1:1 (v/v) ratio and boosted at weeks 3 and 9, with 25 µg of rEBNA-1 in incomplete Freund's adjuvant (IFA). Five mice were injected with 50 µg of EBNA-

1 complexed with 2.5 μg of calf thymus DNA (CTdsDNA) in CFA and boosted at weeks 3 and 9 with 25 μg EBNA-1 complexed to 1.25 μg of dsDNA in IFA. EBNA-1 was complexed to CT dsDNA prior to injection by incubating the protein with dsDNA for 30 minutes at room temperature. In addition, 5 mice were injected with CFA only and boosted with IFA, 5 mice were injected with 2.5μg of CTdsDNA only and boosted with 1.25 μg of CTdsDNA and 5 mice remained uninjected throughout the study. All mice were bled immediately before injection and at weeks 1.5, 4, 10, 12, 15 and 18. The sera obtained, from these mice were tested for anti-EBNA-1 and anti-dsDNA antibodies by ELISA.

2.3 Extraction and Purification of EBNAΔCBS1,2,3

E. coli colonies transformed with pLS001 were selected on Luria-Bertani (LB) plates containing 50μg/ml of kanamycin and grown at 37^{o}C in 50 ml of LB media. Plasmid, pLS001 was previously generated in this laboratory. The plasmid, pLS001, carries the encoding sequence for the carboxyl region of EBNA-1 from amino acid 365 to 641 in the pET28a expression vector. It lacks the three chromosomal binding sites and has an N-terminal His tag allowing for purification on a Ni-NTA column. Cultures were diluted in 490 ml LB and induced with 0.1 mM IPTG and grown for several hours at 37^{o}C to a final OD_{600} of approximately 0.6. Cultures were harvested and re-suspended in lysis buffer (50 mM Tris-HCl, pH 7.8, 250 mM NaCl) containing 1.0 mM PMSF. Cells were sonicated for 15 minutes on ice with a 5 second on pulse, 10 seconds off at 30% amplitude. The cell lysate was cleared by centrifugation at 10,000 rpm for 30 minutes at 4^{o}C. Two mls of Ni^{2+}-NTA beads equilibrated with lysis buffer were added to the cleared supernatant and incubated with gentle rocking at room temperature for 2 hours. The

beads (bound to the recombinant protein) were poured onto a column. They were then washed with wash buffer (50 mM Tris-HCl, pH 7.8, 250 mM NaCl, 60 mM imidazole, and 10% glycerol). The protein, EBNAΔCBS1,2,3 was eluted with 2 mls of elution buffer (50 mM Tris-HCl, pH 7.8, 250 mM NaCl, 250 mM imidazole, 10% glycerol). The eluted recombinant protein, EBNAΔCBS1,2,3 was concentrated and the buffer was exchanged with PBS, 250 mM NaCl using an Amicon Centrifugal filter. EBNAΔCBS1,2,3 was analyzed by 12%SDS-PAGE and Western Blot.

2.4 Injection of mice with EBNAΔCBS1,2,3

Five, 6 week old, female, BALB/c mice were injected with 50 μg of, EBNAΔCBS1,2,3 in CFA 1:1 (v/v) and were boosted 2 times at weeks 3 and 9 with 25 μg of EBNAΔCBS1,2,3 in IFA 1:1 (v/v). Five age and sex matched control BALB/c mice were immunized with the complete rEBNA-1 in CFA and boosted with r EBNA-1 in IFA and 5 mice were used as uninjected age matched controls. The mice were bled immediately before injection and at weeks 1.5, 4, 6, 10, and 12. The sera obtained, from these mice were tested for anti-EBNA-1 and anti-dsDNA antibodies by ELISA.

2.5 ELISAs

2.5a Detection of antibodies to EBNA-1, Sm, LPS, Proteinase 3 and BSA

Diluted serum samples from EBNA-1 injected mice, hybridoma supernatants, or purified monoclonal antibodies were tested for binding to EBNA-1, Sm, LPS, or PR-3 by ELISA as

previously described (102, 105). For the detection of antibodies to EBNA-1, LPS, Proteinase 3, and BSA, Costar plates (Corning Incorporated, Corning, NY) were coated overnight at 4°C with 5.0 μg/ml of antigen in PBS. Costar plates were coated overnight with 5.0 μg/ml of Sm (Immunovision, Springdale, AR) in 0.1M carbonate buffer for the detection of antibodies to Sm. All plates were blocked with 100μl of 1% BSA-PBS for 2 hours at room temperature. The plates were then washed three times with wash buffer (PBS +0.05%Tween-20). Samples (50μl) were then added to each well and incubated at 37°C for one hour. After washing the plates 6 times with wash buffer, 50 μl of 1:1000 dilution of goat anti-mouse IgG-alkaline phosphatase was added to each well and incubated at 37°C for one hour. The plates were washed 6 times again with wash buffer and 50μl of 4-Nitrophenyl phosphate disodium salt hexahydrate (phosphatase substrate, Sigma Aldrich, St.Louis, MO) in substrate buffer (1mM $MgCl_2$, 50mM Na_2CO_3, pH 9.8) was added to each well and O.D. at 405nm was read on a multiscan Titertek ELISA reader.

2.5b Anti ds-DNA ELISA

For the detection of antibodies to dsDNA, Immulon-2 plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with 100 μg/ml of calf thymus dsDNA (CTdsDNA) in PBS. The dsDNA solution was filtered through a 0.22 μ filter (Milipore, Billerica, MA) prior to coating plates to remove ssDNA. For the ssDNA ELISA, CTdsDNA was boiled for 5 minutes to denature the dsDNA and then immediately chilled on ice. Plates were coated with either 100 μl of the dsDNA or ssDNA. DNA was dried on plates at 37°C for 48 hours and washed with distilled water to remove any remaining salts. Then the plates were blocked with 200μl of 1%BSA-PBS for 2 hours at room temperature. The plates were then washed with wash buffer (PBS +0.05%Tween-20) three times. 100μl of diluted serum samples from EBNA-1 injected

mice, hybridoma supernatants, or purified monoclonal antibodies were then added to each well and incubated at 37°C for two hours. After washing the plates 6 times with wash buffer, 100 µl of 1:1000 dilution of goat anti-mouse IgG-AP was added to each well and plates were incubated at 37°C for one hour. The plates were then washed 6 times again with wash buffer and 100 µl of phosphatase substrate was added to each well and then O.D. 405nm was read on the multiscan Titertek ELISA reader.

2.5c Detection of antibody binding to truncated amino or carboxyl fragments of EBNA-1

Purified monoclonal antibodies were tested for binding to truncated amino (LS7 and LS8) and carboxyl fragments (LS9, $EBNA_{452-641}$, $EBNA_{459-619}$, and $EBNA_{459-607}$) of EBNA-1. ELISA plates were coated with 5.0 µg/ml of the purified, truncated recombinant proteins diluted in PBS. Subsequent steps in the ELISA were performed as described above and according to Yadav et al (106).

2.5d Isotype ELISA

ELISA plates were coated with 50 µl of a 1:1000 dilution of either unlabeled goat anti-mouse IgG1, IgG2a, IgG2b or IgG3 (Southern Biotech, Birmingham, Alabama) diluted in PBS. Plates were incubated at 37˚C for one hour and then overnight at 4^{o}C. After blocking the plates with 1% BSA-PBS, monoclonal 3D4 antibody was diluted to 1.5 µg/ml and incubated on the plates for one hour at 37˚C. The plates were then washed 6 times and then 50 µl of a 1:1000 dilution of goat anti-mouse IgG1-AP, anti-IgG2a-AP, anti-IgG2b-AP, or anti-IgG3-AP (Southern Biotech) were added to wells coated with unlabeled anti-IgG1, anti-IgG2a, anti-IgG2b, or anti- IgG3 respectively and incubated for one hour at 37°C. The plates were then washed 6 times and then

color development was measured following the addition of 4-nitrophenyl-phosphate disodium salt as substrate and plates were read at 405 nm on a Titertek Multiscan ELISA plate reader.

2.5e Quantitative ELISA

A quantitative ELISA was performed, to determine the concentration of purified monoclonal IgG or IgM antibodies in hybridoma supernatants (105). ELISA plates were coated overnight with 100µl of a 1:1000 dilution of a goat anti-mouse IgG or an IgM antibody (Southern Biotech). A commercial, purified mouse monoclonal IgG or IgM antibody (Sigma, St Louis, MO) was serially diluted 1:2, from 200 ng/ml to 3.125 ng/ml and used to generate a standard curve. Serial dilutions of monoclonal antibody (MAb) were applied to the anti-IgG or anti-IgM coated wells and the concentration of antibody was extrapolated from the standard curve. Monoclonal antibodies were detected with goat anti-mouse IgG or goat anti-mouse IgM antibody conjugated to AP followed by the addition of 4-nitrophenyl-phosphate disodium salt as substrate.

2.6 Crithidia Assay

Ready to use Crithidia slides from the CrithiDNA Anti-nDNA Antibody Test Kit (Antibodies Inc., Davis, CA), were immunostained either with mouse sera from EBNA-1 injected mice, diluted 1:50 or 3D4 (10µg/ml), 0211(15µg/ml) or 16D2 (10µg/ml). Slides were incubated in a moist, dark chamber for 30 minutes at room temperature (RT). A positive control anti-dsDNA antibody was provided with the kit. A nonspecific monoclonal mouse IgG1 antibody was used as an isotype control for 3D4 and 0211 and a IgM monoclonal antibody was used as control for 16D2 (Sigma). Next, the slides for 3D4 and O211 were extensively washed with PBS and

immunostained for 30 minutes at RT with a 1:250 dilution of biotinylated goat anti mouse IgG (Southern Biotech). This was followed by a 1:500 dilution of Streptavidin-FITC (Southern Biotech) for 30 minutes at RT. The slide for 16D2 was incubated with a 1:1000 dilution of goat anti-mouse IgM-FITC at RT for 30 minutes. Slides were then extensively washed with PBS and Prolong Gold Antifade (Invitrogen, Carlsbad, CA) was added prior to examination by fluorescence microscopy using a Nikon Eclipse microscope, model, TE 2000-S at a magnification of 40 X .

2.7 Western Blot

Proteins were analyzed by SDS-PAGE on a 12 or 15% gel and transferred to a nitrocellulose membrane using a Bio-Rad wet transfer apparatus (BioRad, Hercules, CA). After transfer, the membranes were blocked with 3% Milk-PBS for one hour at RT with shaking. After blocking the membrane was washed three times with wash buffer (PBS, 0.05% Tween-20). The blot was incubated overnight at 4^0C with MAbs 3D4 (1 µg/ml) or a commercially prepared MAb, 0211 (10 µg/ml) or MAb 16D2 (5µg/ml) diluted in PBS. The membrane was washed 6 times in wash buffer (PBS, 0.05% Tween-20). Bound MAbs 3D4 and 0211 were detected by chemiluminescence using HRP-conjugated goat anti mouse IgG (Southern Biotech) diluted 1:20,000 in PBS. Bound IgM, 16D2 was detected with HRP-conjugated goat anti mouse IgM (Southern Biotech) diluted 1:20,000. Molecular weight markers conjugated to *strep-tag* (Precision plus protein WesternC, Biorad, Hercules, CA) were detected with a 1:20,000 dilution of Strep-Tactin-HRP (Biorad). For detection, the substrate working solution was prepared by mixing equal parts of Detection Reagents 1 and 2 (Pierce, ECL Chemiluminescence Western

Blot kit, Rockford, IL). The blot was incubated with working solution for 1 minute at RT and was then exposed to an X-ray film (Kodak, Rochester New York).

2.8 Somatic Cell Fusion

BALB/c mice were immunized intraperitoneally (ip) with 50 µg of rEBNA-1 in CFA and then boosted at 3, 7, and 12 weeks with 25 µg of rEBNA-1 in IFA. The sera obtained from these mice were tested for anti-EBNA-1 and anti-dsDNA antibodies by ELISA. Three days after the last boost, mice were selected for somatic cell fusion and their splenocytes were harvested. Red blood cells were lysed from the splenic population using RBC lysis buffer (15.5 mM NH_4Cl, 1.0 mM $KHCO_3$, 10 mM EDTA pH7.2) at room temperature for 4 minutes. Splenocytes were fused to NSO cells at a ratio of 2:1 using 0.5 ml of 50 % Polyethylene glycol and were grown in HAT selection media (100µM hypoxanthine, 0.4µM aminopterin and 16µM thymidine, DME, supplemented with 20% FBS, 10% NCTC, 1% Pen-Strep, 1% non essential amino acids and 1% glutamine) as described by Iliev et al (107). Hybridoma supernatants were then tested by ELISA for the presence of anti-EBNA-1 and anti ds-DNA antibodies. Hybridomas were subloned twice on 0.4% Sea plaque agarose (Seaplaque Bioproducts, Rockland, ME) plates and were grown at 37°C in a 5% CO_2 incubator. Once the clones were of reasonable size they were picked under a light microscope using sterile pipette tips and were grown in cloning media (DME, 10% J774, 20%FCS, 10% NCTC, 1% Pen-Strep). Supernatants from these subclones were tested again for anti-EBNA-1 and anti-dsDNA antibodies by ELISA as described above.

2.9 Purification of Monoclonal Antibodies

Hybridomas producing IgG MAb were grown in serum free media (Hyclone, Logan, Utah) and were purified on a protein G Sepharose column (Gamma Bind™ Plus Sepharose ™ gel beads, Amersham Pharmacia, Uppsala**,** Sweden). Approximately 400 ml of supernatant were passed over a 0.5 ml protein G column (Biorad, Hercules, CA). The column was then washed with 20 ml of 0.01M NaH_2PO_4, 0.15 M NaCl, and 0.01 M EDTA, pH 7.0. Purified IgG antibody was eluted with 2 ml of 0.1M-glycine pH 2.5, according to the manufacturer's protocol. One ml of the eluted fraction was neutralized with 45 µl of 1M Tris-HCl. The purified antibody was dialyzed overnight in PBS and antibody concentration was determined by a quantitative ELISA.

2.10 Antibody adsorption on dsDNA-cellulose columns

Columns were packed with 0.5ml of calf thymus dsDNA-cellulose or cellulose beads (Sigma, St.Louis, MO) according to the manufacturer's protocol. The columns were washed with 10 mM Tris buffer pH 7.9 containing 1 mM EDTA. Columns were then blocked with 5% FBS-PBS overnight at 4^{o}C. A 1:5000 dilution of week 12, rEBNA-1 injected mouse sera or 5 µg/ml of MAbs, 3D4, 0211 or 16D2 were slowly loaded onto cellulose or dsDNA cellulose columns and allowed to sit for 1 hour at 4^{o}C. The flow through was collected and pre and post adsorbed sera or monoclonal antibody was tested for binding to dsDNA and EBNA-1 by ELISA and Western blot as described above.

2.11 Glomerular Isolation and Binding Assay

The slides for the glomerular binding assay were prepared according to Budhai et al (108). A C57BL/6 mouse was sacrificed and kidneys were collected in PBS in a petri dish. The kidneys were chopped and then put on a 180μm sieve followed by a 100μm sieve and then glomeruli were collected from the top of a 71μm sieve using Leffert buffer (0.1M Hepes, 0.003M KCl, 0.13M NaCl, 0.001M NaH_2PO4, 0.01M D-Glucose, pH 7.4). The glomeruli were then spun at 2300 rpm for 5 minutes at room temperature (RT). They were then digested with 100 μg/ml of collagenase IV-S (Sigma) diluted in 5 mls of Leffert buffer, for 15 minutes at 37°C and then washed with Leffert buffer. The glomeruli were then resuspended in Leffert buffer at 4000 glomeruli/ml and 10μl of the glomeruli solution were put on glass slides followed by 10μl of acetone and slides were allowed to air dry and were stored at -20°C.

The immunostaining procedure has been previously described by Chowdhary et al (109). Prior to immunostaining, the slides were thawed at RT and washed with 1X PBS for 5 minutes. Slides were then blocked with 75 μl of 10% BSA-PBS for 1hr at RT. Then the slides were immunostained with 100μl of 5μg/ml of 3D4 diluted in 1% BSA-PBS and were incubated for 45 minutes at room temperature. The slides were then washed twice with PBS followed by incubation with 1:400 dilution of goat anti-mouse IgG-FITC diluted in 1% BSA-PBS for 45minutes at RT. The slides were then washed again and then 1 drop of DAPI/OCT solution was added. The slides were then visualized using a Nikon Eclipse TE 2000-S microscope at a magnification of 10X and 40X.

2.12 PCR Amplification

The amino and carboxyl regions of EBNA-1 were generated by PCR and ultimately cloned into a pET28a expression vector, which contains a 6X His-tag at the amino end. The pLS8 expression plasmid carries the encoding sequence for the amino terminus of EBNA-1, from the initial Met residue to amino acid position 404 (LS8) and lacks virtually all of the Gly-Ala repeat (Figure 20A). It was prepared by PCR amplification of the EBNA-1 cDNA in plasmid pMRC72, which contains the EBNA-1 coding sequence that lacks the Gly-Ala repeat, using the primers EBV 7 and EBV 6 as indicated in Table1 and Figure 4 (102). The pLS7 expression plasmid also carries the encoding sequence for the amino terminus of the EBNA-1 protein, from the initial Met residue but it terminates at amino acid position 393 (LS7) and lacks the PPPGRRP epitope (aa 398-404) (Figure 20A). It was prepared from pMRC72 using the primers EBV7 and EBV5 as indicated in Table 1 and Figure 4. The pLS9 expression plasmid carries the EBNA-1 encoding sequence for the carboxyl terminus of EBNA-1 from amino acids 410 to 641 (LS9). It was also prepared from pMRC72 using the primers, EBV3 and EBV4 as indicated in Table1 and Figure 4. The primers were synthesized with a restriction site at the 5` end to facilitate cloning. The forward primers were constructed with a Nde I site and the reverse primers were constructed with a Xho I restriction site at the 5` end (Table1B, underlined).

Five truncated PCR fragments (PCR 18-22) were generated from the carboxyl end of EBNA-1 using pLS9 as a template (Figure 22B). The primer pairs used to PCR each fragment are indicated in Table 1 and Figure 4. Forward primers were constructed with a NdeI site at the 5`

end and reverse primers were constructed with a Hind III site on the 5` end to facilitate cloning into the pET28a expression vector. The restriction sites are indicated in Table 1(B).

All PCR amplifications were performed as follows: 95°C for 5 minutes, 30-35 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 54°C and elongation for 40 seconds at 72°C. This was followed by a final elongation at 72°C for 5 minutes.

2.13 Cloning of PCR fragments into plasmid vectors

The PCR products were first cloned into the PCR 2.1 TOPO® cloning vector (Invitrogen, Carlsbad, CA) which served as a holding vector from which the PCR products were sequenced (Macrogen, Rockville, MD) to check for any mutations that might have occurred during PCR. The recombinant TOPO vectors containing PCR products 7-9 were then digested with NdeI and XhoI and recombinant TOPO vectors containing PCR products 18-22 were digested with Nde1 and Hind III (Table 1B). The digested inserts and the vectors were separated on a 1% agarose gel. Inserts 7,8,9,18,19,20,21 and 22 were then extracted from the agarose gel using the Qiagen Gel Extraction Kit (Qiagen, Venlo, Netherlands) and were ligated into the pET28a expression vector (Novagen, San Diego, CA) to generate the plasmids pLS7, pLS8, pLS9, pLS18, pLS19, pLS20, pLS21 and pLS22 respectively. A100 ng of the pET vector was ligated to 0.2 pmol of insert at 16°C overnight using 10 units of T4 DNA Ligase (New England Biolabs, Ipswich, MA). These plasmids were then transformed into a non-expression cell line, XL-1 blue (Agilent Technologies, Santa Clara, California) to ensure the stability of the plasmids. The fragments were resequenced (Macrogen, Rockville, MD) to make sure the inserts were in frame and then

the plasmids were transformed into the BL21 (DE3) expression cell line (New England Biolabs, Ipswich, MA) for isolation of the recombinant proteins.

2.14 Transformation of recombinant plasmids

PCR 2.1 TOPO® cloning vector recombinants were transformed into the One Shot Chemically Competent E. coli cells (Invitrogen, Carlsbad, CA). Following the addition of 2 μl of the recombinant TOPO vector, the competent cells were heat-shocked for 30 seconds at 42°C and transformants were selected by blue-white selection on LB plates containing β-galactosidase plates. The pET28a recombinant plasmids (10 ng) were first transformed into non- expression XL-1 blue competent cells to ensure the stability of the plasmids (Agilent Technologies, Santa Clara, CA) by heat shock for 45 seconds at 42°C. The pET28a recombinant plasmids (25 ng) were next transformed into BL21 (DE3) competent cells (New England Biolabs, Ipswich, MA) by heat shock for 10 seconds at 42°C. All transformants containing pET28a recombinants were selected on LB plates containing 50 μg/ml of kanamycin.

2.15 Purification of recombinant EBNA-1 proteins from E. coli

E.coli transformants expressing the truncated EBNA-1 fragments were grown overnight in 50 ml of LB media containing 1% glucose and 50μg/ml of kanamycin. Overnight cultures were added to 490 ml of LB-kanamycin. Cells were induced with 0.1 mM IPTG and grown for 2-4 hours at 20^{o}C to a final OD_{600} of approximately 0.6. Cultures were harvested and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.8, 250 mM NaCl) containing 1.0 mM PMSF. Cells were

sonicated for 15 minutes on ice with a 4 second on pulse, 6 second off pulse at 30% amplitude. The cell lysate was cleared by centrifugation at 10,000 rpm for 30 minutes at 4^{o}C. Five mls of Ni^{2+}-NTA beads equilibrated with lysis buffer were added to the cleared supernatant and incubated with gentle rocking at room temperature for 2 hours. The beads (bound to the recombinant protein) were separated from the supernatant by centrifuging at 500 rpm for 5 minutes. They were then washed 6 times with wash buffer (50 mM Tris-HCl, pH 7.8, 250 mM NaCl, 60 mM imidazole, and 10% glycerol). Two ml of elution buffer (50 mM Tris-HCl, pH 7.8, 250 mM NaCl, 250 mM imidazole, 10% glycerol) were added to the beads and beads were rocked for 15 minutes. The beads were then removed from the reaction by centrifugation at 500 rpm for 5 minutes. Supernatants containing the recombinant protein were concentrated and the buffer was exchanged with PBS, 250 mM NaCl using an Amicon centrifugal filter. Proteins were analyzed by SDS-PAGE and Western Blot.

Plasmids (vector pET15b) expressing the following EBNA-1 amino acid sequences; $EBNA_{452-641}$, $EBNA_{459-607}$, and $EBNA_{459-619}$ were a generous gift from Dr. Lori Frappier (110). Peptides encoded by these plasmids contained an N-terminal 6X His-tag. These peptides were expressed in Escherichia coli strain BL21 (DE3) and isolated from cell-lysates as described above.

Chapter 3: Results

3.1 Mice injected with purified recombinant EBNA-1 protein develop antibodies to dsDNA

Our laboratory previously demonstrated that mice injected with an EBNA-1 expression vector developed antibodies to dsDNA in addition to EBNA-1 (102). However, not all mice injected with the EBNA-1 expression vector developed these antibodies. Presumably this was because they did not all express a sufficient concentration of EBNA-1 protein to elicit an immune response. To confirm that mice exposed to the rEBNA-1 protein could develop antibodies to dsDNA, I decided to inject them with purified rEBNA-1 protein rather than the expression vector. The rEBNA-1 used for injections was prepared from Sf9 cells infected with a baculovirus vector, expressing EBNA-1 that was obtained from Dr.Lori Frappier (McMaster University, Ontario, Canada). This rEBNA-1 lacks most of the gly-ala repeat region. The MW of the rEBNA-1 protein lacking the gly-ala repeat is approximately 52Kda (Figure 5). The high G-C content in the repeat region has made it difficult to PCR through this region and therefore made it difficult to generate a full length PCR product for cloning. It has also been shown that the gly-ala repeat is not required for the replication, transactivation or segregation functions of EBNA-1 and its presence seems to negatively correlate with a good yield of native protein (104). In addition, the gly-ala repeat has been shown to interfere with antigen processing and MHC class I presentation of EBNA-1 and to inhibit mRNA translation (89, 90).

I also wanted to determine whether a preformed complex of EBNA-1 with ds-DNA would elicit a better anti-dsDNA response than EBNA-1 alone. DNA by itself is not very immunogenic so I

wondered whether by complexing with dsDNA, EBNA-1 would render it immunogenic by acting as a carrier protein and activating EBNA-1 specific T cells. These T cells could then provide help to anti-dsDNA B cells activating them to produce anti ds-DNA antibodies. This phenomenon has been observed in the case of the polyoma T antigen. Moens et al demonstrated that the polyoma T antigen must bind to DNA in order to elicit anti dsDNA antibodies. Mutations in the DNA binding sites in the polyoma T antigen prevented it from complexing with dsDNA and thereby prevented the development of an anti-dsDNA response (111).

I injected five, 6 week old, female, BALB/c mice with 50 μg of rEBNA-1 protein alone, 5 mice with 50 μg of EBNA-1 complexed with 2.5 μg of calf thymus DNA (CT dsDNA) and 5 mice with 2.5μg of CT dsDNA only. Five mice were injected with adjuvant (CFA) only and 5 mice served as uninjected age matched controls. I observed that all 5 mice injected with rEBNA-1, developed IgG antibodies to EBNA-1 within the first 3 weeks of injection (Figure 6A). Similarly, mice injected with rEBNA-1 complexed with CT dsDNA developed IgG antibodies to EBNA-1. No anti-EBNA-1 response was observed in mice injected with CT dsDNA or CFA only or in uninjected mice. In addition, I observed that mice immunized with rEBNA-1 only and rEBNA-1 complexed with dsDNA developed antibodies to dsDNA. Interestingly, there was no significant difference in the anti-dsDNA response between mice injected with EBNA-1 alone and mice injected with EBNA-1 complexed to dsDNA (Figure 6B). This observation suggested that rEBNA-1 complexed with dsDNA does not elicit a better anti-dsDNA response than rEBNA-1 alone. The kinetics of the anti-dsDNA response lagged behind that of the anti-EBNA-1 response. This may be because the anti-dsDNA response developed over time as a consequence of either epitope spreading or somatic mutation. Some mice immunized with adjuvant only, also

developed antibodies to dsDNA but with the exception of one mouse, their levels of anti-dsDNA antibody were never as high as that of mice injected with rEBNA-1. Intraperitoneal delivery of CFA has been shown by others to elicit the production of autoantibodies in mice, including the production of anti-DNA antibodies (112). It is extremely unlikely that the anti-dsDNA response in rEBNA-1 injected mice was due primarily to adjuvant, as our previous DNA based inoculation studies using EBNA-1 expression vectors in the absence of adjuvant, also elicited the production of anti-dsDNA antibodies (102). No anti-dsDNA response was observed in mice injected with CTdsDNA only, consistent with the fact that dsDNA is a poor immunogen.

3.2 EBNA-1 lacking the three chromosomal binding sites is sufficient to elicit an anti-dsDNA response

Since EBNA-1 is a dsDNA binding protein I wondered whether subsequent to injection EBNA-1 was complexing with dsDNA released from apoptotic cells and thereby eliciting an anti dsDNA response. To address this, the laboratory generated an EBNA-1 protein lacking all three chromosomal binding sites (EBNAΔCBS1,2,3). Presumably this prevented the protein from binding to dsDNA (Figure 7A). The EBNAΔCBS 1,2,3 protein was injected into five six week old, female BALB/c mice to determine whether an EBNA-1 protein lacking the major DNA binding sites could still elicit an anti-dsDNA response.

Five age and sex matched control BALB/c mice were immunized with the complete rEBNA-1 and 5 mice were used as uninjected age matched controls. We observed that all 5 mice injected with EBNA-1ΔCBS1,2,3 developed IgG antibodies to EBNA-1 within the first 3 weeks (Figure

7B). This paralleled the response observed in mice immunized with the complete rEBNA-1 protein. Mice also developed antibodies to dsDNA (Figure 7C). At weeks 9 and 12, the anti-dsDNA response was slightly lower in mice injected with EBNA-1ΔCBS1,2,3 than in mice injected with complete EBNA-1, although the difference was not significant (p=0.32). These results suggested that EBNA-1 does not need to complex with dsDNA in order to elicit an anti dsDNA response. However, we cannot rule out the possibility that rEBNA-1 may complex with dsDNA via the Viral DNA binding site (VBS). VBS is the region of EBNA-1 protein that attaches it to viral DNA but this region may have some affinity for mammalian DNA as well.

3.3 Crithidia luciliae Assay

Since I did not observe a difference in the anti-dsDNA response in mice injected with EBNA-1 only or EBNA-1 complexed with dsDNA, I chose to conduct the subsequent studies using the sera from the mice injected with rEBNA-1 only. To confirm the specificity of the anti-dsDNA response, week 12 sera from mice injected with rEBNA-1 were diluted 1:50 and used to immunostain Crithidia luciliae slides. The Crithidia luciliae assay is a highly specific and sensitive test for detection of anti-dsDNA antibodies. The protozoan, Crithidia luciliae, has a condensed mass of dsDNA present in the kinetoplast, which is housed in the mitochondria of the organism. The kinetoplast is free of ssDNA regions and nucleoproteins, therefore the binding of sera to the kinetoplast in indicative of specific reactivity with dsDNA. In Figure 8A, I demonstrated that sera from a week 12 mouse injected with EBNA-1 bound to kinetoplasts. In contrast, sera from an adjuvant immunized mouse did not bind the kinetoplast (Figure 8B). Either the anti-DNA antibodies present in the adjuvant injected mice were of much lower affinity

than the anti-dsDNA antibodies obtained from rEBNA-1 injected mice or they were not specific for dsDNA.

3.4 Pathogenicity Studies

While all mice injected with EBNA-1 developed antibodies to dsDNA, we did not consistently observe features of clinical lupus in these mice. However, the urine of 2 out of 5 mice at 15 weeks post injection with rEBNA-1 showed significantly elevated levels of protein as detected by Uristix strips (Bayer, Leverkusen, Germany) (Table 2). The other 3 mice had protein levels that were around 100 mg/dl. None of the uninjected and adjuvant injected mice had more than trace levels of protein in their urine (Table 2). The kidneys of 3 mice injected with rEBNA-1 were examined for immune complex deposition and one of these mice had evidence of IgG complex deposition (data not shown). This mouse also had the highest level of protein (2000 mg/ml) in the urine as compared to the other EBNA-1 injected mice. However, there were no signs of lupus histopathology. No mice injected with adjuvant only developed immune complex deposition in the kidney. These results suggest that exposure to the EBNA-1 protein and the development of anti-dsDNA antibodies may lead to some kidney pathology.

3.5 Antibodies generated in response to EBNA-1 cross-react with dsDNA

In order to understand the basis of the anti-dsDNA response in mice injected with EBNA-1, I sought to explore whether antibodies to EBNA-1 cross-react with dsDNA. To begin to address this, I adsorbed week 12 sera from 5 rEBNA-1 injected mice onto dsDNA cellulose columns to

determine whether this removed both dsDNA and EBNA-1 reactive antibodies. I assayed the sera of these mice before adsorption and measured the flow through after adsorption, by ELISA. Non specific sticking of antibody to cellulose beads was measured by adsorbing sera first to cellulose only beads. Figure 9, represents the OD 405 nm pre and post adsorbed sera tested by ELISA for reactivity with DNA (A) and EBNA-1 (B), following adsorption onto dsDNA cellulose beads, after the value for non-specific binding to cellulose (~25%) was subtracted. I observed a significant decrease in anti-dsDNA antibody activity in the flow through following adsorption on dsDNA cellulose beads, in mouse 1, 3, 4, and 5 ($p < 0.001$) (Figure 9A). Similarly, I observed a significant decrease in anti-EBNA-1 activity in the sera from mouse 1, 3, 4, and 5, following adsorption on dsDNA cellulose ($p < 0.005$) (Figure 9B). Mouse 2 showed a small decrease in anti-dsDNA and anti-EBNA-1 activity following adsorption on dsDNA cellulose although it was not significant. This was likely because mouse 2 developed a negligible response to dsDNA following injection with rEBNA-1 even though the anti-EBNA-1 response was significant. The observation that the reduction in anti-EBNA-1 reactivity following adsorption on dsDNA cellulose paralleled the reduction in anti-dsDNA reactivity suggested that at least some of the anti-EBNA-1 antibodies cross-react with dsDNA. However, I did not observe a huge decrease in the anti-dsDNA or anti-EBNA-1 response in serum adsorbed over the dsDNA columns. This could be because of the fact that serum is polyclonal and the sera from EBNA-1 injected mice most likely had many different anti-EBNA-1 and anti-dsDNA antibodies, not all of which were cross-reactive. Not all of the anti-dsDNA antibodies adsorbed on the dsDNA column were likely to cross-react with EBNA-1 so many of the anti-EBNA-1 antibodies were not removed from the sera. Another way one can demonstrate cross-reactivity is by a competitive inhibition ELISA in which serum is incubated with increasing concentration of one

antigen to determine whether this inhibits binding of the antibody to the cross-reactive antigen coated on an ELISA plate. However, this was not possible to do with EBNA-1 because it is a dsDNA binding protein and preincubation of EBNA-1 with anti-EBNA-1 antibody might mask direct binding of the antibody to dsDNA coated plates but it wouldn't prevent EBNA-1 (bound to antibody) from binding to the dsDNA coated plates. Therefore, I decided to generate monoclonal antibodies from EBNA-1 injected mice and test them for cross-reactivity with dsDNA.

3.6 Generation of monoclonal antibodies to EBNA- 1 that cross-react with dsDNA

Although, the adsorption studies suggested that some antibodies to EBNA-1 generated in rEBNA-1 injected mice cross-react with dsDNA they did not prove this. To confirm this, I decided to generate monoclonal anti-EBNA-1 antibodies from rEBNA-1 injected mice and test these antibodies for binding to dsDNA by ELISA. To generate monoclonal antibodies to EBNA-1, I fused splenocytes from rEBNA-1 injected BALB/c mice that developed antibodies to both EBNA-1 and dsDNA, to NSO cells. Hybridoma supernatants were screened by ELISA for antibodies to EBNA-1 and dsDNA. Surprisingly, I observed that the majority of supernatants that tested positive for antibody binding to EBNA-1 were also positive for binding to dsDNA (10 out of 14 clones or 71%) suggesting cross-reactivity. Seven clones that secreted antibody to EBNA-1 were subcloned two times to ensure clonality. Supernatants from these hybridomas were then tested for reactivity to EBNA-1 and dsDNA. In addition, they were tested for binding to the blocking agent Bovine Serum Albumin (BSA). Three subclones, secreted monoclonal antibody that reacted strongly with both EBNA-1 and dsDNA and did not bind BSA. Subclone

3D4 is representative of this group (Table 3). One subclone, 3F3, secreted antibody specific for EBNA-1 only. Three other subclones, represented by 9G3, secreted antibody that bound not only to EBNA-1 and dsDNA but BSA as well and was more polyreactive in nature (Table 3). In another fusion, I also obtained an IgM clone, 16D2, which secreted an antibody to EBNA-1 that cross-reacted with dsDNA (Table 3). Unfortunately, some of these hybridomas either stopped secreting antibody during the subcloning or became contaminated with bacteria. However, I successfully subcloned three of them, 3D4 and 16D2, which showed high affinity to EBNA-1 and dsDNA and 9G3 which was weakly reactive to both EBNA-1 and dsDNA in addition to BSA.

I also wanted to determine whether a monoclonal anti-EBNA-1 antibody obtained from a commercial source cross-reacted with dsDNA. I therefore examined the ability of the commercial monoclonal IgG1 anti-EBNA-1 antibody, 0211 (Thermo Fisher Scientific/Pierce, Rockford, IL) to bind dsDNA and observed that indeed this antibody did recognize dsDNA. Table 3 summarizes the reactivity of the panel of representative MAbs to EBNA-1, dsDNA, ssDNA and BSA. I observed that all MAbs that bound to dsDNA also bound ssDNA with a similar affinity.

I next compared the relative binding affinity of MAbs 3D4, 16D2, 9G3 and 0211 to EBNA-1 and dsDNA by ELISA. At a concentration of 5 μg/ml 3D4, 16D2 and 0211 displayed high affinity to EBNA-1 while 9G3 displayed a much weaker affinity (Figure 10A). However, at 10 μg/ml 9G3 was significantly reactive to EBNA-1 relative to an isotype control (data not shown). At 0.625 μg/ml, 3D4 showed a higher affinity to EBNA-1 than 16D2 and 0211(Figure 10B). I also

compared the relative binding affinity of all four of these MAbs to dsDNA and observed that the affinities varied with 3D4 having the highest affinity to dsDNA and 9G3 the weakest (Figure 10C).

For subsequent studies, I chose to focus on the high affinity MAbs, 3D4, 16D2 and 0211 rather than the weaker more polyreactive antibodies such as 9G3. 3D4 and 0211 were of interest because they are both IgG anti-dsDNA antibodies, which is characteristic of lupus autoantibodies. 16D2 was of interest to me because it is an IgM antibody that binds strongly to dsDNA and I wanted to see if its specificity for EBNA-1 was similar to that if IgG antibodies. If so this might suggest that its specificity and cross reactivity was germline encoded rather than acquired in a germinal center response. I chose not to further characterize 9G3 in this study because it also bound to BSA and other blocking reagents, thereby making ELISAs and Western blots difficult to interpret. However, future studies in the lab will examine the polyreactive MAbs since they may be the precursors of more pathogenic autoantibodies seen in lupus (113).

3D4 was isolated and purified from hybridoma supernatant, on a protein G column. Following purification, 3D4 was shown to bind to EBNA-1 by ELISA at concentrations as low as 0.125 μg/ml but not to BSA or P2, a cystovirus protein isolated by Gottlieb et al (114) (Figure 11A). 3D4 was also shown to bind at concentrations as low as 0.25 μg/ml to dsDNA by ELISA (Figure 11B). Using an isotype specific ELISA, I demonstrated that 3D4 is of the IgG1 isotype (Figure 11C).

Cross-reactivity of 3D4 was further demonstrated by adsorption over dsDNA cellulose beads and then testing pre and post adsorbed MAb for binding to dsDNA and EBNA-1 (Figure 12). Adsorption over dsDNA cellulose resulted in almost complete depletion of 3D4 in the flow through as detected by an anti-IgG ELISA (Figure 12A). This was specific as evidenced by the observation that 3D4 was depleted only after adsorption onto dsDNA cellulose and not on cellulose only beads. In addition, adsorption of an IgG1 isotype control antibody on both cellulose and dsDNA cellulose beads did not result in a significant loss of IgG antibody indicating negligible non specific binding. Following adsorption of 3D4 over dsDNA cellulose, the flow through was depleted of reactivity not only to dsDNA but to EBNA-1 as well ,as detected by ELISA, confirming that 3D4 is cross reactive (Figure 12B). In addition, post adsorbed 3D4 showed reduced binding to EBNA-1 compared to pre-adsorbed 3D4, by Western blot (Figure 12C, compare lanes 2 and 4). No binding of pre-adsorbed 3D4 to BSA was observed (lane 3).

Reactivity of 3D4 with dsDNA was confirmed by its ability to recognize the dsDNA containing kinetoplasts of Crithidia luciliae (Figure 13A). An IgG1 isotype control MAb failed to bind kinetoplasts (Figure13B).

3D4 was also tested for pathogenicity by a glomerular binding assay. Glomeruli were isolated from C57BL/6 mice and adhered to glass slides according to Budhai et al (108). Slides were immunostained with 10μg/ml of 3D4 followed by goat anti-mouse IgG-FITC and were visualized by fluorescence microscopy. 3D4 was observed to bind to glomeruli suggesting

pathogenic potential (Figure 14 A and B). An IgG1 isotype control antibody showed no binding to glomeruli (Figure 14 A and B right panels).

Next, I tested MAb, 0211 at a range of concentrations, for binding to EBNA-1 and dsDNA by ELISA (Figure 15 A and B). MAb, 0211 was reactive to EBNA-1 and dsDNA and did not bind to BSA and P2. 0211 was also adsorbed onto a dsDNA cellulose column and post adsorbed antibody was almost completely depleted of reactivity with dsDNA and EBNA-1 as detected by ELISA (Figure 15 C). A reduction in binding of post dsDNA cellulose adsorbed antibody to EBNA-1 was also demonstrated by Western blot (Figure 15D, right panel, compare lanes 2 and 4). No binding of pre-adsorbed 0211 antibody to BSA was observed (lane 3). Specificity for dsDNA was demonstrated by binding to kinetoplasts on Crithidia lucilae slide (Figure 16).

MAb, 16D2 was similarly tested at a range of concentrations for binding to EBNA-1 and dsDNA by ELISA. I observed that even at concentrations as low as 0.625 µg/ml, 16D2 bound to EBNA-1 and dsDNA. (Figure 17A and 17B). Cross-reactivity of 16D2 was further demonstrated by adsorption of the purified MAb over a dsDNA cellulose column and then testing pre and post adsorbed antibody for binding to dsDNA and EBNA-1 by ELISA and Western blot. Post adsorbed 16D2 was observed to be depleted of anti-EBNA-1 and anti-dsDNA reactivity by ELISA (Figure 17C) and Western blot (Figure 17 D). Pre-adsorbed 16D2 bound to EBNA-1 (lane 2) but not to BSA (lane 3) as evidenced by Western blot while post adsorbed antibody did not bind EBNA-1 (lane 4). Reactivity of 16D2 with dsDNA was confirmed by its ability to bind kinetoplasts of Crithidia luciliae (Figure 18A). An IgM control MAb failed to bind kinetoplasts (Figure 18B).

3.7 MAbs 3D4, 16D2 and 0211 were examined for binding to a panel of antigens

MAbs 3D4, 16D2 and 0211 were also examined for binding to a panel of antigens including Sm, lipopolysaccharide (LPS), BSA, and Proteinase-3 (PR-3). Reactivity to Sm was tested because some antibodies to EBNA-1 have been shown to cross react with Sm (95). Reactivity to LPS was examined because LPS has an overall negative charge and I was interested in determining whether 3D4, 16D2 and 0211 cross-reacted with dsDNA because of a charge interaction since dsDNA also has a net negative charge (115). Therefore, I sought to test whether these MAbs would bind to other negatively charged molecules. PR-3 is the target autoantigen in Wegener's granulomatosis and antibodies to PR-3 are a subgroup of classic anti-neutrophil cytoplasmic antibodies (cANCA). At 5 μg/ml, 3D4 and 16D2 displayed negligible binding to Sm relative to BSA (Figure 19 A and B) while 0211 bound moderately well to Sm suggesting some cross-reactivity with this autoantigen as well (Figure 19 C). None of the MAb bound PR-3 or BSA. In addition, none of the MAbs bound LPS suggesting that the basis for cross reactivity with dsDNA was not due to a charge interaction.

3.8 MAbs 3D4, 16D2 and 0211 display differences in reactivity to the amino and carboxyl regions of EBNA-1

To begin to understand whether MAbs 3D4, 16D2 and 0211 recognize the same or different regions of EBNA-1, they were examined by ELISA for binding to three truncated recombinant EBNA-1 proteins, LS7, LS8, and LS9 (Figure 20). These proteins were expressed in E.coli by

pET expression vectors (pLS7, pLS8 and pLS9) and were isolated in this laboratory. These truncated proteins are comprised of the amino or carboxyl regions of EBNA-1. The protein designated LS8, consists of the amino region of rEBNA-1, from the initial Met residue to aa 404 . It lacks most of the Gly-Ala repeat but contains the PPPGRPP region in EBNA-1 (aa 398-404) that was shown by James et al to be homologous to a proline rich epitope in Sm B/B' (116). LS7 is identical to LS8 except that it terminates at aa 393 and therefore lacks the PPPGRPP epitope. The rational for generating the two amino fragments, LS7 and LS8, was to determine whether the proline rich epitope, which is responsible for eliciting cross-reactivity with Sm, is also involved in eliciting cross-reactivity with dsDNA. LS9 consists of the carboxyl region of the rEBNA-1 protein from aa 410 to the terminal aa 641. MAb 3D4 was observed to bind strongly to LS9 but not at all to LS7 or LS8 (Figure 20B). The kinetics of 3D4 binding to LS9 closely paralleled the kinetics of binding to the entire rEBNA-1 protein indicating that this carboxyl region (aa 410-641) is sufficient for optimal recognition by 3D4. This suggests that the cross-reactive epitope recognized by 3D4, is configured within the carboxyl region.

Binding of 16D2 to LS7, LS8 and LS9 paralled the 3D4 response. 16D2 bound strongly to LS9 and displayed negligible binding to LS7 and LS8, suggesting that the cross-reactive epitope recognized by 16D2 also lies within the carboxyl region (Figure 20C).

MAb 0211 was observed to bind all three truncated proteins indicating that it recognizes epitopes in both the amino and carboxyl regions of EBNA-1, however binding to LS7 and LS8 was better than the binding to the carboxyl protein, LS9 (Figure 20D). Interestingly, 0211 bound more

strongly to LS7 than LS8 and since LS7 does not contain the PPPGRPP epitope, this indicates that the proline epitope is not necessary for the binding of 0211 to EBNA-1.

Despite the fact that our results show that 0211 binds to both the amino and carboxyl fragments while 3D4 and 16D2 bind only to the carboxyl fragment, all three antibodies cross-react with dsDNA. Therefore, there could be more than one epitope in EBNA-1 that can act as a mimotope for dsDNA. Alternatively, the epitope (s) in the carboxyl region may be more important for cross-reactivity with dsDNA and since 0211 also binds Sm, the epitope in the amino region may be more important for cross-reactivity with Sm.

3.9 3D4, 0211 and 16D2 bind to a 148 aa core domain in the carboxyl region of EBNA-1 that lacks the negatively charged C-terminal amino acids.

To begin to map a smaller fragment in the carboxyl region of EBNA-1 that contains the epitope recognized by 3D4, 0211 and 16D2, I examined the binding of these MAbs to three truncated carboxyl fragments; $EBNA_{452-641}$, $EBNA_{459-619}$, and $EBNA_{459-607}$ (Figure 21A). These fragments were expressed by plasmids kindly provided to us by Dr. Lori Frappier (McMaster University, Ontario, Canada) (110). We observed that 3D4, 0211 and 16D2 bound all three fragments strongly and did not show diminished binding to these fragments relative to the entire carboxyl region ($EBNA_{410-641}$) (Figure 21 B, C and D). In fact they all, displayed strong binding to the smallest fragment, $EBNA_{459-607}$, suggesting that the cross-reactive epitope lies within this 148 aa region. The carboxyl region of EBNA-1 has a net negative charge due to the high freequency of negatively charged amino acids at the C-terminus (aa 619-641). Twelve out of 22 of the C

terminal amino acids are either glutamic or aspartic acid. Both, EBNA $_{459\text{-}619}$, and EBNA$_{459\text{-}607}$ lack these negatively charged amino acids. Since removal of these negatively charged amino acids did not diminish recognition by 3D4, 0211 and 16D2, this reaffirms that charge interaction is not the basis for 3D4's binding to EBNA-1.

3.10 Peptide mapping of the cross-reactive epitope in carboxyl region of EBNA-1

To further map the epitope in EBNA-1 that is recognized by the cross-reactive MAbs, 3D4, 16D2 and 0211, my strategy has been to generate five overlapping peptides (LS18-LS22) derived from the, smallest reactive carboxyl fragment EBNA$_{459\text{-}607}$ and determine the reactivity of the MAbs to these peptides (Figure 22A). LS18 spans from amino acids 459-558. LS19 spans from amino acids 500-607 and corresponds to the core domain of the viral DNA binding site of EBNA-1. LS20, LS21 and LS22 span from amino acids 459-502, 500-558 and 553-607 respectively. The cDNA encoding each peptide was amplified by PCR (Figure 22B) using the primer pairs indicated in Table 1 and Figure 4 and transformed first into the PCR 2.1 TOPO vector. The TOPO vector served as a holding vector where the corresponding cDNAs were sequenced to check for any mutations that might have occurred during PCR. The recombinant fragments were then cloned into the pET28a expression vector to generate recombinant plasmids pLS18-pLS22, containing inserts of the appropriate molecular weight. They were then digested with NdeI and Hind III and run on a 1% agarose gel (Figure 23). Clones were then sent out for sequencing and all of them were found to be in frame. The fragments were then transformed into the BL21 (DE3) cell line for expression of the proteins in E coli.

I recently began to express several of the truncated proteins in E.coli. Figure 24 demonstrates the induction of peptides LS18, LS19 and LS21. We have not yet examined the expression of LS20 and LS22. Studies are underway to purify these proteins and test them for reactivity with MAbs, 3D4, 0211 and 16D2. Once we determine the peptide that these MAbs recognize, we will then have overlapping 15aa peptides generated from that peptide. We will then screen our cross-reactive MAbs against these overlapping peptides in search for one that is recognized by all of the MAbs and which may serve as a peptide mimetope for dsDNA.

Chapter 4: Discussion

This study demonstrates that mice immunized with rEBNA-1 protein can develop antibodies to dsDNA (106). I observed that EBNA-1 complexed with dsDNA did not elicit a better anti-dsDNA response than mice injected with EBNA-1 only. In order to prove that EBNA-1 does not need to complex with dsDNA to produce anti-dsDNA antibodies, future studies will first need to mutate the three CBSs (CBS1,2 and 3) and the VBS of EBNA-1 making it unable to bind to DNA in vitro. Then we would have to inject this mutated rEBNA-1 protein into mice and determine whether this protein could still elicit an anti-dsDNA response or not.

This study also shows that antibodies to EBNA-1 may actually cross-react with dsDNA (106). Cross-reactivity was demonstrated by generating MAbs to EBNA-1 that were shown to bind strongly to double stranded DNA as well as to EBNA-1. One of these MAbs, 3D4 was observed to bind to mouse kidney glomeruli suggesting that at least some of these cross-reactive antibodies have pathogenic potential. The binding of anti-dsDNA antibodies to glomeruli has been shown to be an important prerequisite to the development of glomerulonephritis a major pathological feature of SLE (56). Nonspecific antibodies of identical isotype to the cross-reactive antibodes were used as controls. However, an isotype identical MAb that recognized EBNA-1 only and did not cross-react with dsDNA would have served as a better control in the glomeruli binding assay because it would have been a more accurate way to demonstrate the importance of the MAb's cross-reactivity in its deposition in the kidney. Unfortunately, such an antibody was not available at the time of the study but will be used in future studies. Also, DNAase treatment of both the glomeruli sections and MAb, 3D4, would have proved beyond doubt that 3D4 is

specifically binding to glomeruli of mouse kidney. Several of the cross-reactive monoclonal antibodies that have been characterized in this study have been shown to recognize the carboxyl region of EBNA-1. Mapping of this region has revealed that the epitope that cross-reacts with dsDNA resides within the EBV viral binding site.

It is not clear whether the cross-reactive anti-dsDNA antibodies that arise upon exposure to EBNA-1 are germline encoded or somatically mutated. I have observed that the anti-EBNA-1 antibodies arise early in the response to EBNA-1 while the anti-dsDNA response is delayed suggesting that cross-reactivity may develop over time as a consequence of somatic mutation. A specific mutation in the variable heavy and/or light chain regions of an anti-EBNA-1 antibody may alter its specificity from one that only recognizes EBNA-1 to one that recognizes dsDNA as well as EBNA-1. However, an observation that is inconsistent with somatic mutation is that one of the cross-reactive MAbs, 16D2, which I have identified, is an IgM antibody. Generally antibodies undergo somatic mutation when they undergo class switching and since this antibody has not class switched it may not have undergone somatic mutation. In future studies, we will need to sequence the heavy and light chain genes of anti-EBNA-1 MAbs obtained from the same mouse. In this way we can determine the geneology of the antibodies that cross-react with dsDNA to see if they were derived from those that bound to EBNA-1 only and arose following somatic mutation.

Another potential reason why the anti-dsDNA response was slightly delayed relative to the anti-EBNA-1 response in mice injected with EBNA-1, may be because of epitope spreading. It may be that early in the response to EBNA-1, the preferred epitopes that are targeted do not elicit a

cross-reactive response to dsDNA. Later in the response, other epitopes in EBNA-1 may be targeted as a result of intra-molecular epitope spreading and these latter epitopes may be the ones that are responsible for the cross-reactive response to dsDNA.

The two MAbs, 3D4 and 0211, characterized in this study are of the IgG1 isotype. This suggests a role for T_H2 cells in providing T cell help for the cross-reactive response to EBNA-1 however, future studies will need to identify additional cross-reactive MAbs and determine their isotype to see if the γ1 heavy chain is the preferred isotype in this cross-reactive response. Another study by Putterman et al, also showed that mice injected with DWEYS, a peptide mimic of dsDNA, lead to high titres of IgG1 anti-dsDNA antibodies (117). The major role of T_H2 cells is to help in the proliferation and differentiation of B cells into antibody secreting cells. The major cytokines released by T_H2 cells are IL-4, 5, 10 and 13. These cytokines are non-inflammatory by nature but they can lead to the production of antibodies of certain isotypes (IgG1) that may preferentially participate in antibody dependent cell cytotoxicity or antibody mediated cellular dysfunction when they bind to an autoantigen on the cell surface. This can lead to apoptosis without recruiting complement and other inflammatory mediators. This hypothesis has been supported by a study from DeGiorgio et al, which showed that anti-dsDNA antibodies could lead to apoptosis by antibody mediated perturbation of cell signaling pathways (41).

It was previously demonstrated that patients with lupus tend to mount an immune response to different epitopes on EBNA-1 than healthy individuals (70-72). While sera from healthy individuals, preferentially react with the gly-ala repeat, sera from lupus patients tend to recognize

epitopes in the amino or carboxyl terminal regions of EBNA-1. It is possible that antibodies to epitopes in these regions may be more likely to cross-react with nuclear autoantigens than antibodies to the gly ala region. It is not known whether lupus patients are genetically predisposed to developing cross-reactive antibodies or whether they have a defect in B cell tolerance leading to failed regulation of the autoreactive B cells producing these antibodies. Most of the monoclonal anti-EBNA-1 antibodies generated in this study were found to cross-react with dsDNA. Very few of them were found to recognize EBNA-1 only. This may be because mice that were selected for fusion had already developed maximal levels of cross-reactive antibodies either due to epitope spreading or somatic mutation. However, it may also be because the EBNA-1 that I used for injection lacks the gly ala repeat. In the absence of this repeat, the response may be biased towards other epitopes in EBNA-1 that may elicit a cross-reactive response.

Antibodies to dsDNA have been shown to cross-react with other self antigens. Studies have shown that anti-dsDNA antibodies that cross-react with other self antigens may deposit in different organs of the body and lead to clinical symptoms of SLE such as glomerulonephritis, and cognitive impairment. A study by Deocharan et al has shown that some anti-dsDNA antibodies cross-react with non muscle α-actinin in the kidney and can lead to lupus nephritis in mice (40). While these cross-reactive antibodies can be eluted from the kidneys of lupus mice, the exact mechanism by which they cause renal tissue damage is not known. DeGiorgio et al have reported, that both murine and human anti-DNA antibodies cross-react with NMDA receptors present in the brain (41). The extracellular domain of the NMDA receptor was found to carry the sequence DWEYS, which has previously been shown to be a molecular mimic of

dsDNA (36). NMDA receptors bind to the neurotransmitter, glutamate and play a role in brain functions such as cognition, memory and learning (118). Evidence suggests that the anti-dsDNA antibodies that cross-react with NMDA receptors in the brain may be responsible for the CNS abnormalities observed in lupus prone mice and patients with SLE (41).

Antibodies to dsDNA have also been shown to cross-react with some microbial antigens. A study by Sharma et al, has shown that human anti-dsDNA antibodies present in the sera of SLE patients, cross-react with phosphorylcholine, a component of the cell wall of *Streptococcus pneumoniae* (119). Polyreactive antibodies which bind to multiple ligands are often the first line of defense against microbial pathogens. Certain viral infections such as EBV, hepatitits, and HIV frequently elicit polyreactive antibodies (120). This is advantageous to the host because the broad reactivity of these antibodies enables them to provide initial protection from the pathogen while the more specific antibodies are developing. However, the danger of these polyreactive antibodies is that they may cross-react with autoantigens or they may be the precursors of more pathogenic autoantibodies as has been observed with autoantibodies from lupus patients (113, 121).

Anti-dsDNA antibodies have been found to cross-react with two peptide mimetopes for dsDNA; DWEYSVWLSN and RLTSSLRYNP. These peptides have previously been identified by the technique of random phage display libraries (36, 37). These peptides are recognized by a majority of SLE patient sera that have anti dsDNA antibodies. A homology search failed to find any region in EBNA-1 that is homologous in sequence to either of these two peptides thus indicating that a different peptide mimetope of dsDNA exists in EBNA-1. While the linear

amino acid sequences of these peptide mimetopes may be different, they may have some yet unknown structural similarity.

Three MAbs, 3D4, 16D2 and 0211, that bind to both EBNA-1 and dsDNA were extensively characterized in this study. Epitope mapping revealed that 3D4 and 16D2 recognize the carboxyl region of EBNA-1, while 0211 recognizes both the amino and carboxyl regions. In addition, MAb 0211 binds moderately well to Sm while 3D4 and 16D2 display negligible binding to Sm. The basis for the cross-reactivity of 0211 with Sm does not seem to be dependent on the proline rich epitope described by James et al, since 0211 binds even stronger to a truncated amino fragment of EBNA-1 lacking this determinant (116). It is not yet clear whether the epitopes in the amino and carboxyl region recognized by 0211 share homology. However, the observation that 0211 binds to both Sm and the amino fragment, while 3D4 and 16D2 bind to the carboxyl region only, suggests that an epitope in the amino fragment may be more important for cross-reactivity with Sm while an epitope in the carboxyl region may be important for cross-reactivity with dsDNA.

Further mapping of the carboxyl region has revealed that 3D4, 16D2 and 0211 bind strongly to a 148 aa fragment in the carboxyl region of EBNA-1 (EBNA-$1_{459\text{-}607}$). This region lacks the negatively charged terminal amino acids of the carboxyl region of EBNA-1. Since removal of these amino acids does not diminish binding to the carboxyl region (Figure 22), this suggests that the basis for antibody cross-reactivity with EBNA-1 and dsDNA is not charge interaction. Further support for this comes from the observation that, none of the MAbs, recognize LPS, which is a negatively charged molecule (Figure 20).

Recently, three additional MAbs have been identified in the laboratory that bind strongly to EBNA-1 only and do not cross react with dsDNA. They bind to the carboxyl fragment, LS9 (aa 410-641) but not to the more truncated fragments, $EBNA_{452-641}$, $EBNA_{459-607}$ or $EBNA_{459-619}$ (data not shown) (Figure 21). These antibodies most likely recognize an epitope that lies upstream of the truncated fragments between amino acids 410-452 or to a conformational epitope that is lost when LS9 is further truncated. These results are also consistent with the observation that the cross-reactive epitope lies within $EBNA_{459-607}$ corresponding to the viral binding site (VBS) of EBNA-1 (Figure 20). It is not known whether the cross-reactive epitope in EBNA-1 is a linear amino acid sequence or is structural. X-ray crystallography has revealed that the VBS has extensive secondary structure making it a potential target for antibody recognition (Figure 25) (122). There are two distinct domains in the VBS; a core domain that mediates protein dimerization and a flanking domain that mediates base contact with dsDNA. The core domain (aa 504-607) contains a ß barrel, α helices and a proline loop and the flanking domain (aa 470-503) contains an α helix and an extended chain (Figure 25). Potentially, the cross-reactive antibodies may recognize a portion of the αhelix that mimics the α helix in dsDNA. Alternatively, the cross-reactive epitope may reside in the proline loop (amino acids 545-553) present in the core domain. This proline loop protrudes out of the secondary structure and could be more accessible for interaction with the antibodies.

My strategy to further define the cross-reactive epitope in the carboxyl region of EBNA-1 was to generate overlapping fragments from $EBNA_{459-607}$ and examine the binding of the cross-reactive antibodies to these peptides. Although this work is still ongoing, I have made the following

predictions; if the MAbs bind to both LS18 and LS19 then this would suggest that the epitope is present in the overlapping region of these peptides, and so the antibodies should also react with LS21 (Figure 22). If the MAbs bind to LS18 and not to LS19 then this would suggest that they bind to a region upstream of the overlap so the epitope should be in the region of peptide 20. If the MAbs bind to LS19 and not to LS18, then it would suggests that the epitope is downstream of the region of overlap, which is peptide LS22. If, the MAbs do not bind to LS18 or LS19 it is possible that the MAbs recognize a structural epitope that is lost when the carboxyl region is truncated. Ultimately, the most definitive way to determine if the epitope is structural, is by X-ray crystallography of the antibody bound to EBNA-1. In this way the points of contact between the peptide and the antibody can be visualized. In future studies, we will further map the cross-reactive epitope based on the outcome of these experiments.

Identifying an epitope in EBNA-1 that is a peptide mimic for dsDNA is important because this peptide may have diagnostic and therapeutic value in SLE. This peptide can potentially be used to screen sera from healthy individuals and SLE patients to determine if there is any significant difference in their reactivity. If the sera from SLE patients preferentially reacts to this peptide while the sera from the healthy individuals does not, then this peptide can be used as diagnostic tool. This peptide can also be used to screen the sera from individuals even before the onset of lupus symptoms to determine if they are at high risk for developing lupus so that treatment strategies can be initiated early. In the long term, this peptide may also be used therapeutically to prevent cross-reactive antibodies from binding to target autoantigens and forming immune complexes, which eventually can lead to tissue damage. A similar strategy has been demonstrated by two studies in which the DWEYS, peptide mimetope, prevented the deposition

of anti-dsDNA antibodies in the kidney and brain of mice (36, 123). Once our laboratory identifies a cross-reactive epitope from the carboxyl region of EBNA-1 then this epitope will be used to screen sera from SLE patients.

In summary, this study demonstrates that exposure to the EBV protein EBNA-1, may elicit antibodies to EBNA-1 that cross-react with dsDNA. These antibodies may have pathogenic potential and lead to the manifestations of SLE. This strengthens the association between EBV and SLE and supports the role of EBV in the etiology of SLE. Mapping the epitope in EBNA-1 that elicits this cross-reactivity may be important in the design of diagnostic strategies that can aid in the prediction of those at risk for developing SLE and may facilitate the development of therapeutic strategies that may protect from the onset of SLE.

Tables

Table 1: Primers used to generate truncated rEBNA-1 fragments

A)

PCR Product	Forward Primer	Reverse Primer	Functional Peptide
7	EBV 7	EBV 5	LS7
8	EBV 7	EBV 6	LS8
9	EBV 3	EBV 4	LS9
18	EBV 12	EBV 17	LS18
19	EBV 14	EBV 15	LS19
20	EBV 12	EBV 13	LS20
21	EBV 14	EBV 17	LS21
22	EBV 16	EBV 15	LS22

B)

Primer	Sequence	Restriction Site
EBV 3	5'-CATATGGGGGAAGCCGATTATTTTGAAT-3'	Nde I
EBV 4	5'-CTCGAGTTACTCCTGCCCTTCCTC-3'	Xho I
EBV 5	5'-CTCGAGTTAAGACCCGGATGATGA- 3'	Xho I
EBV 6	5'-CTCGAGTTATGGCCTTCTACCTGG-3'	Xho I
EBV 7	5– CATATGTCTGACGAGGGGCCAGGT-3'	Nde I
EBV 12	5`- CATATGCGCAAAAAAGGAGGGTGG-3`	Nde I
EBV 13	5`-AAGCTTTTAAGTTCCTTCGTCGGTAGT-3`	Hind III
EBV 14	5`-CATATGGAAGGAACTTGGGTCGC-3`	Nde I
EBV 15	5`-AAGCTTAAGGCAAATCTACTCCATCGTC-3`.	Hind III
EBV 16	5`- CATATGCCGCTAAGGGAGTCCATT-3`	Nde I
EBV 17	5`- AAGCTTTTAAATGGACTCCCTTAGCGG-3`	Hind III

Restriction sites are underlined.

Table 2: Protein concentration in the urine of immunized mice

Immunization regimen	-	trace	30 mg/dl	100 mg/dl	300 mg/dl	2000 mg/dl
Uninjected	3/5	2/5	–	–	–	–
Adjuvant	2/4	2/4	–	–	–	–
EBNA-1	–	–	–	3/5	1/5	1/5

Table 3: Reactivity of representative monoclonal antibodies to EBNA-1, dsDNA ,ssDNA and BSA

MAbs	anti-EBNA-1	anti-dsDNA	anti-ssDNA	anti-BSA
3D4	+++	+++	+++	-
9G3	+	+	-	+
3F3	++	-	ND	-
0211 (Pierce)	++	++	++	-
16D2 (IgM)	+ + +	+ +	++	-

+++ strong binding , ++ moderate binding, + weak binding

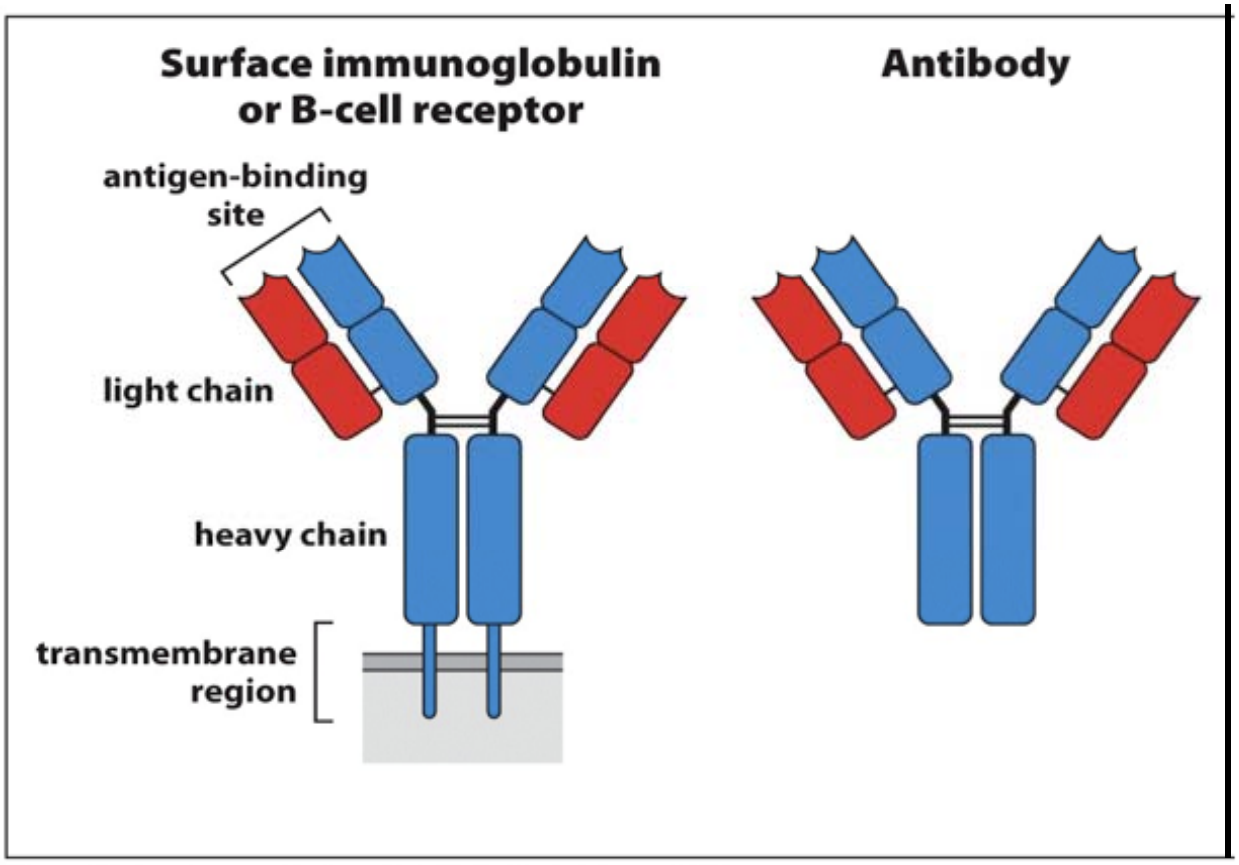


Figure 3.1 The Immune System, 3ed. (© Garland Science 2009)

Figure 1. Structure of an antibody. An antibody consists of two identical heavy and two identical light chains joined together by disulphide bonds. Antibodies can be expressed on the cell surface or can be secreted.

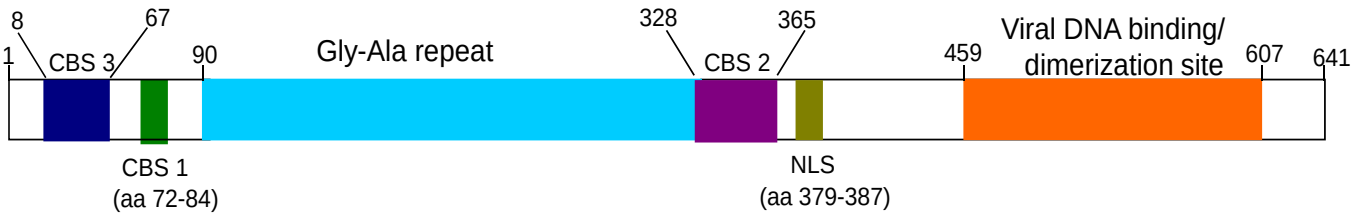
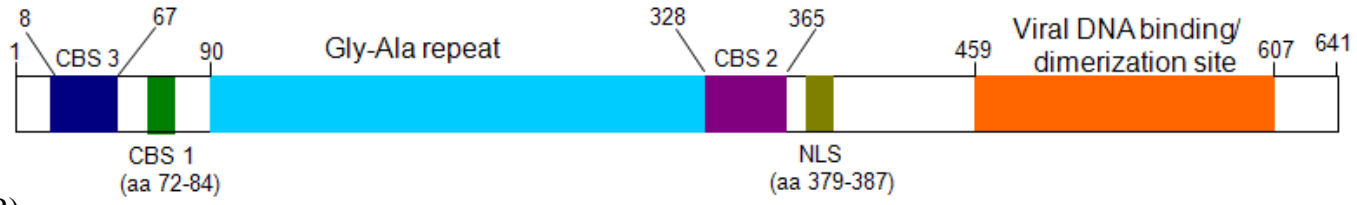


Figure 2. Functional map of the Epstein Barr Virus Nuclear Antigen-1 (EBNA-1). EBNA-1 is a 641 amino acid nuclear protein of EBNA-1. It helps in viral replication and maintenance of viral latency. The three chromosomal binding sites, CBS 1 (aa 72-84), CBS2 (aa 328-365) and CBS 3 (aa 8-67) are indicated. The Viral DNA binding site (VBS) spans from aa 459-607. The gly-ala repeat in EBNA-1 spans from aa 90-328. The Nuclear localization signal (NLS) spans from aa 379-387.

A)



B)

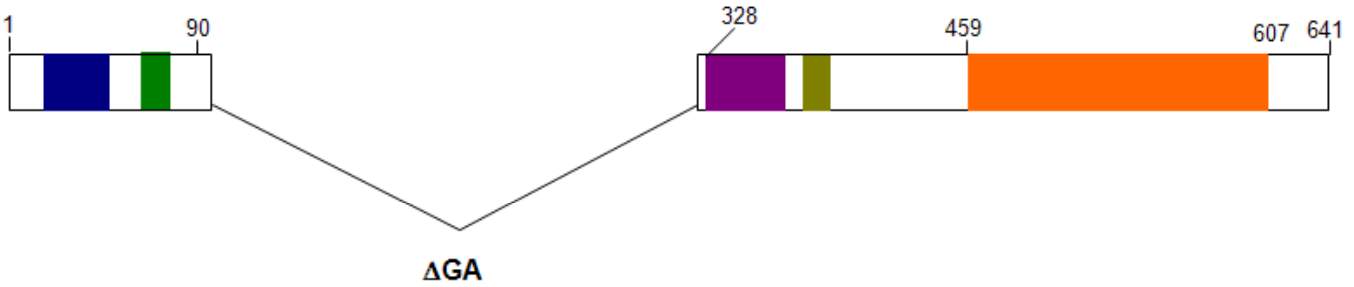
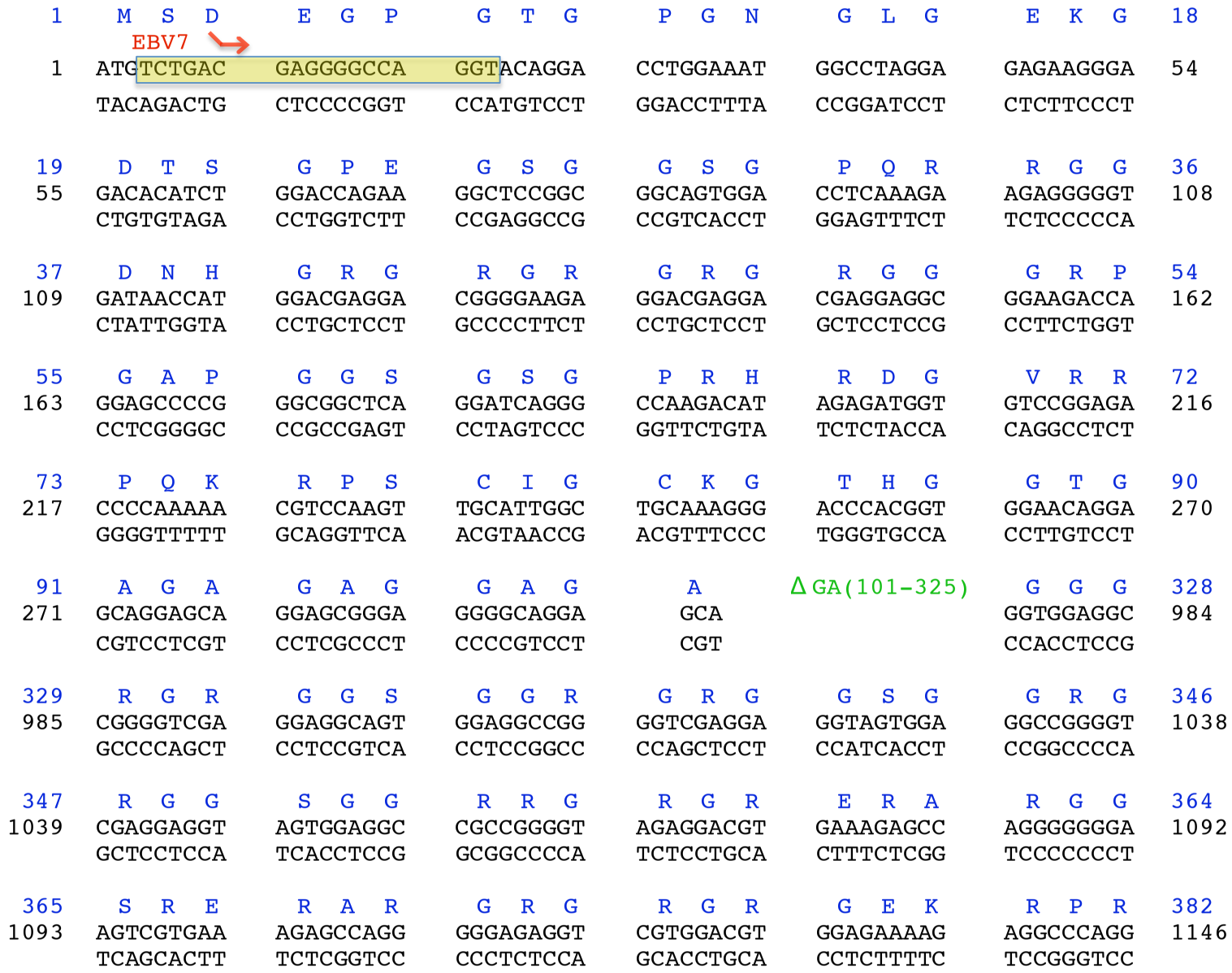
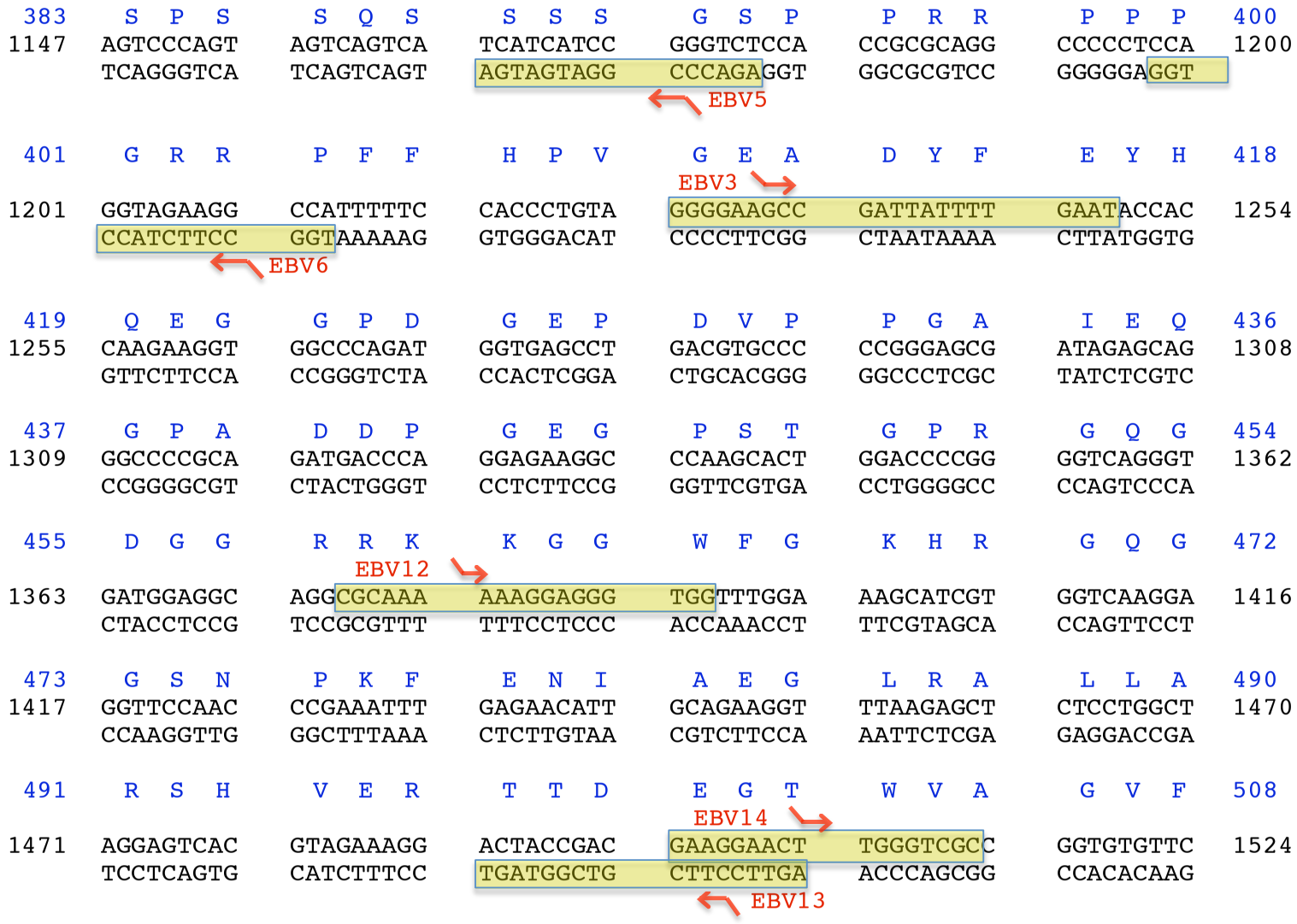


Figure 3. Functional map of EBNA-1 and rEBNA-1 lacking gly-ala repeat. Functional maps of EBNA-1 (A) and the rEBNA-1 used in this study lacks most of the gly-ala repeat (aa101-325) (B).


1 M S D E G P G T G P G N G L G E K G 18
EBV7
1 ATGTCTGAC GAGGGGCCA GGTACAGGA CCTGGAAAT GGCCTAGGA GAGAAGGGA 54
TACAGACTG CTCCCCGGT CCATGTCCT GGACCTTTA CCGGATCCT CTCTTCCCT
19 D T S G P E G S G G S G P Q R R G G 36
55 GACACATCT GGACCAGAA GGCTCCGGC GGCAGTGGA CCTCAAAGA AGAGGGGGT 108
CTGTGTAGA CCTGGTCTT CCGAGGCCG CCGTCACCT GGAGTTTCT TCTCCCCCA
37 D N H G R G R G R G G R G G 54
109 GATAACCAT GGACGAGGA CGGGGAAGA GGACGAGGA CGAGGAGGC GGAAGACCA 162
CTATTGGTA CCTGCTCCT GCCCCTTCT CCTGCTCCT GCTCCTCCG CCTTCTGGT
55 G A P G G S G S G P R H R D G V R R 72
163 GGAGCCCCG GGCGGCTCA GGATCAGGG CCAAGACAT AGAGATGGT GTCCGGAGA 216
CCTCGGGGC CCGCCGAGT CCTAGTCCC GGTTCTGTA TCTCTACCA CAGGCCTCT
73 P Q K R P S C I G C K G T H G G T G 90
217 CCCCAAAAA CGTCCAAGT TGCATTGGC TGCAAAGGG ACCCACGGT GGAACAGGA 270
GGGGTTTTT GCAGGTTCA ACGTAACCG ACGTTTCCC TGGGTGCCA CCTTGTCCT
91 A G A G A G G A G G G 328
271 GCAGGAGCA GGAGCGGGA GGGGCAGGA GCA GGTGGAGGC 984
CGTCCTCGT CCTCGCCCT CCCCGTCCT CGT CCACCTCCG
329 R G R G G S G G R G R G G S G R G 346
985 CGGGGTCGA GGAGGCAGT GGAGGCCGG GGTCGAGGA GGTAGTGGA GGCCGGGGT 1038
GCCCCAGCT CCTCCGTCA CCTCCGGCC CCAGCTCCT CCATCACCT CCGGCCCCA
347 R G G S G G R R G R G R E R A R G G 364
1039 CGAGGAGGT AGTGGAGGC CGCCGGGGT AGAGGACGT GAAAGAGCC AGGGGGGGA 1092
GCTCCTCCA TCACCTCCG GCGGCCCCA TCTCCTGCA CTTTCTCGG TCCCCCCCT
365 S R E R A R G R G R G R G E K R P R 382
1093 AGTCGTGAA AGAGCCAGG GGGAGAGGT CGTGGACGT GGAGAAAAG AGGCCCAGG 1146
TCAGCACTT TCTCGGTCC CCCTCTCCA GCACCTGCA CCTCTTTTC TCCGGGTCC
ΔGA(101-325)


383 S P S S Q S S S S G S P P R R P P P 400
1147 AGTCCCAGT AGTCAGTCA TCATCATCC GGGTCTCCA CCGCGCAGG CCCCCTCCA 1200
TCAGGGTCA TCAGTCAGT AGTAGTAGG CCCAGAGGT GGCGCGTCC GGGGGAGGT
EBV5
401 G R R P F F H P V G E A D Y F E Y H 418
EBV3
1201 GGTAGAAGG CCATTTTTC CACCCTGTA GGGGAAGCC GATTATTTT GAATACCAC 1254
CCATCTTCC GGTAAAAAG GTGGGACAT CCCCTTCGG CTAATAAAA CTTATGGTG
EBV6
419 Q E G G P D G E P D V P P G A I E Q 436
1255 CAAGAAGGT GGCCCAGAT GGTGAGCCT GACGTGCCC CCGGGAGCG ATAGAGCAG 1308
GTTCTTCCA CCGGGTCTA CCACTCGGA CTGCACGGG GGCCCTCGC TATCTCGTC
437 G P A D D P G E G P S T G P R G Q G 454
1309 GGCCCCGCA GATGACCCA GGAGAAGGC CCAAGCACT GGACCCCGG GGTCAGGGT 1362
CCGGGGCGT CTACTGGGT CCTCTTCCG GGTTCGTGA CCTGGGGCC CCAGTCCCA
455 D G G R R K K G G W F G K H R G Q G 472
EBV12
1363 GATGGAGGC AGGCGCAAA AAAGGAGGG TGGTTTGGA AAGCATCGT GGTCAAGGA 1416
CTACCTCCG TCCGCGTTT TTTCCTCCC ACCAAACCT TTCGTAGCA CCAGTTCCT
473 G S N P K F E N I A E G L R A L L A 490
1417 GGTTCCAAC CCGAAATTT GAGAACATT GCAGAAGGT TTAAGAGCT CTCCTGGCT 1470
CCAAGGTTG GGCTTTAAA CTCTTGTAA CGTCTTCCA AATTCTCGA GAGGACCGA
491 R S H V E R T T D E G T W V A G V F 508
EBV14
1471 AGGAGTCAC GTAGAAAGG ACTACCGAC GAAGGAACT TGGGTCGCC GGTGTGTTC 1524
TCCTCAGTG CATCTTTCC TGATGGCTG CTTCCTTGA ACCCAGCGG CCACACAAG
EBV13
509 V Y G G S K T S L Y N L R R G T A L 526
1525 GTATATGGA GGTAGTAAG ACCTCCCTT TACAACCTA AGGCGAGGA ACTGCCCTT 1578
CATATACCT CCATCATTC TGGAGGGAA ATGTTGGAT TCCGCTCCT TGACGGGAA

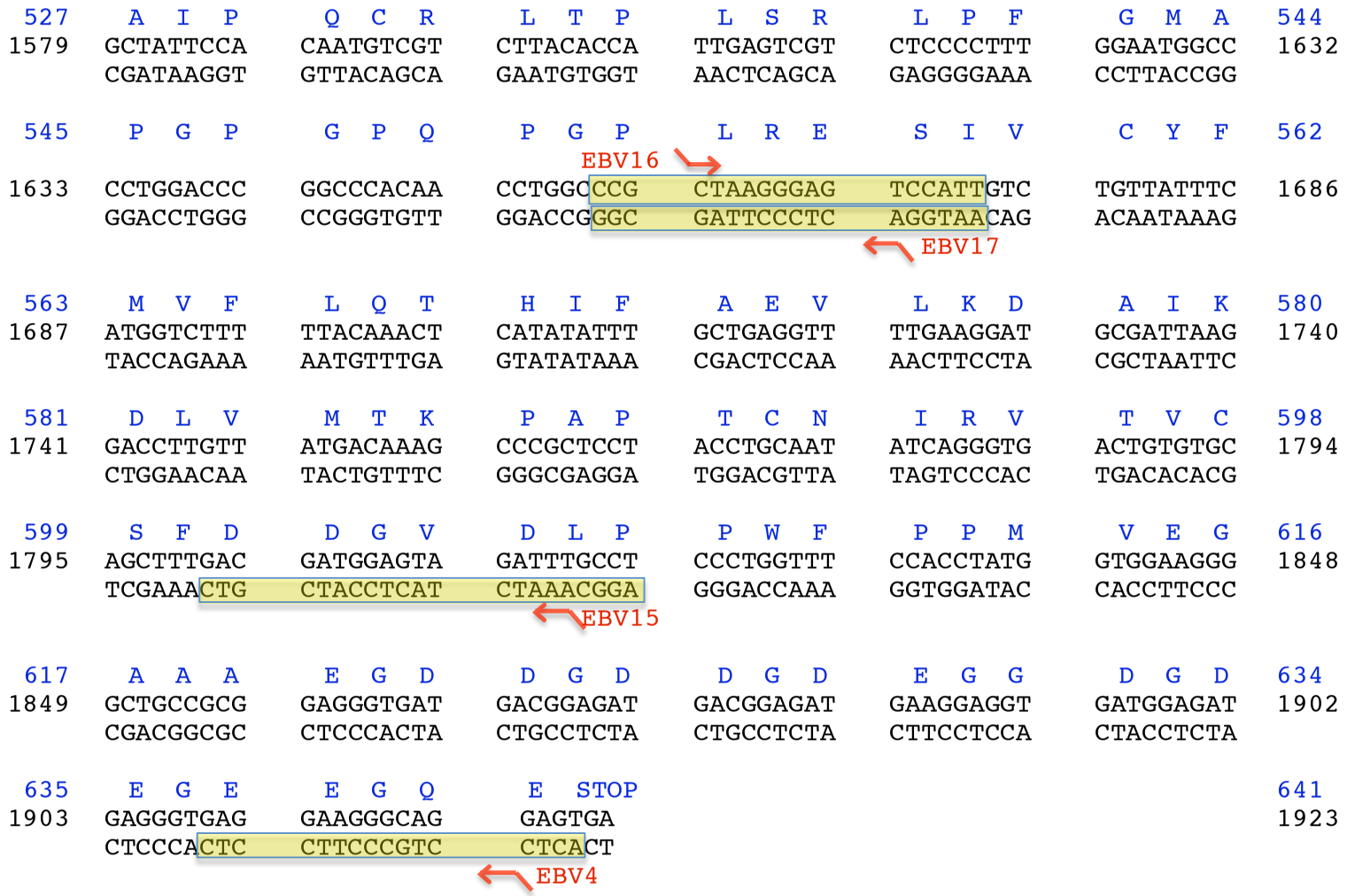


Figure 4. DNA Sequence of EBNA-1 showing the primer positions used in this study. The amino acid corresponding to each codon is indicated above it in blue color and the nucleotide numbering is in black color. All the primers used in this study have been shaded in yellow and the names are indicated.

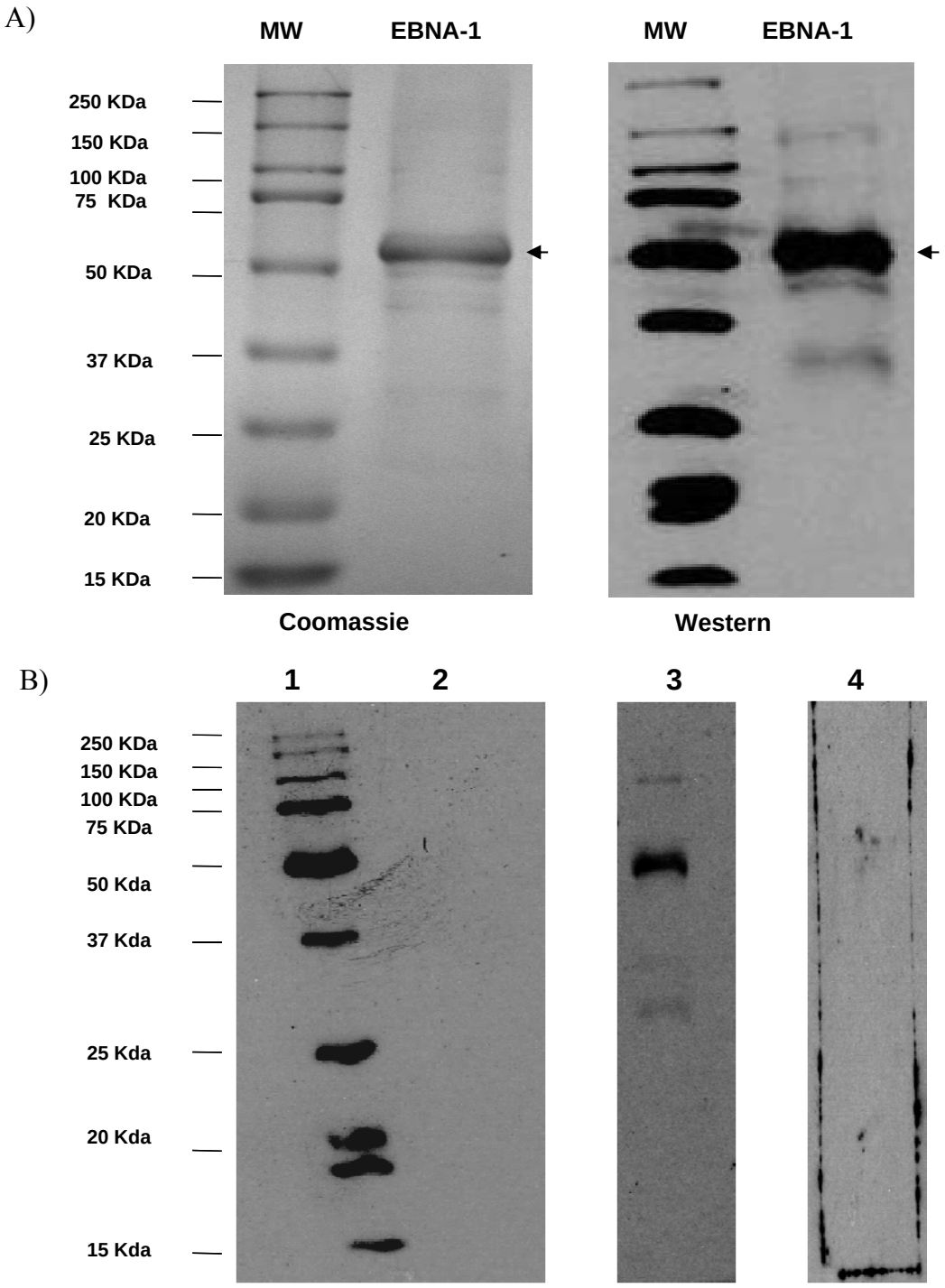
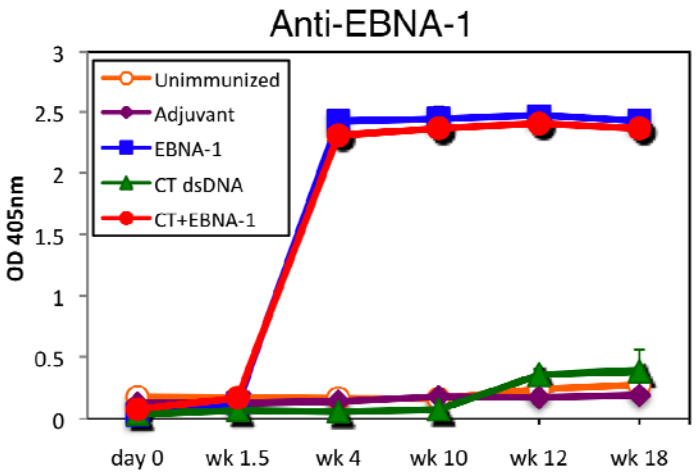


Figure 5. Specific detection of rEBNA-1 protein with a monoclonal anti-EBNA-1 antibody. (A) rEBNA-1 was purified from a baculovirus vector grown in SF9 insect cells. SDS-PAGE of rEBNA-1 stained by Coomassie blue (left panel) and Western blot immunostained with a monoclonal antibody (MAb) to EBNA-1(right panel) and visualized by chemoluminescence. Strep tag MW marker is visualized with Strep-Tactin HRP. (B) Secondary antibody and strep tactin-HRP do not bind non specifically to EBNA-1 . Lane 1; MW Marker. Lanes 2,3,4 contain EBNA-1. Lanes 1 and 2 incubated with Streptactin-HRP only. Lane 3 incubated with 5µg/ml of MAb to EBNA-1 followed by secondary antibody anti-mouse IgG-HRP (1:20,000). Lane 4 incubated with secondary antibody only.

A)



B)

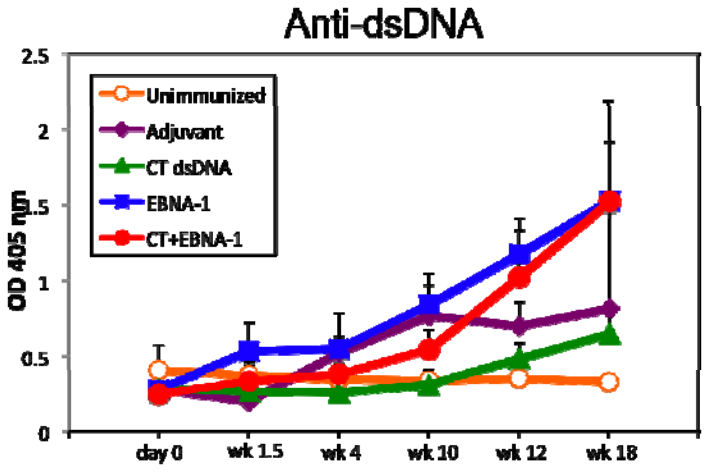
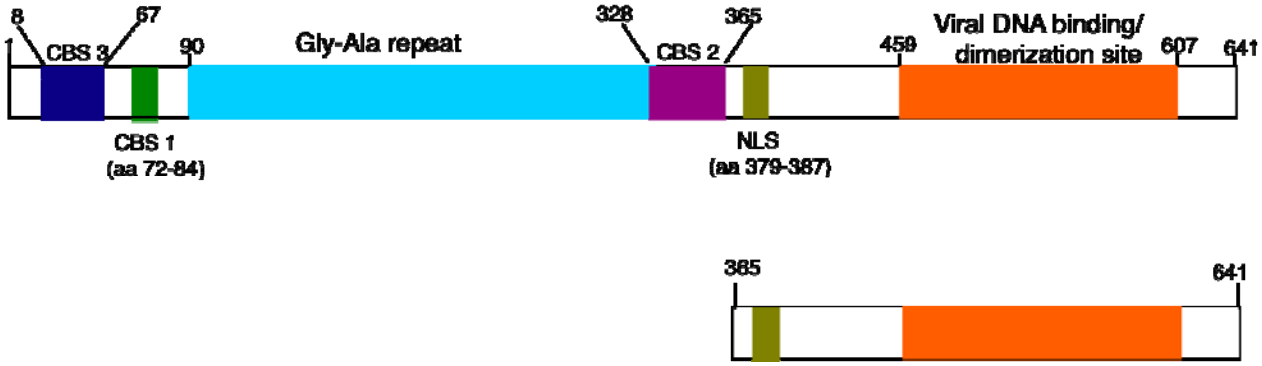
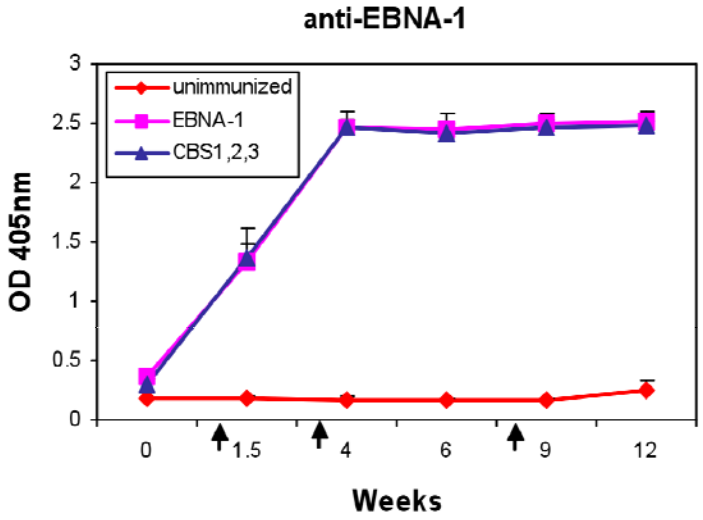


Figure 6. rEBNA-1 injected mice develop antibodies to EBNA-1 and dsDNA. Mice were injected ip with rEBNA-1 emulsified in CFA or EBNA-1complexed to CTdsDNA or CT dsDNA alone, CFA alone or were uninjected. The mice were boosted with rEBNA-1, CTdsDNA complexed to EBNA-1 or CTdsDNA emulsified in IFA or IFA alone boosted at weeks 3 and 9. Mice were bled at weeks 1.5, 4, 10, 12 and 18 and sera tested by ELISA for antibody to EBNA-1 **(A)** and dsDNA (**B).** Results are the average of 5 mice in each group. Standard deviations are indicated.

A)



B)



C)

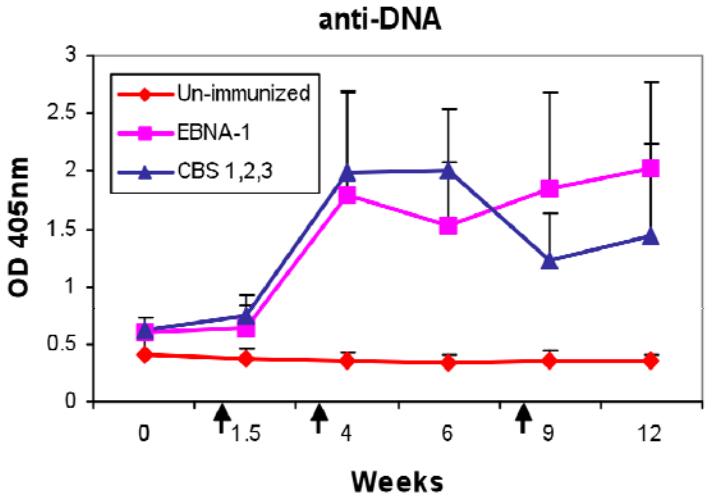
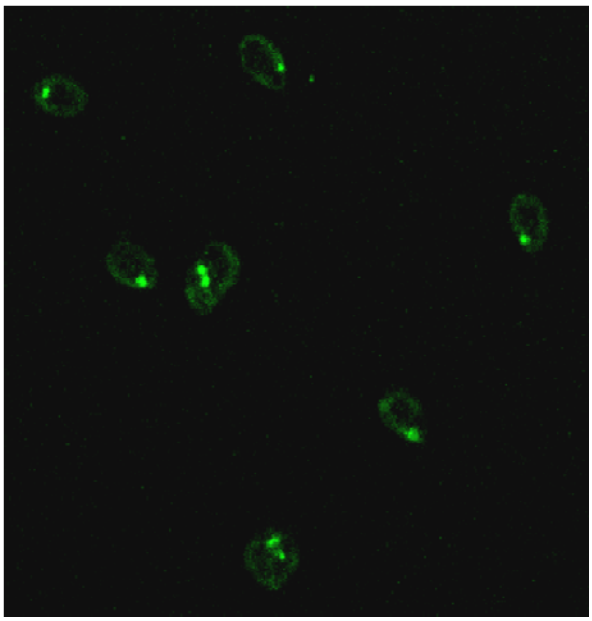
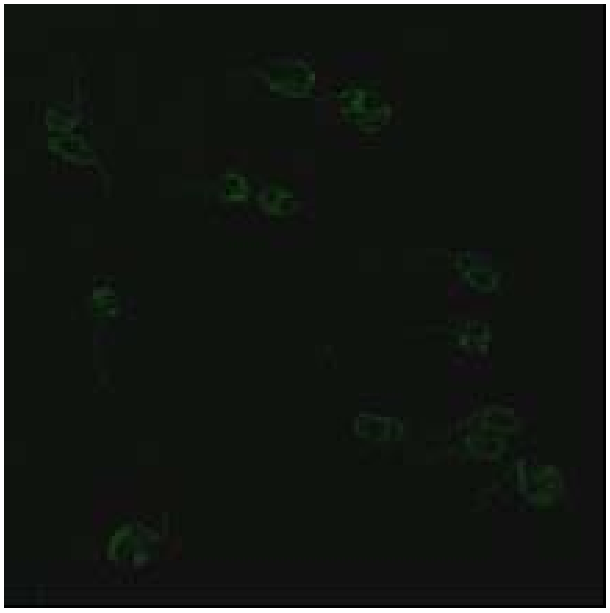


Figure 7 EBNA-1 lacking the three chromosomal binding sites is sufficient to elicit an anti-dsDNA response. (A) EBNAΔCBS1,2,3 lacks the 3 chromosomal binding sites and also the gly-ala repeat. It spans from amino acid 365-641. (B and C) Mice were injected with 50 ug of ΔCBS1,2,3 in CFA and then were boosted in IFA with 25 ug of the protein at weeks 3 and 9. The first injection and subsequent boosts are indicated by an arrow. The mice were bled and the sera was tested for anti-EBNA-1(B) and anti-dsDNA antibodies (C) by ELISA.

A)

B)





EBNA-1

Adjuvant

Figure 8. Sera from EBNA-1 injected mice bind to dsDNA in a Crithidia luciliae assay.
Serum from a week 12 mouse injected with rEBNA-1 (A) or adjuvant only **(**B) was diluted 1:50 and used to immunostain Crithidia luciliae slides. Immunostaining of the dsDNA in the kinetoplast of Crithidia luciliae was observed in (A). Results are representative of 3 r EBNA-1 and 3 adjuvant only injected mice.

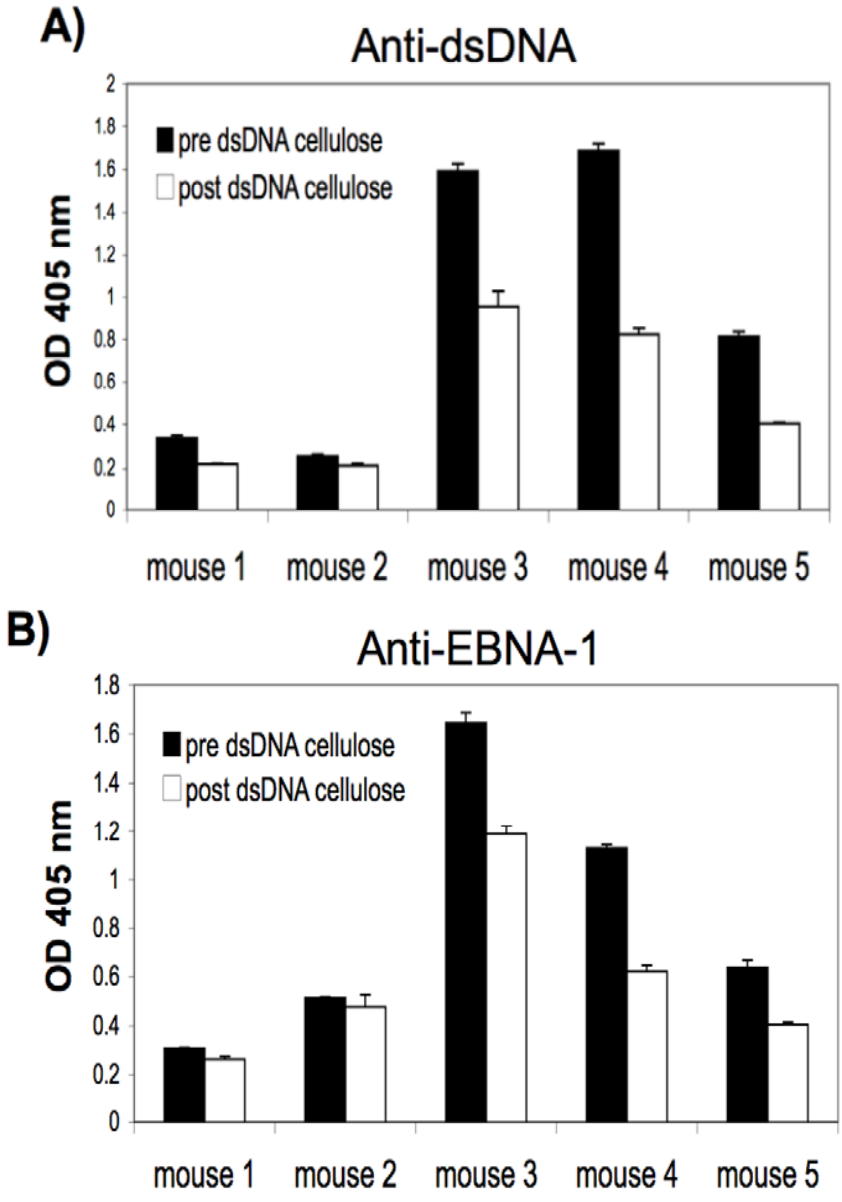


Figure 9. Sera from EBNA-1 injected mice display reduced binding to EBNA-1 following adsorption onto dsDNA cellulose beads. Week 12 sera from five rEBNA-1 injected mice were adsorbed onto dsDNA cellulose beads and pre and post adsorbed sera were tested by ELISA for binding to dsDNA (A) and EBNA-1 (B). Results represent OD 405 nm values after subtraction of non specific binding to cellulose only beads. Standard deviations of triplicate wells are indicated.

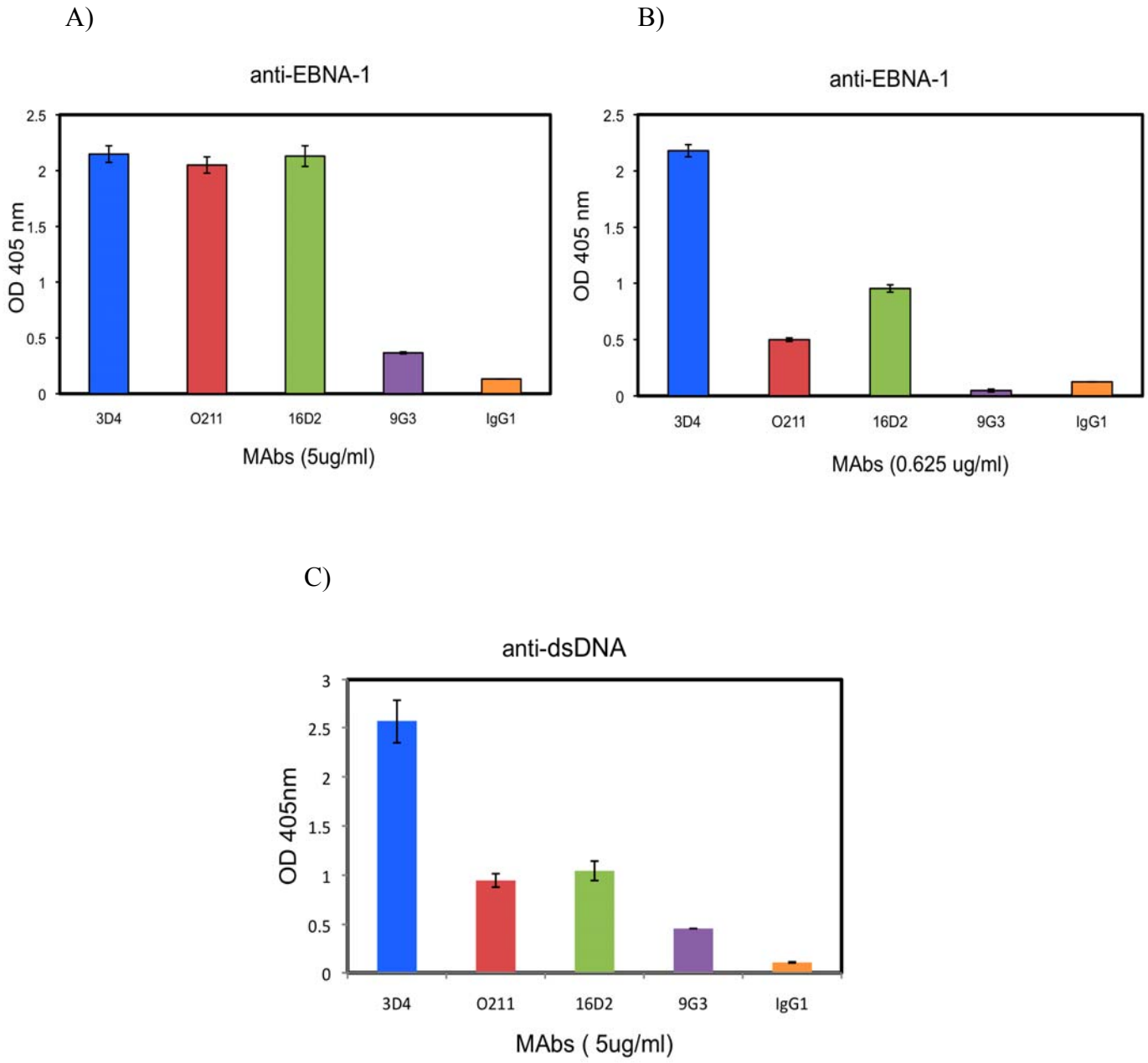
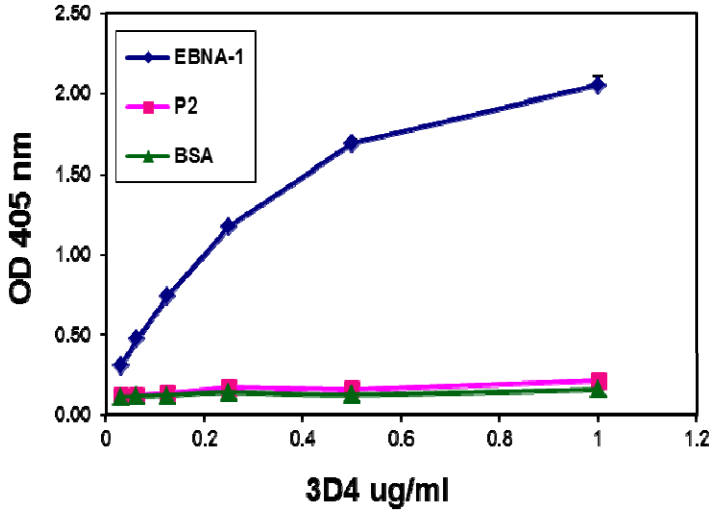
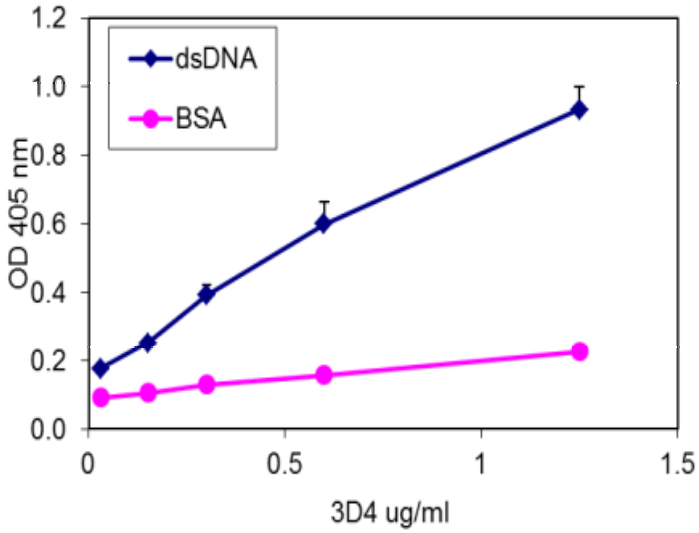


Figure 10. Relative binding of MAbs to EBNA-1 and dsDNA. Monoclonal antibodies (MAbs) were generated from EBNA-1 injected mice and tested by ELISA for binding to EBNA-1 at 5µg/ml (A) or 0.625 ug/ml (B) and to dsDNAat 5µg/ml (C). IgG1 is an isotype control and 0211 is a commercially prepared antibody to EBNA-1.

A)



B)



C)

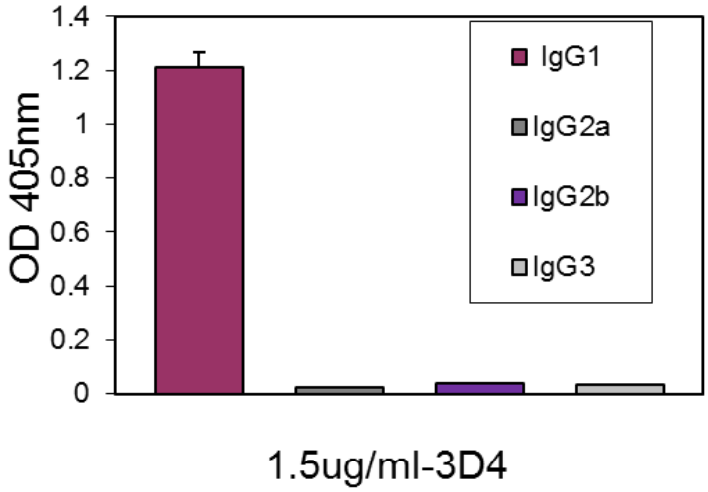


Figure 11 . MAb, 3D4, is specific for both EBNA-1 and dsDNA. (A) Anti-EBNA-1 ELISA. 3D4 was tested by ELISA, at increasing concentrations, for binding to EBNA-1, BSA, and the cystovirus, polymerase protein, P2 (negative control). (B) 3D4 was tested by ELISA for binding to dsDNA at increasing concentrations. Results in A and B are the average of triplicates and standard deviations are indicated. (C) 3D4 antibody is of the IgG1 isotype. ELISA plates coated with unlabeled anti-IgG1, anti-IgG2a, anti-IgG2b, or anti-IgG3 were incubated with 1.5 µg/ml of 3D4 MAb followed by the respective polyclonal isotype specific antibodies conjugated to alkaline phosphatase.

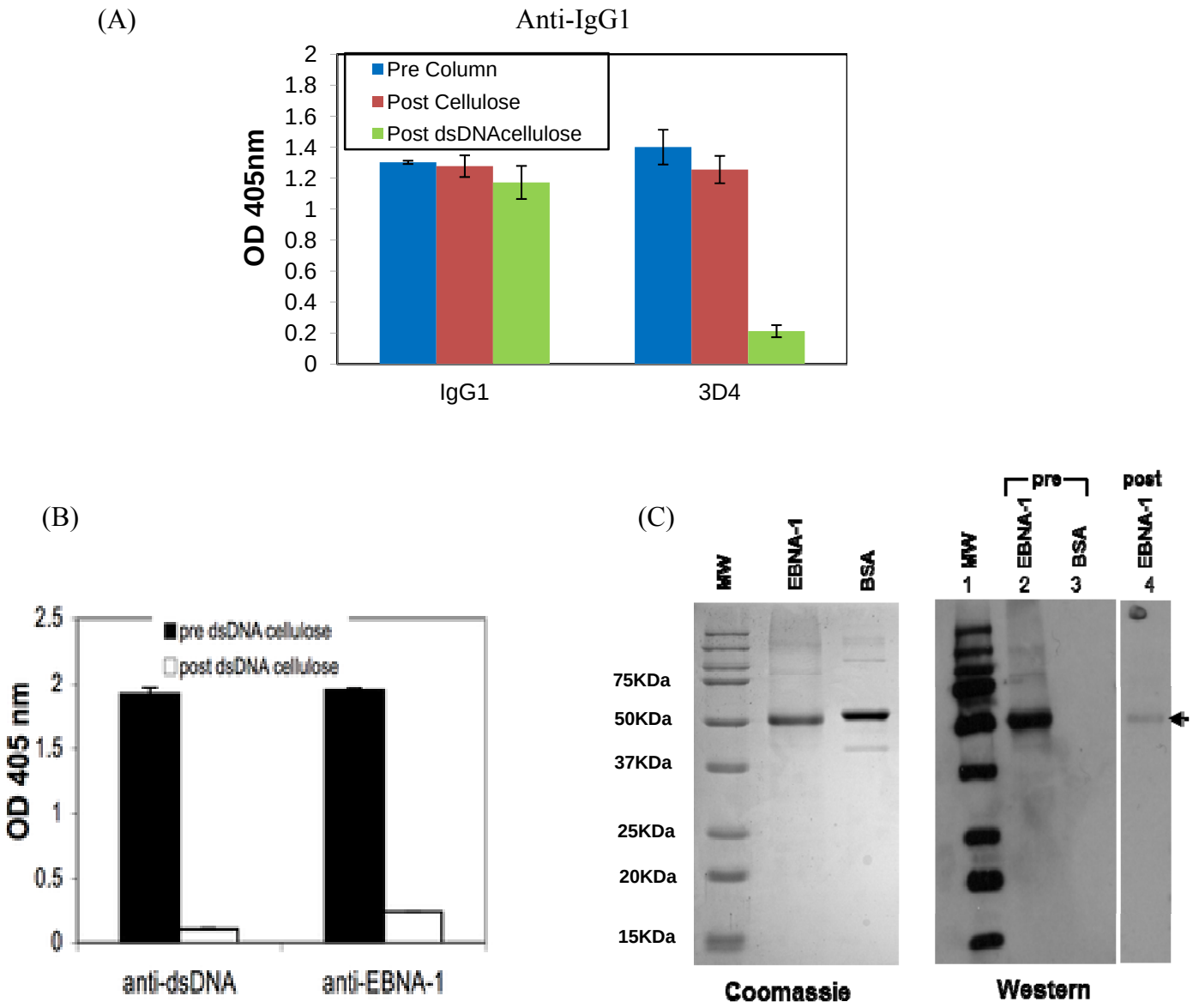
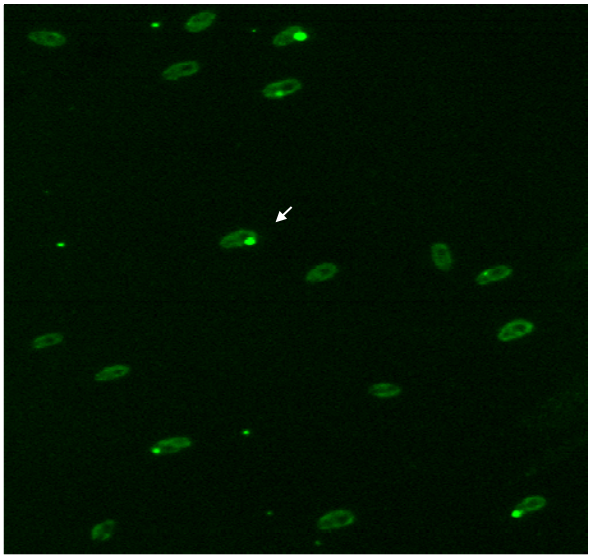
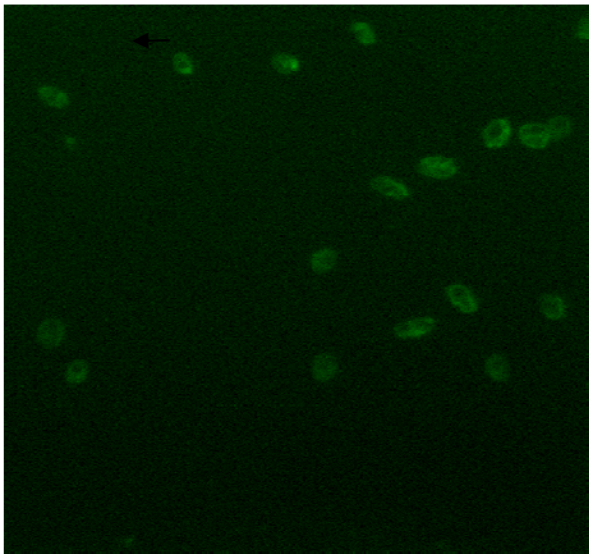


Figure 12. Adsorption of 3D4 on a dsDNA cellulose column removes reactivity to EBNA-1. (A) 3D4 was adsorbed onto cellulose or dsDNA cellulose beads. Pre and post adsorbed MAb were tested by an anti-IgG ELISA (A) or by an anti-dsDNA or anti-EBNA-1 ELISA (B). Results in B represent OD 405nm after the values for any non-specific binding to cellulose beads was subtracted. Standard deviations of triplicate wells are indicated. Pre and post adsorbed MAb were also tested for binding to EBNA-1 by Western blot (C). Post dsDNA cellulose adsorbed 3D4 shows reduced binding to EBNA-1 by Western blot.
Left panel: Coomassie stained SDS-PAGE. Right panel: Western blot: filters were immunostained with pre (lanes 2 and 3) or post adsorbed 3D4 (lane 4) . Molecular weight markers used in Western blot were conjugated to *strep-tag* and were detected with Strep-Tactin-HRP.

A)

B)





3D4

IgG1

Figure 13. 3D4 binds dsDNA in a Crithidia luciliae assay 3D4 at a concentration of 10 µg/ml was examined for binding to Crithidia luciliae slides. (A) Arrow indicates 3D4 binding to kinetoplasts of Crithidia luciliae. (B) IgG1 isotype control antibody, does not bind to kinetoplasts.

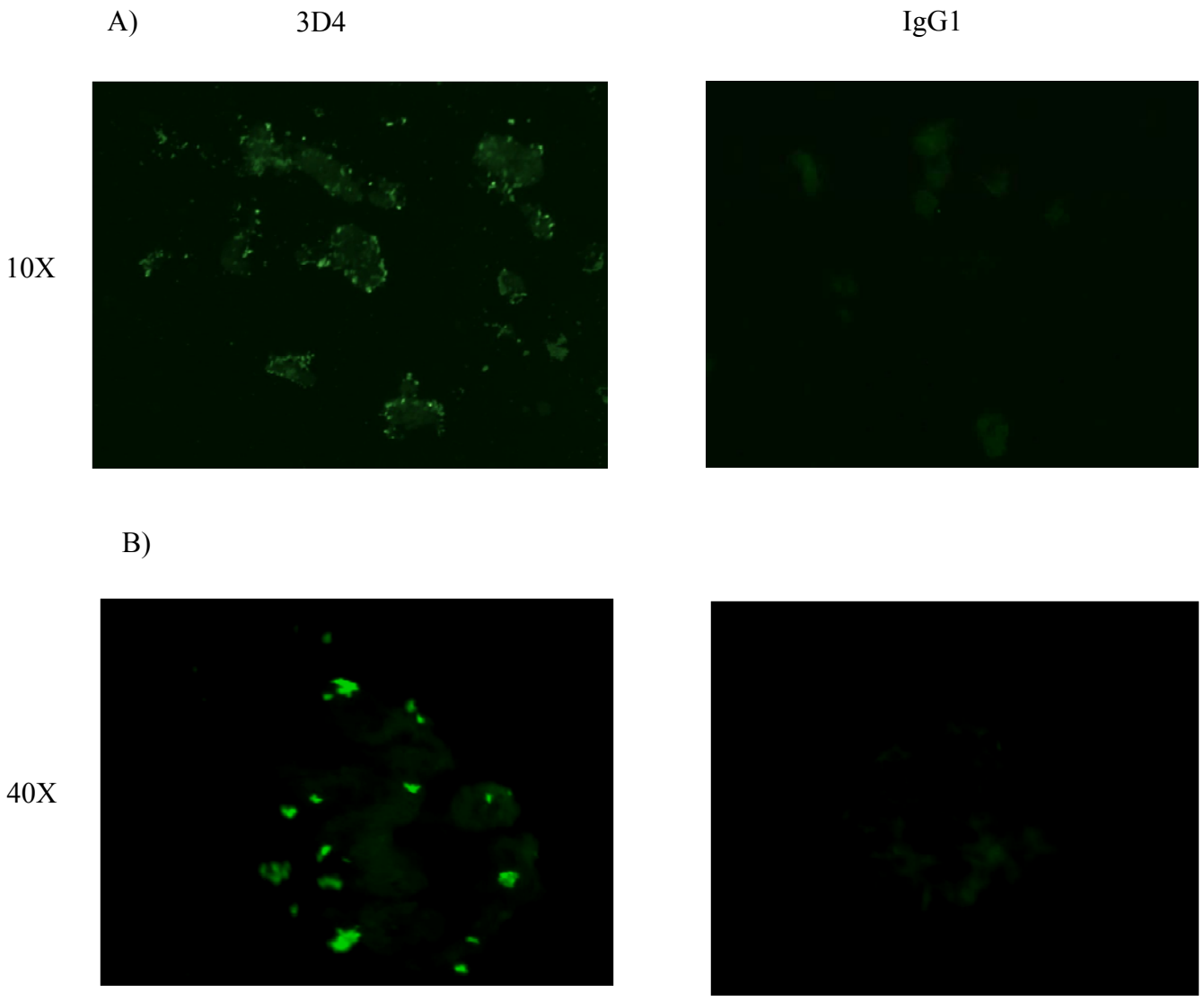


Figure 14. Glomerular binding assay. 3D4 at 10ug/ml was used to immunostain glomeruli from a C57BL/6 mouse at a magnification of 10X (A) and 40X (B). An IgG1 isotype antibody was used as a negative control (right panels). The slides were visualized with a Nikon Eclipse TE-2000S florescence microscope.

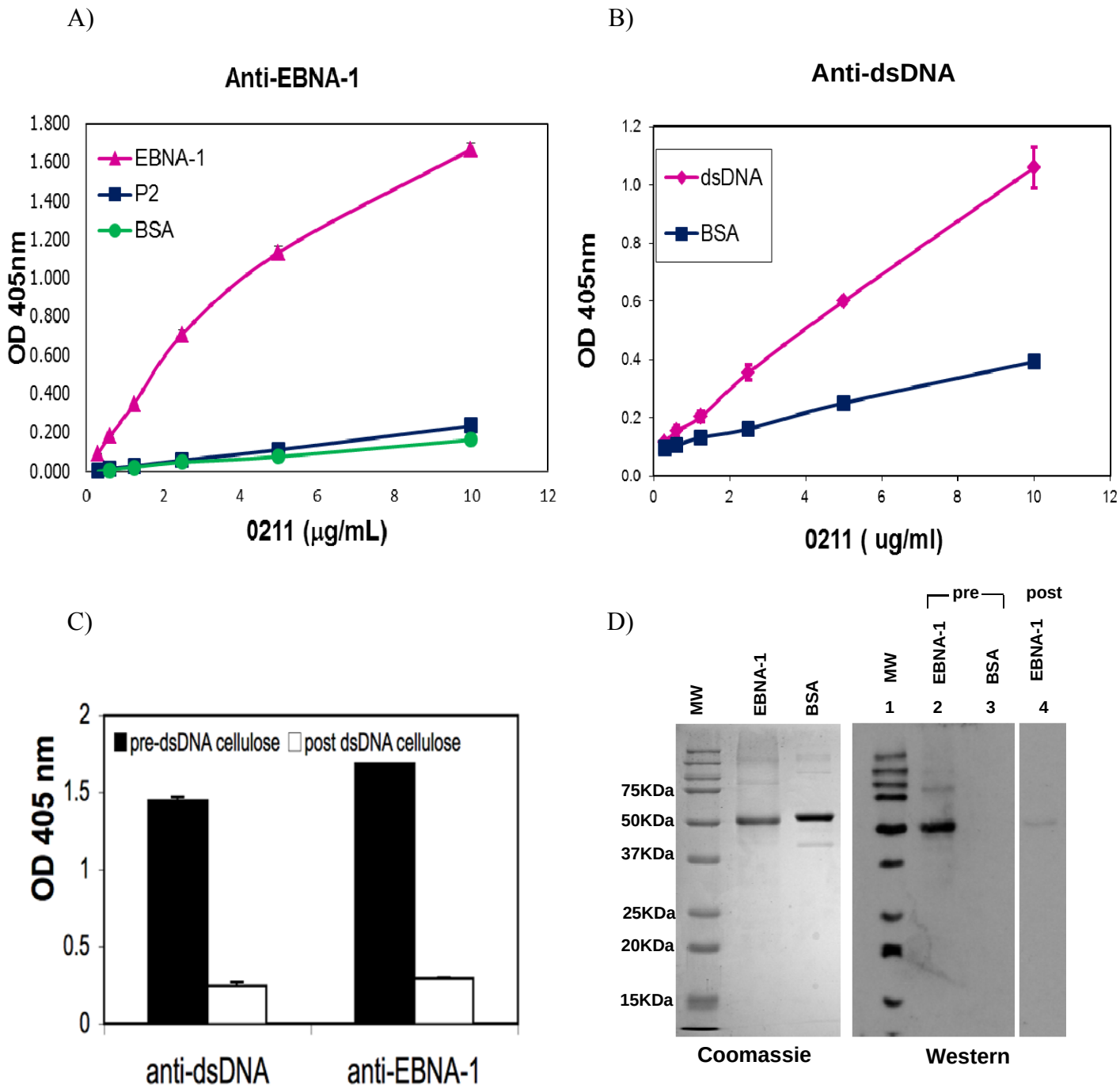
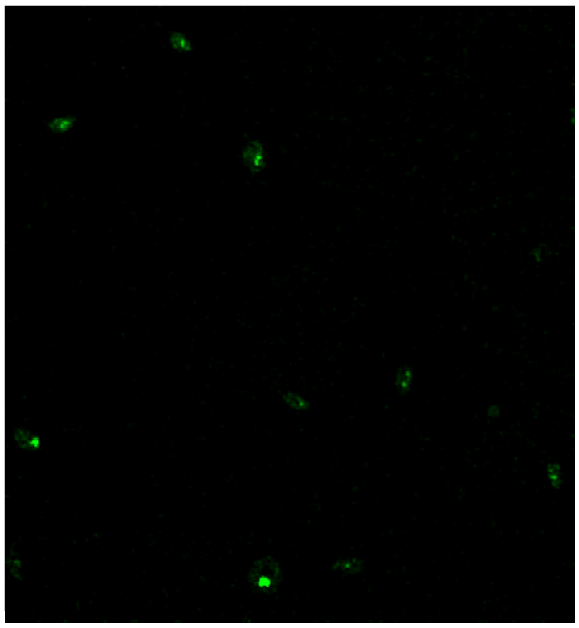


Figure 15. Commercial MAb, 0211 cross-reacts with dsDNA. MAb 0211 binds to EBNA-1 (A) and dsDNA (B) as detected by ELISA but not P2, or BSA.**(C & D)** MAb 0211 was adsorbed onto dsDNA cellulose beads and pre and post adsorbed antibody were tested for binding to dsDNA and EBNA-1 by ELISA **(C)** and Western blot (D). Results in (C) represent OD 405 nm values after subtraction of non specific binding to cellulose only beads. **(D)** Post dsDNA cellulose adsorbed MAb 0211 shows reduced binding to rEBNA-1 by Western blot. Left panel: Coomassie stained SDS-PAGE. Right panel: Western blot: filters were immunostained with pre (lanes 2 and 3) or post dsDNA cellulose adsorbed 0211 (lane 4) as indicated.

A)

0211



B)

IgG1

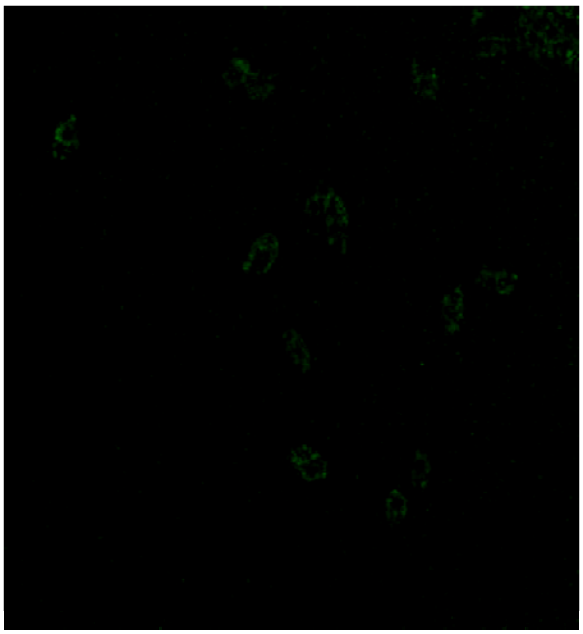


Figure 16. MAb, 0211 binds to dsDNA in a Crithidia luciliae assay. Purified 0211 at a concentration of 15 µg/ml was examined for binding to Crithidia luciliae slides. (A) shows the binding of 0211 to kinetoplasts of Crithidia luciliae (B) IgG1 isotype control antibody, failed to bind to kinetoplasts.

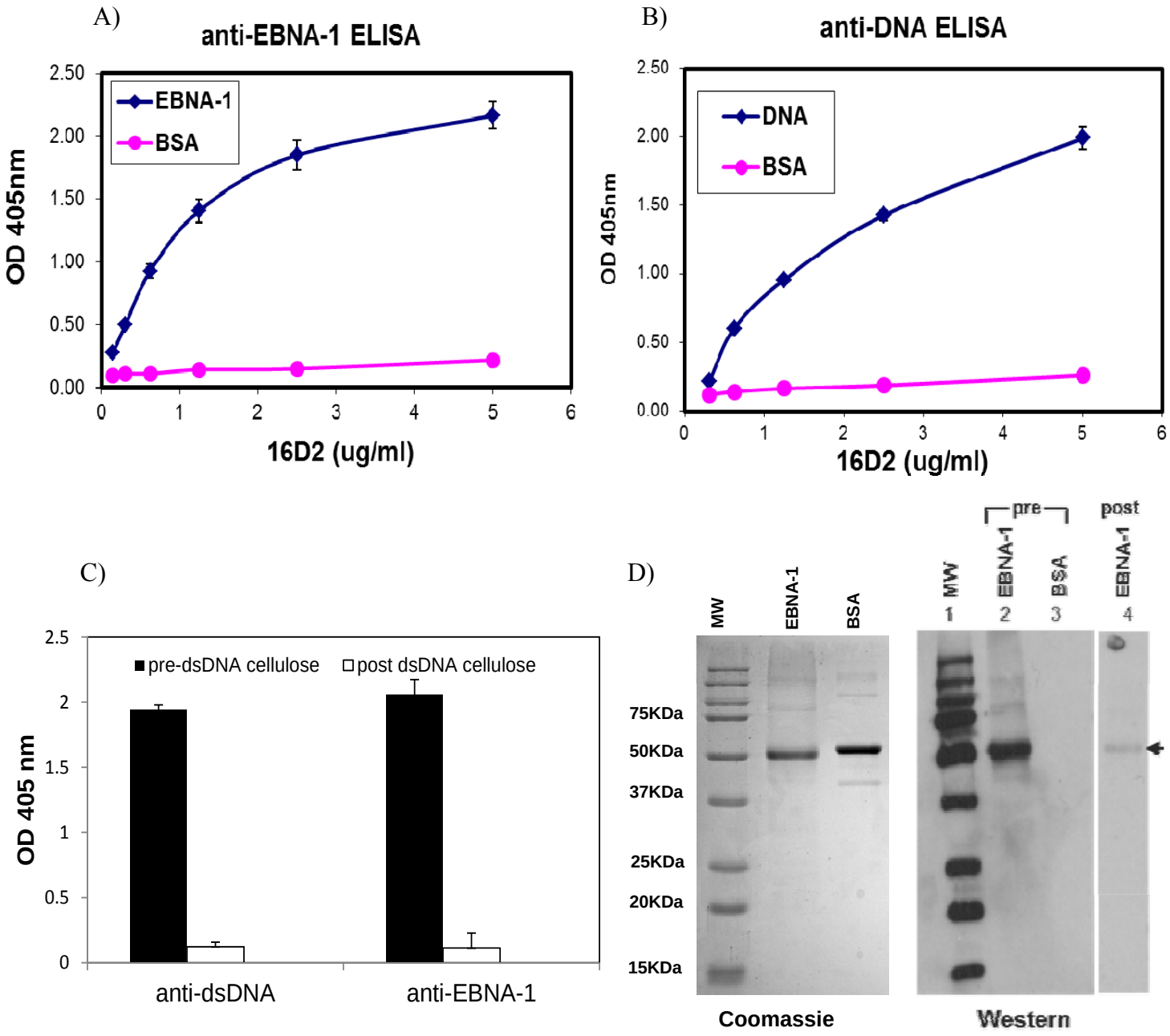
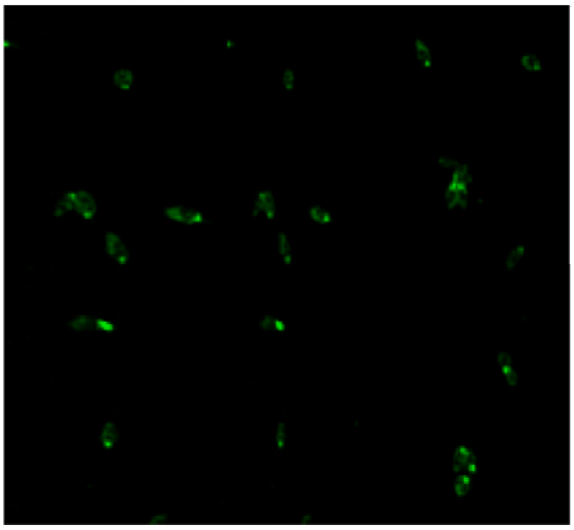


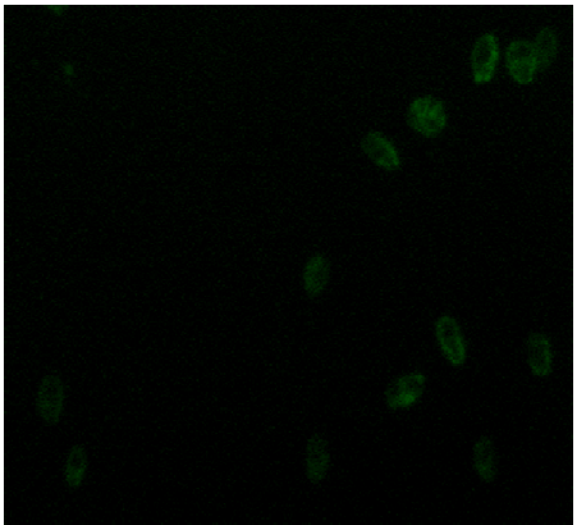
Figure 17. 16D2 cross-reacts with dsDNA. MAb 16D2 binds to EBNA-1 (A) and to dsDNA (B) as detected by ELISA but not to BSA. (C & D) 16D2 was adsorbed onto dsDNA cellulose beads and pre and post adsorbed antibody were tested for binding to dsDNA and EBNA-1 by ELISA (C) and EBNA-1 by Western blot (D). Results represent in (C) OD 405 nm values after subtraction of non specific binding to cellulose only beads. (D) Post dsDNA cellulose adsorbed 16D2 shows reduced binding to rEBNA-1 by Western blot. Left panel: Coomassie stained SDS-PAGE. Right panel: Western blot: filters were immunostained with pre (lanes 2 and 3) or post dsDNA cellulose adsorbed 0211 (lane 4) as indicated.

A)



16D2

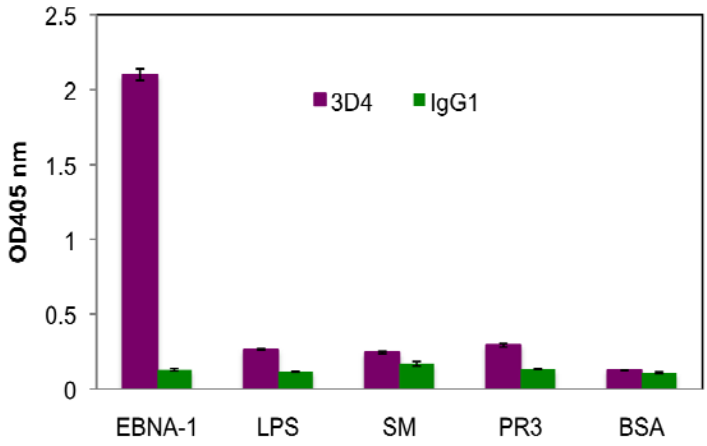
B)



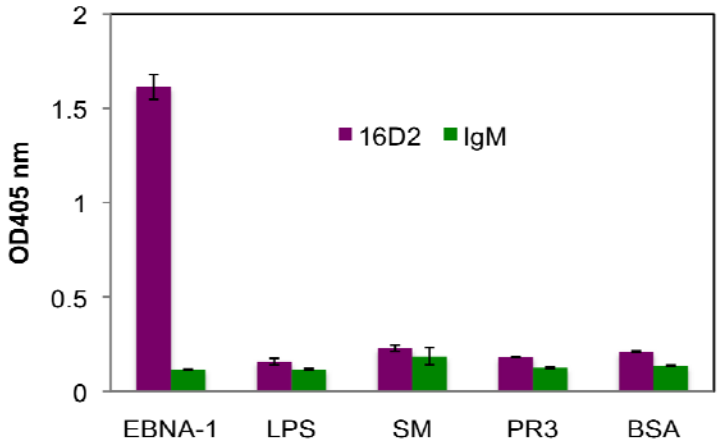
IgM

Figure 18. MAb, 16D2 binds to dsDNA in a Crithidia luciliae assay. 10 µg/ml of 16D2 was examined for binding to Crithidia luciliae slides. (A)16D2 binds to kinetoplasts of Crithidia luciliae (B) IgM control antibody, does not bind to the kinetoplasts.

A)



B)



C)

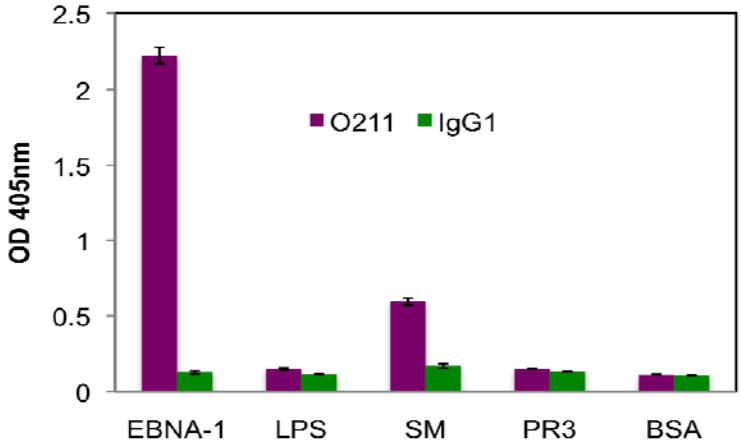


Figure 19. Screening MAbs against a panel of antigens. Binding of MAbs (5ug/ml) to several antigens was examined by ELISA. (A) 3D4 and (B)16D2 do not significantly bind to any antigens examined other than EBNA-1 (C) 0211 binds moderately well to Sm but not to the other antigens tested. An IgG1 isotype control is used for 3D4 and 0211 and an IgM isotype control is used for 16D2.

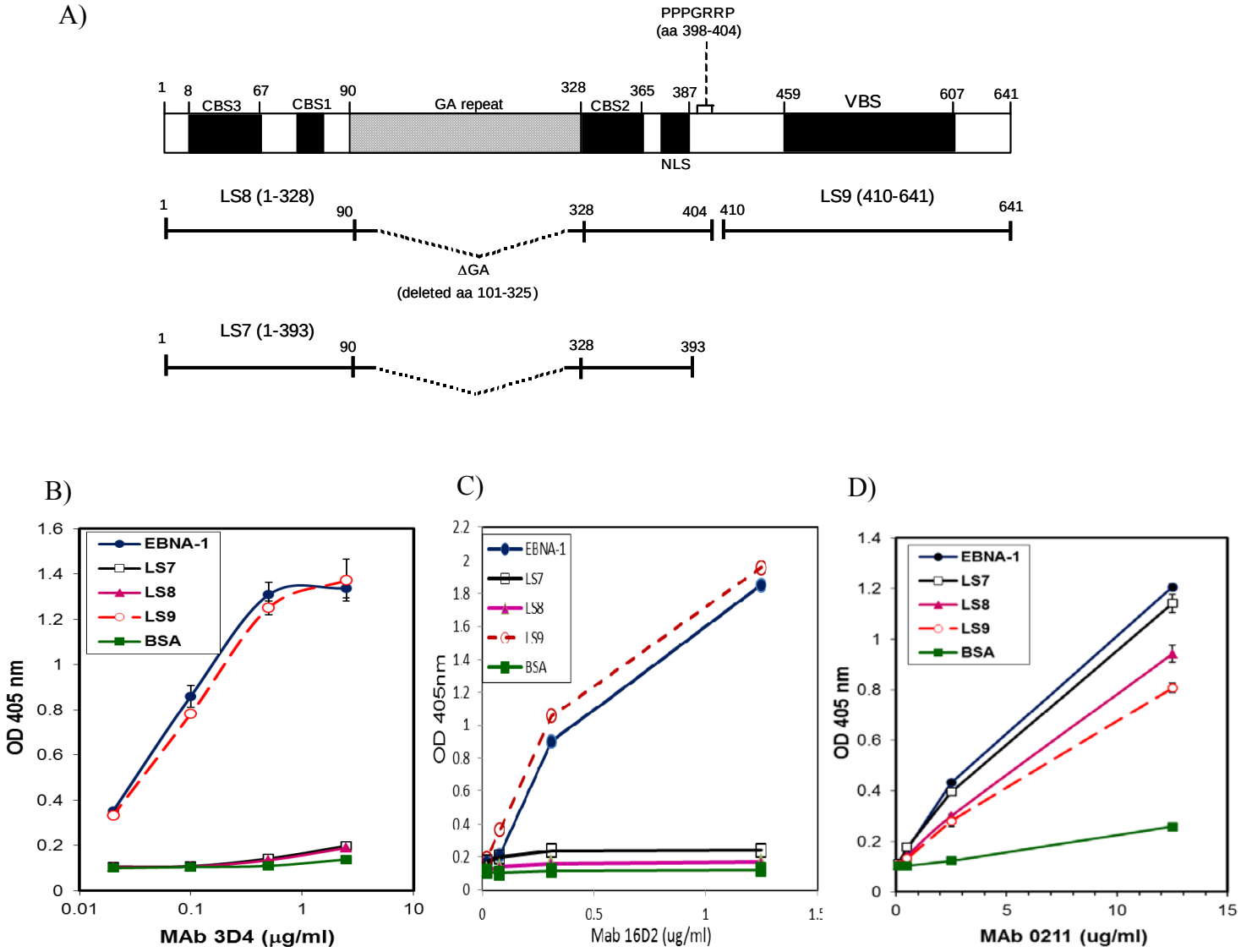
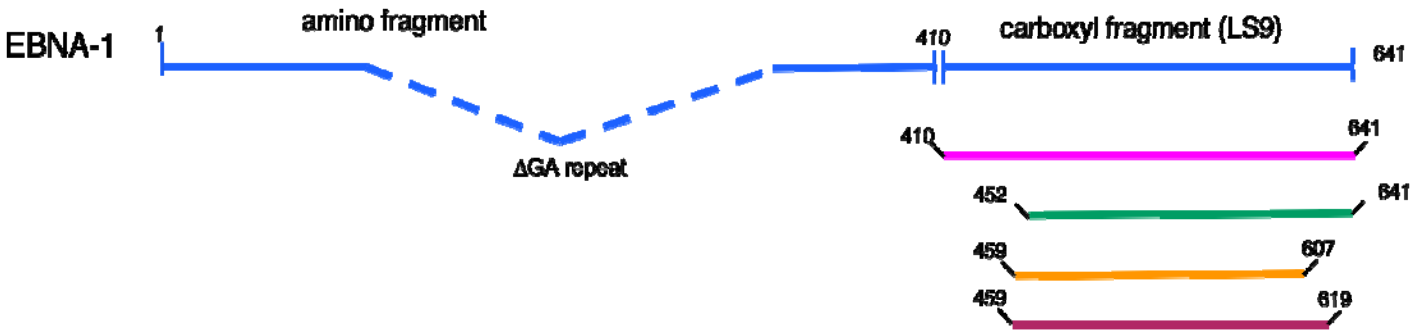
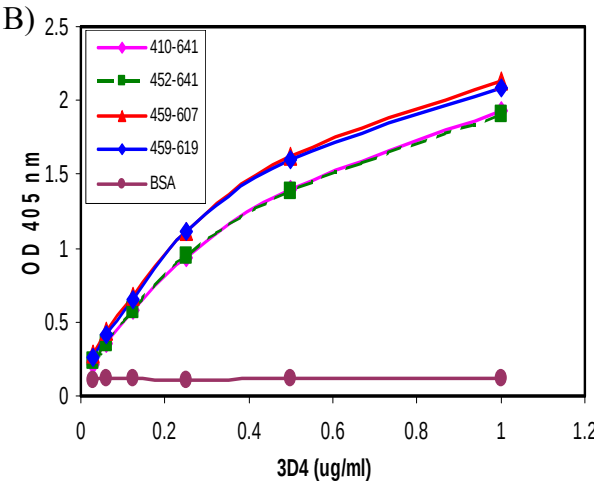


Figure 20. 3D4 and 16D2 bind to the carboxyl region while 0211 binds to both the carboxyl and amino regions of EBNA-1. (A) Functional map of the EBNA-1 protein and maps of amino and carboxyl fragments. LS8 denotes the amino fragment (aa 1-404) lacking most of the GA repeat. LS7 denotes the amino fragment (aa 1-393) lacking the GA repeat and lacking PPPGRRP. LS9 denotes the carboxyl fragment (aa 410-641). (B-D) MAbs,3D4,16D2 and 0211 were tested for reactivity with LS7, LS8 and LS9 by ELISA.

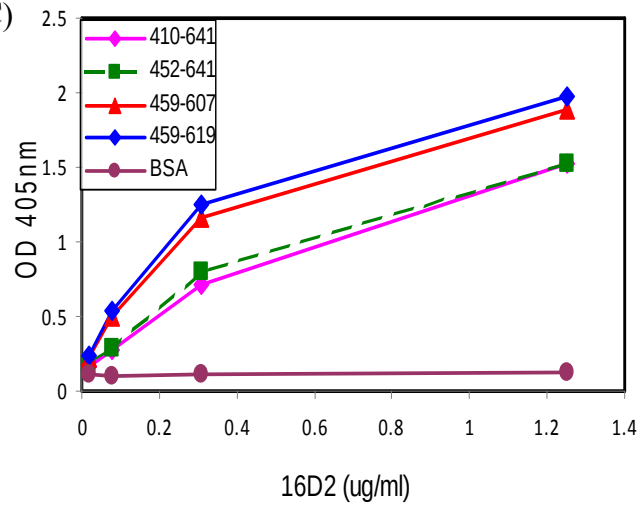
A)



B)



C)



D)

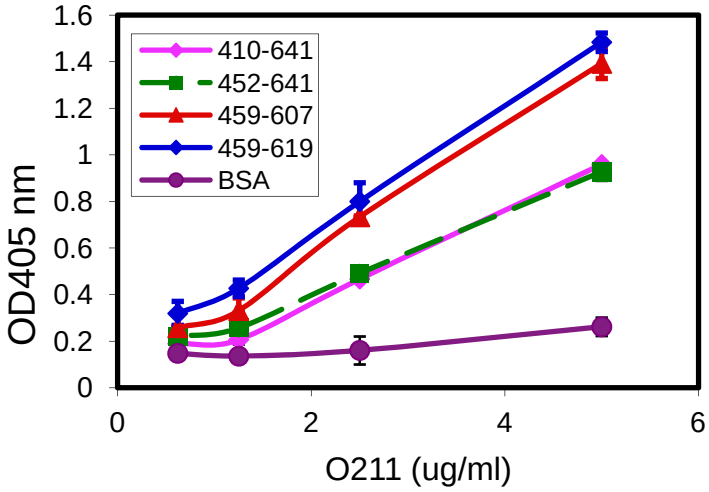


Figure 21. 3D4, 16D2 and 0211 recognize an epitope located between amino acids (aa) 459-607 in the carboxyl region of EBNA-1.(A) Map of EBNA-1 proteins truncated at the 3' or 5' ends of the carboxyl region. (B-D) MAb, 3D4 (B), 16D2 (C) and 0211 (D) were tested by ELISA for binding to the 3 truncated fragments of EBNA-1. All MAbs bind strongly to all 3 fragments.

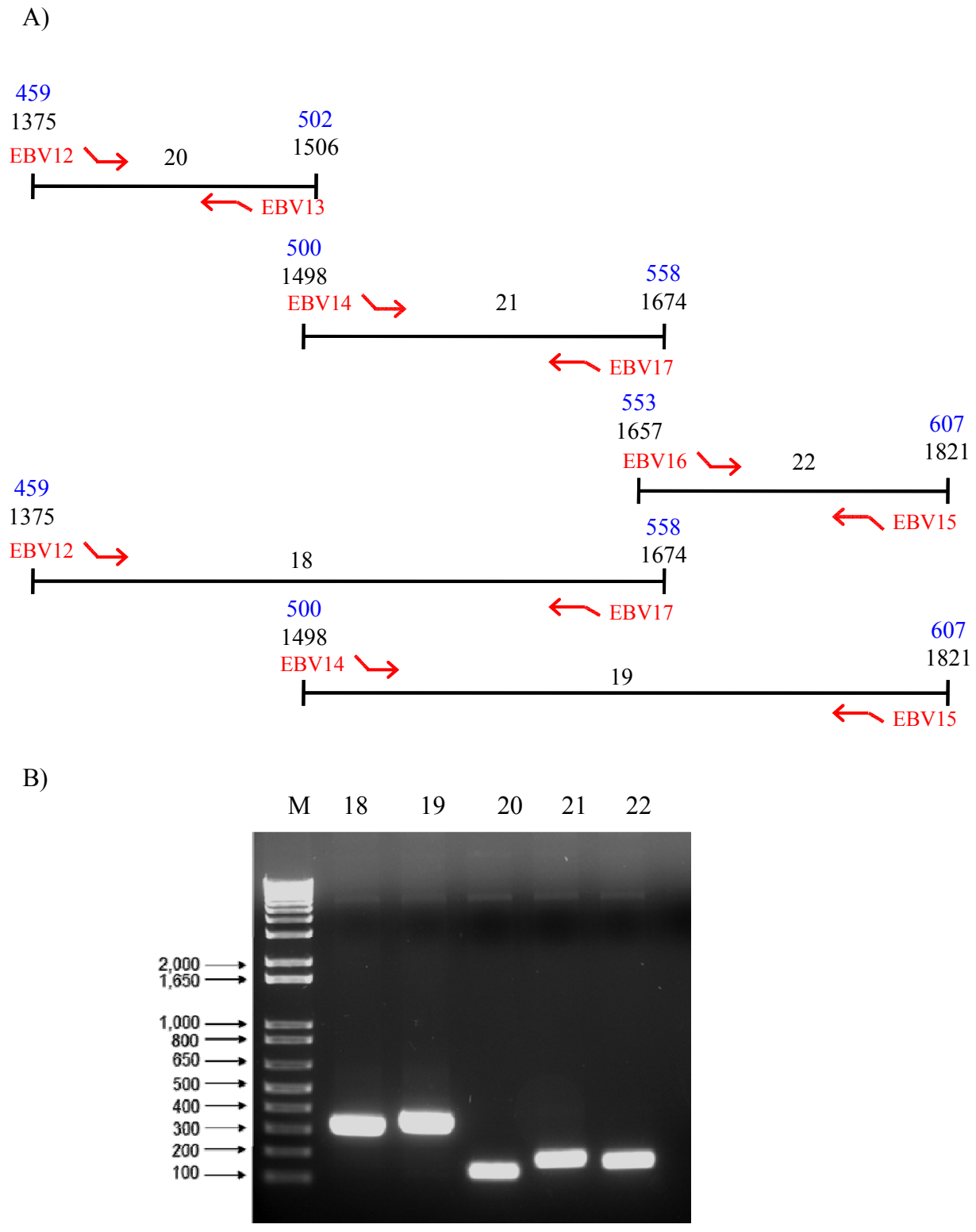


Figure 22. Strategy for further mapping the cross-reactive epitope in the carboxyl region of EBNA-1
(A)Arrows indicate PCR primers used to generate PCR products encoding five peptides derived from the carboxyl fragment , EBNA-$1_{459\text{-}607}$. Black numerals indicate the nucleotide number and blue numerals indicate amino acid number. (B) PCR products generated according to strategy in (A).

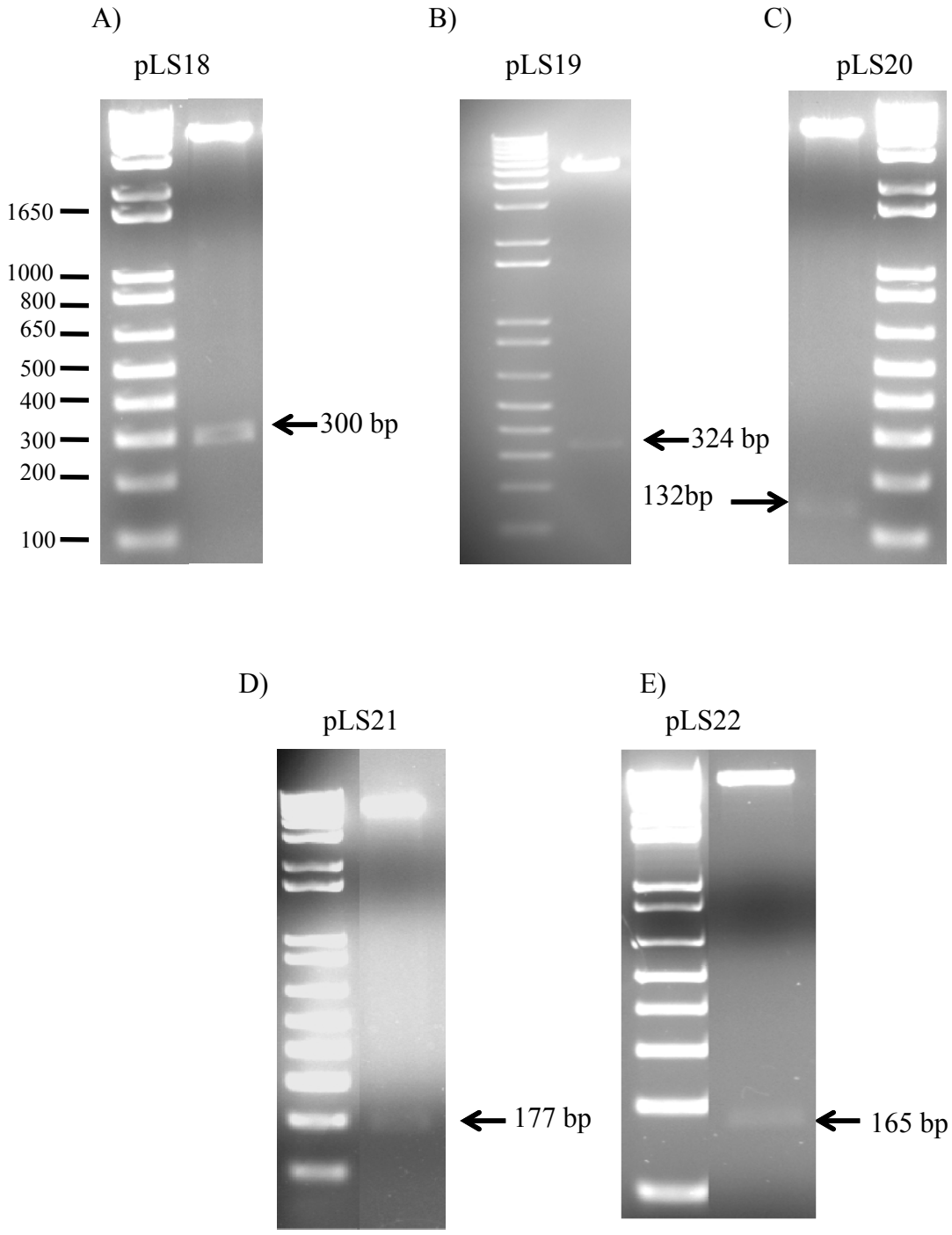


Figure 23. Recombinant pET28a plasmids containing inserts 18, 19, 20, 21 and 22 derived from the carboxyl region of EBNA-1. (A-E) Plasmids (pLS18-pLS22) were digested with NdeI and Hind III and were separated on 1% agarose gel. The inserts are indicated by an arrow.

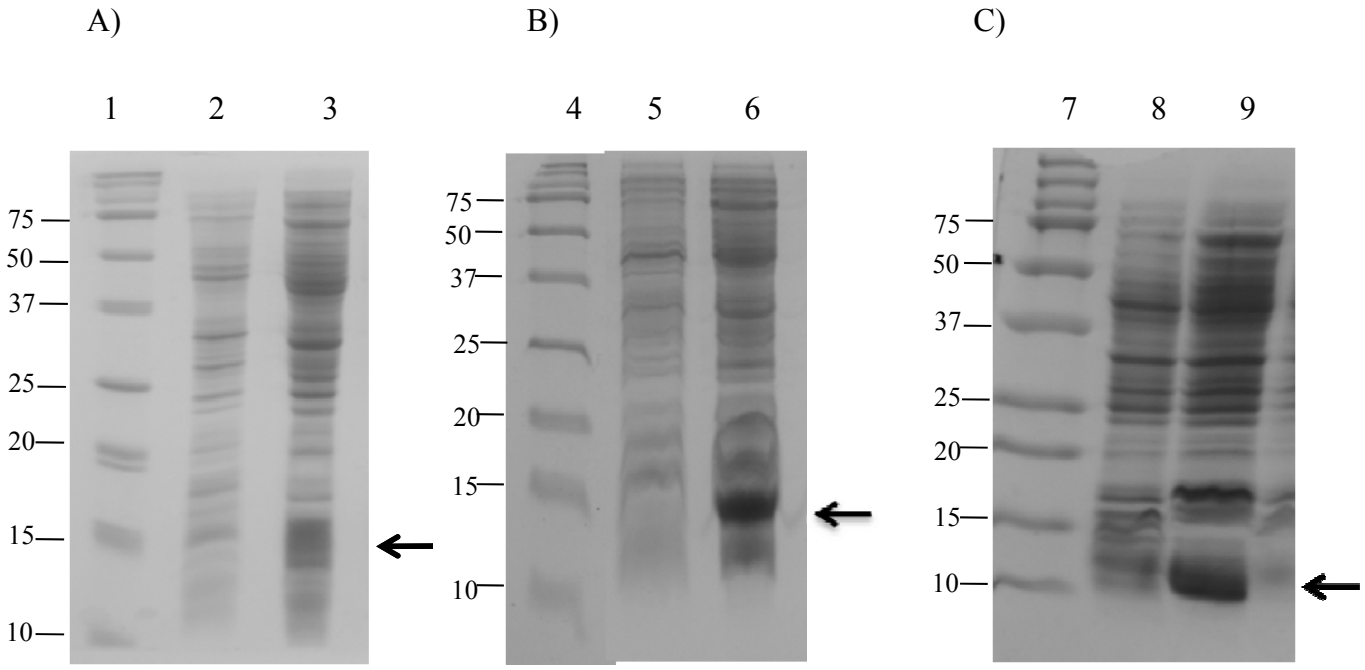
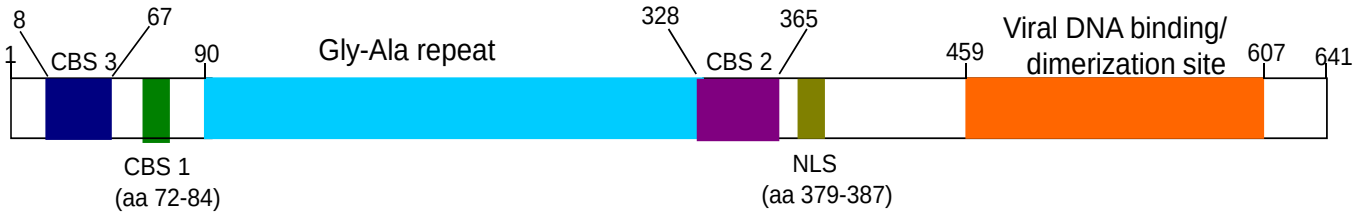


Figure 24. Expression of truncated protein fragments. Expression of LS18 (A) LS19 (B) and LS21(C) following induction with 1mM IPTG (lanes 3,6 and 9). Lanes 1, 4 and 7 are molecular weight markers, Lanes 2, 5 and 8 are uninduced samples. Arrows indicate proteins of expected molecular weight.

A)



B)

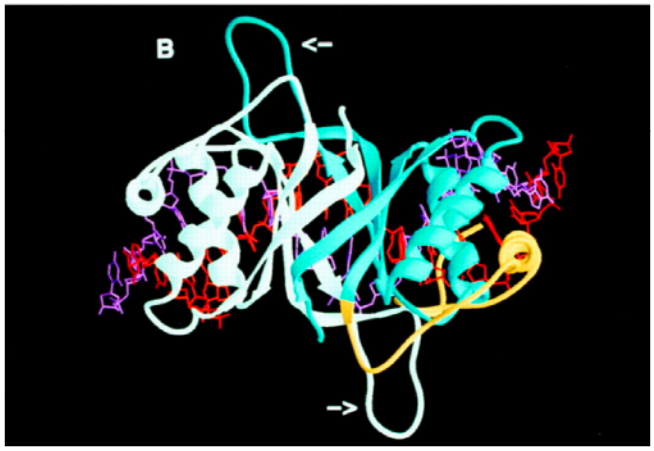


Figure 25. Crystal Structure of Viral binding site of EBNA-1. (A) Functional Map of EBNA-1 showing the Viral binding site (VBS) of EBNA-1 (aa 459-607). (B) Crystal structure of VBS as described by Bochkarev et al. EBNA-1 binds as dimers to 18bp consensus viral DNA sequence. The arrows indicate the proline loops of VBS.

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