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An Epstein-Barr Virus with a 58-Kilobase-Pair Deletion That Includes BARF0 Transforms B Lymphocytes In Vitro

ERLE S. ROBERTSON,* BLAKE TOMKINSON,† AND ELLIOTT KIEFF

Departments of Medicine and Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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A family of Epstein-Barr virus (EBV)-encoded RNAs found in nasopharyngeal carcinoma cells is also present at low levels in some latently infected and growth-transformed B lymphocytes (P. R. Smith, Y. Gao, L. Karran, M. D. Jones, D. Snudden, and B. E. Griffin, J. Virol. 67:3217–3225, 1993). A molecular genetic approach using EBV recombinants was undertaken to evaluate the role of these transcripts in primary B-lymphocyte growth transformation and latent infection. Since the se transcripts arise from a 22-kbp segment of the EBV genome and construction of large deletion mutants is an improbable result after transfection of infected cells with an EBV DNA fragment with a large deletion mutation, a new approach was taken to make a recombinant with the DNA encoding all of the BARF0 RNAs deleted. The approach derives from a recently described strategy for making recombinants from five overlapping EBV cosmid-cloned DNAs (B. Tomkinson, E. Robertson, R. Yalamanchili, R. Longnecker, and E. Kieff, J. Virol. 67:7298–7306, 1993). A large segment of EBV DNA was deleted from the transfected cosmid DNAs by omitting a cosmid which included all of the DNA encoding the BARF0 RNA and by ligating the distal halves of the two flanking cosmids so as to create one cosmid which had ends that overlapped with the other two unaltered cosmids. EBV recombinants with 58 kbp including BARF0 deleted resulted from transfecting the three overlapping EBV DNA fragments into P3HR-1 cells and simultaneously inducing lytic replication of the endogenous, transformation-defective, P3HR-1 EBV. The endogenous P3HR-1 EBV provided lytic infection and packaging functions. EBV recombinants with intact transforming functions were then selected by infecting primary B lymphocytes and growing the resultant transformed cells in lymphoblastoid cell lines. The efficiency of incorporation of the deletion into transforming EBV recombinants was close to that of a known indifferent marker, the type 1 EBNA 3A gene, indicating the absence of significant selection against the deletion. Cells infected with the deleted recombinant grew similarly to those infected with wild-type recombinants and had a similar level of permissiveness for lytic EBV infection. Thus, the BARF0 transcript is not critical to primary B-lymphocyte growth transformation or to latent infection. This methodology is useful for constructing EBV recombinants which are specifically mutated at other sites in the three cosmids and is a step toward deriving a minimal transforming EBV genome.

Epstein-Barr virus (EBV) was discovered over 25 years ago in the search for an infectious etiology for African Burkitt's lymphoma (BL). The virus was soon also associated with nasopharyngeal carcinoma (NPC) (16, 52). Although the etiologic relationship between EBV and BL or NPC is more complicated than initially envisaged, the virus can rapidly and efficiently induce unrestrained proliferation of human B lymphocytes in vitro and in vivo (2, 5, 15, 16, 33–36, 40, 51, 52). Molecular biologic investigations have described 10 genes encoding a set of six nuclear proteins (EBNAs), two integral membrane proteins (LMPs), and two small RNAs (EBERs), which are transcribed in latently infected and growth-transformed cells and are therefore likely to be mediators of latent infection or cell growth transformation (for a review, see reference 24). Many biochemical and phenotypic activities of these genes have been defined (for a review, see reference 24). EBV recombinant-based molecular genetic analysis of the role of specific genetic elements in EBV latency and transformation has indicated that EBNA LP, EBNA 2, EBNA 3A, EBNA 3C, and LMP1 are critical to EBV-mediated B-lymphocyte proliferation at the cellular level, whereas EBNA 3B, LMP2, and the EBERs are not (6, 7, 12, 20, 25–28, 43–48).

Previous recombinant EBV-based molecular genetic evaluations of the role of genes known to be expressed during latency do not exclude the possibility that other parts of the genome may contribute as yet unidentified *cis*- or *trans*-acting functions. This possibility became evident with the finding that EBV DNA mapping at 150 to 160 kbp in the B95-8 genome encodes a hitherto unrecognized family of highly spliced and polyadenylated RNAs in human NPC cells and biopsies and in some latently infected B lymphocytes (3, 4, 11, 12, 19, 37, 42) (Fig. 1). The abundance of the RNAs decreases with the transition to lytic infection, consistent with a role for the RNAs in latency (19). One role could be as a final checkpoint in preventing lytic reactivation from latency since the RNAs are antisense (19, 42) to several immediate-early or early lytic infection RNAs (for a review of lytic infection gene expression, see references 9 and 24). Alternatively, the RNAs may encode a protein since they include at their 3′ end a short *Bam*A DNA fragment-encoded open reading frame (ORF), which has been given the acronym BARF0, consistent with the usual terminology for EBV ORFs (12). Some EBV-infected humans have antibody to in vitro-translated BARF0 polypeptides (12).

We attempted to make EBV recombinants with deletions of the BARF0 ORF by transfecting P3HR-1 EBV-infected cells with an EBV DNA fragment which had a deletion of the

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, Harvard Medical School, Thorn Building, 12th Floor, 75 Francis St., Boston, MA 02115. Phone: (617) 732-7046. Fax: (617) 732-7099. Electronic mail address: esrobert@mbcrr.harvard.edu.

† Present address: Supragen Inc., 1670 Pierce St., Lakewood, CO 80214.

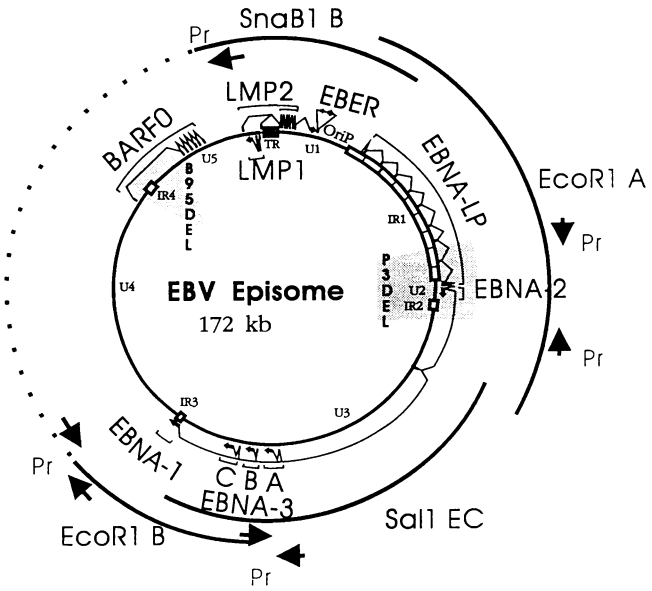

FIG. 1. Schematic diagram of the EBV genome indicating the major latent transcripts (EBNAs, LMPs, and EBERs) and the deletions characteristic of the T1 EBV strain B95-8 and of the T2 EBV strain P3HR-1. The *Eco*RI A, *Sal*I E/C, and *Eco*RI B and *Sna*BI fragments of the deletion cosmid, ESN, which were transfected into P3HR-1 cells, are indicated. The broken line indicates the region of the genome deleted. The BARF0 RNA initiates in U4 about 2 kbp upstream of the B95-8 deletion and ends in U5 about 9 kbp after the deletion. The primers used for PCR analysis are also indicated (Pr).

BARF0 ORF and selecting for recombinants, using a marker rescue of transformation strategy which should have yielded a 10% frequency of recovery of BARF0 deletion mutants (45–48). None of the recombinants that were obtained had incorporated the BARF0 deletion. The failure to obtain the BARF0 deletion recombinant could not be adequately explained by a putative stringent requirement for a BARF0-encoded RNA or protein since the experiments were done under conditions in which approximately 50% of the infected primary B lymphocytes are coinfected with P3HR-1 EBV which should be competent for BARF0 expression (45–48). The failure is more likely due to a low efficiency of recombination caused by the proximity of the BARF0 deletion to a 12-kbp deletion, which is a unique characteristic of the transfected B95-8 strain-derived EBV DNA fragment and not a characteristic of the P3HR-1 EBV genome with which it had to recombine (37, 48) (Fig. 1).

Of the several strategies that have been developed for specifically altering the EBV genome (6, 7, 13, 20, 22, 23, 25–31, 43–48, 50), the most efficient is through transfection of P3HR-1 cells with five, cosmid-cloned, overlapping DNA fragments representative of the complete B95-8 EBV genome (37, 48). The efficiency of this strategy derives from the relative ease of transfecting P3HR-1 cells with large EBV DNA fragments, the inducible permissivity of P3HR-1 cells for EBV replication, and the presence in P3HR-1 cells of an endogenous EBV genome which is competent for lytic EBV replication but has a DNA segment encoding part of EBNA LP and EBNA 2 deleted (6, 7, 13, 14, 17, 18, 29, 33, 38, 41, 43–48). When five overlapping cosmid-cloned EBV DNA fragments from the B95-8 type 1 (T1) EBV strain were used to transfect P3HR-1 cells which harbor a transformation-defective and EBNA LP- and EBNA 2-deleted type 2 (T2) EBV strain, more than 20% of the recombinants, which were selected by lymphoblastoid cell line (LCL) outgrowth and had therefore incorporated the EBNA LP and EBNA 2 genes from the transfected EBV DNA fragments, had incorporated markers from at least two other transfected EBV DNA fragments and almost half of these recombinants had markers only from the transfected cosmid-cloned EBV DNA fragments (48). The 12-kbp deletion, uniquely characteristic of the B95-8 EBV genome (Fig. 1), was thereby incorporated into 10% of the resulting recombinant genomes (48).

The successful incorporation of the B95-8 deletion into 10% of the transforming recombinants in the five cosmid transfections opened the possibility that a substantially larger deletion could be similarly incorporated into EBV recombinants as long as virion "head-full" packaging constraints were not violated. We therefore undertook to make EBV recombinants with deletions of the DNA encoding the entire family of BARF0-related RNAs. Such experiments could also be a step toward deriving a minimal transforming EBV genome.

MATERIALS AND METHODS

Plasmid and cosmid DNAs. The ESN cosmid was derived by ligating the *Eco*RI-*Not*I fragment (residue 95,239 to 117,609) of *Eco*RI to the *Not*I-*Sna*BI fragment (residue 163,415 to 14,294) of *Sna*BI B and to a modified pDVcosA2 (1, 21, 48). The ligated DNA was then packaged into lambda phage and used to infect *Escherichia coli* PLK-A (Stratagene). pSVNaeI Z was used for induction of lytic EBV infection (8). The *Eco*RI A fragment, *Sal*I EC cosmid DNA, and pDVcosA2 were previously described (1, 21, 45–48).

Cell cultures. B95-8 is a marmoset LCL immortalized with a T1 EBV (32, 34). The HH514-16 subclone of P3HR-1 (a gift from George Miller, Yale University, New Haven, Conn.) is infected with the T2 P3HR-1 EBV (17, 33). The Akata cell line is an EBV-positive group I BL cell line, and the BJAB cell line is an EBV-negative BL cell line (48). Cells were maintained in complete medium consisting of RPMI 1640 supplemented with 10 to 15% inactivated fetal bovine serum, 2 mM glutamine, 10 μg of gentamicin per ml, and 50,000 U of penicillin per ml.

Preparation of EBV recombinants. Cosmid DNA was digested with restriction endonucleases to release the EBV DNA. The three cosmid DNAs (20 μg of each) were mixed with 25 μg of pSVNaeI Z DNA, precipitated, resuspended in 400 μl of complete medium, and transfected into 10^7 P3HR-1 c16 cells in a 0.4-cm-gap cuvette (Bio-Rad) at 200 V and 960 μF (45–48). The cells were then immediately diluted into 15 ml of complete medium and incubated at 37°C for 6 days.

Infection of primary B lymphocytes. Primary B lymphocytes were prepared and infected with filtered supernatant (0.45-μm-pore-size) from P3HR-1 cells at 6 days after transfection (45–48). Human peripheral blood mononuclear cells (10^7 cells), depleted of T cells by rosetting with 2-aminoethylisothiouronium bromide-treated sheep erythrocytes at 4°C overnight, were incubated with virus at 37°C for 2 h in 5 ml of complete medium. The infected cells were then diluted in 30 ml of complete medium and plated onto two 96-well microtiter plates at a concentration of 5×10^4 cells per well in a final volume of 150 μl. The plates were then incubated at 37°C in 5% CO_2 for 21 days. The cultures were fed with 100 μl of complete medium and incubated at 37°C for another 7 days. LCLs were macroscopically visible by 4 weeks and were expanded.

PCR. Primers were made on an Applied Biosystems model 391 synthesizer and corresponded to bp 117,520 to 117,543 and bp 163,595 to 163,578 (1) for amplification across the deletion

TABLE 1. Frequency of LCLs infected with an EBV recombinant with a 58-kbp DNA segment deleted

Experiment no.	No. of LCLs	No. with T1 EBNA 3A[a]	No. with the ESN deletion incorporated[b]	No. without WT DNA[c]
1A	59	20	18	5
2A	68	19	19	13
1B	93	30	31	9
2B	68	19	17	11
1C	49	12	6	4
2C	74	21	15	7
1D	84	23	19	4
2D	74	18	16	6
1E	36	5	2	2
2E	54	8	4	2
1F	64	14	14	8
2F	84	21	15	8
1G	41	5	9	3
2G	67	16	20	3
1H	32	5	3	1
2H	34	6	5	3
Total	981	242	213	89

[a] Indicates incorporation of the transfected T1 *Sal*I E/C fragment.
[b] All except 5 of the 213 were also positive for the T1 *Sal*I E/C fragment.
[c] Number infected with an ESN deletion recombinant which lacks WT DNA at the deletion site. WT DNA usually indicates P3HR-1 coinfection.

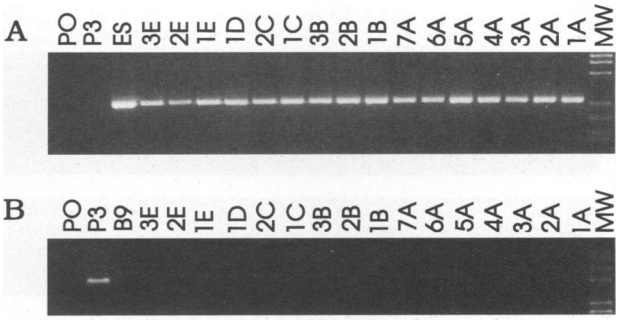

FIG. 2. PCR analysis of LCLs infected with EBV recombinants using primers that amplify a 261-bp fragment across the site of the ESN deletion (A) or a 257-bp fragment across the site of the P3HR-1 deletion (B). PCR with the ESN deletion primers does not amplify the 58-kbp corresponding fragment of P3HR-1. Similarly, PCR with the P3HR-1 deletion primers does not amplify the 6-kbp WT DNA fragment. Neither primer amplifies WT B95-8 DNA. Size markers on the right (lanes MW) are φX174 DNA digested with *Rsa*I. Lanes 1A through 3E are amplifications with DNA from LCLs infected with EBV recombinants. Lanes P3 and B9 contain control amplifications with DNA from P3HR-1 and B95-8 cells. Lane ES contains a control amplification using 10 ng of control cosmid DNA mixed with DNA from an EBV-negative BL line, BJAB. Lanes PO represent control amplifications with primers and no added DNA.

in the ESN cosmid. This oligonucleotide pair was selected by using the primer program version 0.5 (©1991, Whitehead Institute for Biomedical Research) and amplifies a 261-bp fragment across the deletion mutant DNA but does not amplify a fragment from wild-type (WT) DNA. The primers used for amplification of WT DNA within the deletion correspond to bp 117,520 to 117,543 and bp 117,677 to 117,653 (1). These primers amplified a 157-bp DNA fragment from both the P3HR-1 and B95-8 genomes. The primers which flank the P3HR-1 deletion amplify a 257-bp DNA fragment (47–48). The primers which result in amplification of type-distinctive fragments from EBNA 3A were described previously (39, 45–48). The sensitivity of the WT primers was tested by serial dilution of WT LCL DNA in DNA prepared from an EBV-negative cell line, BJAB.

DNA used in PCR was prepared from 10^5 cells. The cells were resuspended in 100 μl of 0.2% isotonic phosphate-buffered saline and heated at 95°C for 15 min. Proteinase K (1/10 volume of 10 mg/ml) was added, and the cells were incubated for 60 min at 55°C. The proteinase K was then inactivated by incubation at 95°C for 30 min. PCRs used a Perkin-Elmer thermal cycler machine with 5 μl of DNA solution and 0.625 U of *Taq* DNA polymerase (Stratagene) in a 25-μl reaction. The amplified products were analyzed by electrophoresis in 1% ME–2% Nusieve (wt/vol)–agarose (FMC) gels in 0.5× TBE buffer (50 mM Tris, 50 mM boric acid, and 0.5 mM disodium EDTA) (pH 8.0). The gels were stained with ethidium bromide and visualized by UV fluorescence.

Immunoblot, Southern blot, and in situ lysis cell gel analyses. EBV proteins were resolved on 8% denaturing polyacrylamide gels and detected by immunoblot with EBV-immune human serum or with S12 monoclonal antibody to EBV LMP1 (gift of D. Thorley-Lawson, Tufts University, Boston, Mass.) or with bz-1 monoclonal antibody (gift of A. Rickinson, University of Birmingham, Birmingham, England) to EBV Z immediate-early protein. Cell DNA was prepared by harvesting 10^7 cells, treatment with proteinase K for 2 h at 55°C, and extraction with phenol and chloroform. The DNA was precipitated, washed with 70% ethanol, and resuspended in 200 μl of TE buffer (10 mM Tris and 1 mM EDTA) containing RNase A. Thirty micrograms of DNA from LCLs was digested for 4 h with the appropriate enzymes and with buffer supplied by the vendors in a 200-μl total volume. The digested DNA was analyzed by electrophoresis through a 0.5% ME-agarose (FMC) gel (20 by 20 cm) run for 20 h at 60 V. For in situ lysis, 2.5×10^6 cells were loaded on 0.75% agarose gels (10). Gel-fractionated DNAs were transferred to an activated nylon membrane (GeneScreen Plus, New England Nuclear) by capillary action overnight. The membranes were then baked in vacuum for 2 h and hybridized with radioactive probes. EBV DNA fragments were labelled with [^{32}P]dCTP (3,000 Ci/mmol), using the random primer system (Stratagene). Hybridizations were with probes of 2×10^6 to 4×10^6 cpm/ml at 68°C for 12 to 18 h.

RESULTS

EBV recombinants from transfection of three overlapping cosmids. The underlying hypothesis of these experiments was that a mini-transforming EBV genome could be assembled from overlapping EBV DNA fragments with specific deletions of a large segment of EBV DNA, by transfecting these fragments into P3HR-1 cells and inducing lytic replication of the endogenous P3HR-1 genome. The P3HR-1 genome would provide replication and packaging functions. EBV recombinants resulting from recombination among the transfected cosmids or between the transfected cosmids and the replicating P3HR-1 genome would be specifically selected for by the ability to transform primary B lymphocytes into long-term LCLs, an ability that the P3HR-1 EBV lacks. LCLs transformed by mini EBV genomes resulting from recombination among the transfected EBV DNAs could then be distinguished from EBNA LP- and EBNA 2-restored and transformation competent P3HR-1 genomes by PCR amplification of DNA across the site of the 58-kbp deletion, by PCR amplification of

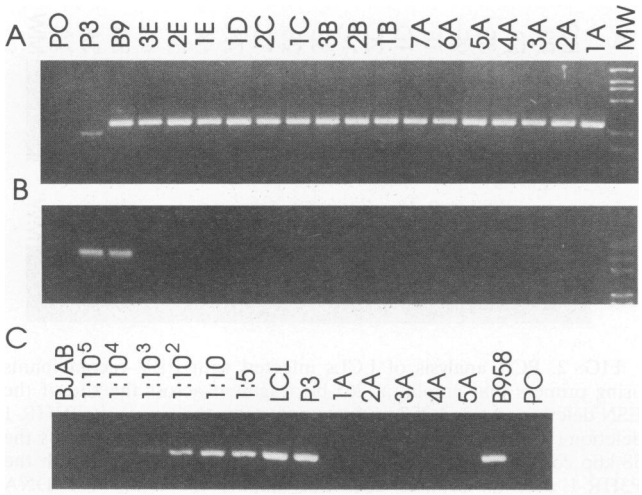

FIG. 3. PCR analysis of LCLs infected with EBV recombinants using primers which amplify a 276-bp fragment from T1 EBNA 3A of the B95-8 strain or a 237-bp fragment from T2 EBNA 3A of the P3HR-1 EBV strain (A) or using primers which amplify a 157-bp fragment specific for WT EBV DNA deleted from the ESN cosmid (B). Lanes P3 and B9 in panel B show specific amplification of the WT EBV genomes to produce a 157-bp DNA fragment. Panel C demonstrates the sensitivity of the WT primers within the deletion, using WT LCL DNA serially diluted in DNA from an EBV-negative BL cell line, BJAB.

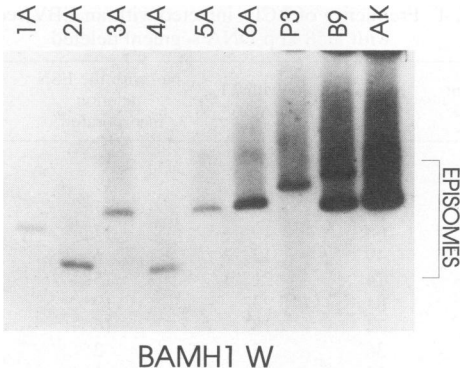

FIG. 4. Southern blot analysis of EBV episomes separated on a 0.75% ME in situ lysis agarose gel, transferred to a nylon membrane, and incubated with a [^{32}P]dCTP-labelled probe made from the EBV *Bam*HI W fragment. Lanes: 1A through 6A, episomes from LCLs transformed by recombinant EBV with 58 kb deleted; P3 and B9, WT EBV-positive cell lines with T2 and T1 EBV episomes, respectively; AK, group 1 cell line containing a WT EBV episome.

WT DNA within the deletion, and by PCR amplifications which detect differences between the T1 and T2 EBNA 3 genes of the transfected DNA and P3HR-1 EBV, respectively (39). Although the BARF0 transcript might be essential for latent infection or growth transformation, the experiments could be done under conditions in which about 50% of the primary B lymphocytes are infected with nonrecombinant P3HR-1 EBV (45–48) which would provide WT BARF0 RNA and protein in *trans*. We were, however, concerned that the resulting genome might be too small to be packaged as a unit-length genome and too large to be packaged as a dimer.

A new cosmid (ESN) was constructed from the T1 B95-8 cosmid EBV DNA library so as to potentially enlarge the 12-kbp, B95-8-unique deletion to 58 kbp, thereby deleting all of the DNA encoding BARF0 RNAs (Fig. 1). The ESN cosmid was constructed by splicing together the *Eco*RI-*Not*I fragment of the *Eco*RI B cosmid and the *Not*I-*Sna*BI fragment of the *Sna*BI B cosmid (Fig. 1). The resulting cosmid had deletions of the right half of *Eco*RI B, all of the *Sal*I EBV DNA fragment, and the left half of the *Sna*BI B cosmid. By deleting nucleotides 117,609 to 163,415 (1), the incorporation of the resulting ESN cosmid into an EBV genome would delete 58 kbp, including the entire BARF0 transcript which maps at nucleotides 150,640 to 160,991 (42). Transfection along with two other cosmids, *Eco*RI A and *Sal*I E/C, might enable transforming EBV genomes to be generated by recombination among the cosmids. This might even be more efficient than recombination among five cosmids, since with pseudorabies virus, four cosmids containing the entire genome were more efficient than five cosmids in reconstituting the genome after transfection into cells permissive for pseudorabies virus replication (49). The three cosmids would have a lytic and a latent infection DNA replication origin, the genes for the essential EBNAs and LMPs, and the *cis*-acting packaging signal (Fig. 1) (reviewed in references 9 and 24).

Sixteen independent experiments were done with two different clones of the ESN cosmid. The number of transforming recombinants recovered with the ESN deletion ranged from 5 to 33% of the total number of infected LCLs that were analyzed, with an average of approximately 22% (213 of 981) in the 16 experiments (Table 1). This is more than twice the frequency (10%) observed for incorporation for the much smaller B95-8 deletion in previous experiments done with transfection of five overlapping cosmid-cloned EBV DNAs. Most of these, 208 of 213, probably resulted from recombination among the three transfected cosmids since they also contained T1 EBNA 3A DNA (Table 1). This frequency of recombination among the transfected cosmid DNA is also more than twice that obtained in five previous cosmid transfection experiments (48). Thus, the increased efficiency for recombination among the three transfected cosmids appears to outweigh any putative difficulty of packaging a smaller recombinant genome. Overall, 42% (89 of 213) of these recombinants were in LCLs that had only deleted genomes and no parental P3HR-1 genomes. Since this is the coinfection frequency expected for WT recombinants (43–48), there is little or no selection in the deletion-infected cells for P3HR-1 coinfection. These results demonstrate that a transforming EBV genome can be reconstructed from three overlapping cosmids and that the BARF0 transcript and protein are not essential for lymphocyte transformation. The data are summarized in Table 1 and will now be described in more detail.

The transformed LCLs were initially analyzed by PCR, using primers specific for amplification across the *Not*I site which joins EBV genome site 117,609 to 163,415 in the ESN cosmid and in those LCLs infected with recombinant virus which had incorporated the ESN deletion. No fragment was amplified from P3HR-1 cells with this primer pair, and a 261-bp DNA fragment was amplified from the ESN cosmid and from 16 representative LCLs which were infected only with recombinants which had incorporated the ESN deletion (Fig. 2A). All 16 LCLs lacked P3HR-1 DNA, as demonstrated by amplification of a 257-bp fragment from P3HR-1 cells but not from the LCLs, using primers which flank the sites at which EBNALP- and EBNA2-encoding DNA are deleted from P3HR-1 (Fig. 1, shaded area, and Fig. 2B).

Using primers (EBV bp 117,520 to 117,543 and bp 117,677 to 117,653) specific for WT DNA within the 58-kbp deletion

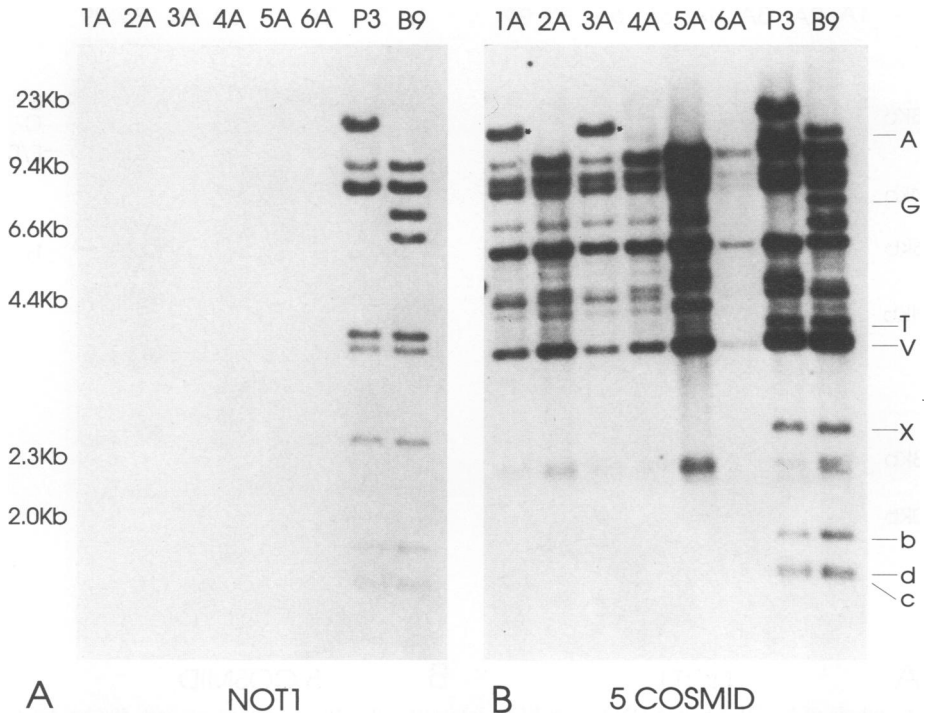

FIG. 5. Southern blot analysis of *Bam*HI-digested DNA from LCLs infected with EBV recombinants with 58 kbp deleted or from cells infected with the T2 P3HR-1 EBV or the T1 B95-8 EBV. Thirty micrograms of LCL DNA or 10 μg of B95-8 or P3HR-1 DNA was digested with *Bam*HI, and the fragments were separated on a 0.5% ME-agarose gel and transferred to a nylon membrane. The probes were [^{32}P]dCTP-labelled EBV DNA. (A) EBV DNA from bp 117,609 to 155,113 was labelled as the probe and is designated *Not*I. (B) All five cosmid clones of B95-8 EBV DNA were labelled as a probe. Blots were first used in panel A and then stripped and rehybridized for panel B. Some of the *Bam*HI fragments deleted from the EBV recombinants (A) can be distinguished from the fragments characteristic of the rest of the genome using the all-cosmid probe (B) and are indicated on the right of panel B. The fragments in the B95-8 lane (B9) in panel A from bottom to top are *Bam*HI c, d, b, X, V, T, G, and A. The positions of lambda DNA *Hin*dIII fragments are shown on the left. The fragments indicated by asterisks in lanes 1A and 3A of panel B hybridize extensively to a terminal repeat probe and probably arise from episomes with expanded copies of the terminal repeat.

(bp 117,609 to 163,415; Fig. 1), a 157-bp DNA fragment was readily amplified from P3HR-1 and from B95-8 cell DNA but not from any of the 16 recombinant genomes or the ESN cosmid (Fig. 3B). This primer pair was then used as the basis for a sensitive PCR which would detect as little as one WT EBV genome in 10^4 cells. Five of the clones shown in Fig. 2 were analyzed in parallel and no WT DNA was detected, excluding any possibility that WT DNA could be contributing to the ability of the EBV genome in these cells to maintain latent infection or cell proliferation. Of the 213 clones which were infected with an EBV recombinant which had the ESN deletion, 89 of 213 or 42% had no detectable WT DNA at this site. Since this is similar to the frequency of P3HR-1 coinfection of WT recombinants in similar experiments (44–49), there is no apparent selection for P3HR-1 coinfection which could provide WT BARF0.

Each of the 981 LCLs was also analyzed by PCR with primers which amplify a 276-bp fragment from the T1 EBNA 3A gene and a 237-bp fragment from the T2 EBNA 3A gene (39). The representative LCLs, which have only the ESN deletion DNA as shown in Fig. 2, are each infected with EBV with only T1 EBNA 3A DNA, as demonstrated by amplification of only the 276-bp fragment. In contrast, a 237-bp fragment did amplify from the P3HR-1 T2 EBNA 3A gene (Fig. 3A). This result indicates that these EBV genomes contain T1 *Sal*I E/C DNA from the transfected cosmid and do not contain T2 P3HR-1 EBNA 3A DNA, as would be expected if they had resulted from recombination with the endogenous P3HR-1 genome. Since these EBV genomes must contain the EBNA 2 gene from the transfected *Eco*RI A cosmid and have been demonstrated to contain the ESN deletion and no WT DNA from that site, the data strongly support the working model that these genomes arose from recombination among the three cosmids.

Overall, of the 981 LCLs, 242 or 26%, were infected with an EBV genome which had incorporated the T1 *Sal*I E/C cosmid, as determined by PCR analysis for T1 EBNA 3A DNA. Of these, 208, or more than 85%, also had the ESN deletion and therefore likely arose from recombination among the three cosmids. The remaining 34 of the 242, or about 15%, are likely the result of recombination of *Eco*RI A and T1 *Sal*I E/C with the P3HR-1 genome. The overall frequency of incorporation of T1 EBNA 3A without the ESN deletion is 34 of 981, or approximately 3%. The frequency of incorporation obtained by second-site homologous recombination with *Eco*RI A and the *Sal*I-digested *Sal*I EC (so as to eliminate a possible overlap effect and interaction with *Eco*RI A) cosmids in cotransfection was about 10%, and a 16% frequency was obtained in the five cosmid transfections (48). Thus, overall, the efficiency of T1 EBNA 3A incorporation was similar to that obtained in the five cosmid transfection experiments, but 85% of the genomes which incorporated T1 EBNA 3A probably arose from recombination among the three transfected cosmids, whereas in the five cosmid transfections, only 40% of the genomes which had incorporated T1 EBNA 3A were likely the result of recombination among the five transfected cosmids (48).

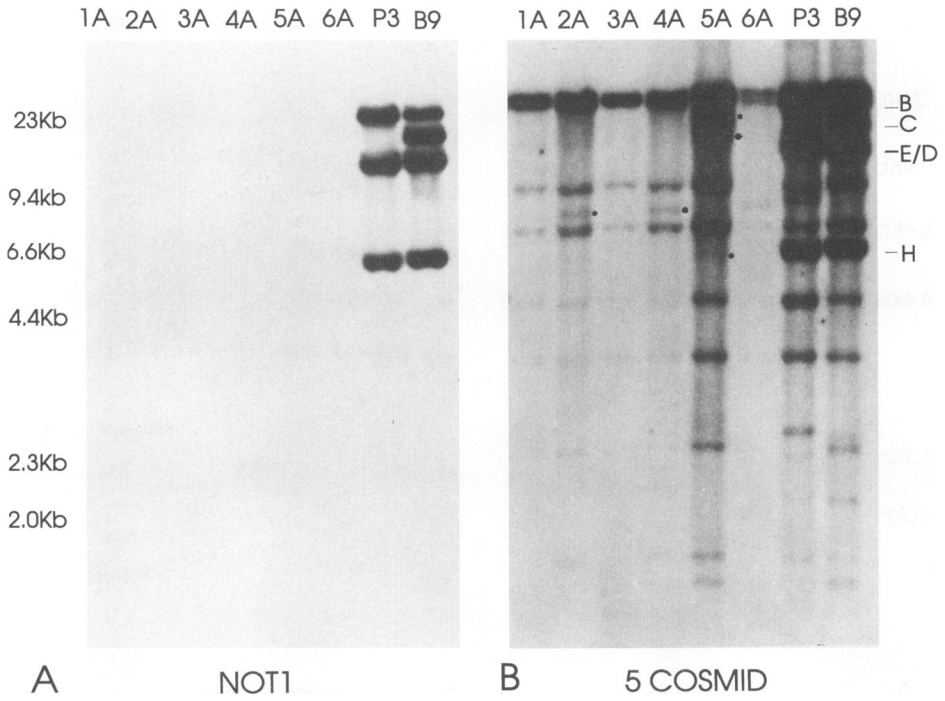

FIG. 6. Southern blot analysis of *Eco*RI-digested DNA from LCLs infected with EBV recombinants with 58 kbp deleted. The deletion results in a fusion between the *Eco*RI B and *Eco*RI D to IJ het fragments, which is >21 kb and migrates close to the *Eco*RI A fragment. The fragments identified by the probe in panel A from the *Eco*RI digest from bottom to top in the B95-8 lane (B9) are H, E, C, and B. The closed circles in panel B indicate anomalous fragments which hybridize to the *Eco*RI A probe. The asterisk indicates a fragment smaller than most of the other *Eco*RI B/D to IJ het fusion fragments, which hybridizes to the terminal repeat probe. The remainder of the description is contained in the legend to Fig. 5.

Southern blot analyses of the EBV genome in LCLs infected with recombinants that lack 58 kbp of EBV DNA by PCR analysis. The structures of the EBV genomes in LCLs which were infected with ESN recombinants were further evaluated by in situ lysis and restriction endonuclease analyses. By in situ lysis, the EBV genomes in most LCLs were similar in size to those of B95-8 or Akata cell episomes (Fig. 4, lanes 1A, 3A, 5A, and 6A). Other LCLs had episomes which were significantly smaller (Fig. 4, lanes 2A and 4A). These results are compatible with a model in which the ESN-deleted EBV genomes can be packaged or can exist in cells as 126-kbp monomers (B95-8 is 172 kbp, and the ESN deletion removes an additional 46 kbp) or as larger episomes, possibly dimers. Linear genomes indicative of progeny EBV DNA are detected in the faster-migrating fraction of the P3HR-1 and B95-8 cell lines but were not detected in the in situ lysis of the ESN EBV deletion recombinant-infected LCLs.

Southern blots of *Bam*HI-, *Eco*RI-, or *Sal*I-digested LCL DNAs were compared with digests of P3HR-1 and B95-8 DNA, using a probe consisting of labelled B95-8 DNA corresponding to bp 117,609 to 155,113, which had been deleted in the construction of the ESN cosmid, a probe from each of the cosmid-cloned EBV DNAs individually, or a probe consisting of all five overlapping cosmids together. The results of blots probed with the B95-8 DNA from bp 117,609 to 155,113 (Fig. 5A, 6A, and 7A) or with all five cosmids are that the bp 117,609 to 155,113 probe identified the expected B95-8 and P3HR-1 *Bam*HI (Fig. 5A), *Eco*RI (Fig. 6A), and *Sal*I (Fig. 7A) EBV DNA fragments and did not hybridize to any fragment of EBV DNA from the ESN recombinant-infected LCLs. These results qualitatively confirm the absence of WT DNA from the ESN deletion EBV recombinant-infected cells as was shown above with a quantitatively more sensitive PCR. These results also significantly extend the PCR results by demonstrating the absence of all of the putatively deleted 58 kbp of DNA. The readily distinguishable fragments which are affected by the ESN deletion are indicated by their letter designations to the right of the same blots probed with the EBV DNA from the five overlapping cosmid EBV DNAs (Fig. 5B, 6B, and 7B). Most of the rest of the fragments of the ESN recombinant DNAs were readily identifiable by their correspondence in size to fragments of the B95-8 genome and were demonstrated to be the expected fragment by their hybridization to individual EBV DNA probes made from each of the cosmids (data not shown). As expected, some fragments did not correspond to fragments of the B95-8 genome and were the result of fusion of two fragments as a result of the ESN deletion. Other fragments which differed from the expected B95-8 EBV DNA fragments were the result of amplification or contraction of an internal or terminal repeat in the ESN deletion recombinant EBV DNA. An example of an amplification of the terminal repeat DNA is the larger than expected *Bam*HI and *Sal*I fragments of EBV DNA in the 1A and 3A LCLs (Fig. 5B and 7B, asterisks). An example of a contraction in repeat number is the smaller than expected *Eco*RI A fragments in the 2A and 4A EBV DNAs (Fig. 6B, dots). The new fragments created by the ESN deletion are a new *Bam*HI fragment, approximately the same size as *Bam*HI E and therefore difficult to distinguish in the blot shown in Fig. 5B; a new *Eco*RI fragment not well resolved from *Eco*RI A (Fig. 6B); and a new *Sal*I fragment which includes the terminal repeat and therefore varies in size and is evident at approximately 9.5 kbp (Fig. 7B). All fragments in the blots probed with a single EBV DNA cosmid clone corresponded to the fragments expected, except for the frag-

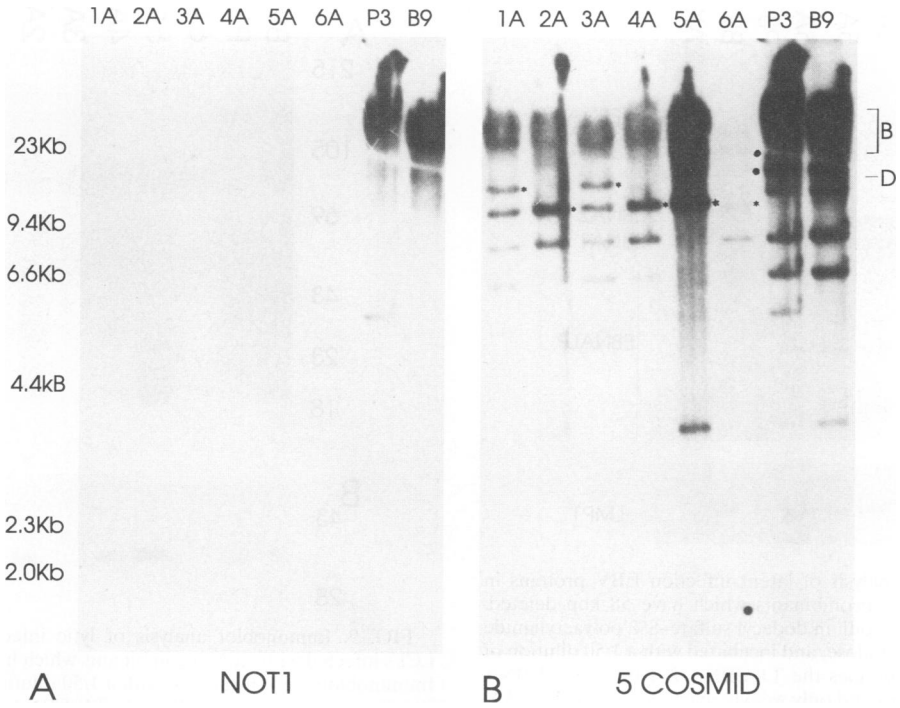

FIG. 7. Southern blot analysis of *Sal*I-digested DNA from LCLs infected with EBV recombinants with 58 kbp deleted. The asterisks in panel B indicate a new fragment in the deletion recombinant-infected cells, which consists of part of *Sal*I B fused to part of *Sal*I D. This fused fragment hybridizes to a terminal repeat probe and varies in size as a result of differences in numbers of terminal repeats. Note that LCLs 1A and 3A have a larger terminal repeat fusion fragment, as shown in Fig. 5. For LCLs 2A and 4A, the terminal repeat fusion fragment is almost as small as the *Sal*I E fragment. The closed circles in panel B indicate anomalous fragments which hybridize to a *Bam*HI W probe. The remainder of the description is contained in the legend to Fig. 5.

ments that were produced by the terminal repeats and the large IR1 repeats which appear to undergo contractions and expansions within the recombinant genomes.

Expression of EBNAs, LMP1, and lytic infection-associated proteins in LCLs infected with EBV recombinants which have only the ESN deletion and T1 EBNA 3A DNA. Since the deletion of 58 kbp of DNA could have an effect on the regulation of EBNA, LMP1, or lytic EBV gene expression as a consequence of removal of BARF0 or a putative *cis*-acting site, immunoblots were done with antisera which could detect these proteins. Since these LCLs were infected with EBV recombinants which likely arose from the transfected T1 EBV cosmid DNAs, expression of T1 EBNAs was expected and the EBNA immunoblots were reacted with human serum specific for T1 as opposed to T2 EBNA 2, EBNA 3A, EBNA 3B, and EBNA 3C. Each of the cell lines had the B95-8-size EBNA 1 and not the P3HR-1-size EBNA 1 (Fig. 8A). Further, each cell line had the T1 EBNA 2, EBNA 3A, EBNA 3B, and EBNA 3C (Fig. 8A). Moreover, the level of expression of EBNA LP, EBNA 2, EBNA 1, EBNA 3A, EBNA 3B, and EBNA 3C (Fig. 8A) was similar to that in B95-8 cells. The size and level of LMP1 expression was also similar to that in B95-8 cells (Fig. 8B). The LCLs varied widely in their level of spontaneous Z expression, as is characteristic of recently established LCLs, with some expressing levels as high as those in B95-8 and P3HR-1 cells (Fig. 9). The size of the Z protein was similar to that of B95-8 and slightly larger than that of P3HR-1 (Fig. 9B). The abundance of the 45- to 55-kDa BMRF1 and BMLF1 early lytic infection proteins correlated with the abundance of Z (Fig. 9A). The level of Z and of early lytic infection proteins expressed in six LCLs infected with ESN recombinant EBV alone was compared with that in six LCLs infected with WT recombinants derived in parallel. A range of Z and early lytic protein expression was observed for both types of infected LCLs without a significant difference between the two groups (data not shown). There was however no 135-kDa BALF2 protein expressed in the LCLs infected with the ESN deletion EBV recombinants (Fig. 9A). This is expected since the BALF2 ORF extends leftward to bp 161,387 and the ESN deletion extends rightward to bp 163,415 (for reviews, see references 9 and 24). Thus, these data confirm the origins of the BZLF1 and EBNA 1, EBNA 2, EBNA 3A, EBNA 3B, and EBNA 3C genes from the transfected T1 B95-8-derived cosmid EBV DNAs and indicate that latent and spontaneous lytic EBV gene expression is similar to that observed in other LCLs. Late lytic cycle EBV gene expression was not assayed, since BALF2 is essential for EBV DNA replication and the deletion of BALF2 would be expected to result in no late gene expression.

DISCUSSION

These experiments demonstrate that transformation-competent EBV recombinants can be constructed by transfecting three overlapping cosmid-cloned EBV DNA fragments into a BL cell line which can be permissive for EBV replication and is already infected with an EBV strain which is replication competent and transformation incompetent. In this background, replication of the endogenous EBV genome supports recombination of the endogenous genome with the transfected DNAs or recombination among the transfected DNAs, resulting in transformation-competent EBV genomes. This is an

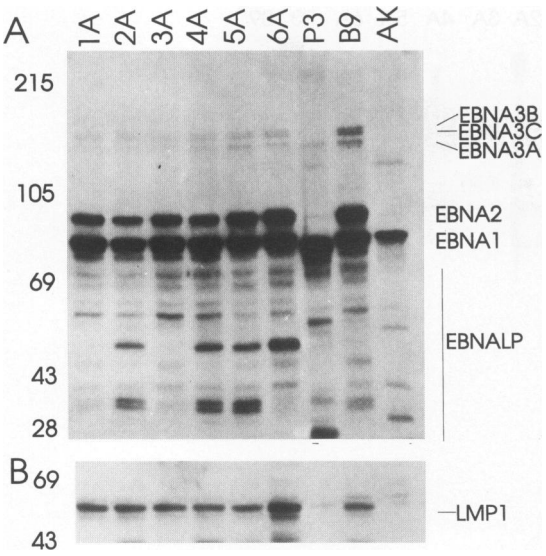

FIG. 8. Immunoblot analysis of latent infection EBV proteins in LCLs infected with EBV recombinants which have 58 kbp deleted. Proteins were analyzed on sodium dodecyl sulfate–8% polyacrylamide gels, transferred to nitrocellulose, and incubated with a 1/50 dilution of a human serum which identifies the T1 EBNA 2, EBNA 3A, EBNA 3B, and EBNA 3C proteins and only weakly reacts with T2 EBNA 3A, EBNA 3B, or EBNA 3C. Lanes P3, B9, and AK contain proteins from control cell lines T2 P3HR-1, T1 B95-8, and the Akata BL cell line which expresses predominantly EBNA 1, respectively. All the recombinant EBV-infected LCLs shown express T1 EBNAs. (B) Immunoblot incubated with the S12 monoclonal antibody to LMP1. The size markers (in kilodaltons) used as standards are prestained and shown on the left.

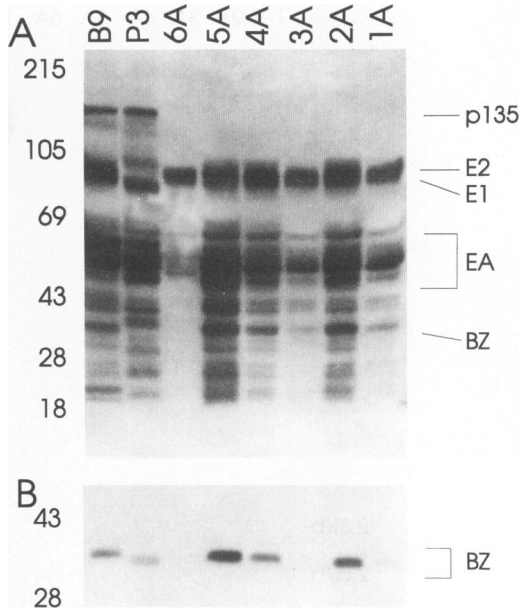

FIG. 9. Immunoblot analysis of lytic infection EBV proteins in LCLs infected with EBV recombinants which have 58 kbp deleted. (A) Immunoblots were incubated with a 1/50 dilution of a human immune serum which identifies EBNA 1, T1 EBNA 2, the 45- to 55-kDa BMRF1 and BMLF1 EA-D complex, the early lytic 135-kDa BALF2 single-stranded DNA binding protein, and the BZLF1 immediate-early protein (for a review, see reference 24). (B) The immunoblot was incubated with BZLF1 monoclonal antibody bz-1.

efficient system for construction of specifically mutated EBV recombinants. The methodology is particularly useful for the construction of recombinants with specific mutations in genes associated with latency and growth transformation and is most useful for the construction of large deletion mutations which will allow definition of the minimal transforming EBV genome. EBV genomes resulting from recombination among the transfected cosmid DNAs were obtained in each of 16 consecutive experiments. These genomes were packaged into virions despite their expected 120-kb size, which is considerably smaller than the 170- to 180-kb WT EBV genome. Some of these recombinants were probably packaged as dimers of the EBV genome, since the episomes in the infected LCLs appear to be as large as WT EBV genomes.

Many of the LCLs produced in these experiments were coinfected with parental P3HR-1 and remain stably coinfected with P3HR-1 over months in culture. The fact that some of these LCLs are coinfected with P3HR-1 is potentially useful. P3HR-1 coinfection enables the recovery of LCLs infected with EBV recombinants with specific null mutations in any essential transforming gene outside of EBNA LP or EBNA 2 (20, 43–48). The relative frequency for obtaining any specific mutation in LCLs with or without P3HR-1 coinfection and the overall frequency of incorporation of EBNA LP and EBNA 2 into the P3HR-1 genome provide internal controls for each experiment. The frequency with which the primary B lymphocytes are coinfected with parental P3HR-1 virus can also be altered by using less of the virus preparation from the transfected P3HR-1 cells for infection of the same number of primary B lymphocytes or by increasing the number of primary B lymphocytes. The recombinant genomes carrying null mutations in essential genes can be characterized in the coinfected LCLs. Further, since the P3HR-1 EBV is replication competent, coinfection allows for induction of lytic replication in the coinfected LCLs and assay of the biologic activity of each of the coinfecting genomes and of secondary recombinants between the coinfecting genomes (20, 43–48).

An obvious deficiency of the system is that recombinants carrying null mutations in essential latency, transforming, or replicative genes cannot be obtained or reactivated to lytic infection and the progeny passaged in primary B lymphocytes without coinfection with the P3HR-1 EBV genome. The ESN recombinants have deletions of an EBV DNA segment from bp 117,609 to 163,415. This segment includes the BARF0 gene and a large number of genes which are normally expressed immediate early, early, or late in lytic EBV infection (for reviews, see references 9 and 24). These include the BALF2 gene, which encodes the single-stranded DNA binding protein; the BALF3 gene, which may encode a glycoprotein transport protein; BALF4, which encodes gp110; BALF5, which encodes the EBV DNA polymerase; BI'LF4, which encodes an immediate-early transactivator; BILF2, which encodes a glycoprotein; BGLF5, which encodes an alkaline DNase; BGLF4, which encodes a protein kinase; and BXLF2, which encodes gp85. Thus, lytic EBV replication is aborted in the LCLs infected with the ESN recombinants alone.

One objective of these experiments was to investigate the role of the BARF0 RNA in latent infection and cell growth transformation. This family of rightward RNAs which appear to initiate at EBV genome coordinate 150,640 (42) was first identified in NPC tumor biopsies and in NPC tumor cells passaged in nude mice (3, 4, 11, 12, 19, 42). NPC is a common

malignancy in some human populations, and almost all anaplastic NPCs are latently infected with EBV. The BARF0 RNAs, EBNA 1 RNA, and LMP1 and LMP2 RNAs are commonly detected in NPC tumors and have also been found in some latently infected B lymphocytes (3, 4, 19, 42). Although our data demonstrate that the BARF0 RNA is not critical for latent EBV infection of B-lymphocytes or cell growth transformation in vitro, BARF0-encoded protein is expressed in vivo (12) and may be important for epithelial or lymphocyte infection in vivo. The BARF0 RNA is also antisense to an immediate-early and several early lytic replication-associated genes and could serve as a "last stopper" for the transition to lytic infection (19, 42). The deletion recombinants that are described in this report cannot encode the sense as well as the antisense transcripts through this region and therefore do not directly test whether the BARF0 RNA counters a putative effect of the opposite-strand transcripts in enhancing the transition to lytic infection. However, the major immediate-early transactivators of lytic EBV infection, Z and R, are not expected to be affected by the deletion or by the BARF0 RNAs (for a review, see reference 24), and Z expression was similar to that in WT LCLs.

Further experiments are in progress with the goal of deleting other parts of the EBV genome to more precisely define the minimal transforming genome. It is likely that the ESN deletion can be extended to the EBNA 1 and LMP1 RNA termination sites and that much of the DNA between the terminal repeat and the long internal repeat can be deleted. In fact, the EBNA 3B (46), LMP2 (25–26), EBER (44), BHRF1 (23, 31), and BCRF1 genes (43) and the *Bam*C latency promoter (43) are not critical for lymphocyte infection or cell growth transformation in vitro. Deletion of most of the rest of the genome could have an adverse effect on processing of the EBNA mRNAs which are essential for episome maintenance or cell growth transformation.

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