Epstein-Barr Virus (EBV) Recombinants: Use of Positive Selection Markers To Rescue Mutants in EBV-Negative B-Lymphoma Cells

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The objective of these experiments was to develop strategies for creation and identification of recombinant mutant Epstein-Barr viruses (EBV). EBV recombinant molecular genetics has been limited to mutations within a short DNA segment deleted from a nontransforming EBV and an underlying strategy which relies on growth transformation of primary B lymphocytes for identification of recombinants. Thus, mutations outside the deletion or mutations which affect transformation cannot be easily recovered. In these experiments we investigated whether a toxic drug resistance gene, guanine phosphoribosyltransferase or hygromycin phosphotransferase, driven by the simian virus 40 promoter can be recombined into the EBV genome and can function to identify B-lymphoma cells infected with recombinant virus. Two different strategies were used to recombine the drug resistance marker into the EBV genome. Both utilized transfection of partially permissive, EBV-infected B95-8 cells and positive selection for cells which had incorporated a functional drug resistance gene. In the first series of experiments, B95-8 clones were screened for transfected DNA that had recombined into the EBV genome. In the second series of experiments, the transfected drug resistance marker was linked to the plasmid and lytic EBV origins so that it was maintained as an episome and could recombine with the B95-8 EBV genome during virus replication. The recombinant EBV from either experiment could be recovered by infection and toxic drug selection of EBV-negative B-lymphoma cells. The EBV genome in these B-lymphoma cells is frequently an episome. Virus genes associated with latent infection of primary B lymphocytes are expressed. Expression of Epstein-Barr virus nuclear antigen 2 (EBNA-2) and the EBNA-3 genes is variable relative to that of EBNA-1, as is characteristic of some naturally infected Burkitt tumor cells. Moreover, the EBV-infected B-lymphoma cells are often partially permissive for early replicative cycle gene expression and virus replication can be induced, in contrast to previously reported *in vitro* infected B-lymphoma cells. These studies demonstrate that dominant selectable markers can be inserted into the EBV genome, are active in the context of the EBV genome, and can be used to recover recombinant EBV in B-lymphoma cells. This system should be particularly useful for recovering EBV genomes with mutations in essential transforming genes.

Epstein-Barr virus (EBV) genetic analyses have been difficult because the *in vitro* host range is restricted to primate B lymphocytes which are largely nonpermissive for EBV replication. Further, recovery of EBV depends on its ability to growth transform normal human B lymphocytes (27). EBV genes which are characteristically expressed in the growth-transformed B lymphocytes are of particular interest because they are likely to mediate B-lymphocyte growth transformation (for a review, see reference 17). Mutations in these genes are likely to interfere with primary lymphocyte growth transformation and be irretrievable in primary B-lymphocyte cultures.

The only genetic studies of EBV growth transformation to date (8, 14, 32) have utilized the transformation-incompetent P3HR-1 EBV strain which has deleted the EBV DNA encoding all of EBV nuclear antigen 2 (EBNA-2) and the 3' unique exons of EBNA-LP (4, 28). Immortalizing EBV can be rescued only when homologous recombination restores the deleted DNA in P3HR-1 EBV (8, 14, 32). By using cloned viral DNA fragments, this approach indirectly demonstrated that EBNA-2 is an essential gene for B-lymphocyte transformation since only DNA fragments containing a functional EBNA-2 gene could reconstitute the lost transforming ability of P3HR-1 (8, 14). This approach is limited because recombinant nontransforming viruses cannot be

recovered or characterized and because mutations outside of the P3HR-1 deletion cannot be positively selected.

Procedures for recovering recombinant EBVs irrespective of their capacity for lymphocyte growth transformation are necessary for further EBV genetic analyses. EBV-negative B-lymphoma cells could also be useful for virus recovery. These cells are not dependent on EBV for their growth and are susceptible to EBV infection. EBV-negative Burkitt lymphoma cell lines stably infected with EBV have been derived following *in vitro* infection and subcloning (5, 7, 11, 18). These converted B-lymphoma cells are latently infected with EBV, express the usual repertoire of latent viral gene products, and acquire phenotypic characteristics associated with primary B-lymphocyte growth transformation (5, 9, 24, 26, 34, 38). EBV replication in these cells has not been extensively studied beyond the observation that early replicative cycle genes are sometimes expressed (10, 11, 19). To develop EBV recombinant molecular genetic strategies, we investigated whether drug resistance genes could be inserted into the EBV genome and used to select for EBV recombinants in B-lymphoma cells. These studies reveal new aspects of EBV infection of B-lymphoma cells and indicate that this system might be useful for EBV genetic analyses.

MATERIALS AND METHODS

Cell lines. B95-8 is an EBV immortalized marmoset B-cell line partially permissive for virus replication (23). IB4 is an

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EBV (B95-8)-infected human lymphoblastoid cell line. BL30 and BL41 are EBV-negative Burkitt lymphoma cell lines. BL30/B95-8 and BL41/B95-8 are clones that are stably infected with EBV after in vitro infection (kindly provided by G. Lenoir [5]). BJAB is an EBV-negative B-lymphoma cell line (kindly provided by G. Klein [22]). Akata is an EBV-infected Burkitt tumor cell line (35). All cell lines were maintained in RPMI 1640 with 10% fetal bovine serum.

Transfection and drug selection. B95-8 cells, 5×10^6 , in 300 μ l of RPMI 1640 with 10% fetal bovine serum at 20°C were mixed with 10 μ g of *Bam*HI-digested pX-gpt plasmid or 10 μ g of undigested pN-Hyg plasmid, electroporated (gene pulser; Bio-Rad) at 960 μ F and 0.20 V, resuspended in 10 ml of media, and incubated overnight at 37°C. Cells were then plated at 5×10^3 cells per well in 96-well, flat-bottom microtiter plates in RPMI 1640 with 10% fetal bovine serum and 10 μ g of hypoxanthine, 160 μ g of xanthine, and 3 μ g of mycophenolic acid (GIBCO) per ml for cells transfected with pX-gpt or 400 μ g of hygromycin (Calbiochem) per ml for cells transfected with pN-Hyg. Less than 50% of the wells were positive for growth in selective media in both cases. Clones were expanded and maintained in selective media.

Induction of virus replication. EBV replication was induced by culture of B95-8 or infected BL41 or BJAB cells at 5×10^5 cells per ml in medium containing 20 ng of 12-*O*-tetradecanoyl-phorbol-13-acetate per ml and 3 mM *n*-butyrate (Sigma Chemical Co.) for 3 days. Efficiency of induction was monitored by cell surface immunofluorescent staining with the 2L10 monoclonal antibody specific for EBV gp350/220 followed by fluorescein isothiocyanate-conjugated goat antiserum against mouse immunoglobulin. Cell-free EBV was obtained by centrifuging induced cultures and filtering the supernatants through a 0.45- μ m filter. The induced cell pellets were harvested for immunoblot analysis.

EBV infection of B-lymphoma cells and primary B lymphocytes. Non-T lymphocytes from an EBV-seronegative adult donor were prepared by density centrifugation with Ficoll-Hypaque and rosetting with 2-aminoethylisothiuronium bromide-treated sensitized sheep erythrocytes as described previously (37). Non-T cells, 10^7 , were resuspended in 10 ml of EBV supernatant for 3 h at 37°C. Cells were then pelleted, resuspended in fresh medium, and plated at 5×10^5 cells per well in 96-well microtiter plates. No outgrowth was observed when 10^7 uninfected cells were similarly treated. BL41, BJAB, or BL30 cells, 5×10^6 , were resuspended with 10 ml of EBV supernatant for 3 h at 37°C, pelleted, resuspended in fresh medium, and cultured overnight. Cells were then plated at 5×10^3 cells per well in flat-bottom, 96-well microtiter plates in the appropriate selective medium at the concentrations described above for transfections.

Plasmids. The simian virus 40 (SV40) early-region promoter-driven guanine phosphoribosyltransferase (SV-gpt) or hygromycin (SV-hyg) gene was obtained as a 1.1-kb fragment from pSV₂gpt (25) or a 1.8-kb fragment from pMZhyg-1 (kindly provided by A. Korman [39]). pX-gpt was constructed by cloning a repaired *Eco*RI/*Bam*HI SV-gpt fragment into the *Sma*I site of the B95-8 EBV *Bam*HI X fragment (pDK96). A repaired *Bam*HI/*Cla*I SV-Hyg fragment was cloned into the *Sfi*I site of the *Bam*HI N_{het} DNA. pN-Hyg was constructed by cloning the *Bam*HI N_{het} fragment including SV-Hyg into PUC18 containing the EBV latent (2.2-kb *Sph*I/*Sac*II fragment of B95-8 EBV *Bam*HI-C DNA [40]) and lytic (1.5-kb *Sac*I/*Taq*I fragment of B95-8 EBV *Bam*HI-H DNA [13]) replication origins.

Nucleic acid and protein analyses. Immunoblots were developed with a pool of EBV immune human sera. Southern

FIG. 1. Flow chart and comparison of experiments. B95-8 is a partially permissive, EBV-infected marmoset B-cell line. BL41, BJAB (BJ), and BL30 are EBV-negative B-lymphoma cell lines.

blots were made from cell DNA (10 μ g) digested to completion with restriction endonuclease and separated on a 0.4% agarose gel or from in situ lysing gels (12). For in situ lysing gels, 2×10^6 live cells were loaded per lane. Agarose with 1% sodium dodecyl sulfate and 50 μ g of proteinase K per ml was located behind the wells, resulting in lysis of the cells in situ when electrophoresis was begun. Electrophoresis was started at 0.5 V/cm (15 V) for 4 h at 4°C and then increased to 3.3 V/cm (100 V). Some 16 to 20 h later, the gel was washed free of SDS with 10 mM sodium phosphate and stained with ethidium bromide to visualize the markers and episomal location. Blots were hybridized with ³²P-labeled, randomly primed probes derived from cloned EBV (B95-8) or SV40 promoter with gpt or hygromycin DNA.

RESULTS

Recombinant EBV genomes carrying the guanine phosphoribosyl-transferase (gpt) gene can be recovered in B-lymphoma cells. The selection of recombinant mutant EBVs in B-lymphoma cells would be facilitated if a positive selection marker linked to the mutation would render infected cells resistant to a toxic drug. While drug resistance genes driven by heterologous eukaryotic promoters can render B-lymphoma cells resistant to toxic drugs, these promoters might not function in the context of a recombinant EBV genome.

In the first series of experiments, drug-resistant EBV was derived from partially permissive B95-8 cells in which a transfected positive selection marker had integrated into the EBV episome. The overall experimental design is outlined in Fig. 1A. The pX-gpt plasmid (Fig. 2) contained the gpt gene under control of the SV40 early promoter inserted into BXLFI, an EBV open reading frame homologous to the nonessential herpes simplex virus type 1 thymidine kinase gene (21). The surrounding EBV *Bam*HI-X DNA could target the marker into the EBV genome in a reading frame expected to be nonessential. The plasmid was transfected into B95-8 cells, and B95-8 clones resistant to mycophenolic acid were selected. If the transfected pX-gpt plasmid had recombined into the EBV genome, induction of virus replication should result in replication of the gpt DNA.

Four mycophenolic acid-resistant B95-8 clones were ana-

FIG. 2. Schematic representation of plasmid constructs used to insert selectable markers into the EBV genome. The linear EBV virion DNA is shown at the top. The DNA has been extended beyond the terminal repeats (TR) to depict the *Bam*HI *N*_{het} fragment from EBV episomes. Major internal repeat regions (IR1, IR2, IR3, and IR4) are indicated. *Eco*RI and *Bam*HI restriction sites and pertinent *Eco*RI and *Bam*HI fragments in the EBV genome are shown on the second line. The pX-gpt plasmid was derived by inserting an SV40 early region promoter-driven guanine phosphoribosyltransferase gene (SV-gpt) into the BXLFI open reading frame in an EBV (B95-8) *Bam*HI-X DNA clone. Numbers shown are EBV genome coordinates of the *Bam*HI fragment and point of SV-gpt insertion. pN-Hyg was constructed by inserting an SV40 early region promoter-driven hygromycin phosphotransferase gene (SV-Hyg) into the first exon of the LMP1 gene in an EBV (B95-8) *Bam*HI *N*_{het} DNA clone. The lytic (Ori-lyt) and latent (Ori-p) EBV replication origins were also included in this plasmid. pN-Hyg contains three *Eco*RI sites as indicated in the vector, the SV-Hyg, and the *Bam*HI *N*_{het} DNA sequences.

lyzed to determine whether the gpt DNA was replicated when virus replication was induced. Southern blot analysis of cellular DNA revealed DNA fragments in one (B95 x-gpt c1) of four clones which displayed a marked increase in gpt hybridization when virus replication was induced (Fig. 3A, lanes 2 and 3). When episomal EBV DNA was separated from chromosomal DNA by an in situ cell lysing gel technique (12), gpt DNA and EBV DNA hybridized to the identical episomal DNA band in this clone (Fig. 4C, lanes 1 and 6), confirming that the gpt DNA had recombined into the EBV episome. As expected, induction of virus replication also resulted in increased gpt hybridization to the more rapidly migrating linear, replicated viral DNA (12) (Fig. 3B, lanes 3 and 4). This indicated that episomes containing gpt DNA could be replicated and cleaved into linear viral DNA.

Further Southern analysis of B95 x-gpt c1 revealed that the gpt DNA had not recombined into the expected EBV genomic BXLFI site. gpt DNA was linked to EBV *Bam*HI-C DNA rather than to fragments adjacent to *Bam*HI-X (data not shown). This mapped the gpt DNA near the left end of the EBV genome. In addition, detection of two *Bam*HI fragments with gpt DNA hybridization suggested that complete or partial duplication of the gpt sequences had occurred (Fig. 3A, lane 2, and Fig. 2).

Since the central question was whether the drug resistance gene would function in the context of the EBV genome regardless of the integration site, EBV induced from B95 x-gpt c1 was used to infect EBV-negative BL41 B-lymphoma cells. Four BL41 clones were derived by mycophenolic acid selection, and all expressed latent EBV nuclear proteins by immunoblotting (representative clone shown in Fig. 4A, lane 3). Southern blot analysis of genomic DNA from a clone, BL41/B95 x-gpt c4, demonstrated that the gpt DNA was in

FIG. 3. Southern blot analysis of SV-gpt DNA insertion into episomal EBV. (A) *Bam*HI-digested cell DNA from B95-8 (lane 1), B95 x-gpt c1 cells (lane 2), or virus replication-induced B95 x-gpt c1 cells (lane 3) was separated by gel electrophoresis, transferred to nylon membranes, and probed with an SV-gpt DNA probe. (B) B95-8, B95 x-gpt c1 cells, induced B95-8, or induced B95 x-gpt c1 cells were loaded into an in situ lysing gel where linear viral DNA (L), episomal DNA (E), and chromosomal or concatemeric viral DNA (localized to the wells at the origin of the gel [O]) were separated by agarose gel electrophoresis. The DNA was transferred to nylon membranes and hybridized to an SV-gpt DNA probe.

FIG. 4. Western blot (immunoblot) and Southern blot analyses of BL41 cells infected with SV-gpt recombinant EBV. (A) A B95-8 EBV-infected LCL (IB4, lane 1), stable B95-8 EBV-infected BL41 (BL41/B95-8 [5], lane 2), or EBV (from B95-8 x-gpt c1)-infected BL41 (BL41/B95 x-gpt c4, lane 3) cells were analyzed by Western blot developed with EBV immune human sera. EBV latent nuclear proteins, EBNA-1 (E1), EBNA-2 (E2), EBNA-3A (E3A), and EBNA-3C (E3C), were detected as indicated. Early replicative cycle proteins (EA) were detected in BL41/B95 x-gpt c4 cells. Relative molecular size markers are 200,000, 110,000, 97,000, 66,000, and 45,000 Da, respectively. (B) Cell DNA (10 μ g) was digested with *Bam*HI, separated by gel electrophoresis, transferred to nylon membranes, and probed with SV-gpt DNA. Molecular size markers shown are 23,100, 9,400, 6,600, 4,400, 2,300, and 2,000 bp, respectively. (C) Cells were analyzed by in situ lysing gel, Southern transfer, and hybridization with a gpt DNA probe (top panel). EBV-negative BL41 cells (BL41) or an EBV-infected lymphoblastoid cell line (LCL) are included. The blot was then stripped and hybridized with an EBV DNA probe (*Bam*HI-V) in the lower panel. Episomal (E), linear EBV (L) DNA, and chromosomal DNA at the top of the gel (O) are shown.

the same context as in the parental B95-8 clone (cf. Fig. 4B, lane 2, and Fig. 3A, lane 2). This demonstrated that the positive selection marker could be transmitted by viral infection and would function to identify a recombinant EBV.

All four EBV-infected BL41 clones contained EBV episomes as determined by EBV and gpt DNA hybridization to Southern blots of in situ lysing gels (Fig. 4C, lanes 4 and 9). EBV episomes were not detected in the parental BL41 cells (Fig. 4C, lane 7) or in BL41 cells derived by subcloning after infection with wild-type B95-8 EBV (Fig. 4C, lane 8).

Although predominately latently EBV infected, these BL41 clones were also partially permissive for replication cycle-associated gene expression. Immunoblots of viral proteins in infected BL41 clones revealed EBNA-1, -2, -3A, and -3C and also the 55- to 60-kDa early replicative cycle proteins (e.g., BL41/B95 x-gpt c4 shown in Fig. 4A, lane 3). This lytic cycle gene expression correlated with the detection of EBV episomes and linear, replicated EBV DNA in all mycophenolic acid-resistant BL41 clones by Southern blots of in situ lysing gels (BL41/B95 x-gpt c4 shown in Fig. 4C, lanes 4 and 9). Viral replication was induced from one recombinant virus-infected BL41 clone, and virus was re-

covered by growth transformation of primary B lymphocytes. The spontaneous and inducible EBV replication in these in vitro-infected B-lymphoma cells was in sharp contrast to the tightly latent EBV infection of previously reported in vitro infected B-lymphoma cells derived by subcloning or prolonged culture (7, 10, 11, 18, 19, 26).

Recombinant EBV and amplicon carrying hygromycin resistance. In a second series of experiments, recombinant drug-resistant EBV was derived from B95-8 cells which contained a positive selection marker in the context of an EBV DNA fragment on a resident plasmid. The overall experimental design is shown in Fig. 1B. In contrast to the first series of experiments, the recombinant event would occur after induction of virus replication and would be detected by screening the EBV-infected, drug-resistant B-lymphoma cells. The pN-Hyg plasmid contained an SV40-driven hygromycin phosphotransferase gene cloned into the EBV *Bam*HI N_{het} fragment (Fig. 2). The EBV latent replication origin (ori-p) was included to provide for plasmid maintenance when this DNA was transfected into an EBV-infected cell (40). The EBV lytic replication origin (ori-lyt) was included to provide for high plasmid copy number upon

induction of viral replication (13). Thus, in this plasmid, larger EBV DNA flanking sequence homology, inclusion of the terminal repeat region in the flanking DNA, plasmid and lytic EBV origins, and high copy number with viral replication may facilitate recombination into the EBV genome. The plasmid was transfected into B95-8 cells, and 12 hygromycin-resistant clones were selected. These clones were pooled, virus replication was induced, and BJAB B-lymphoma cells were infected with the resultant virus. Since the plasmid contained the hygromycin phosphotransferase gene in the context of an EBV DNA fragment encompassing the terminal repeats and the *cis* packaging signals within the terminal repeats, the resultant virus stocks would contain amplicons composed of concatemeric plasmid (33), as well as parental and recombinant EBV.

Of 43 hygromycin-resistant BJAB B-lymphoma cell clones, 20 were positive for EBNA by immunoblot and 8 were analyzed further by Southern blot of restriction endonuclease-digested genomic DNA. Five clones revealed evidence of homologous recombination of the hygromycin gene into the EBV genome. In these cases, the hygromycin gene cloned into the *Bam*HI N_{het} fragment was now linked to the adjacent *Bam*HI A fragment in a common *Eco*RI fragment. EBV *Bam*HI-A DNA hybridizes to two EBV *Eco*RI fragments (*Eco*RI-C and *Eco*RI-D_{het}; Fig. 2 and Fig. 5, lane 10). *Bam*HI-A DNA is not present in the pN-Hyg plasmid and thus should not cohybridize with hygromycin DNA present in pN-Hyg. A representative clone (BJ Hyg c84) shown in Fig. 5, lanes 4 and 7, demonstrated cohybridization of hygromycin and EBV *Bam*HI-A DNA to a common 10-kb *Eco*RI D_{het} fragment, indicating new linkage between the hygromycin DNA and the EBV *Bam*HI-A DNA most likely as a result of homologous recombination of the hygromycin DNA into the *Eco*RI D_{het} fragment of the EBV genome. The *Eco*RI D_{het} fragment varies in size due to heterogeneity in the terminal repeat copy number. The other three clones analyzed were likely coinfecting with wild-type B95-8 EBV and hygromycin-resistant amplicon since *Bam*HI-A and hygromycin DNAs were not linked in the same *Eco*RI fragment. In these clones, *Bam*HI-A hybridized to the nonrecombinant *Eco*RI-C and *Eco*RI D_{het} EBV DNA fragments (representative clone BJ Hyg c65; Fig. 4, lane 5), and hygromycin DNA hybridized to two *Eco*RI fragments predicted by the pN-Hyg plasmid with the smaller fragment variable in size due to different terminal repeat copy number (Fig. 5, lane 5). This series of experiments illustrated a second method for generating recombinant EBV and the efficacy of an SV40 promoter-driven hygromycin gene for positive selection in a different region of the EBV genome.

Thirteen EBV-positive clones, including the eight clones described above, were studied by Southern blot of in situ cell lysing gels to determine whether the EBV genome was episomal. An EBV *Bam*HI-W DNA probe hybridized to the episome in 9 of 13 clones (e.g., BJ Hyg c10, 30, 65, and 84 in top panel of Fig. 6). EBV DNA hybridized to the wells at the origin of the gel and not to episomes in four clones (e.g., BJ Hyg c15, 64, and 91 in top panel of Fig. 6) compatible with integrated EBV DNA. EBV integration in clone 91 was also supported by restriction digest and Southern blot analysis in Fig. 5. Clone 91 contained recombinant EBV demonstrated by cohybridization of hygromycin (Fig. 5, lane 2) and EBV *Bam*HI-A DNA (Fig. 5, lane 9) to a 10-kb *Eco*RI D_{het} fragment. However, the second *Eco*RI fragment detected by a hygromycin probe containing the terminal repeats (Fig. 2, pN-Hyg) was much larger than expected (approximately 14 kb; Fig. 5, lane 2) and was distinct from *Eco*RI-C. This most

FIG. 5. Restriction analysis of infected, hygromycin-resistant BJAB cell DNA. Cell DNA (10 μ g) from B95-8 cells or four infected, hygromycin-resistant BJAB cell clones (BJ Hyg clones 65, 84, 89, and 91) was digested with *Eco*RI and analyzed on duplicate blots hybridized with an EBV *Bam*HI-A DNA (lanes 6 to 10) or a hygromycin DNA (lanes 1 to 5) probe. The EBV *Bam*HI-A DNA probe detected the *Eco*RI-C (C) and *Eco*RI D_{het} (D_{Het}) in *Eco*RI-digested EBV DNA (lane 10; see Fig. 2). The *Eco*RI D_{het} fragment varies in size due to heterogeneity in the terminal repeat copy number. The fainter 8- and 4.4-kb bands in lanes 6, 7, and 8 likely represent hybridization with low levels of vector DNA in the EBV DNA probe.

likely represented a junction fragment with chromosomal DNA created by integration of the EBV DNA through the terminal repeats, as was confirmed by further restriction digest and Southern blot analyses (data not shown). Hygromycin-resistant BL30 cells were derived in a similar manner, and three of three clones had episomal EBV (BL30 Hyg c1, 2, and 3 in top panel of Fig. 6). All three clones were coinfecting with wild-type EBV plus amplicon as determined by restriction digest and Southern blot analyses (data not shown). Thus, EBV infection of B-lymphoma cells in these experiments frequently, but not inevitably, results in EBV persistence as episomes, whether the positive selection marker is in the EBV genome (i.e., recombinant EBV) or not (i.e., EBV coinfection with amplicon).

By immunoblot, the EBV-infected BJAB clones were latently infected and EBNA-1 was expressed in all clones (Fig. 7). However, EBNA-2 and EBNA-3A/3C expression was variable relative to EBNA-1 expression (Fig. 7, lanes 2 and 5 versus lane 11) consistent with the variable EBNA-2 and EBNA-3A/3C latent gene expression seen in Burkitt tumor isolates (30).

Of 20 clones, 11 were partially permissive for EBV early replicative cycle protein expression. Two of the 11 clones which spontaneously express early replication cycle proteins and 1 of the 9 clones which did not spontaneously express

FIG. 6. In situ lysing gel analysis of infected, hygromycin-resistant BJAB and BL30 clones. A Southern blot of an in situ lysing gel was hybridized sequentially with an EBV DNA probe (*Bam*HI-W) (top) or a hygromycin DNA probe (bottom). EBV-negative BL30 (BL30) and stable B95-8 EBV-infected BL30 (BL30/B95-8 [5]) cells were included. Episome (E) and linear viral (L) DNAs and the origin (O) are indicated.

early antigens were studied further to see whether these clones were responsive to induction. Increased replication cycle protein expression was observed after induction of both clones which had spontaneous, low-level early antigen expression (Fig. 7, lanes 3 and 4 versus lane 2; lanes 6 and 7 versus lane 5). Both clones contained EBV episomes. One clone was infected with recombinant EBV (BJ Hyg c84) and one clone was coinfecting with nonrecombinant EBV and amplicon (BJ Hyg c65). A clone with integrated recombinant EBV remained tightly latent despite chemical induction (Fig. 7, lanes 12 and 13 versus lane 11). Both induction-responsive clones expressed EBV late replicative genes as measured by EBV gp350 cell surface immunofluorescence on 1% of the cells (data not shown). Thus, spontaneous and inducible EBV replication was a frequent occurrence in both series of experiments, indicating that the proclivity for EBV replication in these in vitro-infected B-lymphoma cells was not due to the specific recombinant event or selectable marker.

Of the 23 hygromycin-resistant BJAB clones which were negative for EBNA_s by immunoblot, 10 were confirmed to have parental amplicon DNA in the absence of other EBV DNA by Southern blot analysis. Hygromycin DNA hybridized to the expected amplicon fragments, a unique 8-kb fragment, and a smaller, variable sized DNA fragment containing the terminal repeats (representative clone BJ Hyg c89; Fig. 5, lane 3). The EBV *Bam*HI-A DNA probe did not hybridize to any DNA fragments, consistent with the absence of EBV DNA other than the *Bam*HI N_{het} DNA in the amplicon (Fig. 5, lane 8). In the absence of EBNA-1 from a coinfecting EBV genome, the amplicon DNA was not maintained as an episome and was integrated into cellular DNA. Southern blot hybridization of in situ lysing gels revealed hygromycin DNA hybridization only to chromosomal DNA at the origin in four clones (e.g., BJ Hyg c7 and 89 in bottom panel of Fig. 6).

DISCUSSION

These experiments demonstrate that (i) two different toxic drug resistance genes under control of the SV40 promoter and enhancer can be inserted into the EBV genome, (ii) specific EBV DNA segments can be targeted for recombination, (iii) drug resistance genes can be expressed from at least two sites in the EBV genome, (iv) drug resistance genes recombined into the EBV genome enable recombinant genomes to be selected for in EBV-negative B-lymphoma cells, (v) EBV is frequently maintained as an episome in in vitro-infected B-lymphoma cells, and (vi) in vitro-infected B-lymphoma cells are frequently partially permissive for EBV replication and can be induced to produce virus.

These experiments describe a new strategy for generating and identifying EBV recombinants by passage into B-lymphoma cells. Since B-lymphoma cells are not dependent on EBV for their growth and previous experiments demonstrated that both transforming B95-8 EBV and nontransforming P3HR-1 EBV can persist in in vitro-infected B-lymphoma cells (5, 7, 11, 18), the strategy is not likely to be affected by mutations in essential transforming genes. Thus, viruses with mutations in essential transforming genes are likely to be recoverable in B-lymphoma cells so that their genomes can be characterized. Since these experiments also indicate that B-lymphoma cells are frequently partially permissive for virus replication and can be productive after induction, recombinant mutant viruses can be identified, passaged, and phenotypically characterized.

The insertion of the SV40 promoter and enhancer-driven hygromycin phosphotransferase gene into *Bam*HI N_{het} DNA described in these experiments is the first targeted EBV recombination outside of the P3HR-1 deletion. The specific construction placed the SV40-hygromycin DNA into the DNA encoding the first LMP1 exon so that further biological and chemical characterization of the recombinant viruses will be of substantial interest. These experiments do not

FIG. 7. Western blot of infected, hygromycin-resistant BJAB clones before and after induction of viral replication. Akata is a partially permissive EBV-infected Burkitt tumor cell line which spontaneously expresses early replicative cycle antigens. Infected, hygromycin-resistant BJAB clones 65, 84, 89, and 91 before (lane 2, 5, 8, and 11) and after induction with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and 1 mM *n*-butyrate (lanes 3, 6, 9, and 12) or TPA and 3 mM *n*-butyrate (lanes 4, 7, 10, and 13) are shown. Latent EBV nuclear proteins EBNA-1 (E1), EBNA-2 (E2), EBNA-3A, and EBNA-3C (E3A/3C) are indicated. Relative molecular size markers are 200,000, 115,000, 97,000, 66,000, and 45,000 Da. Spontaneous expression of the 55- to 60-kDa early antigens before chemical induction could be observed in BJ Hyg c65 and 84, but not c91, on longer exposures.

specifically address the requirements for efficient homologous recombination in this system. In other systems, the extent of flanking sequence homology and characteristics of the target DNA sequences, such as transcription or replication activity or copy number, affect recombination efficiency (6, 36). Given sufficient flanking sequence homology, it is likely that recombination can be targeted to any site within the EBV genome.

Most studies of *in vitro* EBV infection of B-lymphoma cells have focused on the effects of EBV infection on B-lymphocyte surface markers and growth characteristics (5, 9, 24, 34, 38). Little was known about the EBV genome after *in vitro* infection of B-lymphoma cells, in part due to the difficulty in deriving stable EBV-infected B-lymphoma cell lines by infection and subcloning (7, 10, 11, 18). Most B95-8 EBV-infected B-lymphoma cell clones have few EBV genomes per cell (2, 9, 10) and have usually been nonpermissive for virus replication (7, 10, 11, 18, 19, 26). P3HR-1 EBV infection of B-lymphoma cells has resulted in some replicative cycle gene expression (10, 18), but defective virus may have been an important factor (15). In fact, the resident B95-8 EBV genome in stable BJAB/B95-8 cells was incapable of replication or being packaged since transforming recombinant P3HR-1 EBV, but not wild-type B95-8 EBV, was recovered after P3HR-1 superinfection of B95-8-converted BJAB cells (32). The inability of the B95-8 EBV genome to be replicated and packaged was probably due to EBV integration into cell DNA. A previous study reported that three EBV-converted Ramos clones contained only integrated EBV (3). In the experiments reported here, two previously established B95-8 EBV-infected BL30 and BL41

clones (5) had only integrated EBV DNA since no EBV episomes were detected by *in situ* lysing gel analysis (Fig. 4 and 6), and Southern blot analysis of restriction endonuclease fragments was indicative of integrated EBV DNA (data not shown).

Thus, these studies characterize new aspects of *in vitro* EBV-infected B-lymphoma cells compared with the above observations in that infected cells frequently had EBV episomes, spontaneous early replicative cycle gene expression, and linear replicated viral DNA. These data are compatible with the hypothesis that episomes are necessary for replication cycle gene expression. In addition, episomes are likely to be necessary for late replication cycle gene expression since herpesvirus DNA replication proceeds from a rolling-circle intermediary and late replication cycle gene expression requires viral DNA synthesis (29). Consistent with this hypothesis, we could induce replicative cycle gene expression in two clones with episomal EBV and not in a clone with integrated EBV. Thus, the high frequency of replicative cycle gene expression may be due to EBV episomes present in most of the *in vitro* infected B-lymphoma cell clones reported here. Duration in culture, DNA methylation, and episome copy number are other factors which may contribute to permissive infection.

Why, then, were integrated EBV and tightly latent infection characteristic of previously described *in vitro*-EBV-infected B-lymphoma cells? Previous investigators generally studied uniformly EBV-infected cultures. Integration may have been inadvertently selected for since EBV integration would guarantee uniform EBV genome transmission to all progeny cells. In contrast, since B-lymphoma cells do not

require EBV infection for growth, low episome copy number in in vitro-infected B-lymphoma cells and random episome segregation among progeny cells would result in mixed cultures of EBV-infected and uninfected cells. In the experiments reported here, the uninfected cells were eliminated through drug selection so that clones containing episomal EBV remained uniformly infected. The studies described here were also performed relatively early, 2 to 4 months after infection. There is probably no specific selection initially for episome versus integrated EBV DNA in B-lymphoma cells. In fact, we did observe some clones which contained integrated EBV DNA. These integration events may have occurred from input linear viral DNA or after EBV DNA circularization. Thus, even our clones with EBV episomes may accumulate integrated EBV with prolonged passage in selective media. The frequency of EBV integration from an episome could be ascertained by observing these clones over time.

In primary human B-lymphocyte infection, EBV characteristically persists as an episome even after long-term passage (1, 12, 16). Episomal persistence in growth-transformed B lymphocytes suggests that cell proliferation is episome dependent. LMP2 expression requires EBV episome persistence since mRNAs are encoded across the linear viral DNA termini (20, 31). Thus, the persistence of EBV episomes in primary B lymphocytes could arise because of continuous selection for LMP2 expression. The strategy for recombinant EBV molecular genetic analysis of essential transforming genes described in these studies should facilitate the determination of whether LMP2 and LMP1 are essential for lymphocyte growth transformation.

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