

DNA Viruses - Herpesviruses II (EBV)

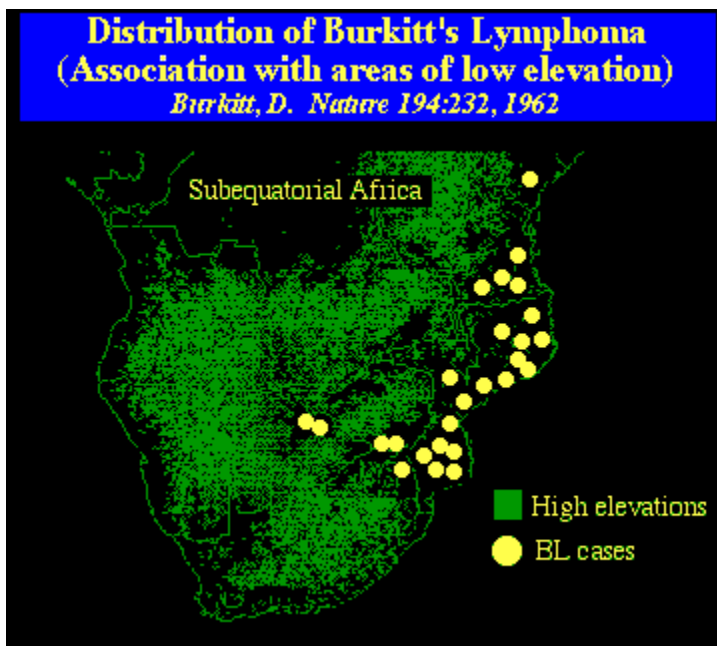
Sugden, Sem. Virol. 5:197-205, 1994

Kieff, Chapter 74 of Fields' Virology, 3rd Edition

Rickinson and Kieff, Chapter 75 of Fields' Virology, 3rd Edition

Discovery of EBV.

EBV was discovered as a result of pioneering work in the 1950s, by Denis Burkitt. Burkitt identified a previously unrecognized form of cancer which affected the jaws of young African children, and he made the crucial insight that the distribution of this common tumor (now known as Burkitt's lymphoma) appeared to be influenced by climatic factors -- notably temperature and elevation. Burkitt theorized that the tumor might be due to a mosquito-borne virus, or arbovirus.



This discovery led Tony Epstein, Yvonne Barr and Burt Achong to examine freshly excised tumor biopsies for the presence of a virus. In 1964, using electron microscopy, they found herpesvirus-like particles in a small number of the biopsied cells, and they subsequently established that this was in fact a new virus. Epstein-Barr virus was thus identified as the first candidate human tumor virus.

EBV is a member of the lymphocryptovirus genus of gammaherpesviruses. Related viruses exist in all Old World primates, including *Herpesvirus pan* and *Herpesvirus*

papio. These viruses share a tropism for **B lymphocytes**, and have a propensity to **oncogenicity**. A particularly valuable animal model for EBV is the infection of rhesus macaque monkeys with the rhesus lymphocryptovirus (Rhesus LCV). This induces an mononucleosis-like syndrome in the monkeys with virus persistence and shedding in oropharyngeal secretions.

Biological properties of EBV: An overview

Like other herpesviruses, EBV infects non-dividing cells. However, in the case of EBV, these cells are primary B lymphocytes, which EBV latently infects with very high efficiency, both *in vivo* and *in vitro*. The virus triggers these cells to start to proliferate, and then sustains their proliferation through the expression of specific growth-promoting genes which have the ability to render EBV tumorigenic. In total, latently infected B cells express only about 10% of the EBV genome, and only rarely support the lytic phase of EBV's life cycle. This latent infection of B cells by EBV represents the best understood example of herpesvirus latency. These notes therefore focus largely on EBV latency.

Latent EBV infection of B cells

The steps in EBV infection of B cells are as follows. First, EBV attaches itself to B cells, via the interaction of the EBV gp350/220 envelope glycoprotein with the **CD21** molecule on the B cell (aka CR2, for complement receptor 2), which serves as the cellular receptor for EBV and also as the receptor for the C3b component of complement. *Note that additional attachment receptors must exist since (1) virus attachment to epithelial cells can occur independently of CD21, and (2) gp350/220-negative mutants of EBV are still capable of infecting B cells and epithelial cells.*

Second, membrane fusion occurs, allowing entry of the EBV particle into the host cell cytoplasm. These post-attachment events involve the interaction of a viral glycoprotein complex (including the viral gH and gL homologs, as well as the gp42 molecule) with MHC class II molecules (including HLA-DR) on B cells. For epithelial cell infection, the gH/gL complex interacts with an unknown ligand, in a gp42-independent manner.

Following virus entry, the EBV particle is then dis-assembled and the genome is transported to the nucleus, where a state of viral latency is rapidly established. This is associated with the **activation, proliferation and immortalization of the latently infected B cell**.

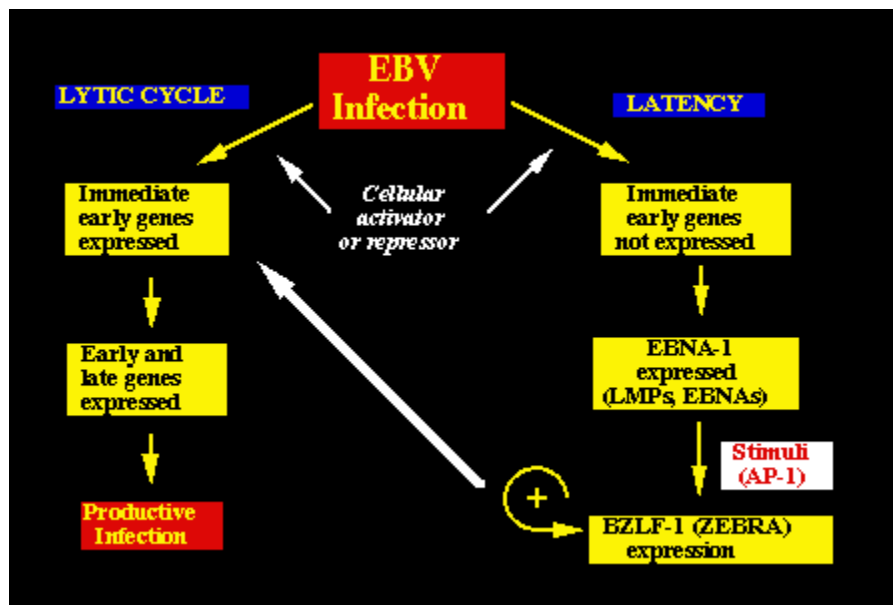
The remarkable ability of EBV to efficiently induce B cell growth transformation to permanent lymphoblastoid cell lines is a multistep process. First, EBV induces mature resting B cells to become activated, and to enter the cell cycle (going from a G₀, or resting state, to G₁). This initial step occurs as a result of EBV-mediated cross-linking of the the CD21 molecule, and is associated with secretion of Ig (immunoglobulin) and with

cell clumping, which increases local cell density and occurs as the result of the upregulation of cellular adhesion molecules. Second, the EBV-infected cells begin to proliferate in a manner that depends on high cell density and on the autocrine production of B cell growth-promoting cytokines. Over time, the EBV+ cells continue to proliferate and evolve into more rapidly growing cells that are less dependent on autocrine growth mechanisms.

EBV lytic cycle.

In vivo, EBV replication occurs most efficiently in epithelial cells. However, EBV replication also occurs spontaneously in a small fraction of the latently infected B cell population, as the result of viral reactivation. The signals responsible for this are unknown, although EBV replication can be triggered in a fraction of latently infected B cells *in vitro*, using phorbol ester (a protein kinase C activator) or by expression of a powerful immediate early transactivator of EBV lytic infection, the EBV-encoded **BZLF1** protein.

EBV Life Cycle



Overview of EBV pathogenesis.

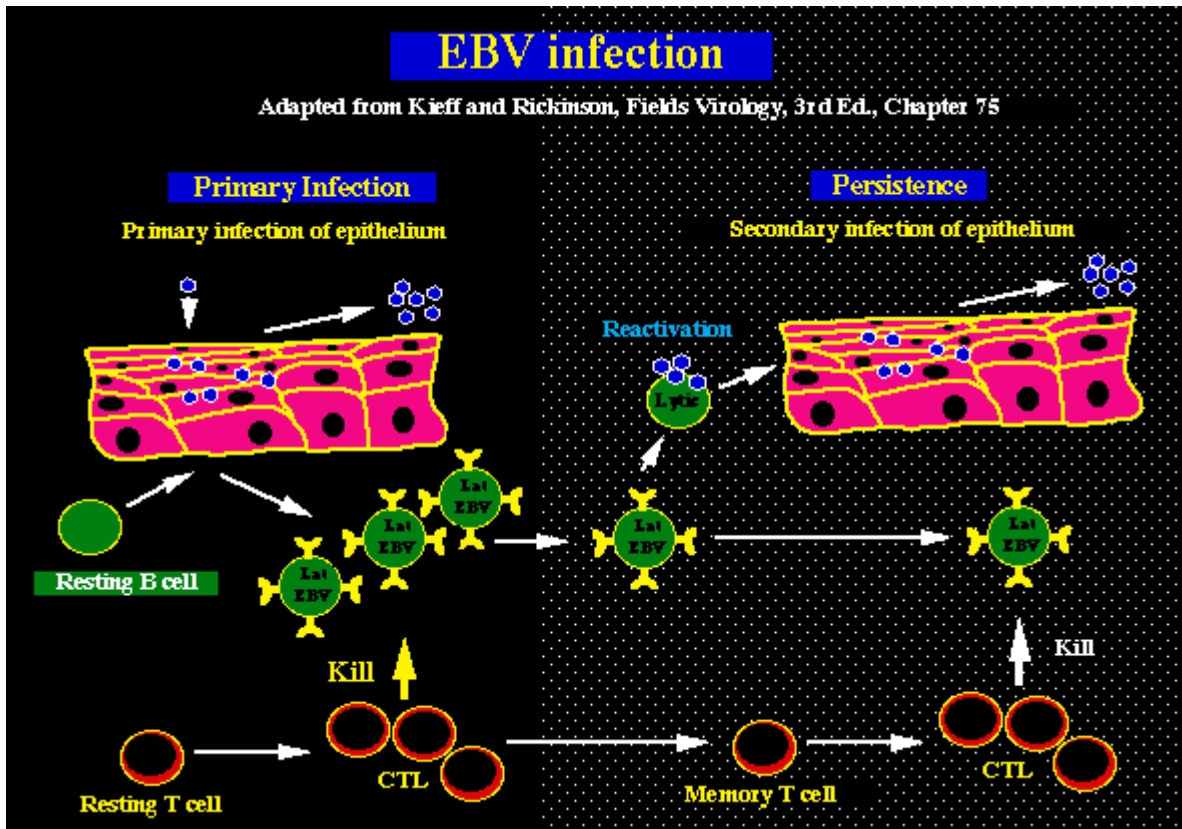
EBV associated diseases often arise from a failure of the host immune response to control the proliferation of latently infected cells. This is the opposite of what happens in other herpesviruses, where the problem is a failure to respond to lytic infection, and this correlates with the fact that latent infections by EBV predominate *in vivo*, with lytic-phase infection occurring in very few cells.

Pathogenesis of EBV infections. EBV infection is usually asymptomatic in childhood, and about 90% of adults are positive for the virus. EBV is spread by saliva and the virus initially infects oropharyngeal epithelial cells, where it replicates efficiently. The virus then infects B cells, as they pass through the oropharynx and this results in the establishment of latent viral infection in B cells. EBV persists lifelong in B cells, with roughly 1 in 10^5 to 10^6 of B cells being positive for the virus.

EBV infection of B cells is associated with the rapid proliferation and expansion of EBV+ B cells during primary viral infection. Normally, this EBV-driven proliferation of B cells is brought under control by cytotoxic T cells (CTLs). This commonly results in an **infectious mononucleosis (IM)**, particularly in young adults. However, in certain individuals, the initial EBV-driven B cell proliferation is not adequately contained, and this may result in fatal IM, which occurs particularly in males with X-linked lymphoproliferative disorder (**XLP**).

During the persistent stage of EBV infection (i.e., following the resolution of the acute infection), the virus primarily infects long-lived memory B cells in the periphery. This is thought to allow the growth-promoting genes of the virus to be switched off to create a site of persistent infection in vivo, without causing disease or providing antigenic targets for the immunosurveillance (*Joseph et al. J. Immunol. 165:2975, 2000*).

A note on XLP: Recent data have revealed that some persons with XLP develop fulminant infectious mononucleosis without ever becoming infected with EBV. This suggests that these individuals have some fundamental defect in T/B cell homeostasis, following viral infections. This view is consistent with the fact that the genetic defect in XLP is in a gene called SAP, which is thought to regulate T cell activation and homeostasis; SAP may also regulate B cell homeostasis.



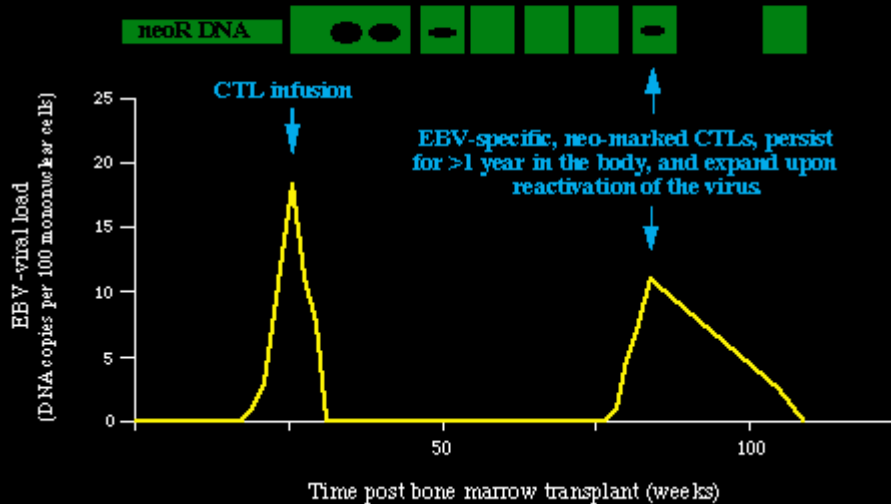
Immunoblastic lymphomas

Latent EBV infection of B cells is also associated with rapid tumor development in immunocompromised individuals, such as bone marrow transplant recipients and persons with AIDS (of whom about 10% will develop B cell malignancies). These immunoblastic lymphomas may, however, be amenable to treatment by adoptive transfer of virus-specific cytotoxic T lymphocytes (CTLs). In one study, Helen Heslop and her colleagues showed that infusions of gene-marked EBV-specific CTLs resulted in a restoration of immune responses against EBV in immunocompromised patients at risk for the development of EBV lymphoproliferative disease (see figure). EBV-specific CTLs were isolated and propagated *in vitro*, marked with the NeoR gene and then introduced into the host at approx. 25 weeks post-transplant (when there was a rise in the EBV load). The EBV load fell dramatically in response to the infusion. One year later, a second period of EBV reactivation was associated with a re-amplification of the NeoR-marked CTL population. The re-expansion of the EBV-specific CTL population once again led to control of the reactivated infection, demonstrating the importance of the CTL response in controlling EBV reactivation, and showing that adoptive CTL transfer may have therapeutic value.

Restoration of immunity to EBV by adoptive transfer of gene-modified EBV-specific cytotoxic T cells (CTLs)

Heslop et al. Nature Med. 2:551, 1996

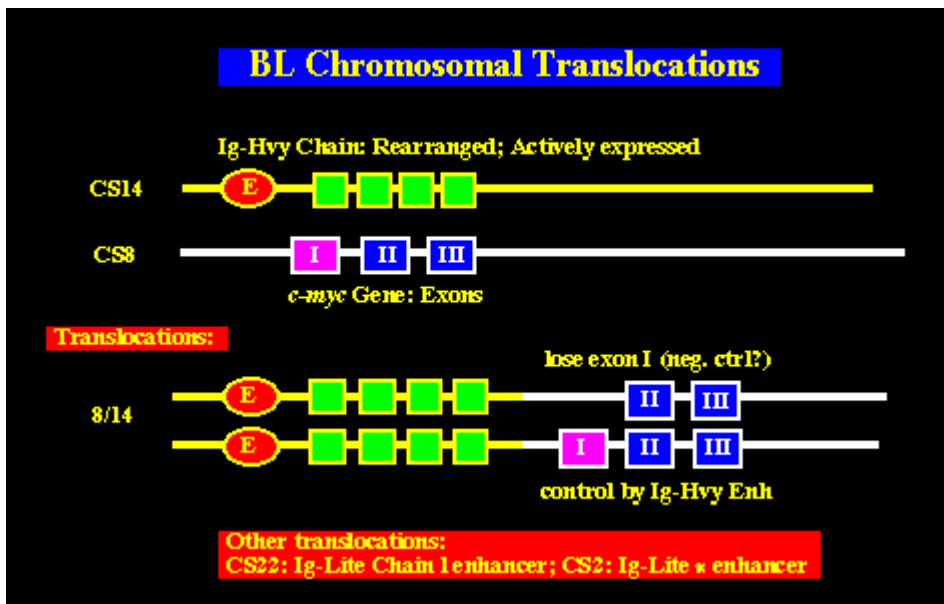
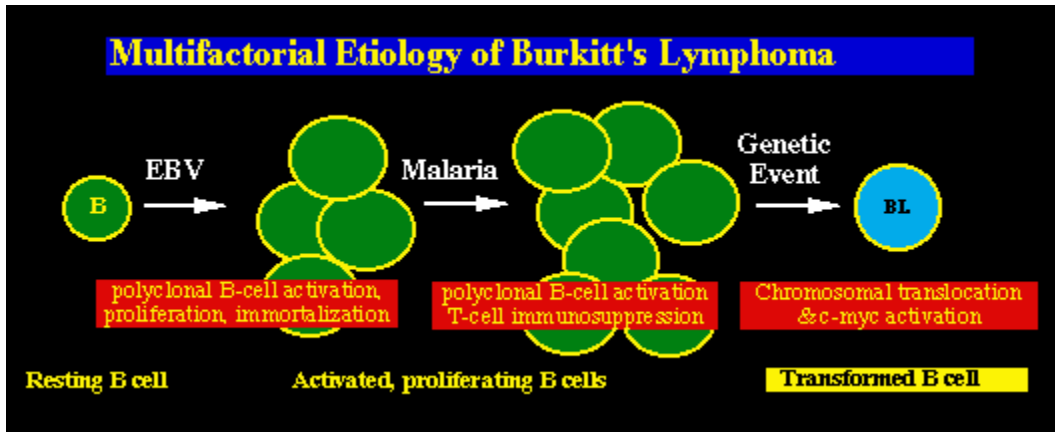
Relevance: EBV lymphomas develop in ~30% of bone marrow transplants from unmatched family members or from matched unrelated donors



Burkitt's lymphoma.

Burkitt's lymphoma (BL) has likewise been linked to immunosuppression in AIDS, and to malarial infection in the case of endemic BL. The latter phenomenon explains Burkitt's original finding that climatic factors are involved in the etiology of endemic BL, since temperature and elevation influence the distribution of the mosquitoes which carry malaria. All cases of Burkitt's lymphoma are also marked by the presence of specific chromosomal translocations, which result in the activation of the *c-myc* proto-oncogene. The majority of these involve a reciprocal translocation between chromosome 8 at or near the site of the *c-myc* locus, and the immunoglobulin heavy chain locus on chromosome 14.

Multifactorial pathogenesis of endemic BL



Other EBV associated tumors include Hodgkin's disease (a common malignant lymphoma), certain unusual types of T cell lymphoma, and an epithelial tumor that develops many years after initial virus infection and which is common in Southeast Asia - nasopharyngeal carcinoma.

Selected EBV-associated tumors

Tumor	Subtype	Latent Period	EBV Positivity
Immunoblastic lymphoma	Fatal IM	< 6 months post EBV	100%
	Tr-associated	< 6 months post Tr	100%
	AIDS-related	5-10 years post HIV	70-80%

Burkitt's lymphoma	Endemic	3-8 years post EBV	100%
	AIDS-related	3-8 years post EBV	30-40%
Hodgkin's disease	Various	10-30 years post EBV	30-90%
T cell lymphoma	Nasal	> 30 years post EBV	100%
Nasopharyngeal carcinoma		> 30 years post EBV	100%

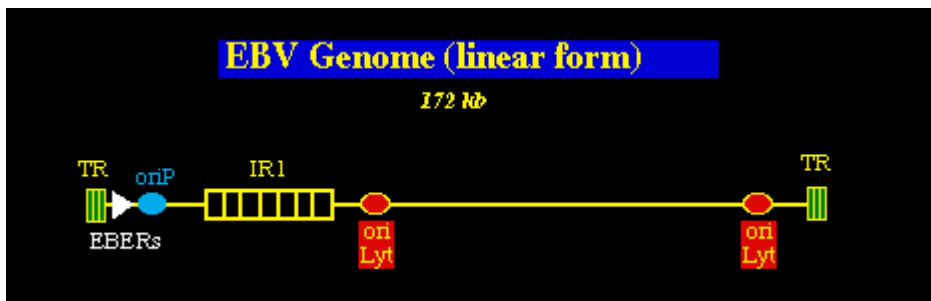
IM: Infectious mononucleosis; Tr: Transplant

EBV genome

EBV was the first herpesvirus to be completely sequenced, in 1984. It is 172 kbp in size, and contains a single long unique region that is interspersed with a number of internal repeats and flanked by terminal direct repeats at either end. The largest of the internal repeats is known as IR1 and corresponds to repeating Glycine-Alanine motif that is part of the EBNA1 protein.

Note that two distinct types of EBV (EBV-1 & EBV-2) have been identified, which have extensive homology and are equally common in different geographical areas and populations. The significance of these different virus types remains uncertain.

EBV genome (linear form)



EBV gene expression during viral latency.

EBV devotes a large amount of genetic information to establishing and maintaining the latent state in mitotic B cells. At least 11 EBV genes are expressed during latent infection. Two of these encode small, non-polyadenylated and non-coding RNAs (EBER 1 and EBER 2), six encode nuclear proteins (EBNA 1, 2, 3A, 3B, 3C, and LP) and three encode membrane proteins (LMP 1, 2A, and 2B). Together, these proteins lead to cellular immortalization, which involves both increased cellular survival and increased cellular proliferation.

EBV immortalizing genes.

Four EBV latent genes have been shown to be required for detectable proliferation of infected cells. These are: EBNA2, LMP1, EBNA3A and EBNA3C. A fifth protein, EBNA1, is also required for maintenance of the EBV genome in latently infected cells.

These EBV immortalizing genes have three important common themes. First, they are highly spliced and their coding elements are dispersed through genome. In comparison, the EBV lytic genes are more compact and less highly spliced. Second, although the coding elements of the EBV latent genes are scattered, the *cis*-acting control elements that regulate their expression (promoters, enhancers) are clustered, and are not dispersed. Third, no homologs of the EBV immortalizing genes can be found in the alpha- and beta-herpesviruses.

Functions of EBV Latent Genes

Gene	Function
EBNA-1	Latent phase viral DNA replication: binds the viral latent origin, <i>oriP</i> . EBNA1 also activates transcription of other latent genes. It is minimally antigenic due to a GlyAla repeat.
EBNA-2	Transcriptional activator of viral and cellular genes: Targeted to DNA by the cellular J kappa recombination signal sequence binding protein (RBPJ) -- <i>this is analogous to HSV VP16 which also cannot bind DNA directly</i>
LMP-1	Transcriptional activator: Engages signaling proteins for the tumor necrosis factor receptor family (TRAFs), activates NF- κ B & mimics CD40 signals to cause B cell activation and differentiation. Prevents apoptosis by inducing <i>bcl-2</i> (and NF/ κ B).
EBNA-3C	Transcriptional activator of CD21, LMP1. May allow fine control of EBNA2 (it binds RBPJ and stops it from contacting DNA)
EBNA-3A	Contributes to the initiation of cellular proliferation in EBV infected B cells -- uncertain how. Like EBNA-3C, it binds RBPJ.

Patterns of EBV latent gene expression.

There are several patterns of EBV latent gene expression. One may involve EBV genome maintenance in resting B cells. This can occur in the absence of EBNA-1 expression, since there is no need for EBV to replicate its genome in a resting B cell. However, it is not clear how an EBV-positive B cell is able to re-enter the resting state.

Other types of EBV latency include:

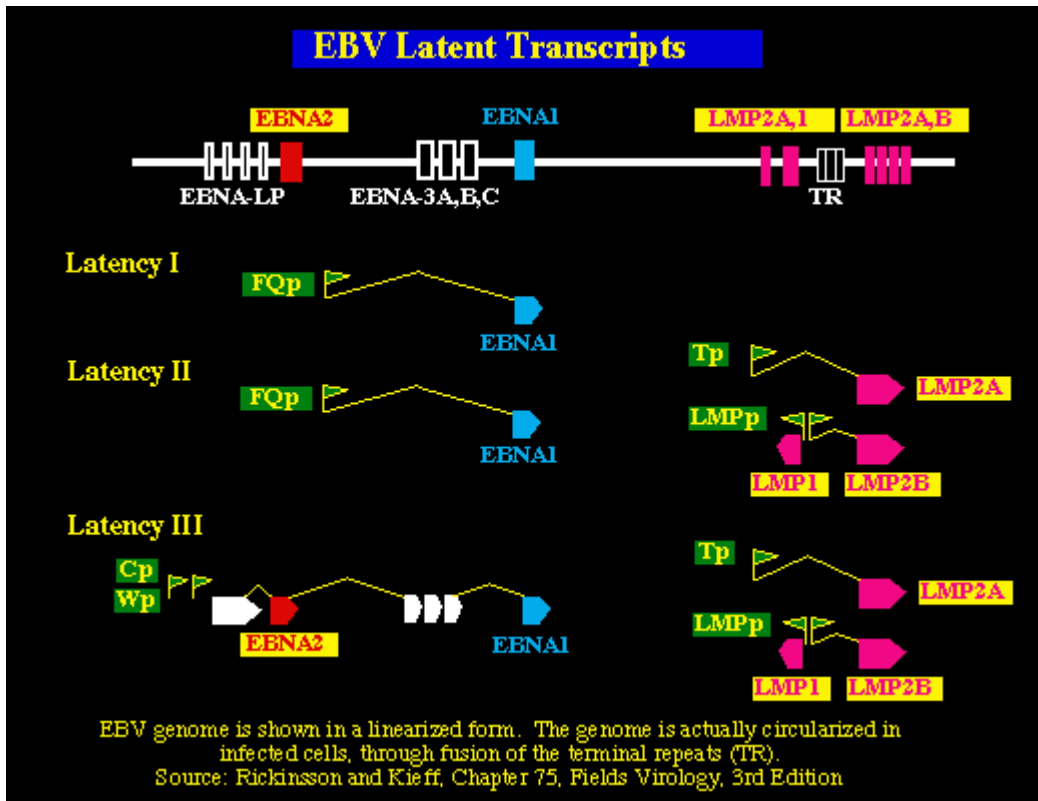
Type I latency: this is typical of fresh BL biopsy specimens, and is characterized by the expression of only the EBERs and EBNA1. This is thought to be the minimum possible level of EBV gene expression which can support viral genome maintenance in a dividing host cell (since EBNA-1 is required for genome maintenance). As a result there is almost no target for immune recognition *in vivo* -- a phenomenon that is exacerbated by the fact that EBNA1 is a very weakly antigenic protein.

Type II latency is typical of NPC tumors. In this case, the LMP proteins are expressed, in addition to EBNA1 and the EBERs.

Type III latency is typical of normal (non-cancerous) cells that are latently infected by EBV, as well as EBV-immortalized lymphoblastoid cell lines and long-term passaged BL tumor cells. In this case, all 11 viral latent genes are expressed.

Regulation of EBNA expression. The EBNAs are expressed from one of three known promoters, and are derived by splicing from huge primary transcripts (up to ~100 kb). A distinct promoter (FQp) is used to express EBNA-1 in type I and type II latency (FQp is ~50 kb closer to the EBNA-1 ORF than are Wp or Cp). In type III latency, the Wp and Cp promoters are used to express all six EBNAs. These promoters are used in a mutually exclusive fashion.

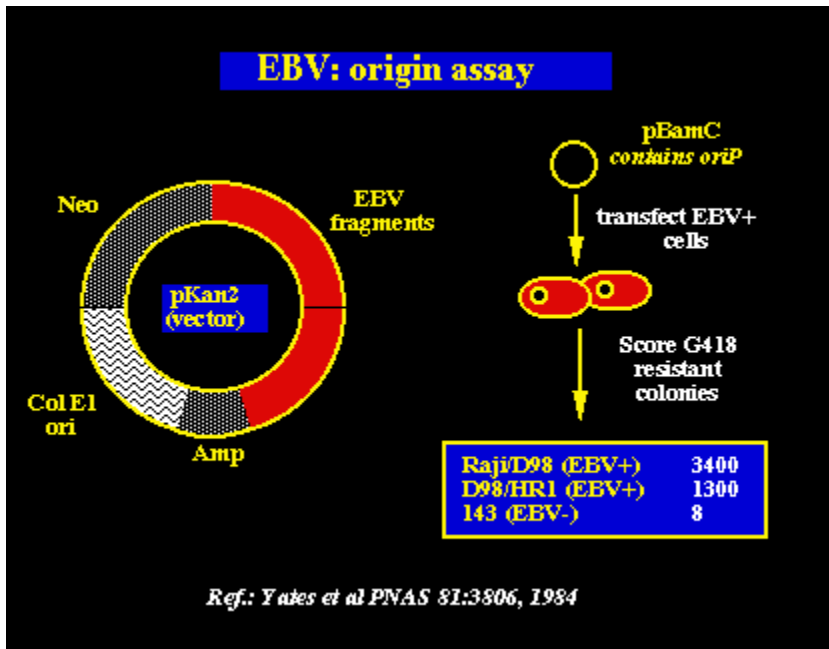
According to a model proposed by Strominger, the Wp promoter is used to express EBNA-2 and EBNA-LP (only) during the initial phase of EBV infection in B-cells. As EBNA-2 accumulates, it triggers a switch to the nearby Cp promoter, which allows the synthesis of the other EBNA proteins. *In tumor cells, a switch to the distant FQp promoter occurs somehow at a later time, leading to the synthesis of EBNA-1 alone.*



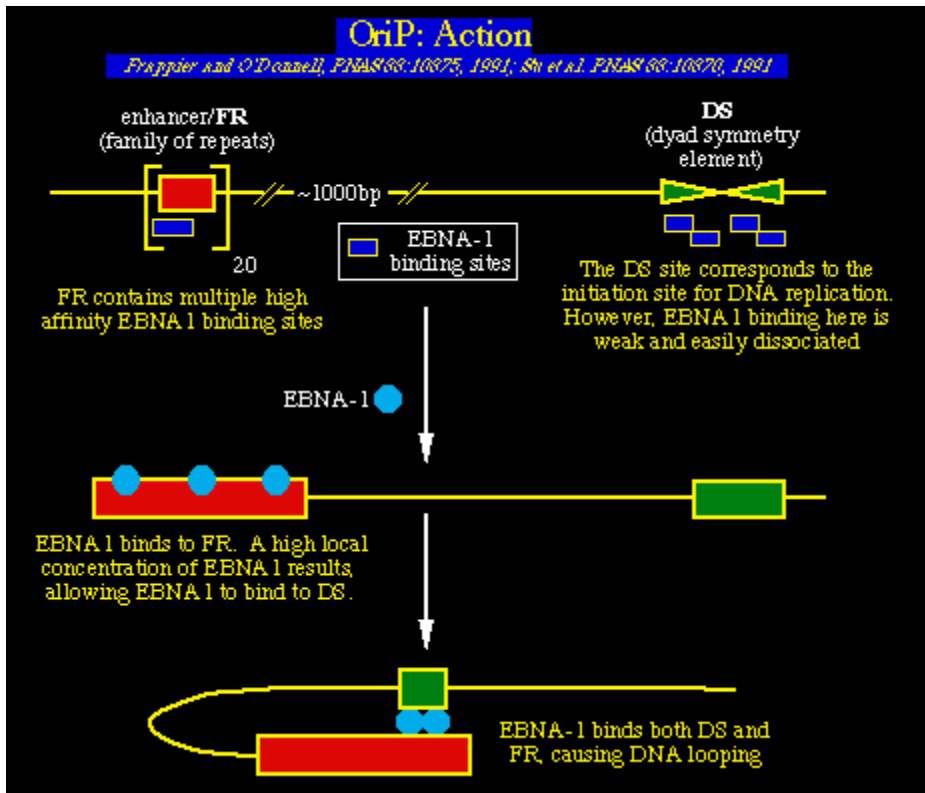
OriP and latent phase EBV DNA replication

OriP is the origin of DNA synthesis for episomal EBV DNA in latently infected B cells. Its activity is absolutely dependent on the expression of EBNA1, since it also allows plasmid replication in primate cells which express only EBNA1 (and no other EBV genes). *OriP* also allows for the proper segregation of replicated EBV episomes to daughter cells, upon cell division. *OriP*'s activity most likely also requires one or more cellular proteins, since *oriP* does not allow plasmid replication in non-primate cells which express EBNA1.

OriP was originally identified by John Yates and Bill Sugden, who cloned a library of EBV DNA fragments into pKan2, a G418-selectable plasmid which encodes the Neo^R gene. They then transfected the library into EBV-infected (EBV+) and EBV-uninfected (EBV-) cells. In EBV+ cells, only, plasmids containing the BamC DNA fragment (which includes *oriP*) were maintained with high efficiency, resulting in a large number of G418 resistant, plasmid-containing, colonies.



OriP is composed of two elements. One is the FR site (a family of repeats) and the other is the DS (dyad symmetry) element, which is located about one kilobase away. DNA replication actually starts at the DS element, which is somewhat surprising in that the DS element binds EBNA1 much more weakly than the FR element. However, studies by Lori Frappier, Bill Sugden and their colleagues revealed that FR acts a replication enhancer. Specifically, FR binds EBNA1 very efficiently. The result is that EBNA1 accumulates at a site on the EBV genome (FR) which is very close to the DS site. This allows EBNA1 to then contact the DS element, while also interacting with FR (this happens because EBNA1 can bind to itself as well as to DNA).



Structure of EBNA1: Aled Edwards and his colleagues have succeeded in determining the crystal structure of the EBNA1 protein, both alone and bound to DNA. It turns out that EBNA1 binds to DNA as a dimer, and that the EBNA1 dimer is both very stable and very rigid. Thus, when the two EBNA1 molecules within each dimer bind to two adjacent protein binding sites, such as those present in the DS element, the DNA is forced to undergo a structural distortion. This is believed to be crucial to the initiation of DNA replication, since it helps to unwind the viral DNA at the DS element, thereby facilitating the initiation of DNA replication at this site.

Minimal antigenicity of EBNA1: All latent EBV expression requires the production of EBNA1. Thus, this represents an important potential target for immune recognition. Indeed, recognition of EBNA1+ cells would lead to elimination of the latent reservoir of EBV infection. For this reason, it is not surprising that EBNA is a uniquely weak immunogen. This property appears to be due to the presence of an extended glycine-alanine (GA) repeat domain within the protein, which inhibits in *cis* MHC class I-restricted antigen presentation. This GA repeat appears to mediate its effect by interfering with the proteasomal processing system. This is required for the presentation of endogenous cellular antigens via the class I pathway (*Shapiro, Nature Med. 4:939, 1998*).