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# SPECIFICALLY MUTATED EPSTEIN-BARR VIRUS RECOMBINANTS: DEFINING THE MINIMAL GENOME FOR PRIMARY B LYMPHOCYTE TRANSFORMATION

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## INTRODUCTION

In the past 30 years since the discovery of Epstein-Barr Virus (EBV) in Burkitt lymphoma (BL) cells growing in culture, the epidemiology and role of this Herpes virus in benign and malignant disease, and the relevant molecular biological properties of the virus in establishing latent infection in B lymphocytes, in causing proliferation of the latently infected cells *in vitro*, and in replicating in B lymphocytes, have been extensively investigated. However, relatively little has been done to correlate the molecular biological observations with EBV recombinant molecular genetics which could provide a critical and independent approach to defining the role of viral genes in specific aspects of virus infection and its effects on cell growth. For the most part, the lag in recombinant EBV molecular genetics arose because of the poor permissivity of B lymphocytes for EBV replication and the lack of an alternative, more permissive host cell. Recently, however, substantial progress has been made in the development of strategies for specifically mutating EBV genes and for creating EBV recombinants. These strategies have resulted in significant advances in understanding the role of specific EBV genes in lymphocyte infection and in cell growth transformation. This review will focus on latent EBV infection and B lymphocyte growth transformation. The relevant biology and biochemistry of EBV infection, the general strategies for constructing EBV recombinants, and the significant results of the application of these strategies to the study of the role of specific EBV genes in latent infection and growth transformation will be described.

## BACKGROUND

First, the relevant medical and molecular biology (for more extensive reviews and primary references see Liebowitz & Kieff, 1993; Miller, 1989; Epstein & Achong, 1979, 1986; Farrell, 1992; Henle & Henle, 1979; Klein, 1987; Middleton *et al.*, 1991; Moss *et al.*, 1992; Rogers, Strominger & Speck, 1992; Thomas, Allday & Crawford, 1991; Magrath, 1990; Amen *et al.*, 1986, and also contributions by Rickinson, Farrell and Klein in this volume). EBV is transmitted in saliva. Infection begins in the oropharyngeal epithelium which is permissive for virus replication. In the course of primary infection, as many as 10% of the circulating B lymphocytes become infected. These cells are largely non-permissive for virus replication and are probably the important site of long-term latent EBV infection and of periodic reactivation for reseeding of virus on to the oropharyngeal epithelium. Initially, natural killer cells, and later, CD8<sup>+</sup> cytotoxic T lymphocytes eliminate almost all virus infected B lymphocytes. In adolescence, this phase of infection and immune response results in the manifestation of acute infectious mononucleosis. After primary EBV infection, only about 1 in 10<sup>5</sup>-10<sup>7</sup> peripheral blood B lymphocytes is latently infected with EBV. When these latently infected cells are cultured *in vitro*, or when normal B lymphocytes are infected with EBV *in vitro*, the latently infected cells will continuously proliferate. These latently infected cells are tumorigenic in nude mouse brain or in SCID mice. The effect on B lymphocyte proliferation is also seen in cotton top tamarins where inoculation of a large dose of EBV results in an acute polyclonal lymphoproliferative disease. A related disease occurs in humans whose cytotoxic T lymphocytes specific for EBV transformed lymphocytes have been destroyed or rendered functionally incompetent or in humans with X linked or sporadic genetic susceptibility to EBV infection. Thus, although latent infection of B lymphocytes is associated with the expression of virus genes which can cause cell proliferation, in the normal host the proliferating latently infected cells are well contained and provide a long term reservoir of virus from which infection can reactivate, can spread to the oropharyngeal epithelium, and can infect non-immune hosts.

When primary B lymphocytes are infected with EBV *in vitro*, the virus expresses six nuclear proteins or EBNAs, two integral membrane proteins or LMPs and two small RNAs or EBERs. The EBNAs, LMPs and EBERs are expressed in some latently infected cells *in vivo* since normal infected humans have continuously circulating T lymphocytes which specifically recognize peptides derived from each of the EBNAs and LMPs (with the possible exception of EBNA 1) in the context of class I histocompatibility molecules. EBNA or LMP mRNAs have also been detected in the peripheral blood of normal seropositive people. Further, these genes are expressed in latently infected cells in EBV associated lymphoproliferative disease. However, the full complement of EBNAs and LMPs may not be expressed

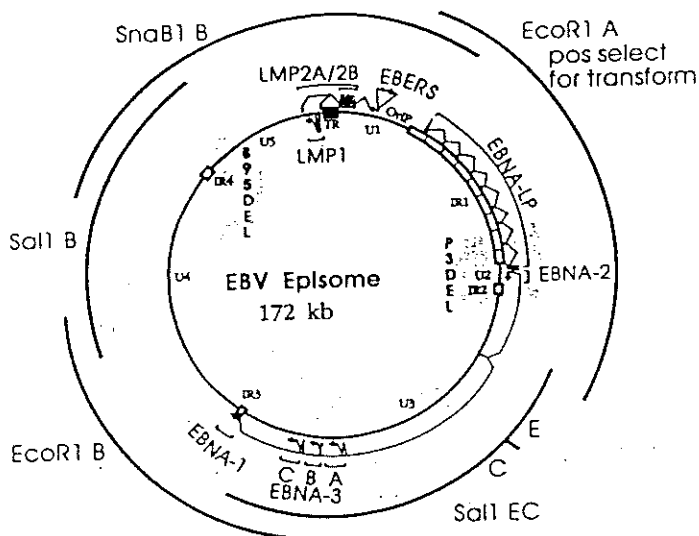


Fig. 1. EBV episome, transcripts, and mRNAs in latent B lymphocyte infection. Largely unique (U1-U5) and highly repetitive internal (IR1-4) or terminal (TR) repeat DNA domains, the episome DNA replication origin and termination site (ori P) and the location of exons encoding EBV nuclear proteins (EBNA 1, 2, 3A, 3B, 3C, LP) or membrane proteins (LMP 1, 2A or 2B) genes are indicated by dark boxes. The EBNA mRNAs are spliced as indicated from a single transcript. Transcription is shown as it initiates from the IR1 or Bam W promoter. In many infected lymphocytes, after expression of EBNA LP and EBNA 2, the Bam C promoter in U1 dominates EBNA transcription. Alternative splicing then leads to expression of all of the EBNAs. EBNA-LP is encoded by repeating exons (W1 or W2) from IR1 and two short unique exons from U2. Translation of EBNA LP is dependent on a splice between the exons downstream of the W or C promoters and the first W1 exon. RNAs with a 5b shorter first W1 exon have an ATG at the beginning of the EBNA LP open reading frame and express EBNA LP. RNAs with a longer W1 exon are not translated to EBNA LP. These RNAs have a long untranslated leader and EBNA 2, 3A, 3B, 3C or EBNA 1 from alternative open reading frames at the end of their respective mRNAs. The EBERs are two small, non-polyadenylated RNAs which are transcribed in latent or productive infection. LMP 1 and LMP 2B are transcribed in opposite directions under control of promoters separated by only 200 bp. The promoters share the same EBNA 2 response element and are both up regulated by EBNA 2 expression. Another clockwise promoter downstream of the LMP 1 polyadenylation site transcribes LMP 2A. LMP 2A also has an upstream EBNA 2 response element. The cosmids used in one cosmid marker rescue of transformation experiments (EcoRI A) or in the first second site marker rescue experiments (SalI C) or in the five cosmid genome reconstruction experiments are shown.

in all latently infected cells. Another state of latent infection has been noted in BL cells where, frequently, EBNA 1 is the only EBNA which is expressed. If a similar state of latency can occur in a normal B lymphocyte, the infected cell would not be recognized as foreign by most EBV immune CD8<sup>+</sup> cytotoxic B lymphocytes.

The key steps in initiation of latent infection in primary B lymphocytes *in vitro* (Fig. 1) are:

1. Within 8 hours after infection, the viral genome circularizes, and RNA

is transcribed under control of the Bam W or IR1 latency promoter. The RNA is processed into two multiply spliced mRNAs which differ only in the second exon acceptor site and which encode either EBNA LP from the multiply spliced first exon or EBNA 2 from a terminal exon. Immediate early or other viral genes expressed in the course of lytic infection are not expressed during latent infection.

2. Within 24 hours, EBNA 2 turns on EBV promoters for LMP1 and LMP2 and cell promoters for CD21, CD23 and c-fgr. The EBNA latency promoter in some cells shifts upstream to a Bam C or U1 latency promoter (probably due to EBNA 2 responsive elements around the Bam C promoter). EBNA transcription now extends through at least two-thirds of the R strand of the viral genome and the EBNA transcript is processed into RNAs encoding EBNA LP, EBNA 2, EBNA 3A, EBNA 3B, EBNA 3C and EBNA 1.
3. Within 48 hours, all the EBNAs and LMPs are expressed. The expression of many B lymphocyte genes is induced by EBNA 2 or LMP 1 or by other EBNAs. EBNA 3A, 3B and 3C are three distantly related genes which evolved from a single progenitor since the three are distantly homologous, are tandemly located in the EBV genome, are similarly processed into a short and long exon at the end of EBNA mRNAs, and encode proteins of approximately 1000 amino acids. EBNA 3C can induce CD21 expression in B lymphocytes. LMP1 and LMP2 localize to a patch in the plasma membrane. LMP1 has transforming effects on immortalized rodent fibroblasts including loss of contact inhibition, serum independence, anchorage independence and nude mouse tumorigenicity. LMP1 also induces expression of many B lymphocyte activation and adhesion molecules and of Bcl-2 and A20, two proteins which have anti apoptotic effects in B lymphocytes. Much of this activation is probably mediated by NF-kb interaction. LMP2 interacts with src family tyrosine kinases (Burkhardt *et al.*, 1992).
4. By 36-48 hours, cell DNA replication begins. Cell division follows. The EBERs are then expressed. The EBV episome is maintained and amplified through cell division in response to EBNA 1 binding to the EBV ori-p element and the recruitment of cellular factors. Viral DNA synthesis initiates at the dyad symmetry component of the origin and terminates at the family of repeats component of the origin. (Both components being composed of repeats of the same EBNA 1 cognate sequence).
5. The EBNAs, LMPs and EBERs continue to be expressed and the cell continues to proliferate.

## OBJECTIVES

Recombinant EBV-based molecular genetic experiments were initiated in order to evaluate the relative importance of the EBNAs, LMPs and EBERs in latent infection and cell growth transformation, and to define critical domains and specific sites in these molecules which can be used to further delineate essential biochemical interactions.

*Background of the experimental strategy*

The approach is based on previous studies of herpes simplex and pseudorabies virus molecular genetics, on the unique ability of EBV to establish latent infection and to transform B lymphocytes into cell lines (LCLs), on strategies for transfection of B lymphocytes, and on strategies for the induction of lytic EBV infection in latently infected cells. The important principles of those previous experiments are:

1. The transfection of herpes simplex virus (HSV) DNA or of overlapping clones of pseudorabies virus (PRV) DNA into cells can result in virus replication.
2. Transfection of HSV DNA into cells along with a mutagenized fragment of HSV DNA results in a high frequency of incorporation of the transfected DNA fragment into the correct site in the progeny viral genomes. Transfection of cells with a DNA fragment and infection with HSV results in a lower frequency of incorporation of the mutagenized fragment into the correct site in the viral genome.
3. Lymphoblasts can be transfected by electroporation.
4. Lytic EBV infection can be induced with a low efficiency in latently infected cells with phorbol esters, or butyrate, or by transfection of cells with an expression vector for the z immediate early transactivator of lytic EBV infection.

Thus, the strategies which evolved were based on lymphoblast transfection, on induction of partial permissivity for EBV replication, on the expectation that a transfected EBV DNA fragment would recombine with the replicating EBV genome in lytically infected cells and on the ability to identify EBV infected primary B lymphocytes by their continuous proliferation into LCLs. This last point raises the most important theoretical barrier at the start of this investigation. How can mutation be studied in a gene which is essential to the processes of latent infection and cell growth transformation, when the only way of obtaining recombinants is through infection of primary B lymphocytes and the clonal derivation of LCLs?

*'Gain of function', transforming, EBV recombinants*

The first recombinant EBV molecular genetic experiments avoided the conceptual barrier of inability to isolate non-transforming mutants by

creating 'gain of function' transforming mutants, using the P3HR-1 EBV strain which has a deletion involving two nuclear proteins. There was considerable precedent for this approach. P3HR-1 is a BL derived cell line which is one of the most spontaneously permissive cell lines for lytic EBV infection. The P3HR-1 EBV is replication competent, but the resulting virus is unable to transform primary B lymphocytes. The P3HR-1 EBV genome is deleted for a DNA segment which includes the last two exons of EBNA LP and the entire EBNA 2 exon. A favourite working hypothesis was that the deletion involving EBNA LP and EBNA 2 was the basis for the inability of P3HR-1 to growth transform primary B lymphocytes. P3HR-1 EBV can induce lytic EBV infection in another Burkitt lymphoma-derived cell line, Raji, which carries a replication defective endogenous EBV genome containing two deletions. The resulting virus stocks could transform primary B lymphocytes. The resultant LCLs contain EBV genomes which have markers characteristic of the P3HR-1 genome except for restoration of the deleted DNA segment that encodes EBNA LP and EBNA 2.

The first recombinant EBV molecular genetic experiments were done by transfecting into P3HR-1 cells a cloned wild type (WT) EBV DNA fragment which spanned the P3HR-1 deletion, inducing replication and infecting primary B lymphocytes with the resultant virus (Hammerschmidt & Sugden, 1989; Cohen *et al.*, 1989). The infected primary B lymphocytes were then either plated in soft agarose over fibroblast feeder layers or seeded into multiple micro well plates. In both sets of experiments, the conditions for the growth of newly transformed primary B lymphocytes were near optimal, and 'gain of function', transforming, EBV recombinants were clonally derived in clones of resultant LCLs. The recombinants had the restriction endonuclease digestion products expected for EBV P3HR-1 DNA which had incorporated the WT DNA at the former deletion site. The procedures were standardized so that the number of transformants obtained following transfection with a WT EBV DNA fragment spanning the deletion was similar among repeated experiments. To evaluate specifically the roles of EBNA LP and EBNA 2 in the rescue of transformation competence, mutations were made in the EBNA LP and EBNA 2 open reading frames within the cloned transfected DNA fragments. Transfection of P3HR-1 cells with cloned EBV DNA fragments which span the site of the P3HR-1 deletion and which had been specifically mutated by insertion of a stop codon or by deletion of substantial parts of the EBNA 2 open reading frame gave no recombinants which could induce B lymphocytes to grow into LCLs. An interesting correlative series of experiments was based on the observation that there are two EBV strains which have diverged in their EBNA 2, EBNA 3A, 3B and 3C genes. Type 1 (T1 or A type) strains have a much greater ability to transform primary B lymphocytes. EBV recombinants constructed by transfection of P3HR-1 cells with WT DNA having the T1 EBNA 2 exhibited the T1 EBV transforming phenotype; while replacement

of the EBNA 2 open reading frame with T2 EBNA 2 results in a T2 transforming phenotype. The consistent failure of at least two DNA clones with specifically mutated EBNA 2 open reading frames to yield LCLs in multiple experiments, the consistent positive results with WT constructs transfected in parallel and the type specific effects of EBNA 2 prove that EBNA 2 is critical to the ability of EBV to transform primary B lymphocytes.

An extended series of these marker rescue experiments demonstrated that the procedures were sufficiently reproducible to determine the role of specific sites and domains of EBNA 2 in enabling EBV to transform primary B lymphocytes and in transactivating LMP1 expression (Cohen, Wang & Kieff, 1991). Two independent constructs of 11 linker insertions or 15 deletions were tested and found to fall into four groups. Ten deletions and one linker insertion resulted in inactivation of transformation marker rescue following transfection of P3HR-1 with the specifically mutated DNAs. Since several of these deletions overlap with each other, or with the inactivating linker insertion, only four domains are defined by these experiments as being essential. Two deletions and one linker insertion resulted in a marked reduction in transformation marker rescue. One deletion and one linker insertion resulted in a small reduction in transformation marker rescue. Two deletions and eight linker insertions had no effect. All mutations which affected marker rescue of transformation also had a similar decreased activity in transactivation of LMP1 expression in transient transfection assays. In these transient assays, the level of EBNA 2 protein expression was similar to WT EBNA 2. These results are consistent with the hypothesis that the essential role of EBNA 2 in enabling EBV to transform B lymphocytes is mediated by gene transactivation.

Subsequent biochemical studies have focused on the essential EBNA 2 domains (numbered 1-4 from amino to carboxy termini). Region 4 includes an acidic domain which, when fused to the gal 4 DNA binding domain, can transactivate a promoter with upstream gal 4 binding sites in transient assays in B lymphocytes (Cohen & Kieff, 1991). Mutation of tryptophan 454 in the acidic domain to a serine results in loss of the transactivating and transforming activity (Cohen, 1992). The acidic transactivating domain of HSV VP16 can substitute for the EBNA 2 domain with similar transformation and transactivation efficiencies. Region 3 includes a gly-arg repeat domain which interacts with many proteins and with nucleic acids. Among nucleic acids, the arg-gly element interacts preferentially with poly-rG. Among proteins there is preferential interaction with histone, H1. Curiously, deletion of the arg-gly repeat element markedly reduces transformation efficiency but substantially increases the efficiency of transient transactivation by EBNA 2 of the LMP1 promoter. These data are consistent with the arg-gly repeat having regulatory effects on transactivating activity which are important to the role of EBNA 2 in cell growth transformation.

Similar studies of EBNA LP indicate that the last two exons are critical to efficient transformation of primary B lymphocytes (Mannick *et al.*, 1991). Although an initial experiment with a deletion encompassing the last two exons of EBNA LP resulted in only a modest effect on the efficiency of transformation marker rescue from P3HR-1, subsequent experiments with similar deletions or with a stop codon inserted at the beginning of the penultimate or ultimate exons resulted in a reduction of more than 95% in the efficiency of transformation marker rescue. The transformants which were obtained grew poorly, tended to differentiate toward Ig secretion and tended to be more permissive for EBV replication. When virus was recovered from the LCLs, the recombinant virus was deficient in ability to transform primary B lymphocytes, and feeder layers facilitated LCL outgrowth. These results are most compatible with the hypothesis that WT EBNA LP is a transactivator of a lymphokine which has autocrine B cell growth factor activity. However, a mechanism of this kind has yet to be confirmed, and other data suggest that the amino terminal repeat domains of EBNA LP can interact inefficiently with Rb or p53 *in vitro*, raising the possibility that EBNA LP may have a low affinity interaction with a tumour suppressor gene (Szekely *et al.*, 1993).

*Marker rescue of transformation as a positive selection strategy for EBV recombinants*

Marker rescue of transformation by transfection of WT EBV DNA into P3HR-1 cells enables EBV recombinants to be selected specifically by their newly acquired ability to cause infected primary B lymphocytes to proliferate into LCLs. This is a sensitive and powerful selection strategy. Even with primary B lymphocytes from seropositive humans, the background outgrowth of LCLs is far less than 1%. When infected cells are plated at less than 0.5 transforming unit per micro well, most LCLs result from a single transforming event and are infected with a single recombinant. Since the transforming recombinants arise from homologous recombination between the transfected DNA and P3HR-1 EBV DNA, specific mutations in the DNA outside of the P3HR-1 deletion might be carried into the recombinant EBV genomes, depending on the precise point of recombination between the transfected DNA and the P3HR-1 EBV DNA. Several studies have used this strategy of making a mutation outside of the P3HR-1 deletion in the transfected, marker rescuing, DNA fragment, in the expectation that some of the recombinants will have incorporated the physically linked mutated DNA segment.

The first EBV recombinant molecular genetic experiment constructing a mutation outside of the P3HR-1 deletion was designed to evaluate the role of the EBERs (Fig. 1, Swaminathan, Tomkinson & Kieff, 1991). For these experiments, the EBER genes were deleted from a 50kb WT EBV DNA



fragment which extends from 6 kb 5' to the EBERs through the P3HR-1 deletion (39–46 kb 3' to the EBERs) and ends 4 kb 3' to the deletion. The mutant fragment was used to transfect P3HR-1 cells and the resultant virus was used to infect primary B lymphocytes in a clonal transformation assay. Approximately 20% of the resultant LCLs were infected with a recombinant which was deleted for the EBERs. These LCLs did not differ from their WT-infected counterparts in their transformed cell growth characteristics, EBNA or LMP expression, or spontaneous lytic replication. EBER deleted recombinants also did not differ from WT recombinants in their sensitivity to interferons or in the permissivity of their interferon-treated LCLs for vesicular stomatitis virus replication (Swaminathan *et al.*, 1992). Thus, the role of the EBERs in EBV infection remains an enigma.

Other pertinent recombinants that have been made using mutations linked to the P3HR-1 transformation marker include those with specific mutations in BHRF 1 (Marchini *et al.*, 1991) or BCRF 1 (Swaminathan *et al.*, 1993), two other genes which had been implicated in latent infection or cell growth transformation. BHRF 1 is distantly but colinearly homologous to *bcl-2*, which has an anti-apoptotic effect in B lymphocytes. Although BHRF 1 is expressed early in lytic infection, RNAs that include BHRF 1 have been identified in latently infected cells. Further, BHRF-1 is transiently expressed following serum refeeding of latently infected Raji cells. Thus, BHRF 1 could have an anti-apoptotic effect in latent or early lytically infected B lymphocytes. To investigate this possibility, EBV recombinants with specifically mutated BHRF 1 open reading frames were constructed. Since BHRF 1 maps less than 2 kb 3' to the P3HR-1 deletion, most transformation rescued recombinants incorporated the specific mutations in BHRF-1 that were introduced into the rescuing DNA fragment. EBV recombinants with a stop codon in the 24th codon of BHRF 1, or with a complete deletion of BHRF 1, were able to latently infect and transform primary B lymphocytes and the transformed cells were indistinguishable from WT recombinant-infected cells in their growth *in vitro*. A slightly different approach had yielded similar results (Lee & Yates, 1992). Since BHRF-1 is expressed at high levels early in lytic EBV infection when *bcl-2* expression might be expected to be shut off, attempts were made to explore the hypothesis that BHRF 1 might protect cells from apoptotic death during lytic infection. Recombinants containing a stop codon in BHRF-1 did not, however, differ from wild type recombinants in their progression from latent through early lytic to late lytic infection, despite incubation of the infected cells in media with low serum, a condition which induces B lymphocyte apoptotic cell death. Thus, although the function of BHRF 1 remains an enigma, the recombinant EBV molecular genetic experiments exclude a role for BHRF 1 in latent, lytic or growth transforming EBV infection of primary B lymphocytes *in vitro*.

In contrast to the distant homology between BHRF 1 and *bcl-2*, BCRF 1 is

84% homologous to the human IL10 gene. IL10 can enhance the growth and differentiation of primary B lymphocytes *in vitro* and BCRF 1 also has this activity. Although BCRF1 is not expressed at high levels in latent infection, the BCRF1 gene is located between the EBERs and the EBNA promoters so that a low level of expression may be possible. To investigate the role of BCRF 1 in EBV infection, two specifically mutated EBV recombinants were constructed. The first contains a termination triplet in place of codon 116 of the BCRF 1 open reading frame, and the second contains a deletion of the entire open reading frame and surrounding sequence extending from bp 9535-12870. The frequency of incorporation of the BCRF 1 stop codon mutation into recombinant virus (as assayed in the resultant LCLs) was 28%. This approximates to the frequency expected for a mutation 30kb away from the marker rescuing DNA. (BCRF 1 is 3 kb closer to the positive selection marker than the EBERs, and EBER mutations were incorporated into 20% of the recombinants; BCRF 1 is 30 kb further away from the positive selection marker than BHRF 1, which is incorporated into >50% of the recombinants). The BCRF 1 stop codon or deletion mutant recombinants were not different from WT recombinants in latent or lytic infection or in cell growth transformation. Importantly, however, B lymphocytes lytically infected with BCRF 1 stop codon mutant recombinants induced very significant levels of gamma interferon in human peripheral blood mononuclear cell cultures, while lymphocytes infected with WT BCRF 1 recombinants induced little or no interferon. These results indicate that BCRF 1 has a more readily demonstrable role in inhibiting gamma interferon induction than in facilitating infected B lymphocyte outgrowth into LCLs. Other experiments with murine or human IL 10 or with BCRF 1 *in vitro* (Stewart & Rooney, 1992), or *in vivo* (Kurilla *et al.*, 1993) point to effects on interferon, NK and cytotoxic T cell responses which are likely to be biologically relevant to EBV infection *in vivo*.

*Second site marker rescue enables the construction of mutations at any site in the EBV genome and determination of the role of other EBNAs and LMPs*

Two observations made during the construction of EBV recombinants with mutations in DNA physically linked to the transformation marker led to new strategies for constructing and testing mutations in other EBV genes. First, the frequency with which transformation marker rescued recombinants had incorporated a linked mutation that was >30 kb away from the positive selection marker was 20% or more. The mutation was not only separated from the marker by a long DNA segment, but the intervening DNA consists almost entirely of multiple copies of a 3 kb repeat which would be expected to decrease genetic linkage to the selected marker. The hypothesis was therefore considered (and proven to be correct) that the high frequency of

incorporation of the mutation was not completely dependent on (the distant) physical linkage to the selected marker. Secondly, the LCLs which arose following infection of B lymphocytes with recombinant viruses derived from P3HR-1 were frequently found to contain the parental P3HR-1 virus in addition to the transformation competent recombinant. This indicated that a sufficient excess of parental P3HR-1 EBV was released following transfection of P3HR-1 cells that many of the primary B lymphocytes which were infected with a recombinant EBV were also co-infected with P3HR-1 EBV (Fig. 2). Furthermore, this episome co-infection state frequently continued through many cell divisions, despite the lack of dependence on the co-infecting P3HR-1 EBV for infected cell proliferation. A potential consequence of this phenomenon was that mutations could be constructed in any gene, essential or not, outside of the P3HR-1 deletion because P3HR-1 co-infection of the transformed cells might provide the WT function. Since the P3HR-1 EBV titre approximated to the number of infected primary B lymphocytes used in a typical experiment, only about half of the cells infected with recombinant EBV were co-infected with P3HR-1. If the mutation were in a gene which is not critical for latency or growth transformation, co-infection would not be a uniform occurrence. If the mutation were in a gene essential for latency or growth transformation, then primary B lymphocytes infected with a mutant recombinant would grow into LCLs only if they were co-infected with P3HR-1 EBV (Fig. 2). The recovery of the mutant recombinant in the co-infected LCL would enable the recombinant to be characterized and then passaged to fresh primary B lymphocytes to further evaluate whether the specifically mutated recombinant genome required P3HR-1 co-infection for cell growth transformation (Fig. 2). A dependence on the WT gene from P3HR-1 could be verified by demonstrating that the B-lymphocytes infected with the second generation mutant EBV would grow into LCLs only when P3HR-1 EBV was provided exogenously. Additional proof of the role of the specific mutation in creating the dependence on P3HR-1 co-infection, as opposed to other random changes which might have occurred elsewhere in the genome, would derive from showing that independently derived specifically mutated recombinants consistently required P3HR-1 co-infection, while WT recombinants derived in parallel did not. A further line of evidence arises from the likelihood that induction of lytic EBV infection in the original co-infected LCL would result in recombination between the specifically mutated recombinant which would be WT for EBNA LP and EBNA 2 and the co-infecting P3HR-1 EBV which is WT for the specific gene and deleted for EBNA LP and EBNA 2 (Fig. 2). Secondary recombinants which are competent for latent infection would be able to transform primary B lymphocytes into LCLs and would be identified in the subsequent PCR analysis because they would not have the specifically mutated EBV DNA segment. If the defect were elsewhere in the mutated recombinant EBV genome, and not a

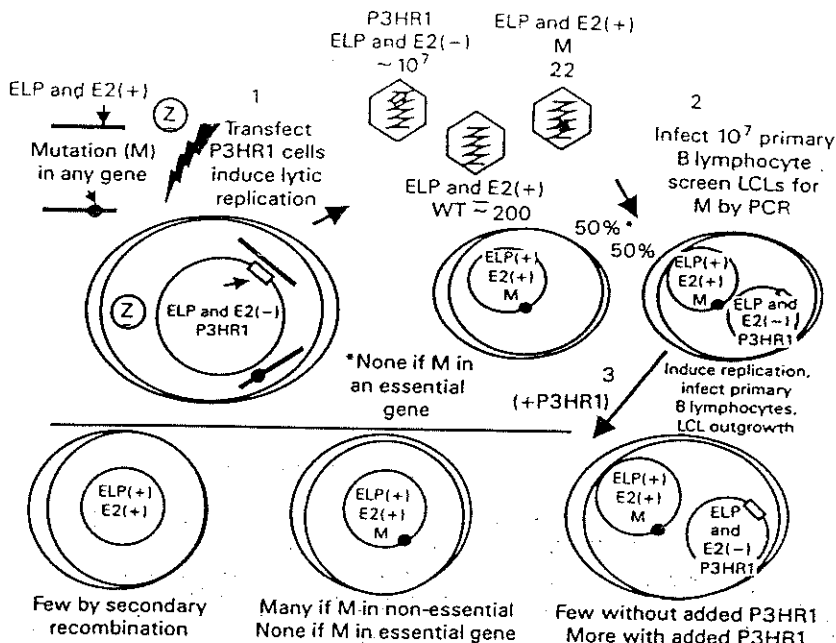


Fig. 2. Schematic depiction of the strategy used for generating EBV recombinants from P3HR-1 cells. In general, EBV recombinants are generated by transfecting latently infected B lymphocytes with cloned EBV DNA fragments and inducing lytic EBV infection in the transfected cells. The progeny virus is used to infect primary B lymphocytes or non EBV infected BL lymphoblasts. The experiments depicted in the diagram start with P3HR-1 cells which contain an EBV genome deleted for the last two exons of EBNA LP and the neighboring exon which encodes EBNA 2. The P3HR-1 EBV genome is defective and unable to initiate growth transformation of primary B lymphocytes unless the EBNA LP/2 deletion is restored by transfection with a wild type EBV DNA fragment which spans the deletion. When such a DNA fragment is transfected into P3HR-1 cells and lytic replication is induced, about 10<sup>7</sup> P3HR-1 virus and 10<sup>2</sup> restored recombinant virus is produced. In a typical experiment, the virus preparation is used to infect 1-2X10<sup>7</sup> primary B lymphocytes. The infected primary B lymphocytes are then plated into 1000 micro wells. The 100-200 wells which have a lymphocyte infected with a restored WT EBNA LP/2 recombinant are able to grow into lymphoblastoid cell lines. About half of the recombinant infected cells are initially co-infected with non recombinant P3HR-1 virus. The co-infected P3HR-1 genomes are lost as the resultant cell lines are maintained in culture over several months. A second, non-linked, cosmid EBV DNA fragment undergoes homologous recombination with the WT EBNA LP/2 recombinant genomes in lytically infected P3HR-1 cells with 10-12% efficiency. Thus, mutations engineered into a second non-linked cosmid EBV DNA fragment will wind up in 10-12% of the infected cell lines. If the mutation is in an essential transforming gene, the frequency of isolating the recombinant is only reduced by 50% since co-infecting P3HR-1 EBV will provide the WT gene in trans. To evaluate further whether the mutated recombinant EBV can infect and growth transform primary B lymphocytes, lytic infection can be induced in most co-infected LCLs and the virus plated onto primary B lymphocytes. Aggregates of virus can result in a small number of cells which are co-infected with both the specifically mutated recombinant and P3HR-1 EBVs. However, most primary B lymphocytes will be infected with the specifically mutated recombinant EBV or with P3HR-1 EBV or with a secondary recombinant. If the mutation affects the ability of the recombinant to establish latent infection or to growth transform primary B lymphocytes, no LCLs will grow out which are infected with the mutated recombinant alone. The mutated recombinant can, however, be demonstrated to be present in the virus preparation by adding sufficient exogenous P3HR-1 virus to infect most of the primary B lymphocytes so as to provide the WT gene function by complementation.

consequence of the specific mutation, phenotypically WT recombinants would be generated which would still have the specific mutation and would lack WT DNA at that site.

These principles were validated in a series of experiments in which a 30 kb WT T1 EBV DNA fragment which included the EBNA 3A, 3B and 3C genes was transfected into P3HR-1 cells along with the transformation marker rescuing DNA fragment and an expression plasmid for the z immediate early gene, to activate lytic EBV infection in the co-transfected cells (Tomkinson & Kieff, 1992a). Since P3HR-1 is a T2 EBV strain, and there are multiple type specific differences between the T1 and T2 EBNA 3A, 3B and 3C genes, incorporation of the T1 transfected DNA into P3HR-1 could be readily assayed by PCR. The resultant virus pool was plated on to primary B lymphocytes which were then distributed into a number of micro wells that exceeded by more than two-fold the number of expected transformation marker rescued recombinants. The surprising and important result was that 10–12% of the EBV recombinants had incorporated the T1 EBNA 3A and 3B genes. The EBNA 3C gene is near the end of the transfected DNA fragment and was incorporated into only 2% of the recombinants. Restriction endonuclease analysis revealed that most recombinants had the sites expected from a model in which the T1 EBNA 3 genes had been incorporated into the recombinant EBV genomes as a result of homologous recombination. As expected, about half of the recombinants were also co-infected with parental P3HR-1 EBV. When these co-infected LCLs were passaged for many months in culture, the co-infecting P3HR-1 EBV genomes were usually lost. When lytic infection was induced in the cells which were infected with only T1 EBNA 3A, 3B and 3C recombinant virus, the efficiency with which this virus replicated, established latent infection and growth transformed primary B lymphocytes was identical to T2 EBNA 3 control recombinants. Thus, the observed recombination results establish a baseline efficiency for incorporation of an unselected marker (T1 EBNA 3) into recombinant EBV genomes which had also incorporated the DNA fragments containing EBNA LP and EBNA 2.

Several parameters of these experiments have a bearing on the formulation of a model that might explain the finding that 10–12% of the transforming recombinants had incorporated the type 1 EBNA 3s (the unselected marker). First, a 5–10 fold molar excess of the z expression vector (relative to the marker rescuing EBNA LP-EBNA 2 and type 1 EBNA 3 DNA fragments) is used to induce lytic replication in the P3HR-1 cells. z expression is induced in about 5% of the transfected P3HR-1 cells. A cell which takes up the z expression plasmid is also likely to take up the other DNA fragments. However, since the marker rescuing and the type 1 EBNA 3 encoding DNA fragments are larger and less abundant than the z expression plasmid, <5% of the cells probably take up these plasmids. An origin for lytic DNA replication is located near EBNA 2 on the marker

rescuing DNA fragment and that fragment is therefore likely to replicate (and recombine) preferentially in a transfected cell in which lytic replication is induced. In a typical experiment,  $10^7$  P3HR-1 cells are transfected and the virus which is produced includes approximately  $10^7$  parental P3HR-1 (which can be independently assayed by its ability to rescue transformation defective mutants in LMP1 or EBNA 3C by co-infection as will be described below). The number of recombinants which have incorporated the EBNA LP-EBNA 2 transformation marker fragment and, which therefore transform primary B lymphocytes into LCLs, is  $1-2 \times 10^2$ . The efficiency of incorporating this fragment is 1 in  $10^5$  ( $10^2$  transforming recombinants among  $10^7$  P3HR-1 EBV). Of these, 10-12% have incorporated type 1 EBNA 3. Having inefficiently incorporated the first DNA fragment, 10-12% of these recombinants incorporated the second fragment, even though that fragment lacks an origin of DNA replication. Thus, an EBV genome which has recombined with one transfected DNA fragment is now particularly able to recombine with a second DNA fragment. This indicates that there is a limiting step in the generation of recombinants such as an enzyme, enzyme complex or site, which having been engaged by a viral genome tends to remain associated with that DNA through successive recombination events. This phenomenon is likely to extend to cells infected with any herpes virus; and may extend to other recombination events.

Exploiting the phenomenon of second site homologous recombination, specific mutated EBV recombinants were constructed with a stop codon inserted into codon 111 of EBNA 3B (Tomkinson & Kieff, 1992b) or into codon 20 of LMP2A (Longnecker *et al.*, 1992). The truncation of EBNA 3B after 110 of the 938 EBNA 3B codons had no effect on the ability of the recombinant virus to latently infect, growth transform or lytically replicate in primary B lymphocytes *in vitro*. No parameter of latent infection, including other EBNA and LMP gene expression, episome copy number or spontaneous transition to lytic infection was affected.

Similar results were obtained with LMP2A. An LMP2A mutation was constructed in an 8 kbp EBV DNA fragment by inserting a stop codon after codon 19 of the LMP2A open reading frame. The efficiency of incorporation of this mutation into marker rescued recombinants was 6%, a significant reduction compared with that of WT T1 EBNA 3A or 3B genes which were incorporated into 10-12% of the recombinants from a 30kb EBV DNA fragment. All of the initial LCLs that were infected with the LMP2A mutant were also infected with non-recombinant P3HR-1. However, when lytic infection was induced in these co-infected LCLs, the resulting mutant progeny readily transformed primary B lymphocytes without added P3HR-1. The mutated recombinant virus could then be serially passaged without P3HR-1. Infection of primary B lymphocytes was unaffected by the mutation. LCLs transformed with LMP2A-mutated EBV recombinants grew as well as those infected in parallel with WT control recombinants. Lytic

infection could be induced as readily in the LCLs infected with the LMP2A as in LCLs infected with the wild type recombinant following transfection with a  $\alpha$  expression plasmid. Since LMP2A interacts with B lymphocyte src family tyrosine kinases and with LMP1, a lack of effect on cell growth transformation was unexpected and the growth properties of the cells infected with the mutant recombinant were extensively compared with those of cells infected in parallel with WT recombinants. No significant differences were observed in LCL growth, either *in vitro* or in SCID animals. Similarly, no difference was found with LMP2 mutated recombinants which were deleted and out of frame with regard to all of LMP2 after the first 120 codons or were truncated after the fifth transmembrane domain (Longnecker *et al.*, 1993a,b). Thus, LMP2 is completely non-critical for latent infection or growth of primary B lymphocytes *in vitro*.

However, a marked difference was observed when surface Ig was cross linked on cells infected with LMP2 mutants (Miller, Longnecker & Kieff, 1993a; Miller *et al.*, 1993b). Cells infected with recombinants that could not express LMP2A (the unique amino terminus of which is critical to the interaction with src family tyrosine kinases) were similar to primary B lymphocytes in their intracellular free calcium rise following surface Ig cross linking, while cells infected with WT recombinants had little or no response to sIg cross linking. The rise in intracellular free calcium in the cells infected with mutant recombinants was associated with induction of EBV replication, while EBV replication was only minimally induced in cells infected with WT recombinants. No difference was observed between mutant and WT infected cells when lytic infection was induced with calcium ionophore and TPA which bypass the signal transducing effects of sIg associated src family tyrosine kinases. Thus, LMP2A plays a key role in preventing activation of lytic EBV infection in response to sIg cross linking. This is quite likely to be critical to the survival of latently infected cells in the peripheral circulation where they may encounter cross linking of sIg or of other receptors in response to antigen or other activating ligands. Activation of lytic infection in that milieu would result in termination of latency, cell death as a consequence of lytic infection or of T cell cytotoxicity and virus death as a consequence of release into immune serum.

Second site homologous recombination has also been used to demonstrate that EBNA 3A, EBNA 3C and LMP1 are essential for latent infection and cell growth transformation. The experiments with EBNA 3A and 3C were done in parallel with those evaluating EBNA 3B (Tomkinson, Robertson & Kieff, 1993a), while the experiments with LMP1 used a DNA fragment similar in size and map coordinates to that used for the LMP2 mutations (Kaye, Izumi & Kieff, 1993). Over 600 EBV recombinants were obtained following eight independent transfections of P3HR-1 cells with either of two independently derived 30 kbp T1 EBV DNA fragments which had been specifically mutated by inserting a stop codon after EBNA 3A

codon 302. The frequency of obtaining LCLs infected with EBNA 3A mutated recombinants was 1.4% among the 637 EBV recombinant infected LCLs compared with a 10% frequency of obtaining WT T1 EBNA 3A recombinants in control experiments done in parallel. The cells infected with T1 EBNA 3A mutants were all co-infected with P3HR-1. All lost the EBNA 3A mutation as they were expanded over the first several months in culture, with the exception of one in which the mutant EBNA 3A DNA had not replaced the WT EBNA 3A. Thus, against an expected number of 63 mutated EBNA 3A recombinants, and while obtaining the expected number of WT EBNA 3A recombinants in control experiments done in parallel, only one mutant EBNA 3A recombinant was stably maintained in LCLs and that mutant resulted from an unusual recombination event which would likely result in expression of the WT type 2 EBNA 3A gene. These experiments indicate that EBNA 3A is critical or essential to latent infection or growth transformation and that the amino terminal 302 amino acids of the 944 in WT T1 EBNA 3A had a dominant negative effect on the out growth of co-infected LCLs, resulting in continuous selection for a cell infected with a secondary recombinant which lacks the mutated T1 EBNA 3A.

Because EBNA 3C maps close to the end of the DNA fragment used to construct EBNA 3 recombinants, the expected frequency of obtaining recombinants which have incorporated WT EBNA 3C is only 2-3%. A stop codon insertion after codon 365 of the 992 EBNA 3C codons was incorporated into 2% of the resulting recombinants. Five independent LCLs were obtained containing T1 EBNA 3C mutants. Each LCL also carried the WT T2 EBNA 3C gene, presumably due to co-infection with P3HR-1 EBV. One of the five lost the EBNA 3C stop codon mutation within the first few months in culture, and two others lost the mutation over the next few months in culture. Of the two LCLs which stably maintained both mutant and WT EBNA 3C, one contained the mutated T1 EBNA 3C and WT T2 EBNA 3C genes in the same EBV genome; while the other was stably co-infected with a recombinant containing the mutated T1 EBNA 3C and WT EBNA LP-EBNA B2 genes and with P3HR-1 EBV which is WT for T2 EBNA 3C. When virus replication was induced in this latter LCL and the resultant 0.22u filtered virus used to infect primary B lymphocytes, all of the LCLs which grew out were infected with secondary recombinants which had the WT EBNA LP-EBNA 2 and the WT T2 EBNA 3C DNA. One of these progeny-infected LCLs had been infected with a secondary recombinant which had retained the WT T1 EBNA 3A gene and had replaced the mutated T1 EBNA 3C with the WT T2 EBNA 3C from the P3HR-1 genome. This specific replacement of the mutated EBNA 3C segment of the T1 EBNA 3 DNA fragment indicates that the fragment is not incidentally defective outside the specific EBNA 3C mutation. Furthermore, the recombinant containing the mutated EBNA 3C and WT EBNA LP-EBNA 2 genes was present in the virus preparation released from the co-infected LCL, but



failed to transform primary B lymphocytes on its own since this recombinant was readily recovered in co-infected primary B lymphocytes when high titre exogenous P3HR-1 EBV was mixed with the virus preparation. Thus LCLs were never obtained in the absence of a WT EBNA 3C gene and these data indicate that WT EBNA 3C is critical to the establishment of latent growth-transforming infection in primary B lymphocytes.

Three types of specifically LMP1 mutated EBV recombinants have been constructed. Two have a stop codon inserted after codon 9 or 84 of the LMP1 open reading frame; while, the third has the same linker inserted into an LMP1 intron to serve as a WT control. Eight independent stop codon 9, three independent stop codon 84 and 5 WT stop codon control recombinants were made. Although four of the five LCLs infected with control recombinants were co-infected initially with P3HR-1, after eight months of continuous cell growth these cells had lost the co-infected P3HR-1 genomes, and had only the WT stop codon recombinant genome. In contrast, all 11 LCLs containing open reading frame stop codon recombinants were initially co-infected with P3HR-1; of the seven which could be grown continuously in culture for 18 months all retained P3HR-1 co-infection. When virus replication was induced in the LCLs that were co-infected with the stop codon control recombinants and with P3HR-1, and the progeny virus was used to transform primary B lymphocytes, many of the new LCLs contained the control recombinants alone. In contrast, when virus was passaged from the seven LCLs that carried the LMP1 mutants all LCLs (>300 were analysed) which arose from the infection of primary B lymphocytes were either co-infected with P3HR-1 or were infected with a secondary recombinant which had acquired the WT LMP1 gene from P3HR-1. Of 172 LCLs that were infected with secondary recombinants, none had retained the LMP1 open reading frame mutation. Four of the original open reading frame recombinant and P3HR-1 co-infected LCLs could be induced to make a sufficient amount of virus so that a complementation test could be done with exogenously added high titre P3HR-1. In each experiment, the added P3HR-1 virus complemented the open reading frame stop codon recombinant and primary B lymphocytes were transformed into LCLs which were co-infected with the open reading frame recombinant and with P3HR-1.

Interestingly, the recombinants containing a stop codon at position 9 expressed abundant cross reactive proteins that were products of reinitiation of translation at codons 44, 89 or 129. These proteins did not form patches in the plasma membrane or inhibit the growth of the co-infected LCLs, indicating that LMP1 initiated at codon 44 does not interact with a putative down stream effector of LMP1 which is in limiting abundance. Taken together, these data provide strong evidence for an essential role for LMP1 in primary B lymphocyte growth transformation. Further, the data point to the amino terminal cytoplasmic domain and the first two transmembrane domains as being critical to LMP1 interaction with the plasma membrane

and to the function of LMP1 in transforming primary B lymphocytes to LCLs.

Subsequent experiments with mutated EBV recombinants which have alterations in the LMP1 amino terminal cytoplasmic amino acid sequence reveal that there is little, if any, sequence specificity in this part of LMP1, beyond a requirement for a hydrophilic sequence which can properly tether the first two transmembrane domains. These experiments strongly favour the model that the principal function of the amino terminus is to correctly position the first two transmembrane domains, and that correct positioning of the transmembrane domains is sufficient for transformation by the six transmembrane domains and the carboxy terminal cytoplasmic domain. Thus, a key component of LMP1's role in cell growth transformation probably derives from interaction with a membrane-associated growth regulating cell protein.

#### *Non-EBV-infected B lymphoma cells as hosts for isolating and replicating EBV recombinants*

All of the above experiments are dependent on the use of transformation marker rescue to identify and isolate EBV recombinants by their ability to cause primary B lymphocytes to grow into LCLs. While this is a powerful and easy selection, non-transforming mutations in EBNA LP or EBNA 2 cannot be derived; and non-transforming mutations in other genes can only be derived by providing the WT gene *in trans*. In almost all of the above experiments, the WT gene is provided by P3HR-1 co-infection, which complicates the physical analysis of the recombinant genome and also complicates the genetic analysis. Attempts to transcomplement using simpler expression vectors for expression of the WT gene have so far not been successful (except in the case of EBNA LP), presumably because of the stringent regulation of EBNA and LMP gene expression in LCLs. Non-EBV-infected BL cells are infectable with EBV *in vitro* and were therefore a possible host for isolating EBV recombinants. These cells are not dependent on EBV for their growth, and transforming or non-transforming recombinants should therefore be obtainable. There were two obvious problems: First, how to identify or select for the cell infected with the EBV recombinant. Several positive selection markers which convey resistance to toxic drugs have been used for selection of drug resistant transfected BL cells. However, whether the usual promoters for expression of these genes would work in the context of the virus genome was uncertain. Secondly, there was considerable uncertainty about whether lytic EBV infection could be reactivated from an *in vitro* infected BL cell line. Several BL cell lines derived from EBV-positive BL can be induced to replicate EBV *in vitro*. However, EBV-negative BL cells, after infection with EBV *in vitro*, are

notable for their integration of EBV DNA and their inability to be activated for lytic infection.

SV40 promoter and enhancer-driven hygromycin phosphotransferase (hyg) or guanine phosphoribosyltransferase (gpt) open reading frames proved to be expressed at effective levels from several different sites in the EBV genome; and to have minimal effects on EBV gene expression (Wang, Marchini & Kieff, 1991; Marchini *et al.*, 1992a; Marchini, Longnecker & Kieff, 1992b; Marchini, Kieff & Longnecker, 1993; Lee *et al.*, 1992; Lee & Yates, 1992; Longnecker *et al.*, 1993b). One effect noted is a local alteration in EBV gene expression so that a gene ordinarily expressed in lytic infection such as DJLMP1 is expressed in latent infection from EBV genomes which have the SV40 promoter and enhancer nearby. Most importantly, EBV genomes with expression cassettes for either hyg or gpt could be selected specifically in non-EBV infected BL cells or primary B lymphocytes using toxic drug resistance. A surprising finding was that the EBV genome persisted as an episome in the *in vitro* infected BL cells. Most of the infected cells expressed only EBNA 1. Some expressed all of the EBNA's and LMP's. These two types of latent EBV infection were noted among separate clones of the same cell line infected with cloned recombinant virus. BL cells varied in their efficiency for latent infection and conversion to toxic drug resistance. BJAB cells were most efficient for recovery of recombinant virus, BL41 and BL30 cells slightly less efficient and Louckes cells still less efficient. The titre of recombinant virus which was fully transforming and carried a positive selection marker was about 10 fold higher in primary B lymphocyte transformation assays than in BJAB conversion to toxic drug resistance. In some non-EBV infected BL cells, reactivation of lytic EBV infection was very rare. Even in response to z expression and phorbol ester treatment, which was the most effective inducer, lytic infection was hardly ever activatable in BJAB cells. BL41 and BL30 cells were more permissive and Louckes cells tended to be most permissive. Lytic infection could be induced in a substantial fraction of some clones of infected Louckes cells. EBV negative BL cells have been used to segregate and characterize non-transforming EBNA 2 null mutants of EBV which were created by transfection of P3HR-1 cells with DNA fragments carrying specific EBNA 2 mutations. Non-EBV infected BL30 cells have also been infected with the transformation defective P3HR-1 genome which had been altered by recombination with an EBV DNA fragment carrying a SV40-hyg cassette. The recombinant EBV-infected cells were then used as a host for creation of new EBV recombinants using a transformation marker rescue protocol similar to that used for marker rescue from P3HR-1 cells. In all these experiments, the principal limitation of the BL cells is the lower efficiency of obtaining cells infected with recombinant virus and the difficulty in inducing lytic EBV infection in the BJAB cells which are the most efficient for recovery of recombinant virus.

*Reconstitution of EBV from sub-genomic DNA fragments*

Based on the experience that transfection of susceptible cells with sub-genomic fragments of PRV results in lytic virus infection, overlapping cosmid libraries have been constructed from the B95-8 EBV strain and many attempts have been made to obtain lytic EBV replication in non-EBV infected BL cells by transfection with the cosmid DNAs and a z expression plasmid. These experiments have so far not resulted in the generation of transforming EBV.

In a parallel series of experiments, the five overlapping cosmids were transfected into P3HR-1 cells along with the z expression plasmid (Fig. 1, Tomkinson *et al.*, 1993b). Previous experiments had established that P3HR-1 cells can be transfected with cosmid size DNA fragments, that transfection with z expression plasmids can induce permissivity for replication of the endogenous P3HR-1 EBV genome, that intermolecular recombination can occur in these cells and that recombinant virus that contains the EBNA LP-EBNA 2 marker rescuing DNA (and parental P3HR-1 EBV) will be found in the LCLs which grow out following infection of primary B lymphocytes with the virus from the transfected P3HR-1 cells. When primary B lymphocytes were infected with the resulting virus and the resultant LCLs were screened for markers which distinguish between the transfected cosmid T1 EBV DNA and the T2 P3HR-1 EBV the important results were:

1. Approximately 10% of the recombinants had markers only of the transfected cosmid DNA; while the other 90% were derived in part from P3HR-1 DNA.
2. Overall, the T1 EBNA 3A gene was incorporated into 26% of the recombinants as opposed to only 10-12% when the T1 EBNA 3 carrying cosmid was transfected into cells with the transformation marker rescuing fragment alone. Of the 26% frequency of T1 EBNA 3 incorporation, 10/26 were due to the recombinants which consisted only of transfected DNA. The rest (16/26) were due to recombinants which had markers from only two transfected cosmid DNAs (4/26), markers from only three transfected cosmid DNAs (6/26) or markers from only four transfected cosmid DNAs (6/26). Of recombinants which had a marker from at least 1 EBV DNA fragment, 26% had a marker from a second DNA fragment, and more than 80% of those that had a marker from a second fragment had a marker from a third. Thus, these experiments provide further evidence that a molecule which has undergone one recombination event is likely to go on to a second, third, fourth or fifth event. Of important practical significance was the finding in these experiments that markers near the end of a cosmid fragment were incorporated into recombinants at a frequency of at least 12%, as was a large deletion in one of the fragments (the B95

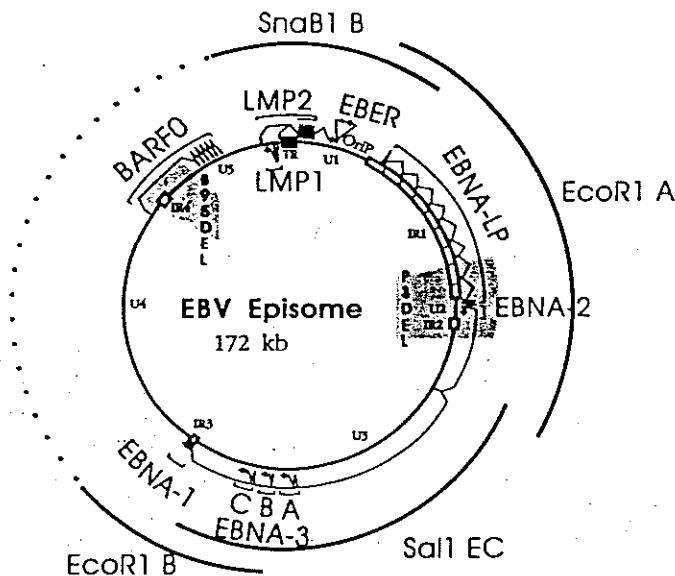


Fig. 3. Strategy for creating minimal EBV genomes sufficient for establishing latent growth transforming infection in primary B lymphocytes. Essential regions of the EcoR1 B and SnaB1 B fragments have been fused into a single cosmid and transfected into P3HR-1 cells along with the EcoR1 A and SalI E/C cosmids. P3HR-1 provides replication functions and some of the progeny virus is generated by recombination among the three cosmids. The BARFO transcript is shown on the schematic diagram of the EBV genome. The BARFO open reading frame is in the last exon.

-8 EBV genome from which the cosmid libraries were made is deleted for 14kb of DNA relative to other EBV genomes). The frequency of incorporation of the same large deletion in a two cosmid transfection experiment was nearly zero. Thus, this procedure offers the highest efficiency for producing recombinant EBV genomes with a specific mutation at any site.

#### *Derivation of a minimal transforming EBV genome*

The relatively high frequency of reconstruction of the EBV genome following five-cosmid transfections into P3HR-1 cells, including as one cosmid a cosmid with a large deletion, opened the possibility of making large deletions at any site in the EBV genome and thereby defining the minimal EBV genome for primary B lymphocyte growth transformation. Since all of the EBNA's and LMP1 are included in the part of the EBV genome between 168 kb and 110 kb in the EBV genome map, the rest of the genome between 110 kb and 168 kb should be dispensable for primary B lymphocyte growth transformation (Fig. 3). This smaller genome could potentially be reconstituted from three overlapping cosmids following transfection into

P3HR-1 cells which would provide the missing lytic replication functions. Such a small genome could fall below the minimal size for packaging and might be too large for packaging as a dimer. Furthermore, a transcript which includes a sizeable open reading frame (BARF0) is entirely encoded by DNA between 145 kb and 163 kb in latently infected lymphoid or nasopharyngeal carcinoma cells (Smith *et al.*, 1993). The appropriate constructs to test the possibility that this part of the genome could be deleted and be packaged as an extensively deleted EBV genome were made and the experiment was then attempted. More than 20% of the resulting LCLs were infected with an EBV recombinant consisting only of the three cosmids. As expected for LCLs established by infection with virus from a P3HR-1 cell transfection, about half the LCLs were co-infected with P3HR-1 EBV. Most importantly, half of the LCLs were infected only with a genome which resulted from recombination among the three transfected cosmids and lacked EBV DNA extending from 115–165 kb. These experiments demonstrate that the BARF0 transcript is not essential for primary B lymphocyte latent infection or cell growth transformation. Based on these experiments, further deletions have been made in the EBV genome using the new three cosmid transfection scheme for *in situ* construction of a transforming EBV. An additional 20 kb DNA segment has been deleted between the EBNA 2 and EBNA 3 encoding DNAs.

#### CONCLUSIONS

Over the past five years, initial forays in recombinant EBV molecular genetics have led to the derivation and application of a number of strategies for constructing specifically mutated EBV recombinants. Aspects of these strategies, such as second site homologous recombination are likely to be useful in constructing mutations in other herpes virus genomes and may be useful in cell molecular genetics. This application of these strategies to the analysis of how EBV transforms primary B lymphocytes has revealed critical roles for EBNA LP, 2, 3A and 3C and LMP1, has led to the definition of key components of these genes and has demonstrated that most of the rest of the genome is not critical for primary B lymphocyte growth transformation *in vitro*. Mutants in EBNA 1, an important gene in maintaining the EBV episome in latent infection, have not as yet been evaluated. Genes which are expressed in latently infected B lymphocytes and which appear to be non-critical for latent infection or cell growth transformation *in vitro* are still likely to be important in latent infection *in vivo*. One of these, LMP2A, is likely to play a critical role in regulating reactivation from latency *in vivo*.

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