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BHRF1 of Epstein-Barr Virus, Which Is Homologous to Human Proto-Oncogene *bcl2*, Is Not Essential for Transformation of B Cells or for Virus Replication In Vitro

MAY-ANN LEE AND JOHN L. YATES*

Department of Human Genetics, Roswell Park Cancer Institute, Buffalo, New York 14263

Received 14 May 1991/Accepted 26 December 1991

The Epstein-Barr virus (EBV) genome contains an open reading frame, BHRF1, that encodes a presumptive membrane protein with sequence similarity to the proto-oncogene *bcl2*, which is linked to human B-cell follicular lymphoma. Potential roles for BHRF1 in EBV's ability to growth transform human B cells and to replicate in B cells in culture were investigated by generating EBV mutants that lack most of the open reading frame. This was accomplished by recombination of plasmids carrying mutations in BHRF1 with the transformation-defective EBV strain P3HR1. Because BHRF1 resides close to the deletion in P3HR1 that renders this strain transformation defective, B-cell transformation could be used to select for recombination events in the region. B-cell clones were established by recombinants which lacked most of the BHRF1 open reading frame, although most of these initial B-cell transformants also carried nonrecombinant (BHRF1$^+$) P3HR1 genomes, at levels ranging from a fraction of a copy to four copies per cell. Secondary B-cell transformants that lacked BHRF1$^+$ EBV at detectable levels were found to release transforming, BHRF1-deficient EBV at levels that were within the normal range for EBV-immortalized B-cell clones. These studies demonstrate that BHRF1 is nonessential for growth transformation of B cells and for virus replication and release from these cells in culture.

Epstein-Barr virus (EBV) is associated with nasopharyngeal carcinoma, B lymphomas, and other lymphoproliferative disorders in humans. In vitro, EBV establishes a latent infection in resting B lymphocytes and transforms these cells into indefinitely proliferating blast cells. EBV's involvement in lymphoproliferative disorders is most likely a result of this capacity to stimulate the proliferation of B cells when infecting them latently (reviewed in references 15 and 19).

Several EBV gene products are believed to contribute to B-cell growth transformation, either directly or indirectly. All of the gene products that are expressed in latently infected, growth-transformed B-cell lines have been generally considered as candidates for involvement in the process and, in sum, sufficient for it. These gene products include two membrane proteins, six nuclear proteins (or antigens; EBNAs), and two abundant small RNAs, called EBERs (15). Genetic proof for their contributions to growth transformation exists for only two of these gene products: EBNA2, which is essential for growth transformation (6, 9), and EBNA-LP (or EBNA4), which makes an important, but not essential, contribution to the outgrowth of B cells in culture (9). EBNA2, and to a lesser extent EBNA3C, can cause the induction of antigens associated with B-cell activation when expressed in particular EBV-negative Burkitt's lymphoma cell lines (37, 38). EBNA2 may also be required for the expression of several of the other EBV genes that are expressed latently (25, 40). The product of another latently expressed gene, the latent membrane protein, is strongly suspected of playing a direct role in supporting B-cell growth because it can also contribute to the induction of activation antigens and, moreover, can transform the growth properties of rodent fibroblast cell lines (3, 35–37). EBNA1 is likely to play at least a supporting role in growth transformation of B cells by sustaining the autonomous maintenance of the circularized EBV genome (43). Activities for the remaining latently expressed proteins have not been reported.

The assumption that the genes mentioned above (i.e., those that are invariably expressed in EBV-established B-cell lines) are together sufficient for transformation of B cells requires the following caveat: additional genes might be necessary during the initial stages of infection but unnecessary later. This situation could exist for biological reasons; certain EBV genes might be expressed only early after infection, serving to establish the patterns of gene expression that define latency but not to maintain them, or acting to initiate B-cell proliferation but not to sustain it. Or the situation might arise for the artifactual reason that as EBV-transformed B cells adapt to growing in culture, variants could emerge that have obviated the need for one or more viral gene product, whose expression could then be lost. These considerations seemed apt for BHRF1 because of several published findings.

BHRF1 has significant colinear sequence similarity with the proto-oncogene *bcl2*, which has been implicated in the development of B-cell follicular lymphoma in humans (5). Expression of *bcl2* from retrovirus vectors or from transgenes in mice led to expanded resting B-cell populations and contributed to B-cell neoplasia (16–18, 29, 34). In vitro, B or T cells expressing *bcl2* were found to survive longer in low serum or when stressed (20, 23, 24, 33), in one such example by avoiding the rapid, apoptotic cell death that would otherwise ensue following deprivation of an essential growth factor (12). In group I EBV-positive Burkitt's lymphoma cells, a restricted state of EBV latent gene expression exists and the cells are prone to apoptosis. Forced expression of an introduced copy of *bcl2* or an introduced EBV latent membrane protein gene, which induced *bcl2* expression, rendered these cells resistant to apoptosis (11).

It is conceivable that BHRF1 possesses *bcl2*-like activi-

* Corresponding author.

ties, but the appropriate functional tests of BHRF1 have not been reported. The 24-kDa Bcl2 protein was found to associate with the mitochondrial inner membrane (12). The 17-kDa BHRF1-encoded protein is associated with membranes in the cytoplasm (21) but has not been localized further. The regulation of BHRF1 expression appears to be complex and is somewhat controversial. Abundant expression of BHRF1 mRNA and protein appears early after lytic induction (1a, 10, 21), suggesting a function for BHRF1 during virus replication. In addition, at lower levels, at least two forms of BHRF1-containing mRNAs whose expression levels appeared unrelated to the productive cycle were detected by Northern (RNA) analysis of RNA from several EBV-transformed B-cell lines, including a tightly latent one (1a). One such form of BHRF1-containing mRNA carries the spliced leader sequences characteristic of the EBNA family of latently expressed mRNAs (1a, 4, 21). However, neither the BHRF1 protein nor an mRNA with known potential to direct its synthesis has been detected in latently infected cells (21).

Reports from two laboratories suggested a method of isolating EBV carrying mutations in BHRF1 (6, 9). These laboratories showed that EBV strain P3HR1, which cannot transform B cells because of a deletion that removes EBNA2 coding sequences, can generate transforming virus by genetic recombination with plasmids carrying EBV sequences spanning the deleted region. Because the region deleted in P3HR1 is only 2 kb away from BHRF1, this presented a method of introducing deletions affecting the open reading frame into a viral strain that would otherwise have a normal phenotype. It was found that viruses mutant for BHRF1 could readily be obtained by this method and propagated in the absence of helper virus by the sequential isolation of immortalized B-cell clones.

MATERIALS AND METHODS

Cell lines and culture. The subclone HH514, clone 16, of P3HR1, which lacks the rearranged defective viral genomes carried by P3HR1 (22), was used throughout this work and is referred to as HH514-16. All cell lines were grown in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum and 5% iron-supplemented calf serum (HyClone, Inc.).

Transformation of B cells. Leukocytes from the peripheral blood of normal adult donors were separated from erythrocytes by centrifugation over Histopaque (Sigma), depleted of monocytes by adherence to plastic culture dishes, and then depleted of T cells by the method of rosette formation with 2-aminoethylisothiouronium bromide-treated sheep erythrocytes (14). In some experiments, the step of depleting monocytes was omitted without a noticeable effect on the results. Although some of the blood donors were seropositive for EBV viral capsid antigens, B cells from such individuals were used in these studies only if they did not give rise to transformants at a detectable frequency without the addition of EBV. Cells from two different donors were used to test each supernatant in each experiment, with little variation in the numbers of transformants observed.

Typically, 5×10^6 HH514-16 cells suspended in 0.6 ml of complete medium were electroporated with 3 μg of the test plasmid plus 3 μg of pCMV-BZLF1 (8) to induce the lytic cycle. Electroporation was done by using a Bethesda Research Laboratories Cell-Porator set at 330 μF and 300 V. Cells were cultured in 10 ml of medium, which was collected after 4 days and passed through a 0.22-μm-pore-size filter. B cells (6×10^4 to 8×10^4) were added to each well of 24-well culture plates along with 0.5 ml of growth medium and 0.1 ml of cell supernatant to be tested for transforming virus. Human fibroblasts, lethally gamma irradiated with 3,000 rads, were also included in the culture dishes at a 1:3 or 1:4 split to serve as feeder cells. Some of the data in experiments 1 and 2 of Table 1 were obtained by testing the electroporated cells for release of transforming virus by cocultivation with B cells. Four days after electroporation, the cells were lethally irradiated, and 5×10^4 cells were added along with B cells to each well of a 24-well plate. Results were very similar to those obtained with the cell supernatants. Cultures were observed for up to 2 months for the growth of transformants.

Plasmids. The plasmids indicated in Fig. 1 were constructed from plasmids pW^{2}YHSalG (43) and pHEBo (31) by placing the EBV DNA indicated between the *Bam*HI and *Sal*I sites of pHEBo. In p530, sequences between the *Bcl*I site at 53767 and the *Bam*HI site at 54853 were deleted. For p531, between the same *Bcl*I and *Bam*HI sites was placed a 2.9-kb *Bam*HI fragment carrying the *neo* gene between the human cytomegalovirus (CMV) immediate-early (IE) promoter and the simian virus 40 small-t-antigen splice sites and early polyadenylation site. This CMVIE-neo selective marker was constructed by excising the *cat* gene from pCATwt760 (28) by using *Xba*I and *Hpa*I and replacing it with a *Bgl*II-to-*Hpa*I fragment from pSV2neo (27) carrying the *neo* gene.

DNA analysis. Cells were lysed in sodium sarcosine in buffer containing EDTA and proteinase K; DNA was then isolated by phenol-chloroform extraction followed by precipitation with ethanol (26). After digestion with restriction enzymes, the DNAs were recovered by phenol extraction and ethanol precipitation. The final concentrations of the digested DNAs were determined with a DNA fluorometer (Hoefer Scientific Instruments) to ensure that equal amounts of digested DNA from each sample would be analyzed. DNA samples (5 μg each) were electrophoresed on 0.7% agarose gels; Southern analysis was done as described previously (42).

RESULTS

Transformation proficiency can be restored to the transformation-defective strain P3HR1 by recombination with cloned EBV DNA spanning a deletion in the P3HR1 viral genome (6, 9). The strategy for testing the potential requirement for BHRF1 in B-cell transformation by EBV was to generate transformation-proficient recombinants of P3HR1 by using DNA carrying mutations in BHRF1, which happens to reside close to the P3HR1 deletion. BHRF1 is approximately 2 kb away from the deleted region, so a deletion introduced into a recombinant virus could be complemented by a coinfecting nonrecombinant P3HR1 genome. Were BHRF1 to be required for B-cell transformation, recombinants lacking BHRF1 would be observed in B-cell transformants only in the company of a complementing viral genome.

A plasmid, p529, was constructed for these studies so that it contained EBV DNA spanning the P3HR1 deletion and extending 8.4 kb beyond BHRF1, as illustrated in Fig. 1. A 1.1-kb deletion removing all but the carboxy-terminal 31 codons of BHRF1 and 0.6 kb of 5′-flanking sequences was introduced between *Bcl*I and *Bam*HI sites. Because BHRF1 is over 2 kb away from the P3HR1 deletion, it was expected that recombination might often occur between BHRF1 and

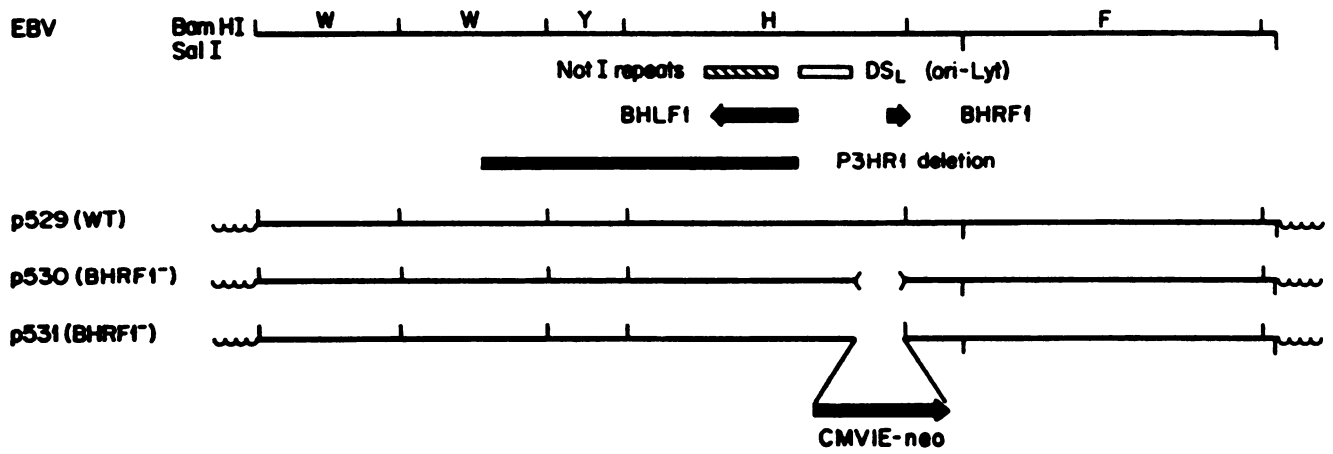

FIG. 1. Schematic diagram of part of the EBV genome (top), extending rightward from within the 3.1-kb internal repeat (*Bam*HI fragment W), and of the EBV DNA present within plasmids constructed for this study (shown below). Positions of the *Not*I repeats, DS_L (*ori-Lyt*), leftward and rightward open reading frames BHLF1 and BHRF1 (2), and the region deleted in strain P3HR1 (13) are indicated. The plasmids consist of the EBV sequences shown linked to pHEBo (31), indicated by the wavy lines, at its *Bam*HI and *Sal*I sites. The deletion introduced into BHRF1, indicated by < >, and the substitution of CMVIE-neo DNA for most of BHRF1 are also shown. *Bam*HI and *Sal*I cleavage sites are indicated by upward and downward marks from the horizontal lines, respectively.

the region deleted in P3HR1, restoring transformation proficiency without introducing the intended BHRF1 mutation into the recombinant viral genome. For this reason, a selective marker, CMVIE-neo, was introduced into the same restriction sites used to generate the deletion in BHRF1. Because CMVIE-neo confers to mammalian cells resistance to G418, the selective marker permitted quick determination of whether the recombinant virus carried by a B-cell clone had acquired the desired substitution.

Generation of recombinant viral genomes carrying deletions in BHRF1. Each plasmid of Fig. 1 was mixed with pCMV-BZLF1 (8), which expresses BZLF1 to induce the EBV lytic cycle (7), and introduced by electroporation into the P3HR1 subclone HH514-16. After 4 days in culture, approximately 2.5% of the cells from each electroporation were observed by immunofluorescence to express viral capsid antigens, compared with 0% of cells without electroporation, indicating that electroporation was successful and that lytic viral replication had been induced. Filtered growth media from the cell cultures, or the cells themselves following lethal gamma irradiation, were combined with B lymphocytes from adult peripheral blood and plated in 24-well culture dishes over gamma-irradiated human fibroblasts. The numbers of wells which produced proliferating B-cell clones for four experiments are presented in Table 1.

The BHRF1-deleted plasmid, p530, consistently yielded fewer transformants (13% of wells positive) than did its parent plasmid, p529 (60% positive). This difference did not likely result from the absence of BHRF1 function since p531, which carries the CMVIE-neo construct within the same BHRF1 deletion, gave rise to transformants as efficiently (72% positive) as did the wild-type plasmid. These perhaps minor differences in transformation efficiencies could have arisen because of effects that the deletion or substitution could have had on the rate of recombination between the plasmids and the EBV genome (see Discussion).

Because of the 2-kb distance separating the P3HR1 deletion and BHRF1, transformation-proficient recombinants could have been generated with p530 and p531 without the transfer of the BHRF1 deletion to the viral genome. The CMVIE-neo selective marker which was substituted for most of BHRF1 in p531 allowed for a quick examination of several B-cell transformants obtained by recombination with this plasmid. All of 29 transformants tested for resistance to G418 at 1,500 μg/ml (~750 μg of active drug per ml), a concentration sufficient to kill cells of other EBV-established B-cell lines, were fully resistant. Since p531 also carries the hygromycin B resistance gene within the vector portion of the plasmid (not shown in Fig. 1), these same transformants were also tested for resistance to hygromycin B; none expressed noticeable resistance. These results suggest that the transforming virus present in these cell lines resulted from double homologous recombination events between the P3HR1 genome and p531, flanking the P3HR1 deletion on the left and flanking the *Bam*HI site within the BHRF1 gene on the right.

Analysis of recombinant viral genomes. For each plasmid used to generate recombinants, several B-cell transformants from individual wells were expanded in as few generations as possible to approximately 20 million cells, and their DNAs were isolated for Southern analysis. Analysis of *Bam*HI-digested DNAs of three clones obtained by using p530 showed that in all three the BHRF1 deletion had been incorporated into the viral genome. This deletion removed the *Bam*HI site between the H and F fragments, resulting in

TABLE 1. Generation of transforming P3HR1 virus recombinants by using plasmids carrying mutations in BHRF1

Plasmid	No. of wells positive/no. tested in expt:				Total	% of wells positive
	1[a]	2	3	4		
p529 (wild type)	55/96	56/96	28/48	35/48	174/288	60
p530 (BHRF1⁻)	8/72	11/72	9/72	5/48	33/264	13
p531 (BHRF1⁻)	52/72				52/72	72
None[b]	0/72		0/48	0/48	0/168	0

[a] For experiment 1, electroporated HH514 cells were cocultivated with B cells. For the other experiments, filtered cell supernatants from electroporated cells were used for infection.

[b] As for the electroporations with the test plasmids, pCMV-BZLF1 DNA was present for these control electroporations.

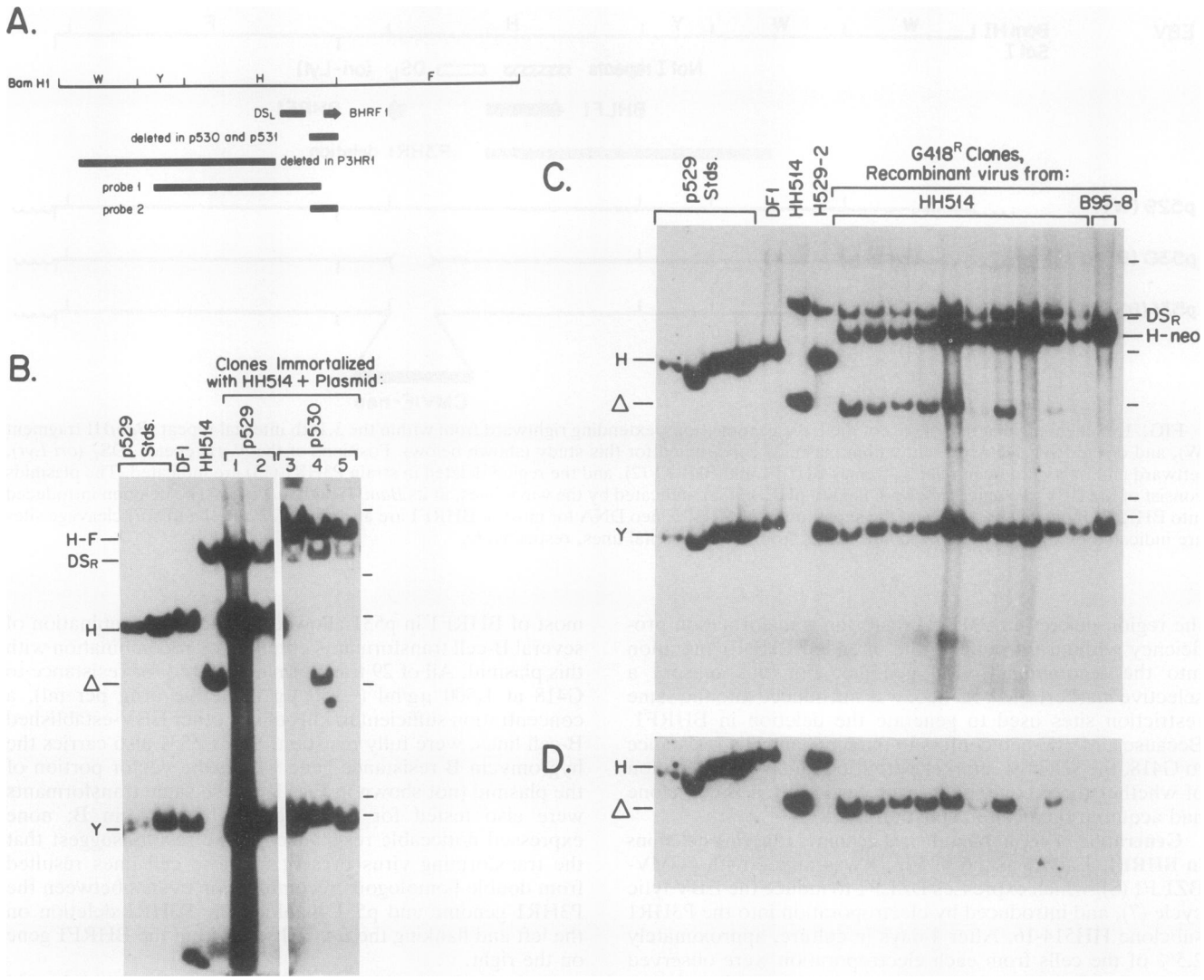

FIG. 2. Southern analysis of *Bam*HI-digested DNA of B-cell lines established by using recombinant viruses harboring deletions in BHRF1. (A) *Bam*HI cleavage sites, relevant genetic features, and probes 1 and 2. Probe 1 is a 6.6-kb *Hpa*I fragment; probe 2 is the 1.1-kb *Bcl*I-to-*Bam*HI DNA deleted in p530 and p531. (B) Southern analysis of 5 μg of cell DNA from B95-8-immortalized clone DF1, from HH514, and from B-cell clones obtained by recombination of P3HR1 with the indicated plasmid, performed with probe 1. As standards, in the first two lanes, 52 and 156 pg of p529 DNA, corresponding to two and six molecules per cell-equivalent of DNA, were mixed with salmon sperm DNA analyzed in parallel to provide standards for quantification. The expected DNA fragments are indicated at the left (see text). Marks at the right indicate the positions of DNA size standards, with sizes (from the top) of 27.5, 9.4, 6.6, 4.4, 2.3, and 2.0 kb. (C) Similar analysis of DNA from B-cell lines established by using viruses conferring G418 resistance, obtained by recombination of p531 with P3HR1 or with B95-8, as indicated. H529-2, also analyzed in panel B, is a clone established by recombination of P3HR1 with p529 (containing wild-type DNA). The p529 standards correspond to (from left to right) 1, 3, 9, and 18 molecules per cell. (D) Region of the blot from panel C after being stripped and reprobed with probe 2, the 1.1-kb *Bam*HI-to-*Bcl*I DNA that was replaced with the CMVIE-neo DNA in p531.

the appearance of a large, H-F *Bam*HI fragment (Fig. 2B). The probe used, a 6.6-kb *Hpa*I fragment indicated as probe 1 in Fig. 2A, also detected the adjacent Y *Bam*HI fragment, which was restored to these viral genomes by recombination. The probe spanned *ori-Lyt*, which is duplicated in EBV strains other than B95-8 and called DS_L and DS_R (for duplicated segments left and right). Thus, a 10-kb *Bam*HI fragment spanning DS_R was also detected in strains carrying the P3HR1 viral genome or recombinants derived from it. Similar analysis of *Bam*HI-digested DNAs from 10 of the B-cell lines obtained with p531 confirmed the presence in all cases of the 7.8-kb, altered *Bam*HI fragment H linked to the CMV-neo DNA (labeled H-neo in Fig. 2C).

Most of the clones established by the BHRF1-deleted recombinants also appeared to carry nonrecombinant P3HR1 genomes, as indicated by the presence of the 4.1-kb *Bam*HI fragment that spans the P3HR1 deletion. As seen in Fig. 2B, the P3HR1 deletion fragment was detected at several copies per cell in one of the clones obtained by using p530 (lane 4). In the cell lines established by using p531, the deletion fragment appeared to be present in all 10 examined (Fig. 2C and D). This is seen most clearly in Fig. 2D, which shows the relevant portion of the blot of Fig. 2C after it had been reprobed with probe 2 (the *Bcl*I-to-*Bam*HI DNA segment that equals the BHRF1 deletion of p531; Fig. 2A) because this probe detects the *Bam*HI H fragment and the

deletion fragment of P3HR1 (Δ in Fig. 2) equally. In one clone, the number of P3HR1 nonrecombinant genomes, about three to four per cell, appeared to exceed the number of recombinant genomes, about two per cell (Fig. 2C and D, leftmost G418^r clone). With three of the clones, the P3HR1 deletion fragment was present at much less than one copy per cell.

Examination of DNA from two cell lines established by recombination with the wild-type plasmid, p529, showed that one did not carry detectable copies of nonrecombinant P3HR1 genomes (H529-2; Fig. 2C and D); for the other, lane background due to the very high number of recombinant EBV genomes present (100 to 200 per cell; data not shown) made it impossible to determine whether a low number of nonrecombinant genomes was also present. However, in similar studies by this and other laboratories, nonrecombinant P3HR1 genomes are commonly found coinfecting B-cell lines immortalized by P3HR1 recombinants (see Discussion). This finding indicates that as these experiments are commonly performed, nonrecombinant virus is present in sufficient quantities to infect a significant fraction of the B cells, while recombinants are present at much lower levels, usually 10 or fewer transforming units per ml in our studies (not corrected for the cloning efficiency of transformed B cells).

The fact that P3HR1 was found to be present at significantly less than one copy per cell in some of the cell lines established by infection with a BHRF1-deficient virus demonstrates that BHRF1 is not essential to sustain the proliferation of latently infected B cells after 2 to 3 months in culture, the times at which DNA was isolated from the cell lines for analysis. However, these observations alone do not exclude the formal possibility that BHRF1 might be required during the initial infection of B cells. The low numbers of recombinant virus present in the supernatants made it impractical to reduce the multiplicity of infection as a test of this hypothetical contribution by the nonrecombinant P3HR1. However, by obtaining secondary and tertiary B-cell transformants with virus released from an initial transformant, it was possible to show that the BHRF1-deficient EBV could propagate and immortalize B cells in the absence of the parental virus.

BHRF1-deleted EBV can immortalize B cells and propagate in the absence of detectable helper genomes. EBV-immortalized B-cell lines typically release little or no virus into the culture medium, because very few cells enter into the productive phase of infection and because most virus that is released remains associated with the cells (41). One of the B-cell lines carrying a virus in which CMVIE-neo replaced BHRF1, H531-7, was found to release transforming virus at the low levels typical of EBV-transformed B-cell lines. Two secondary B-cell transformants were established by infecting 10^6 B cells with 0.5 ml of culture medium of H531-7 after passing the medium through a 0.22-μm-pore-size filter. As expected from the very low multiplicity of infection, the two secondary transformants did not carry the nonrecombinant P3HR1 genome at detectable levels (limit of detection, less than 0.03 copy per cell; Fig. 3, 2°H531 clones 1 and 2).

By using the more sensitive method of cocultivation to detect transforming virus (30, 41), many more secondary transformants were obtained, and several were examined for viral DNA content and for release of transforming virus. Of seven clones examined, four contained no detectable P3HR1 genomes, two clones contained 2 and 4 P3HR1 genomes per cell, and one carried approximately 0.03 P3HR1 genome per cell, just above the detection limit for the Southern analysis.

TABLE 2. Cocultivation assay for release of transforming EBV by B-cell lines carrying BHRF1$^-$ EBV with and without P3HR1 as a potential helper virus

Cell line	Estimated no. of P3HR1 genomes/cell	No. of wells positive/no. tested[a]					
		10^5	10^4	10^4, induced[b]	10^4	10^3	10^2
2°H531-3	0	7/12	1/6	5/12			
2°H531-6	0	5/12	1/6	5/12			
2°H531-8	0	11/12	0/6	5/12			
2°H531-9	0	0/12	0/6	1/12			
2°H531-4	0.03	5/12	0/6	1/12			
2°H531-5	4	11/12	1/6	2/12			
2°H531-7	2	NT	0/6	NT			
B95-8	NA[c]				6/6	6/6	5/6

[a] Cocultivation of B cells with the indicated number of γ-irradiated cells of each cell line per well was done as described in Materials and Methods. NT, not tested.

[b] Cells were treated with inducing agents 3 mM sodium butyrate and 30 nM TPA for 3 days and then washed with medium before cocultivation.

[c] NA, not applicable.

The results of the Southern analysis are summarized in Table 2; the autoradiographs are shown in Fig. 3 for secondary clone 3, carrying no detectable P3HR1 genomes, and for secondary clone 4, carrying 0.03 P3HR1 genome. (The signal for the latter was clearly visible, though faint, in the original autoradiogram but lost during photographic reproduction.) All secondary transformants carried the BHRF1-deficient EBV genome at 7 to 20 copies per cell. It is not surprising that during cocultivation, a cell in the process of releasing EBV might often pass on more than one virus particle to a recipient cell, although this was not known previously for EBV. Six of the seven secondary transformants were tested with or without treatment with sodium butyrate and TPA (tetradecanoyl phorbol acetate), which can induce productive EBV replication in some cell lines. All four cell lines that lacked detectable BHRF1$^+$ virus released transforming EBV, and all but one did so within the range of one transforming event per 10^4 to 10^5 donor cells without inducers. Butyrate plus TPA appeared to increase moderately the amounts of virus released from some of the lines. Four tertiary H531 clones were analyzed for the presence of EBV genomes, three obtained from two secondary clones that lacked P3HR1 genomes and one obtained from secondary clone 4 which contained 0.03 P3HR1 genome per cell. The P3HR1 genome could not be detected in any of these tertiary transformants, while all carried the altered H-neo fragment; the Southern analysis for three of these clones is shown in Fig. 3. One of the tertiary clones was tested subsequently in the cocultivation assay and found to release transforming virus. These experiments clearly demonstrate that BHRF1-deficient EBV can replicate and immortalize B cells without requiring a helper virus.

For the two secondary transformants that carried P3HR1 genomes at more than one copy per cell, one released transforming EBV at levels similar to those of three of the four secondary transformants that lacked detectable P3HR1 genomes (Table 2). The other was tested only at 10^4 cells per well, but since all wells were negative, it is unlikely that this cell line released much more transforming virus than did any of the others.

The secondary and tertiary transformants all had the appearance and clumping tendency typical of EBV-immortalized B-cell lines and grew at typical rates, with population doubling times of 2 to 3 days. All cell lines were resistant to

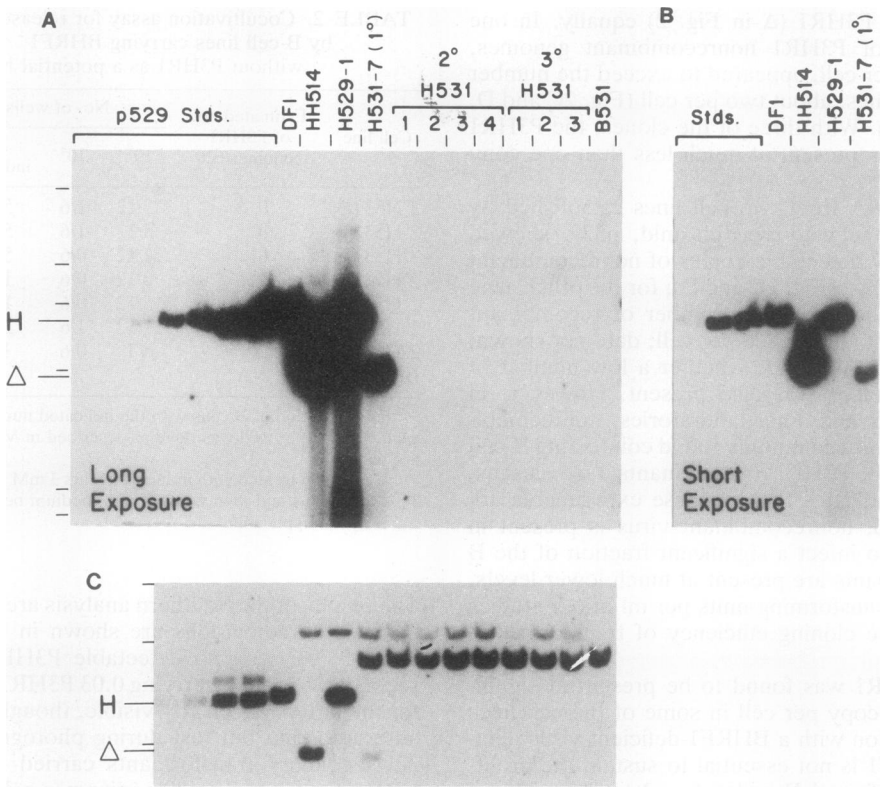

FIG. 3. Southern analysis of EBV genomes present in secondary and tertiary B-cell transformants obtained with BHRF1-deficient EBV. Five micrograms of DNA from each cell line, except H529-1 (0.5 μg), was digested with *Bam*HI and analyzed. Symbols are as described for Fig. 2. (A) Blot probed with the 1.1-kb DNA of the BHRF1 deletion (probe 2; Fig. 2A); (B) much shorter exposure of the central portion of the same blot; (C) similar blot probed with a plasmid carrying the *Bam*HI H segment of EBV. Increasing amounts of *Bam*HI-digested p529 were added as standards corresponding to (from the left) 0.03, 0.05, 0.1, 0.5, 1, 5, and 10 copies per cell. The 7.0-kb DNA seen in the plasmid standards in panel C is the vector portion of p529. The lowest three standards were omitted from this blot. A signal from the lowest standard was clearly visible on the original X-ray film of the blot in panel A.

G418, and the resistant phenotype was maintained if cells were propagated for months in the absence of the drug.

Replacement of BHRF1 with the CMVIE-neo selective marker in strain B95-8. Since the CMV-neo selective marker could be substituted for BHRF1 in the EBV genome and express G418 resistance without interfering with B-cell transformation, we tested whether this substitution could be introduced into the B95-8 strain of EBV by recombination and isolated by G418 selection in immortalized B cells. B95-8 cells were coelectroporated with p531 and pCMV-BZLF1. After 4 days in culture, the fraction of cells expressing viral capsid antigens increased from approximately 4% to 8%. Filtered cell supernatants were used to infect B cells in five 24-well culture dishes, using 0.1 ml of supernatant and 8×10^4 B cells per well. After 3 weeks, at which time transformants were growing vigorously in all wells, G418 was added at 1,500 μg (~750 μg of active drug) per ml. One $G418^r$ clone was identified and expanded for analysis of its DNA. In a *Bam*HI digest of this DNA, the recombinant H-neo fragment was present, as expected, in multiple copies per cell-equivalent of DNA (Fig. 2C and D, rightmost lanes). The normal *Bam*HI H fragment of B95-8 could also be detected clearly, but at a level of only one copy per 20 cells, indicating that the clone had been infected initially by nonrecombinant virus also (Fig. 3, lane B531; the band may not be visible after reproduction). Release of transforming virus from this cell line was not detected in the cocultivation assay.

DISCUSSION

The main finding of this work is that EBV mutants lacking almost all of the BHRF1 open reading frame can immortalize human B cells and replicate in these cells in culture. B cells immortalized by BHRF1-deleted EBV alone, in the absence of detectable BHRF1-containing EBV genomes, were found to release transforming virus at levels that are within the range typical of EBV-immortalized B cells. The normal frequency with which EBV-infected B-cell clones escape latency to release transforming virus varies widely among clones, from $1/10^3$ cells in a population to less than $1/10^6$ (30, 41). Because of this natural variation and because only a limited number of clones were examined, these studies do not rule out small quantitative contributions of BHRF1 to either phase of EBV infection, growth transformation of B cells or virus replication.

In isolating the BHRF1-deficient EBV recombinants, most initial B-cell transformants also carried the parental P3HR1 viral genome because of the large excess of parental virus present during the initial infection. Under such conditions that a large fraction of cells are initially infected with parental virus which can complement the mutant being generated, the number of transformants obtained with a given mutant may not be meaningful. There are good reasons to believe that the deletion and substitution mutations introduced into BHRF1 could have affected the rates of recom-

bination of the plasmids with the viral genome. Since the studies of this report were performed, we have found that the activity of *ori-Lyt*, which is adjacent to BHRF1, greatly stimulates the rate of recombination between the plasmids and EBV DNA (our unpublished studies). Previously it was found that a deletion removing DNA just to the right of DS_L (right of 53820, similar to the BHRF1 deletion in p530) significantly reduced replication activity; in addition, the CMV IE enhancer was found to restore activity to a deletion from the left extending into DS_L (8). Thus, a reduced rate of recombination should be expected with p530, as perhaps reflected in the observed reduction in the number of transformants, and it would not be unexpected for the CMV enhancer to have restored the recombination rate in p531.

During these studies, it was observed that a cell line carrying both BHRF1-deficient EBV genomes and $BHRF1^+$ P3HR1 genomes often would, after cocultivation with B cells, yield B-cell clones carrying both EBV genomes. This observation prompted us to compare the efficiency with which the secondary clones carrying or lacking P3HR1 genomes would yield immortalized B-cell clones following cocultivation with B cells. A significant contribution of BHRF1 either to replication and release of virus from cells or to efficiency of immortalization and outgrowth of B cells would result in significantly fewer B-cell transformants being obtained from cocultivation with cell lines lacking P3HR1 genomes compared with those carrying this potential helper virus. Although only a small number of cell lines with and without P3HR1 genomes were available for this analysis (Table 2), the seven lines under comparison all had been established from the same individual at the same time and had been in culture for the same length of time. While studies with a much larger number of cell lines would be required to support a null hypothesis, it may be stated that the results failed to suggest a conspicuous contribution of the $BHRF1^+$ P3HR1 genomes to the numbers of B-cell transformants obtained.

It is a matter of curiosity that P3HR1 genomes were so readily detected in the initial B-cell lines transformed by virus lacking BHRF1. In similar studies, the P3HR1 genome was detected at much less than one copy per cell in at least one of five clones in a study of BHLF1 (our unpublished data) and in one clone obtained with a recombinant deficient in EBNA-LP (1). In a recent study in which B-cell clones were established with P3HR1 recombinants lacking the EBER genes, 5 of 14 clones transformed by an EBER-deleted genome also carried the EBER-containing (P3HR1) genome as well (32). In the case of the cell lines established with viruses with deletions in BHLF1 or EBNA-LP, no apparent benefit would be provided by the nonrecombinant P3HR1, since P3HR1 does not carry these genes intact. Apparently, in these experiments performed in different laboratories, a significant fraction of all of the B cells were infected with the parent P3HR1 virus, and a coinfecting P3HR1 genome was often maintained gratuitously in the cells that acquired a transforming, recombinant viral genome.

What may be unusual about the cell lines initially obtained by using BHRF1-deficient recombinants is that the P3HR1 genome was often present at one or more than one copy per cell (7 of 13 clones), whereas this was never observed in the isolation of other recombinant viruses that were generated from P3HR1 in our laboratory (0 of 7). Too few studies of this kind have been performed for the significance of this difference to be evaluated. (For the generation of the EBER-deficient recombinants, it was not reported whether the coinfecting P3HR1 genomes were present at more or less than one copy per cell [32].) Little is known about the dynamics of amplification and loss of EBV genomes after infection of B cells. It is conceivable that BHRF1 could provide a slight growth advantage to EBV-infected B cells during the early stages of clonal outgrowth, although no differences were apparent in the growth properties of B-cell lines carrying or lacking viral BHRF1 once the cell lines were established.

Through similarly designed experiments, Swaminathan et al. (32) recently constructed EBV mutants that lacked the EBER genes but appeared normal in the transformation of B cells and in virus replication. Presumably, conditions exist during infection of humans when BHRF1 or the EBERs contribute either to EBV replication or to survival of infected cells. It is possible that at some stage of infection of humans, EBV-infected B cells are prone to apoptosis, for example, when entering the productive phase of infection, and that BHRF1 functions as does *bcl2* in promoting survival of the infected cells (see the introduction).

The demonstration that a selective marker replacing BHRF1 could be recombined into the B95-8 genome, with the mutant virus subsequently identified as a drug-resistant B-cell clone, suggests a general method for the disruption of any EBV gene by homologous recombination. If the selective marker disrupted a gene required for B-cell transformation, drug-resistant B-cell clones would be obtained only if the clones were coinfected with a nonrecombinant, helper virus. For the EBV recombinants generated in these studies, as well as the EBER-deleted mutants constructed by Swaminathan et al. (32), the plasmids used for homologous recombination carried the EBV lytic replication origin, *ori-Lyt*. Recent studies have shown that homologous recombination between introduced plasmids and the EBV genome is greatly stimulated by the presence of *ori-Lyt* on the plasmid; homologous recombination targeted to distant regions of the EBV genome also can be obtained efficiently if *ori-Lyt* is placed on the plasmid carrying EBV DNA from the region of interest (15a). A similar method was reported recently by Wang et al., who found that certain Burkitt's lymphoma cell lines can be used for the isolation and study of EBV mutants carrying selective markers (39). The development of methods such as these should permit full genetic analyses of the potential of EBV to replicate and to transform B cells.

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