The Epstein-Barr Virus BamHI C Promoter Is Not Essential For B Cell Immortalization In Vitro But Greatly Enhances B Cell Growth Transformation

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Epstein-Barr virus (EBV) infection of B cells leads to the sequential activation of two viral promoters, Wp and Cp, resulting in the expression of six EBV nuclear antigens (EBNAs) and the viral Bcl2 homologue BHRF1. The viral transactivator EBNA2 is required for this switch from Wp to Cp usage during the initial stages of infection. EBNA2-dependent Cp transcription is mediated by the EBNA2-response element (E2RE), a region that contains at least two binding sites for cellular factors; one of these sites, CBF1, interacts with RBP-JK which then recruits EBNA2 to the transcription initiation complex. Here we demonstrate that the B cell specific transcription factor BSAP/Pax5 binds to a second site, CBF2, in the E2RE. Deletion of the E2RE in the context of a recombinant virus greatly diminished levels of Cp-initiated transcripts during the initial stages of infection, but did not affect the levels of Wp-initiated transcripts or EBNA mRNAs. Consistent with this finding, viruses deleted for the E2RE were not markedly impaired in their ability to induce B cell transformation in vitro. By contrast, a larger deletion of the entire Cp region did reduce EBNA mRNA levels early after infection and subsequently almost completely ablated LCL outgrowth. Notably however, rare LCLs could be established following infection with Cp-deleted viruses and these were indistinguishable from wild type-derived LCLs in terms of steady state EBV gene transcription. These data indicate that, unlike Wp, Cp is dispensable for the virus’ growth transforming activity.
Epstein-Barr virus (EBV), a B lymphotropic herpesvirus aetiologically linked to several B cell malignancies, efficiently induces B cell proliferation leading to the outgrowth of lymphoblastoid cell lines (LCL). The initial stages of this growth transforming infection are characterized by the sequential activation of two viral promoters, Wp and Cp, both of which appear to be preferentially active in target B cells. In this work we have investigated the importance of Cp activity in initiating B cell proliferation and maintaining LCL growth. Using recombinant viruses we demonstrate that, while Cp is not essential for LCL outgrowth in vitro, it enhances transformation efficiency by more than 100-fold. We also show that Cp, like Wp, interacts with the B cell specific activator protein BSAP/Pax5. We suggest that EBV has evolved this two promoter system to ensure efficient colonization of the host B cell system in vivo.
INTRODUCTION

Epstein-Barr virus (EBV), a lymphotropic herpes virus linked to a number of human lymphomas, efficiently transforms resting B cells in vitro into permanent lymphoblastoid cell lines (LCLs). Such LCLs are driven to continuously proliferate through the coordinated action of a limited set of viral genes; these include the six nuclear antigens (EBNA1, 2, 3A, 3B, 3C and LP), the viral Bcl2 homologue BHRF1, three latent membrane proteins (LMP1, LMP2A, LMP2B), two small non-polyadenylated RNAs (EBERs) and series of miRNAs (1).

The early stages of this B cell transformation process are characterized by the sequential activation of two viral promoters (2). The initiating event is activation of Wp, a viral promoter present in each of the 4-8 tandemly arranged BamHI W repeats, which is dependent on the B cell specific transcription factor BSAP/Pax5 (3). At early time points, these Wp-initiated transcripts lead to the expression of BHRF1, and the two nuclear antigens EBNA2 and EBNA-LP. Subsequently EBNA2 transactivates an alternative EBNA promoter Cp and this switch in promoter usage is accompanied by the expression of the remaining nuclear antigens EBNA1, EBNA3A, EBNA3B and EBNA3C and the LMPs (1).

Much work has focused on the identification of sequences that govern Cp activity in infected B cells. Of these, the EBNA2 response element (E2RE) situated between positions -429 and -245 relative to the Cp transcription start site is the most critical (4-7). Genetic and biochemical studies have defined two binding sites within the E2RE, termed CBF1 and CBF2, which interact with cellular transcription factors (4, 8). Several groups demonstrated that the CBF1 site binds RBP-JK (9-12), a component of the Notch signalling pathway. RBP-JK subsequently recruits EBNA2 to the promoter which simultaneously abrogates RBP-JK mediated repression of Cp while stimulating viral transcription through the EBNA2 transactivation domain (11, 13, 14). While the CBF2 site has been less well characterized, one study reported that this sequence interacts with the AU-rich element RNA-binding
protein 1 (AUF1), also known as hnRNPD (15). In addition, early DNase I footprinting studies revealed that the E2RE sequence from -362 to -327, which includes the CBF2 site, was specifically protected by an unidentified B lymphocyte-specific transcription factor (7). Taken together with the observation from in vitro reporter assays that Cp, like Wp, is preferentially active in B cells (16), these findings suggest that a B cell specific factor may regulate Cp activity. However, both RBP-JK and AUF1 are ubiquitously expressed and therefore this B cell specificity remains unexplained. Other studies have also identified a number of promoter-proximal sequences critical for Cp activity. These include sites for NF-Y, Sp1 and C/EBP which contribute to EBNA1-dependent activation of Cp (17, 18); E2F1, ARID3A/Bright and Oct-2 which act as a bridging complex between Cp and oriP-bound EBNA1 (19); and the cell cycle regulatory proteins E2F1 and Rb which interact with the histone H3K4 demethylase LSD1 (20).

While the above studies have been invaluable in identifying factors that regulate Cp activity, it remains unclear to what extent Cp is required for the virus’ B cell growth transforming activity. In this context, we have previously used recombinant EBV technology to demonstrate that a functional Wp is absolutely required to initiate B cell growth transformation and for the outgrowth of virus-transformed LCLs. By contrast, previous studies with recombinant EBV genomes lacking either the the entire Cp promoter or just the E2RE indicated that Cp is dispensable for LCL formation (21-24). However many of these earlier Cp studies used recombinant viruses generated by linked transformation marker rescue of the non-transforming P3HR1 virus, in which the EBNA2 deletion was repaired at the same time as the introduction of Cp mutations. Notably this approach is unable to investigate the early events during the infection process or quantitatively measure the effects of Cp mutations on B cell transformation efficiency, because of the lack of productively infected cell lines carrying the recombinant EBV genomes.
Following our own finding that Wp activity is dependent on BSAP/Pax5 (3), the present work re-examines the B cell specific nature of Cp and more accurately quantifies the role of Cp during B cell transformation. To this end we used the B95.8 strain-derived BAC system (25) to generate recombinant viruses with defined deletions within the Cp region. Our findings indicate that viral sequences in addition to the E2RE appear to contribute to Cp activation in the context of the virus genome, and that while Cp is not absolutely essential for B cell transformation, Cp activity greatly enhances the efficient outgrowth of EBV-transformed LCLs.

MATERIALS AND METHODS

Ethics statement

This research was approved by The Black Country Committee of the UK National Research Ethics Service (REC reference:14/WM/0001).

Electrophoretic mobility shift assays (EMSA)

The preparation of nuclear extracts and the in vitro binding assay conditions have been described previously (26). The E2RE probe was a $^{32}$P-ATP end-labelled PCR-generated sequence corresponding to nucleotides −430 to −330 relative to the Cp transcription start site. The wild type and mutant RBP-JK (CBF1) and CBF2 binding site sequences used as competitors have been described previously (8, 27). Supershift reactions were carried out with a goat polyclonal BSAP (Pax5 (C-20)) antibody (Santa Cruz Biotechnology) (28). In vitro translated (IVT) proteins were generated from pSG5-BSAP (29) and pu1093-5 (RBP-JK) (30) plasmids using the TNT Rabbit Reticulocyte System (Promega).

Recombinant viruses
Recombinant EBV genomes containing Cp deletions were created from the B95.8 strain-derived EBV bacterial artificial chromosome (BAC) construct 2089 (25), and its 2W derivative (31) by homologous recombination. Shuttle vectors designed to delete the entire Cp promoter region from -429 to +846 (CpKO) or just the E2RE sequences from -429 to -245 (CpE2REKO) were created from a pBluescript vector pBS-Cp containing BamHI C sequences from 7315 to 14558 (32) and a tetracycline resistance cassette. pBS-CpKO was created by deleting the EagI-MscI fragment (coordinates 10801-12190, (32)) while pBS-CpE2REKO was made by deletion of the EagI-Sacl fragment (coordinates 10801-11089). The flanking sequences acted as regions for homologous recombination with the relevant BAC following their introduction into recombinase-expressing E.coli. (33). As a control, a revertant virus containing a wild type Cp sequence was made by homologous recombination between pBS-Cp and the 8W-CpKO BAC. BAC integrity and W repeat number was confirmed by restriction enzyme digestion and visualisation of bands on ethidium bromide stained 0.8% agarose gels following either standard or field inversion gel electrophoresis.

The recombinant BACs were transfected into HEK-293 using Lipofectamine 2000 (Invitrogen) and clones selected in the presence of 100µg/ml Hygromycin B. Individual clones were selected for their ability to produce high titres of virus following transfection and induction of lytic cycle with BZLF1 and BALF4 expression plasmids. Virus supernatants were harvested, purified by density gradient centrifugation (Optiprep, Axis Shield) and virus titration carried out using quantitative PCR (QPCR) as previously described (34).

**B-cell infection**

B-cells were positively selected from apheresis cones (NHSBT, Birmingham) using CD19 Dynabeads (Invitrogen) followed by detachment with CD19 Detachabead (Invitrogen) according to the manufacturer’s protocol. Isolated B-cells were incubated overnight with recombinant virus preparations at known multiplicities of infection (MOI). The attachment of
the recombinant virus to the B-cells was assessed by binding assays, as previously described (35).

**Analysis of EBV gene transcription**

RNA was prepared from cells using a Nucleospin II kit (Fisher). Residual genomic DNA was removed using a DNAfree kit (Ambion) and cDNA synthesised using a QScript kit (VWR) according to the manufacturers’ instructions. EBV transcripts were measured in aliquots of cDNA using either a standard 96 well QPCR assay format (36) or a high throughput 48:48 Dynamic Array Integrated Fluidics Circuit (Fluidigm) (37). For 48:48 arrays, cDNA samples were subjected to an initial 12 rounds of pre-amplification with primers directed against 45 EBV and 3 endogenous control human cellular sequences. The QPCR array was then performed on a 1 in 5 dilution of the pre-amplified product in a Biomark HD instrument (Fluidigm) using primers and probes directed towards the same 48 target sequences. The data was analysed with Biomark Real-Time PCR Analysis Software Version 2.0 (Fluidigm). In both standard and 48:48 array QPCR experiments, the AQ-plasmid (37), containing a single copy of each amplicon was used to generate standard curves, permitting absolute quantification of transcript numbers.

**Transformation assays**

B cell transformation assays were carried out in 96 well plates with fixed cell numbers and varying virus doses as previously described, and wells with growing colonies were scored after 6 weeks (3, 31).
RESULTS

The B cell specific activator protein BSAP/Pax5 binds to the Cp promoter

While a number of transcription factors have been shown to be involved in the regulation of Cp (Fig. 1A), the molecular basis of the preferential B cell specific activity of Cp described in the literature (6, 16) and confirmed in our own unpublished Cp reporter assays is currently unclear. Since our previous studies had shown that the B cell specificity of Wp was mediated by BSAP/Pax5 (3, 28), we considered whether the same factor also interacted with Cp sequences.

The transcription factor BSAP/Pax5 plays an essential role in B cell development and is expressed from the pro-B cell stage through to mature B cells, but is absent in terminally differentiated plasma cells (38-41). Interestingly, previous DNAse I footprinting analysis suggested that a region of the E2RE spanning the CBF2 site interacted with a B cell restricted factor. Notably the CBF2 binding site in Cp bears a striking sequence similarity to the high affinity BSAP binding site D within Wp (bold type in Fig. 1B), as well as homology to the known BSAP site within H2B 2.1, the lower affinity Wp Site B and the degenerate BSAP consensus sequence (grey shading, Figure 1B) (42). We therefore carried out a series of electrophoretic mobility shift assays (EMSAs) to determine whether BSAP/Pax5 bound to the Cp promoter (Fig. 1C). Incubation of a radiolabelled E2RE probe with nuclear extract derived from a non-B cell line CEM lead to the formation of a single protein-DNA complex C1, whereas incubation with nuclear extract from a B cell line Rael yielded the same complex and two additional slower migrating complexes C2 and C3. This cell type specific pattern of protein-DNA complexes was confirmed using nuclear extracts from four other non-B cell lines and six additional B cell lines (data not shown). The formation of this C1 complex seen in both CEM and Rael nuclear extracts was totally blocked by pre-incubation with the RBP-JK/CFB1 oligonucleotide competitor, but not the mutated RBP-JKmut or
CBF2 competitors. Thus the C1 complex likely corresponds to the ubiquitous factor RBP-JK bound at the CBF1 site. Regarding the two B cell specific complexes, C2 formation was specifically inhibited by the CBF2 competitor while complex C3 was abrogated by the RBP-JK and the CBF2 competitors. These findings strongly suggest that the C3 complex contains RBP-JK bound to the CBF1 site and a second, B cell restricted, factor bound to the CBF2 site.

To investigate whether the B cell specific complexes C2 and C3 contained BSAP/Pax5, the EMSAs were repeated in the presence of a Pax5 antibody. Addition of the anti-Pax5 antibody greatly reduced the formation of both complexes C2 and C3 (Fig. 1C), clearly indicating that these complexes contain BSAP/Pax5. Note that the anti-Pax5 antibody had no effect on complex C1 containing RBP-JK. BSAP/Pax5 binding was confirmed in subsequent experiments using in vitro translated (IVT) RBP-JK and BSAP proteins. Thus incubation of IVT RBP-JK and BSAP proteins, either singly or in combination, reproduced the same pattern of protein-DNA complexes observed in the presence of Rael nuclear extract (Fig. 1C). Taken together, these data indicate a novel interaction between BSAP/Pax5 and the CBF2 site.

**Generation of recombinant EBV genomes carrying Cp deletions**

In order to address the contribution of Cp sequences to the initiation and maintenance of B cell growth transformation, we constructed a series of recombinant viruses carrying either a 1.3 kb deletion spanning the entire Cp region from -429 to +846 (CpKO), or a deletion that removed just the E2RE sequences from -429 to -245 which included the newly identified BSAP/Pax5 site (CpE2REKO) (Figure 2). These recombinant EBV genomes were created from the B95.8-derived bacterial artificial chromosome (BAC) construct 2089 which contains 11 copies of the major BamHI internal repeat, or a 2W derivative carrying just two
BamHI W copies (3, 25, 31). As previously reported (31), we observed a tendency for the 2089-derived BACs to lose BamHI W repeats during the recombination process in bacterial (data not shown). Since our earlier studies had demonstrated that the number of BamHI W repeats markedly affects the efficiency of B cell outgrowth, with a minimum of five copies being necessary for optimal growth transformation (31), we therefore selected a series of 2089-derived recombinant BACs each containing 8 copies of BamHI W (denoted by 8W, 8WCpKO and 8WE2REKO) for use in subsequent experiments (Figure 2). As a control, we also created a Cp-revertant in the context of an 8 BamHI W repeat-containing BAC (8WCp-rev) by homologous recombination of wild-type Cp sequences with the 8WCpKO BAC. In parallel, two corresponding series of CpKO and E2REKO recombinants were also created in the background of the 2W BAC (31).

BACs containing the desired mutations were introduced into HEK-293 cells and the transfected cells selected with hygromycin, before screening individual clones for high levels of virus production. Density gradient purified virus stocks from at least two different HEK-293 clones were made for each recombinant. No consistent difference was found in virus titre produced from the different clones, indicating that the introduced Cp mutations had no effect on virus production (data not shown). Likewise there were no consistent differences between the different virus preparations in virus-binding assays (35).

**EBV latent cycle transcription following primary B cell infection**

We initially examined the early transcriptional events following B cell infection. CD19-positive B cells were infected at a MOI of 100 with the panel of wild type and Cp recombinant EBVs, and aliquots of infected cells were harvested at intervals of 1, 2, 3, and 5 days for RNA analysis. In order to comprehensively screen viral transcripts in small numbers of infected cells, viral RNA was quantified by QPCR analysis using a 48:48 Dynamic Array.
(Fluidigm) which allows the simultaneous amplification of 48 samples with 48 different QPCR assays in individual nanolitre scale reactions (37). In contrast to earlier studies, absolute quantification of each transcript was achieved using standard curves derived from pre-amplified dilutions of the AQ-plasmid which contained a single copy of each target amplicon.

The data in Fig 3 show a subset of results from one representative experiment measuring EBV gene transcription in B cells infected with the 8W series of recombinant viruses. Wp-initiated transcripts were detectable with 8W, 8WE2REKO and 8WCp-rev viruses at 1 day post infection and reached similar levels by day 5, with up to 4000 Wp transcripts per ng of RNA (Fig. 3A). However, maximal levels of Wp transcripts were about 4-fold lower in the 8WCpKO virus infected cells. While we were unable to detect Cp-initiated transcription in cells infected with the 8WCpKO virus, Cp-transcripts from the 8W and 8WCp-rev viruses were readily detectable at 2 days post infection and steadily increased throughout the time course, eventually reaching 4,000-5,000 transcripts per ng RNA at day 5 (Fig. 3A); note these values were similar to that seen for Wp transcripts. In sharp contrast, Cp-initiated transcription in cells infected with 8WE2REKO virus followed a similar temporal pattern to that seen with the wild type virus, but the absolute levels of transcription were over 10-fold lower. Thus the 8WE2REKO virus is clearly impaired in its ability to activate Cp during the early stages of the infection process. Notably however the levels of Wp-initiated transcripts with this 8WE2REKO virus were comparable to those in the wild type 8W and revertant 8WCp-rev viruses, indicating that Wp activity has not increased to compensate for reduced levels of Cp activity.

Since our assays measure absolute copy numbers of viral transcripts, we were able to directly compare the levels of Cp and Wp-initiated transcripts in infected cells. To allow a clearer comparison of promoter usage between different viruses, Wp and Cp levels were
expressed as a percentage of the combined number of Wp and Cp transcripts (Fig. 3B). In the case of the 8W, 8WE2RE and 8WCp-rev viruses, Wp activity accounted for 95-100% of the transcripts at day 1 post infection. In the cases of 8W and 8WCp-rev, the proportion of Wp-initiated transcripts fell substantially at day 2, which was accompanied by a concomitant increase in Cp transcripts to around 30-40% of the overall promoter usage, while at day 3 and day 5 post infection the proportion of Cp and Wp usage was approximately similar. By contrast, Wp remained the dominant promoter throughout the time course in cells infected with the 8WE2REKO virus, accounting for more than 90% of the transcripts.

Wp and Cp drive the transcription of a long primary transcript which is subsequently processed to generate the individual EBNA mRNAs. At early times post infection, the most abundant mRNA products in 8W (wild type) infected cells were EBNA2 and BHRF1 (Figure 3C); transcripts encoding EBNA1 and EBNA3A (Figure 3C), as well as EBNA3B and 3C (data not shown) were also detectable but at much lower levels. Notably, despite the lack of efficient Cp activation, deletion of the E2RE did not reduce the overall levels of EBNA and LMP transcripts in infected cells. In contrast, all the latent genes were transcribed at lower levels in the 8WCpKO infected cells, consistent with the diminished levels of Wp activity and the complete absence of Cp activity. Note that while LMP1 and LMP2A transcript levels remained relatively low during the first five days post infection, as these transcripts only reach maximal expression at later times, the lower level of LMP1 in the 8CpKO virus is consistent with reduced expression of EBNA2.

Efficiency of B cell transformation

The early transcriptional events observed in the preceding experiments suggested that whilst the E2RE is necessary for efficient activation of Cp transcription, it did not impact on latent gene expression during the early stages of EBV infection. We therefore investigated
the importance of the E2RE for subsequent cell transformation and establishment of LCLs. B cells were infected at a series of different multiplicities of infections (MOIs) before seeding 10^4 cells into wells of a 96 well plate. Cultures were examined after 6 weeks for the presence of transformed foci. The results (Fig. 4A) are shown for two different clones of each of the 8W series of viruses. The 8W wild type and 8WE2REKO virus both transformed B cells very efficiently; the MOI required for 50% transformation was less than 1 for both 8W wild type clones, and 0.5 and 4.0 for the two 8WE2REKO clones. In contrast, the 8WCpKO virus failed to yield any B cell transformants in this experiment (Fig. 4A).

In previous work, we had shown that viruses with only two copies of the BamHI W repeat region were impaired for Wp/Cp activation and B cell transformation (31). We therefore examined whether deletion of the E2RE would have a more pronounced effect on B cell transformation in the 2W background. Note that these transformation assays were carried out at the same time and on the same batch of primary B cells as the above assays with the 8W series of viruses. As observed for the 8W recombinant viruses, the 2W wild type and 2WE2REKO viruses had almost identical transformation efficiencies, albeit very much lower than for the corresponding 8W viruses (Fig. 4B). In the particular experiment, there were insufficient wells with transformed foci to calculate the MOI required for 50% transformation. As expected from the 8WCpKO result, deletion of the entire Cp region in the context of this 2W virus also completely ablated the virus’ growth transforming capacity.

Although the 8WCpKO viruses failed to yield B cell transformants in the majority of the 96 well transformation assays described above, occasionally we were able to establish LCLs from rare wells showing transformed foci when B cells were infected at the highest virus doses. Furthermore, although the 2WCpKO virus never showed transformation in any of the 96 well assays, we were able on some occasions to establish LCLs from the 2WCpKO virus, as well as the 8WCpKO virus, from bulk cultures of infected primary B cells.
cell lines took longer to establish than the LCLs made with the other viruses as, from microscopic examination, they represented the outgrowth of a very small number of transformed cells, unlike the situation with wild type and E2REKO viruses where the majority (8W) or many (2W) of the cells in each well were transformed. However, once established and the cultures had been expanded, there was no obvious difference in the growth rates between the CpKO LCLs and the others. The absence of Cp transcripts from the 8WCpKO and 2WCpKO LCLs, together with hygromycin resistance, confirmed that these were genuine CpKO LCLs and not rare spontaneously transformed LCLs derived from EBV infected donor B cells.

**EBV gene transcription in LCLs established with recombinant Cp viruses**

To investigate whether the Cp mutations affected the steady state levels of Wp and Cp transcripts in established transformed lines, we screened an extensive panel of LCLs generated from different wild type and mutant viruses; these included twelve 8W wild type LCLs, four 8WE2REKO LCLs, four 8WCpKO LCLs, five 2W wild type LCLs, three 2WE2REKO LCLs and three 2WCpKO LCLs. Three observations can be made from this data (Figure 5). First, there was a broad range in the absolute numbers of Wp- and Cp-initiated transcripts in wild type 8W and 2W LCLs. These data emphasize the fact that Wp activity is readily detectable in established LCLs and contributes significantly to the overall levels of EBNA transcription even in the presence of a wild type Cp sequence. Moreover the relative levels of Cp and Wp usage are not significantly altered by the number of Wp copies present in EBV genome. Second, deletion of the E2RE always resulted in Wp becoming the dominant promoter, with only very low levels of Cp transcripts seen in LCLs derived from 8WE2REKO and 2WE2REKO viruses. Thus the pattern of promoter usage seen in the first few days after infection with E2RE-deleted viruses was maintained in the established LCLs.
Third, in the absence of any detectable Cp transcription, Wp activity does not apparently increase to compensate.

We then extended the analysis on a subset of these LCLs, two of each type, using a high throughput QPCR array to screen a broader range of EBV latent and lytic cycle transcripts. These data (Fig. 6) are expressed as EBV transcript copy numbers normalized to cellular PGK1 expression. As noted above, there was considerable variation in the individual transcript levels between different LCLs but the values were within the ranges previously reported in a panel of LCLs analyzed using this technology (37). Importantly, with the exception of Cp activity, there were no consistent differences in the levels of any latent or lytic transcripts that could be attributed to the Cp mutations. In conclusion, while deletion of the entire Cp region or just the E2RE greatly reduces Cp activity during the initial stages of B cell infection and dramatically decreases transformation efficiency, rare LCLs carrying these mutant viruses transcribe the full spectrum of growth transforming genes at levels comparable to LCLs derived from a wild type EBV strain.

**DISCUSSION**

EBV-induced B cell transformation is characterized by the sequential activation of two viral promoters which drive the expression of six EBV nuclear antigens and the viral Bcl2 homologue BHRF1 in latently infected cells. The first promoter to be activated immediately after infection is Wp, present in each of the tandemly-arranged BamHI W repeat sequences. Several cellular factors regulate transcription initiation from Wp, including the B cell transcription factor BSAP/Pax5 which is absolutely essential for both Wp activation and B cell transformation in vitro (2, 3, 31). These Wp-initiated transcripts lead to the expression of EBNA2 and the subsequent activation of an alternative EBNA promoter Cp, located in the upstream BamHI C region. This raises an important question, namely to what
extent does EBV-induced growth transformation depend on the activity of Cp, both in the early stages of the infection process and in established LCLs.

The starting point for this work was a re-examination of a previous observation that Cp reporter constructs are preferentially active in B cell lines (16, 34). This B cell specificity is achieved, at least in part, by interactions of available cellular factors with the E2RE (6). In this study, we identified sequence homology between the BSAP/Pax5 consensus site and sequences within the Cp E2RE, and subsequently demonstrated that BSAP/Pax5 binds to the E2RE in vitro (Fig. 1). This finding supports the results of earlier DNase I footprinting studies which revealed the binding of a lymphocyte specific factor to this region (7), and is also consistent with a recent ChIP-Seq analysis of BSAP/Pax5 binding across the entire EBV genome (43). This newly-identified BSAP binding site overlaps the CBF2 site, a region previously shown to be critical for Cp activity (27). Notably a mutation within the CBF2 site (27), previously shown to diminish Cp activity, disrupted BSAP/Pax5 binding (Fig. 1C).

Intriguingly an earlier study reported that the CBF2 site interacted with the cellular factor AUF1/hnRNPD (15), a result at odds with the present work since AUF1 is ubiquitously expressed. Although we were unable to reproduce the AUF1 interaction using EMSA (data not shown), we cannot exclude the possibility that under certain conditions AUF1 is recruited to Cp as part of a larger transcription factor complex which includes BSAP/Pax5. One such multi-protein complex containing the B cell transcription factor Oct2, ARID3A and E2F1 has been shown to bind to Cp sequences from -149 to -109 and mediates EBNA1-dependent activation of Cp (19). Importantly our data demonstrate a novel interaction between BSAP/Pax5 and the E2RE which provides a mechanistic basis for the apparent B cell specificity of Cp.

In the next series of experiments we sought to examine the effects of specifically deleting the E2RE sequences in the context of a recombinant virus. Note that it has
previously been reported that E2RE-deleted viruses are still capable of inducing B cell transformation (23, 24). However these earlier studies relied upon marker rescue experiments using P3HR1-derived recombinants and were limited in their quantitative analyses of early events during the infection process and the efficiency of LCL outgrowth. In the present work, we have used purified recombinant viruses of known titres generated using the EBV BAC system, quantified promoter usage and gene expression from early times post B cell infection through to establishment of LCLs using Q-PCR, and rigorously monitored LCL outgrowth at a range of MOIs (3, 31, 35). Our data confirm that the E2RE plays an important role in efficiently activating Cp during the early stages of infection (Fig. 3), but also show that the E2RE-deleted mutant virus was still capable of transcribing latent transcripts at similar levels to wild-type virus. Consistent with these observations, E2RE-deleted recombinants were not markedly impaired in B cell transformation assays in either the 8W or 2W virus backgrounds (Fig. 4).

Notably the absolute levels of Wp-initiated transcripts induced by our E2REKO viruses, either at early stages post-infection or in established LCLs, were not elevated compared to wild-type viruses (Figs. 3A and 5A), suggesting that Wp activity does not simply compensate for reduced levels of Cp transcription. This result contradicts an earlier finding that Wp transcription is increased in viruses lacking the E2RE (24, 44), but is consistent with another report which analyzed the effect of a point mutation in the CBF1/RBP-JK binding site (23). Interestingly, LCLs derived from E2RE-deleted viruses still retained low levels of Cp activity, indicating that additional viral sequences, such as the interaction of oriP with EBNA1, can also contribute to Cp transcription in the context of the viral genome. Indeed a previous study using a recombinant EBV deleted for amino acids 65-89 of EBNA1, has demonstrated the importance of EBNA1 in the activation and maintenance Cp of transcription (45). Overall, our data argue that while the binding of RBP-JK and
BSAP/Pax5 to Cp are not essential for EBV-induced B cell transformation, these proteins may cooperate with factors bound to other regions of Cp (19, 46) ensuring optimal Cp activity and the expression of the latency III program of viral gene expression in infected B cells.

In contrast to the situation with the E2RE-deleted viruses, recombinants lacking the entire Cp region transformed B cells at least 100-fold less efficiently than wild type virus (Fig. 4). Given that E2RE- and Cp-deleted viruses are both highly defective for Cp activity, the dramatic difference in the transforming capacity of these two mutants is surprising. However this disparity may reflect the fact that Cp-deleted viruses expressed 3-4 fold lower numbers of Wp-initiated transcripts during the early stages of infection, leading to correspondingly reduced levels of EBNA and BHRF1 transcripts at days 1-5 (Fig. 3). Note there is no evidence for other unintentional mutations being present in these Cp-deleted viruses, since Wp levels were restored in a Cp-KO revertant (Fig. 3A). We speculate that the large 1.4 kb genomic deletion has potentially disrupted the nucleosomal architecture of the oriP/Cp/Wp region or altered the position of enhancer sequences, with a concomitant reduction in Wp activity (47).

Despite being greatly impaired for B cell transformation, rare LCLs carrying Cp-deleted viruses could be established and, following expansion, they proliferated in culture at similar rates to wild type LCLs. These LCLs provide important clues regarding the relative contribution of Wp and Cp during B cell transformation. First, our data support previous studies demonstrating that Cp is dispensable for LCL outgrowth while also clearly showing that the presence of a functional Cp promoter greatly increases transformation efficiency. Second, Cp-deleted LCLs exclusively used Wp to drive EBNA expression yet expressed steady state levels of latent transcripts similar to those seen in wild type LCLs and E2RE-deleted LCLs (Figs. 5B and 6). In a recent study (37), we described a method of estimating
the absolute number of EBV transcripts on a per cell basis. Using the same approach, the most abundant transcripts in this LCL panel were EBNA2 (median 87 transcripts per cell), LMP1 (median 98) and latent BHRF1 (median 46), while EBNA1, EBNA3A and LMP2A transcripts were present at much lower levels; importantly these values are comparable to those seen in a larger panel of LCLs (37). These findings argue that during clonal selection following infection with a Cp-deleted virus, there is a strong pressure to maintain sufficient levels of Wp-driven latent gene expression to support LCL growth. Third, it has been suggested that the switch from Wp to Cp usage is required to ensure efficient transcriptional elongation of the primary EBV transcript encoding the EBNA3 and EBNA1 genes located at least 70kb downstream of the promoter (48). Our data suggests that, under certain conditions, Wp-initiated transcripts are capable of efficiently driving transcription of the downstream EBNA genes.

The present findings differ from an earlier study which reported that Cp deletion did not affect B cell transformation efficiency (21, 22). This previous study used a virus carrying a larger 3.3 kb deletion, spanning from upstream of the GRE to beyond the Cp transcription start site (Fig 1A), and relied on the rescue of transformation-competent recombinants derived from P3HR1. Recovery of LCLs carrying these Cp-deleted viruses was apparently no less efficient than with a control wild-type virus (22). In addition, cell-free supernatant containing Cp-deleted recombinant virus produced from such LCLs infected and transformed fresh B cells as efficiently as wild-type virus (21, 22). These discrepancies may reflect technical differences between the two studies. While the earlier study used crude virus preparations, our virus stocks were density gradient purified and accurately titred by QPCR to ensure that equivalent MOIs were used in all experiments, while our virus transformation assays were done over a range of MOIs. The P3HR1-rescued virus may also have contained second site mutations which could have been selected during the initial rescue by B cell
transformation. Additionally the BACs used here are based on the B95.8 EBV strain, which has a deletion which removes approximately 12kb of sequence compared to wild type viruses and the recombinants produced in the P3HR1 system. Importantly, however, both studies concur that Cp activity is dispensable for B cell transformation and demonstrated that established LCLs carrying wild-type or Cp-deleted viruses had similar levels of latent gene expression (21, 22).

EBV efficiently colonizes the B cell system in vivo through the transient expression of a series of growth transforming genes which together induce B cell proliferation and ultimately drive the expansion of a pool of latently infected cells. This growth transforming programme involves the sequential activation of two viral promoters, Wp and Cp, which are highly conserved among primate lymphocryptoviruses (49-51), suggesting that these promoters have evolved as part of the virus’ strategy to establish lifelong persistence. By recruiting a key B cell transcription factor, BSAP/Pax5, both of these promoters may be optimized to ensure high levels of viral gene transcription in the infected target cell. While the present work confirms that Wp activity is sufficient for LCL outgrowth, the efficiency of B cell transformation is greatly enhanced by the presence of functional Cp sequences.

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REFERENCES


FIGURE LEGENDS

FIG 1 BSAP/Pax5 interacts with the CBF2 binding site. (A) Schematic representation of the linear EBV genome indicating the multiple repeats of the BamHI W region each containing a copy of Wp, and the second upstream EBNA promoter Cp. Also shown are the locations of the EBV latent genes and the latent origin of replication oriP. The inset is a schematic illustration of the Cp/Wp region indicating the relative positions of known cellular transcription factors. (B) Alignment of the BSAP/Pax5 consensus binding sequence with BSAP/Pax5 binding sites from the H2B 2.1 promoter, the low affinity Wp site B, the high affinity EBV Wp site D, and the Cp CBF2 binding site. Shaded nucleotides indicate positions conserved in the BSAP/Pax5 consensus, while nucleotides in bold font are conserved between Wp site D and CBF2. (C) Protein-DNA complexes formed by incubating the 100bp radiolabelled E2RE probe with nuclear extracts either from the T cell line CEM or the B cell line Rael-BL, either alone (-), or in the presence of the indicated oligonucleotide competitors. Supershifts were performed by the addition of a Pax5 antibody. The last three tracks indicate protein-DNA complexes formed by incubating the E2RE probe with IVT RBP-JK and BSAP proteins.

FIG 2 Construction of recombinant EBV genomes carrying Cp deletions. (A) Schematic illustration of the parental 8W recombinant BAC containing 8 copies of the BamHI W repeat region. (B) Linear representation of the Cp/Wp region indicating the size and position of the Cp deletions introduced into the 8W BAC and the location of known transcription factor binding sites. 8WE2REKO is deleted for sequences from -429 to -245 relative to the Cp transcription start (which include the EBNA2 response element), while 8WCpKO is deleted for the entire Cp promoter region from -429 to +846.
Primary B cells were exposed to 8W wild type, Cp-deleted or revertant viruses at 100 MOI and EBV gene expression was quantified at 1, 2, 3 and 5 days post infection using a 48:48 Dynamic PCR array. (A) Absolute copy numbers of Wp- and Cp-initiated transcripts expressed per ng total RNA. (B) Relative activity of Wp (open bars) and Cp (solid bars) expressed as a percentage of the total number of Wp and Cp transcripts. (C) Absolute copy numbers of selected EBV latent gene transcripts expressed per ng total RNA. Histograms represent the mean and standard error of triplicate RNA samples independently pre-amplified and tested by Q-PCR.

FIG 4 Transformation efficiencies of recombinant viruses. (A) Number of wells from a 96 well plate showing transformed foci within 6 weeks following exposure to the 8W wild type or Cp-deleted viruses at a range of MOIs. Virus preparations from two independent HEK 293 producer clones were assayed for each recombinant. The number in brackets indicates the MOI required for transformation in 50% of wells. (B) Results of transformation assays using 2W, 2WE2REKO and 2WCpKO viruses.

FIG 5 Wp and Cp activity in established LCLs infected with recombinant viruses. (A) Expression of Wp- and Cp-initiated transcripts in a range of established LCLs carrying the indicated EBV genomes determined by standard 96 well QPCR and normalized to cellular GAPDH expression. (B) Relative activity of Wp (open bars) and Cp (solid bars) expressed as a percentage of the total number of Wp and Cp transcripts in the same LCLs as panel (A).
FIG 6 EBV gene transcription in established LCLs infected with recombinant viruses. EBV transcripts from a subset of the wild type (W), E2REKO (E) and CpKO (C) 8W and 2W LCLs were quantified using a 48:48 Dynamic PCR array and the data normalized to cellular PGK1 expression. Data are shown for Wp- and Cp-initiated transcripts; the latent transcripts encoding BHRF1 (containing the Y2-HF splice), EBNA2, EBNA3A, EBNA1 (U-K splice), LMP1 and LMP2A; and lytic transcripts encoding BZLF1 and BHRF1 (containing the H2-HF splice). Histograms represent the mean and standard error of triplicate RNA samples independently pre-amplified and tested by Q-PCR.