Epstein–Barr virus-induced B-cell transformation: quantitating events from virus binding to cell outgrowth

Claire Shannon-Lowe,1 Gouri Baldwin,1 Regina Feederle,2 Andrew Bell,1 Alan Rickinson1 and Henri-Jacques Delecluse2

Correspondence
Alan Rickinson
A.B.Rickinson@bham.ac.uk
1CR-UK Institute for Cancer Studies, The University of Birmingham, Vincent Drive, Birmingham B15 2TT, UK
2German Cancer Centre, Department of Virus Associated Tumours, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany

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Epstein–Barr virus (EBV) infection and growth activation of human B cells is central to virus biology and disease pathogenesis, but is poorly understood in quantitative terms. Here, using virus at defined m.o.i., the different stages of this process at the single-cell level are followed in vitro. Virus binding to the B-cell surface, assayed by quantitative PCR, is highly efficient, particularly at the low m.o.i. values that most likely reflect physiologic events in vivo. However, only 10–15 % of bound virus genomes reach the cell nucleus, as visualized by sensitive fluorescence in situ hybridization (FISH) assay; viral genomes acquired per cell nucleus range from 1 to >10, depending on the m.o.i. Thereafter, despite differences in initial genome load, almost all nuclear genome-positive cells then go on to express the virus-encoded nuclear antigen EBNA2, upregulate the cell activation antigen CD23 and transit the cell cycle. EBNA2-positive cells in the first cycle post-infection then grow out to lymphoblastoid cell lines (LCLs) just as efficiently as do cells limiting-diluted from already established LCLs. This study therefore identifies EBV genome delivery to the nucleus as a key rate-limiting step in B-cell transformation, and highlights the remarkable efficiency with which a single virus genome, having reached the nucleus, then drives the transformation programme.

INTRODUCTION

Epstein–Barr virus (EBV), a human lymphocryptovirus, preferentially infects B lymphocytes through interactions between the major viral envelope glycoprotein gp350 and the complement receptor CR2 (Fingeroth et al., 1984), and between a second glycoprotein gp42 and human leukocyte antigen (HLA) class II molecules (Li et al., 1997; Wang & Hutt-Fletcher, 1998). Both interactions are known to be required for efficient B-cell infection and are dispensable for the much less efficient entry of EBV into other cell types, such as epithelial cells (Borza & Hutt-Fletcher, 2002; Li et al., 1995). Infection of resting B cells in vitro leads to the expression of six Epstein–Barr nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and -LP) and three latent membrane proteins (LMPs 1, 2A and 2B) that act co-ordinately to drive the infected cell into cycle, leading to the outgrowth of permanent lymphoblastoid cell lines (LCLs) (Kieff & Rickinson, 2001). Growth-transforming infections of B cells in vivo are seen transiently in infectious mononucleosis patients undergoing primary EBV infection (Anagnostopoulos et al., 1995; Kurth et al., 2000; Niedobitek et al., 1997). Assuming that this is also true of subclinical primary infections, transformation therefore appears to be central to the process whereby EBV, an orally transmitted virus, colonizes the naïve host. This virus-driven expansion of the infected B-cell pool is eventually contained by T-cell surveillance, but not before some infected cells have down-regulated viral gene expression and entered the memory B-cell pool as resting cells (Babcock et al., 1998). By contrast, in T cell-immunocompromised patients, impaired control of viral transformation can lead to fatal lymphoproliferative disease (Nalesnik, 1997).

The events of B-cell transformation in vitro have been studied by many groups, almost all using high-titre virus preparations in an attempt to synchronize events in as large a number of cells as possible (Allday et al., 1989; Finke et al., 1987; Henderson et al., 1977; Hurley & Thorley-Lawson, 1988; Mark & Sugden, 1982; Moss et al., 1986; Sugden & Mark, 1977; Thorley-Lawson & Mann, 1985). Apart from...
early studies using Gardella gel analysis to track the fate of input virus genomes and to measure their delivery to the nucleus (Hurley & Thorley-Lawson, 1988), most published work has concentrated on events as they later unfold in the successfully infected cell. With the high virus doses routinely used in such experiments, most if not all viral antigens are detectable in B-cell cultures within 2 days of infection and are accompanied by upregulation of the B-cell surface-activation marker CD23; cellular DNA synthesis is initiated by day 3, and microscopic colonies of proliferating B lymphoblastoid cells are visible within 1 week (Allday et al., 1989; Finke et al., 1987; Moss et al., 1986; Thorley-Lawson & Mann, 1985).

However, many quantitative aspects of B-cell infection and transformation still remain to be determined, particularly following exposure to lower virus doses that are more likely to reflect those experienced in the natural situation in vivo, in which the virus is transmitted to an infant early in life, usually from its mother, who as a long-term carrier herself will be shedding low levels of EBV in the saliva. New approaches to these questions become possible with the development of quantitative PCR-based assays of EBV genome number (Junying et al., 2003; Murray et al., 2003), which can in principle be used to determine the genome content of virus preparations, and of fluorescence in situ hybridization (FISH) techniques capable of visualizing the successful delivery of virus genomes into cells (Delecluse et al., 1993). Here, we use these approaches alongside single-cell-based assays of virus latent antigen expression, B-cell activation, cell cycle transit and outgrowth to look again at the rate-limiting steps in the process of B-cell transformation.

**METHODS**

**Quantification of virus production.** Virus preparations were made from 293 cell clones carrying a recombinant B95.8 EBV genome by co-transfection with BZLF1 and BALF4 expression vectors (Delecluse et al., 1998; Neuhierl et al., 2002). Supernatant medium harvested on day 3 post-transfection was used as a virus source in all experiments. These preparations were assayed for EBV genome content by quantitative (Q-) PCR amplification within the BALF5 gene (Junying et al., 2003). Full details of these procedures and of experiments confirming the DNase resistance (i.e. encapsidated nature) of EBV genomes in virus preparations is given in the Supplementary Information.

**Quantification of virus binding to primary B and T cells.** Adult peripheral blood mononuclear cells were prepared from buffy coats (kindly donated from the National Blood Service, Birmingham) and B cells positively selected using M-450 CD19 Dynabeads (Dynal); primary T cells were prepared by negative selection using anti-CD19, -CD16, -CD14, -CD11b (OKM1) and -glycophorin monoclonal antibodies (MAbs) combined with M-450 sheep anti-mouse immunoglobulin G (IgG) Dynabeads (Dynal). B-cell preparations were routinely >99% CD20+ and T cell preparations >99% CD3+. From each population, 10^6 cells were exposed to EBV preparations at m.o.i.s of 0-50 and cultured as above. Cultures were harvested daily up to 6 days post-infection and stained for CD23 expression with a phycoerythrin (PE)-conjugated anti-CD23 MAb (Pharmpingen), and both CD23 levels and CFSE were determined by fluorescence-activated cell sorting (FACS) analysis. In some experiments, cells were sorted on day 5 post-infection into CFSE high (i.e. non-divided) and CFSE low (i.e. divided) cell fractions by FACS, and aliquots of cells from the two fractions were assayed separately for expression of the CD23 marker and for expression of EBNA2 by MAb staining, as above.

**Quantification of outgrowth to LCLs.** Primary B cells were infected with EBV at m.o.i.s of 1-100 and cultured as above. The cultures were harvested at 3 days post-infection, an aliquot stained for EBNA2, and the remainder seeded into U-bottomed microtitre plate wells at cell densities calculated to deliver 1, 3, 10, 20, 60 and 200 EBNA2-positive cells per well. In each well, γ-irradiated human embryo fibroblasts and 10^7 γ-irradiated cells of an allogeneic LCL (transformed with a BZLF1 knockout virus and therefore incapable of virus production) served as a feeder layer (Feederle et al., 2000). Cultures were maintained for 4-6 weeks and successful outgrowth to an LCL visually scored.

**RESULTS**

**Virus binding assays**

Fresh preparations of B and T cells were exposed to EBV at known m.o.i.s for 3 h at 4 °C, cells were harvested, and viral DNA copies bound per cell determined by a Q-PCR assay that quantitated both viral and cellular DNA copies (Junying et al., 2003), which simultaneously quantitates both viral and cellular genome copies. Some experiments used virus preparations first incubated for 1 h at 37 °C with varying dilutions of an anti-gp350 MAb known to block gp350/CRI2-dependent virus binding (Thorley-Lawson & Gelilinger, 1980); other experiments used a gp350 knockout recombinant EBV strain (Janz et al., 2000) as the virus source.

**Quantification of virus genome delivery.** EBV genome delivery to the nucleus of virus-exposed cells was assayed between 1 and 3 days post-exposure using FISH with an EBV genome-specific probe generated by nick translation of the EBV cosmid cm302-21 of 51 kb (Polack et al., 1984), using methods previously developed for Marek's disease virus genome detection (Delecluse et al., 1993). Cells of the EBV-negative BJAB cell line served as negative controls, and cells of the Namalwa (two EBV genome copies per cell) and Raji (up to 50 genome copies per cell) BL cell lines served as positive controls.

**Quantification of viral gene expression.** Following infection, primary B cells were cultured in RPMI 1640 medium with 10%, v/v, fetal calf serum (culture medium) at 10^6 cells per 2 ml well. Cells were harvested at varying time points up to 6 days post-infection, washed in PBS, dropped onto glass slides, air-dried and then fixed in methanol/acetone (1:1, v/v) at −20 °C. Slides were stained with the EBNA2-specific MAb PE2, as described by Young et al. (1989), followed by a Cy3-conjugated anti-mouse IgG secondary antibody (Stratech).

**Quantification of cell activation and cell-cycle entry.** Freshly isolated primary B cells were extensively washed, resuspended at 2×10^7 cells ml^{-1} in PBS and exposed to an equal volume of 5 μM 5-(and-6)-carboxyfluorescein diacetate (CFSE) for 10 min at 37 °C, after which labelling was stopped by adding an equal volume of pre-warmed culture medium and incubation at room temperature for 1 min. After further extensive washing in PBS, cells were infected with EBV preparations at m.o.i.s of 5-50 and cultured as above. Cultures were harvested daily up to 6 days post-infection and stained for CD23 expression with a phycoerythrin (PE)-conjugated anti-CD23 MAb (Pharmingen), and both CD23 levels and CFSE were determined by fluorescence-activated cell sorting (FACS) analysis. In some experiments, cells were sorted on day 5 post-infection into CFSE high (i.e. non-divided) and CFSE low (i.e. divided) cell fractions by FACS, and aliquots of cells from the two fractions were assayed separately for expression of the CD23 marker and for expression of EBNA2 by MAb staining, as above.
et al., 2003). Fig. 1 (upper panels) shows the levels of virus binding detected. There was efficient binding to B cells, especially at the lower m.o.i.s, with mean values of 0.8 genomes bound per cell at an m.o.i. of 1; the equivalent values at m.o.i.s of 10 and 100 were 6 and 25 genomes per cell, respectively. In the same assay, virus binding to T cells never exceeded 0.6 genomes per cell, even at the highest m.o.i. Binding to B cells was abrogated by pre-exposure of the virus to a neutralizing anti-gp350 MAb, whereas the low baseline level of binding to T cells was only marginally affected by this treatment (Fig. 1, middle panels). Furthermore a gp350-knockout EBV assayed in parallel showed dramatically reduced B-cell binding, down to the same low baseline levels as seen on T cells whether using this knockout or the wild-type virus (Fig. 1, lower panels). This demonstrates that the Q-PCR assay is indeed measuring specific, gp350-dependent binding of EBV virions to the B-cell surface.

**Virus genome delivery to the nucleus**

We next monitored virus genome delivery to the nucleus by FISH assay with a fluorescein-labelled EBV cosmid probe capable of detecting individual EBV genomes. Fig. 2(a) illustrates the specificity of the assay, with multiple EBV copies in the nuclei of the reference Raji-BL line and two copies in the nuclei of the reference Namalwa-BL line; the EBV-negative BJAB cell line served as one of many negative controls tested. Fig. 2(b) illustrates the spread of results obtained from normal B cells 1 day post-infection at an m.o.i. of 100, with individual cells observed carrying 1, 6 and 11 genomes respectively. The averaged results from four
such experiments are summarized in Fig. 3, showing the percentage of cells observed with particular numbers of nuclear EBV genomes 1 day after exposure to EBV at different m.o.i.s. At an m.o.i. of 1, around 10% of cells were EBV genome-positive in the FISH assay and almost all of these carried a single genome. By contrast, almost 50% of cells were EBV genome-positive at an m.o.i. of 10 and almost 90% of cells were positive at an m.o.i. of 100. EBV genomes are again detected as green spots on a background of blue DAPI staining for total DNA. p.i., Post-infection.

Fig. 3. Distribution of EBV genome numbers detectable per cell in primary B cells assayed 1 day post-infection using m.o.i.s of 1, 10 and 100. Results are expressed as the percentage of all cells either with no detectable genomes (left of dotted line) or with between 1 and a maximum of 18 genomes, as shown. Note that very similar percentage values were obtained when the assay was repeated on aliquots from the same cultures taken on days 2 and 3 post-infection. The mean number of genomes per genome-positive cell was 1.0 for an m.o.i. of 1, 2.4 for an m.o.i. of 10 and 4.6 for an m.o.i. of 100.

**Initiation of viral antigen expression and cellular activation**

We monitored the same cultures for cells expressing EBNA2, one of the earliest markers of viral infection in B cells. Fig. 4 illustrates the results of EBNA2 MAb staining at day 3 post-infection in cultures exposed to m.o.i.s of 1, 10 and 100, showing the increased numbers of EBNA2-positive B cells with increased virus dose. Note that at day 3 the EBNA2-positive cells are almost all recognizable as larger blast-like cells when viewed under bright-field illumination, whereas EBNA2-negative cells resemble small resting lymphocytes. The results from EBNA2 staining at days 1, 2 and 3 post-infection are summarized in Fig. 5. The induction of EBNA2 positivity was virtually complete by day 2 and in this experiment occurred in 10, 40 and 80% of cells, respectively, after exposure to m.o.i.s of 1, 10 and 100. Importantly, Fig. 5 also shows analysis of the same experiment using FISH to determine the percentage of cells which acquire the virus genome in the nucleus. While on day 1 post-infection there are clearly more genome-positive cells than EBNA2-positive cells, the two percentage values are essentially indistinguishable on days 2 and 3,
suggesting that almost all cells in which the viral genome accesses the nucleus go on to express EBNA2.

Given that expression of the CD23 B-cell activation marker is known to be directly upregulated by EBNA2 (Wang et al., 1987, 1990), in subsequent experiments we compared these two markers for their appearance over the same three-day period post-infection and found that the percentage of CD23-positive cells was consistently higher than that of EBNA2-positive cells, particularly on days 1 and 2 post-infection (data not shown). This reflects the fact that virus binding to CR2 at the cell surface will itself induce transient, low-level CD23 expression in resting B cells (Gordon et al., 1986; Roberts et al., 1996). Beyond day 3, CD23 was predominantly high level and showed closer correlation with EBNA2-positive cell numbers (see below).

**Initiation of cell proliferation**

In further experiments we stained resting B cells with CFSE before exposing the cells to EBV at known m.o.i.s, and then followed the progress of cells through subsequent mitotic divisions by tracking the reduction of CFSE label. Fig. 6 shows the results of such an experiment, conducted with m.o.i.s of 0.5, 5 and 50, where MAb staining on day 3 identified, respectively, 3, 22 and 56% of cells as EBNA2-positive. Note that on day 3 there was still no evidence of cell division in any of the infected cultures, each CFSE profile showing a single peak like that of the uninfected B cells. By day 6, cell division had occurred in all these cultures and there was no evidence that using low doses of virus in any way delayed the response to infection. Thus, cells that were actively infected at an m.o.i. of 0.5 or 5·0 had completed between two and five cell cycles by day 6. Interestingly, at the higher m.o.i. of 50, although more cells had become actively infected, cell proliferation monitored on day 6 was slightly less advanced, with CFSE peaks showing that the cells had completed between one and four cell cycles only (see Fig. 6). We consistently found that the first cell division was delayed by 24 h until day 5 post-infection at high virus doses (data not shown).

Fig. 7 illustrates the correlation observed between cell cycle transit as reflected by CFSE levels, cellular activation as

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**Fig. 4.** Immunofluorescence staining for EBNA2 in primary B cells cultured for 3 days following infection at m.o.i.s of 1, 10 and 100; uninfected cultures served as a control. Parallel bright-field images are shown to illustrate the correlation between EBNA2 expression and enlargement to a blast cell.

**Fig. 5.** Combined results of FISH assays of EBV genome delivery and of immunofluorescence assays of EBNA2 expression in cultures of primary B cells sampled on days 1, 2 and 3 post-infection. Results are expressed as the percentage of EBV genome-positive and EBNA2-positive cells following infection at m.o.i.s of 1, 10 and 100. Uninfected cultures were consistently negative in both assays.
reflected by high-level expression of the CD23 B-cell activation marker, and viral infection as reflected by EBNA2 staining. In this experiment, primary B cells were CFSE-labelled on day 0 before EBV infection at an m.o.i. of 20, then cultured for 5 days, and the cells were sorted by FACS into CFSE high (non-divided) and CFSE low (divided) cell fractions. Essentially, all the divided cells were CD23-positive, whereas non-divided cells were CD23-negative. When aliquots of these same cell fractions were stained for EBNA2, > 95% of divided cells were detectably EBNA2-positive, whereas the non-divided population contained only rare (about 1%) EBNA2-positive cells.

**Outgrowth to established LCLs**

Finally, we assessed the capacity of infected B cells for outgrowth to established LCLs. Resting B cells were exposed to EBV at m.o.i.s of 1, 10 and 100, and an aliquot of each
culture was stained to determine the percentage of EBNA2-positive cells on day 3, immediately prior to the first round of cell division as detected by CFSE staining. Cells from aliquots of the same cultures were then seeded onto U-bottomed wells on γ-irradiated feeder layers, at the numbers required to deliver 1, 3, 10, 20, 60 and 200 EBNA2-positive cells per well. Successful outgrowth of the EBNA2-positive input cells was monitored at 4–6 weeks. Fig. 8(a) shows mean results from four such experiments on independent B-cell preparations. While outgrowth from a single EBNA2-positive cell was rare, inputs of 3 and 10 EBNA2-positive cells led to successful outgrowth in 20–30 % and 70–80 % of replicate cultures, respectively. Note that similar values were obtained whether the infected cells had been exposed to a low or high m.o.i. originally, again implying that absolute genome load was not a major determinant of outgrowth potential. For comparison, we then tested EBV-transformed cells within newly established LCL populations for outgrowth under the same limiting dilution conditions. Fig. 8(b) illustrates results obtained from three such LCL populations that are representative of six lines tested in all; such cells consistently showed a similar outgrowth efficiency to that observed for EBNA2-positive cells seeded in their first cell cycle post-infection.

**DISCUSSION**

Assays that can accurately quantitate the progress of EBV infection in B cells will become increasingly important as each step in the infection process is subjected to genetic analysis with viral mutants. A first step towards such quantification is to conduct experiments at defined m.o.i.s. Our approach, using Q-PCR to determine virion content, represents a significant advance on earlier studies using electron microscopy to enumerate virus particles (Henderson *et al*., 1977; Nemerow & Cooper, 1984;
Seigneurin et al., 1977) or DNA reassociation kinetics (Sugden et al., 1979) and Gardella gel analysis (Gardella et al., 1984) to enumerate virus genome copies in virus preparations. We combined this approach with a Q-PCR assay of virions bound to the target-cell surface in order to measure the efficiency of CR2-dependent virus binding to B cells. At the highest m.o.i. of 100, we found a mean of 25 genomes bound per cell, a value not dissimilar from that reported in an earlier study using a high-titre EBV preparation and Gardella gel analysis of bound genomes (Hurley & Thorley-Lawson, 1988). Interestingly, we found that binding becomes even more efficient at lower m.o.i.s, with 60 and 80% of all input genomes binding to the surface at m.o.i.s of 10 and 1, respectively. Thereafter, to begin to quantify the post-binding steps in EBV infection, we developed a valuable FISH assay, which is capable of visualizing individual virus genomes that reach the cell nucleus. This showed that infection with m.o.i.s of 1, 10 and 100 led to 10, 40 and 80% of cells acquiring EBV genomes in the nucleus, with the mean number of genomes per positive cell being approximately 1, 2-4 and 4-6, respectively. Relating these values back to the Q-PCR assay of virus binding suggests that, at all three m.o.i.s, 10–15% of surface-bound virions achieve nuclear entry. This is the first study visualizing the fate of input virus genomes in this way, and the results clearly indicate that failure of surface-bound virions to deliver EBV genomes to the nucleus is a key rate-limiting step in B-cell transformation.

Remarkably, combining the FISH assay with other markers of viral infection showed that a large majority of the cells in which an EBV genome reaches the nucleus will go on both to initiate viral antigen expression, as determined by EBNA2 staining, and to activate cell growth, as determined by high-level CD23 staining and stepwise reduction in CFSE labelling. Thus, at all three m.o.i.s tested, the number of EBNA2-positive cells detected at day 3 post-infection, immediately before the first round of cell division, almost exactly matched the number of EBV genome-positive cells detected by FISH. Furthermore, on day 3, most if not all of these EBNA2-positive cells were morphologically identifiable as large lymphoblasts and clearly distinct from the small EBNA2-negative population. We found that CD23 itself is a less reliable marker of active infection in this early period since, as already described (Gordon et al., 1986; Roberts et al., 1996), EBV binding to CR2 on the resting B-cell surface will itself induce transient low-level CD23 expression. This almost certainly explains our findings that the percentage of CD23-positive cells appearing within the first few days of infection exceeds that of EBNA2-positive cells. Indeed, we carried out a number of control experiments using Q-PCR-titrated doses of P3HR1 virus, an EBNA2-deleted EBV strain which is non-transforming and which does not induce any transit through the cell cycle (Miller et al., 1974; Rooney et al., 1989). P3HR1 virus binding induced significant levels of CD23 which were detectable on days 1 and 2 post-exposure (data not shown).

![Fig. 8. Efficiency of outgrowth of EBV-infected primary B cells to permanent cell lines.](image-url)
Importantly, we found no evidence for the existence, as reported in earlier studies (Thorley-Lawson & Mann, 1985), of a substantial population of EBNA-positive cells which failed to activate CD23 expression or to move into cycle. However, in agreement with that earlier study, we found that the population of EBNA (in our case EBNA2)-positive, CD23 high cells did indeed consist of those destined to proliferate. Indeed, if these cells (identified in the earlier studies by CD23 positivity and in our case by EBNA2 staining) are reseeded in limiting dilution on day 3 post-infection before their first cell division, then their capacity for outgrowth to an established line is the equal of that shown by limiting-dilution seedings of recently established LCL cells themselves. The cloning efficiency of both cell populations, measured as 5–10 % under the conditions used in our assays, is likely to be an underestimate, since optimization of cell-culture conditions with auxiliary activation signals has been reported to achieve even more efficient outgrowth from limiting-dilution cultures (Traggiai et al., 2004). The important point is that the outgrowth potential of cells in their first cell cycle following EBV infection is essentially indistinguishable from that of established LCL cells. We infer that EBV confers the capacity for unlimited growth on B cells very early post-infection.

An important objective in the present study was to follow infection at low m.o.i.s, rather than at the high, non-physiologic virus doses used in most earlier work. Interestingly, we found that at the single-cell level, growth induction occurred at least as quickly at m.o.i.s of 0.5–1 as it did at m.o.i.s of 5–10, and indeed slightly quicker than at m.o.i.s of 50–100. The 24 h delay in movement through the first cell cycle seen at higher viral doses could reflect competition between cells immediately post-infection for extracellular factors required for activation from G0, or possibly an effect caused by the acquisition of multiple EBV genomes per nucleus. Defining the conditions under which only a single genome (visualized by FISH) is delivered per infected cell also allowed us to follow genome load over time in progeny cells. Transformation assays carried out at such low m.o.i.s, in the presence of acyclovir to prevent any subsequent EBV lytic replication and reinfection in vitro, showed that cells of the resultant LCL harboured multiple EBV episomes per cell (data not shown). This confirms that amplification of the episomal EBV genome does occur in latently infected cells as part of the transformation process (Hurley & Thorley-Lawson, 1988; Sugden et al., 1979), albeit through as-yet unknown mechanisms. Indeed, our results showing equivalent efficiencies of outgrowth from EBNA2-positive cells on day 3 post-infection at an m.o.i. of 1 (before genome amplification) and of cells from recently established LCLs (after genome amplification) indicate that the amplification process itself is not rate-limiting for transformation.

The two most significant findings of the present study both concern early events in the transformation process. One is the identification of nuclear genome delivery as a key rate-limiting step. Thus we estimate that for EBV infection of small resting B cells, some 10–15 % of all bound viruses deliver the genome to the cell nucleus. Although equivalent studies have not been carried out in any other herpesvirus system, the above estimate is in the same range as might be predicted for herpes simplex virus (HSV) infecting permissive cell lines in vitro, based on the observation that the virus particle : plaque-forming unit ratio in permissive cells is typically between 1 in 10 and 1 in 50 (Frenkel et al., 1975; McLauchlan et al., 1992; Smith, 1964; Watson et al., 1963) and that plaque-forming units themselves underestimate the number of cells initiating HSV infection by two- to threefold (Everett et al., 2004).

However, the main obstacles limiting efficient intracellular herpesvirus transport and genome delivery to the nucleus are not known. One of the few quantitative studies involving electron microscopic analysis of HSV infecting Vero cells at very high m.o.i.s (Sodeik et al., 1997) observed fusion of the viral envelope with the plasma membrane and suggested that the majority (60 %) of capsids thus entering the cytosol were transported to the nuclear membrane within 4 h, although the efficiency of subsequent genome release into the nucleus was not determined. Direct parallels between HSV and EBV need to be drawn with caution, however, since EBV entry into small resting B cells occurs not by plasma-membrane fusion but by endocytosis of the virus bound to its receptor CR2 (Nemerow & Cooper, 1984; Tanner et al., 1987), followed presumably by a fusion event at the endosomal membrane releasing the capsids into the cytosol. It will need a detailed study of these events in resting B cells to identify where in the process input viruses are lost; we cannot exclude the possibility that some virions within our EBV preparations, though carrying the viral genome and capable of binding target cells, were nevertheless defective in virion components required for virus entry and transport.

A second significant finding of the present work is the remarkable efficiency with which a single EBV genome, having reached the B-cell nucleus, then drives the cell growth transforming programme. Early studies demonstrating the single-hit kinetics of transformation made it clear that a single EBV genome could initiate the transformation process (Henderson et al., 1977; Sugden & Mark, 1977), but it was never appreciated that essentially every cell that receives one virus genome in its nucleus is then driven into growth. We ascribe this efficiency, at least in part, to the nature of the BamH1 W promoter, which is responsible for initiating viral gene transcription in resting B cells (Woitschlaeger et al., 1990). This promoter is highly dependent upon B-cell-specific factors that are present in resting B cells (Tierney et al., 2000), and its activity may be further augmented by factors induced as a result of CR2 binding (Bohnsack & Cooper, 1988; Sugano et al., 1997). Moreover, multiple copies of the promoter are present within the EBV genome through its representation in each BamHI W repeat. If, as available evidence suggests (Finke
et al., 1987; Rooney et al., 1989), the incoming virus genome uses all available copies of the promoter to drive EBNA transcription, then this would serve to optimize the chances of an introduced viral genome initiating the transformation process.

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