JOURNAL OF VIROLOGY, Feb. 1992, p. 780–789
0022-538X/92/020780-10$02.00/0

Vol. 66, No. 2

Second-Site Homologous Recombination in Epstein-Barr Virus: Insertion of Type 1 EBNA 3 Genes in Place of Type 2 Has No Effect on In Vitro Infection

BLAKE TOMKINSON* AND ELLIOTT KIEFF

Departments of Microbiology and Molecular Genetics and of Medicine, Harvard University, 75 Francis Street, Boston, Massachusetts 02115

Received 23 August 1991/Accepted 17 October 1991

This study was undertaken to develop a general strategy for the introduction of mutations into specific sites in the Epstein-Barr virus (EBV) genome. Previous approaches were limited by the need for physical linkage of the transfected EBV DNA fragment to a positive selection marker. In our experiments, a positive selection marker was introduced into one site in the EBV genome and a distant, nonlinked, marker was introduced into another site. Each marker was on a large EBV DNA fragment and was inserted into the genome by transfection into cells carrying a resident EBV genome. The resident EBV genome was simultaneously induced to replicate by using a cotransfected expression plasmid for the EBV immediate-early transactivator, Z (J. Countryman, H. Jenson, R. Seibl, H. Wolf, and G. Miller, J. Virol. 61:3672–3679, 1987; G. Miller, M. Rabson, and L. Heston, J. Virol. 50:174–182, 1984). Eleven percent of the resultant EBV genomes which incorporated the positive selection marker also incorporated the nonlinked marker. Both markers uniformly targeted the homologous EBV genome site. In this way novel EBV recombinants were constructed in which the EBV type 1 EBNA 3A, EBV type 1 EBNA 3A and 3B, or EBV type 1 EBNA 3A, 3B, and 3C genes were introduced into a largely type 2 EBV genome, replacing the corresponding type 2 gene(s). No difference was observed in primary B-lymphocyte growth transformation, in latent EBV gene expression, or in spontaneous lytic EBV gene expression. These new recombinants should be useful for ongoing analyses of the type specificity of the immune response.

Molecular genetic studies of the Epstein-Barr virus (EBV) genome have been limited by the in vitro host range restriction to primate B lymphocytes and the poor permissivity of B lymphocytes for virus replication. Most molecular genetic approaches used for other herpesviruses require large quantities of virus or of intact virus DNA as well as in vitro replication of progeny virus so that recombinants can be selected, identified, or cloned. Two approaches have recently been used to generate recombinant EBVs. Both approaches utilize and extend the principle of high-frequency homologous recombination between replicating herpesvirus DNA and homologous transfected DNA (28). The first strategy uses a transformation-defective (26), deleted (13) virus genome, resident in the P3HR-1 Burkitt's lymphoma cell line (12, 26a), as one parent and a transfected recombinant EBV DNA fragment which spans the deletion as the other parent (6, 7, 11). When virus replication is induced in these transfected cells, large quantities of parental P3HR-1 EBV are produced along with up to several hundred homologously recombined EBV genomes (6). The recombinant genomes are usually restored for the deleted DNA and are therefore uniquely able to cause growth transformation or immortalization of primary B lymphocytes (6, 7, 11, 31). Clones of transformed B lymphocytes carrying a single recombinant EBV genome can thereby be recovered (6, 7, 11, 17, 18, 31, 32), characterized (6, 7, 11, 17, 18, 32), and in most instances passaged to other primary B lymphocytes following induction of virus replication (17, 18, 32). This strategy has been developed and exploited to create recombinant EBVs with mutations in or around the EBV nuclear antigen 2 (EBNA 2) or EBNA LP genes, which are critical to infected B-lymphocyte outgrowth and are deleted from P3HR-1 virus (6, 7, 11, 17). The strategy has also been used to introduce mutations into adjacent EBV genes, exploiting restoration of growth transformation as a linked positive selection marker (18, 32). The approach is limited to genes which can be physically linked to the deleted DNA segment which is essential to the restoration of the ability of P3HR-1 to transform primary B lymphocytes. The second strategy uses a cell line harboring a nondefective EBV genome and transfection of a recombinant EBV DNA carrying a linked positive selection marker (37). This latter strategy would be applicable to any EBV gene as long as the neighborhood of the gene was compatible with a functioning positive selection marker. In fact, mutations could, in theory, be made in essential transforming genes since the recombinant EBV genomes could be selected, characterized, and recovered from non-EBV-infected B-lymphoma cells (37). A weakness of this latter strategy is that the inserted positive selection marker not only must be functional in the neighborhood of the EBV gene, but also must not affect EBV infection.

We now demonstrate that an EBV genome which has recombined with an EBV DNA fragment containing a positive selection marker will also frequently recombine with a second, nonlinked cotransfected EBV DNA fragment. Restoration of the P3HR-1 EBV-transforming ability was used as the nonlinked positive selection marker. The rate of cotransfer of a cotransfected, nonlinked second marker was investigated, and the resulting recombinant EBVs were characterized. The surprisingly high frequency with which second-site homologous recombination occurs remarkably

* Corresponding author.

extends the ease with which mutations can be made in the EBV genome.

The nonlinked second fragment which was investigated contains the EBNA 3A, 3B, and 3C genes. This fragment was chosen because EBNA 3A, 3B, and 3C and EBNA 2 are the genes which differ most extensively between the two naturally occurring and biologically different EBV types (1, 9, 29). Type 1 EBV strains transform primary B lymphocytes with a higher efficiency than type 2 strains, and the type 1-transformed lymphoblastoid cell lines (LCLs) are more able to sustain their growth at low cell concentrations (7, 27). Otherwise isogeneic recombinant EBVs carrying type 1 EBNA 2 in a type 2 genome are similar in their effects on cell growth to type 1 EBVs, indicating that EBNA 2 is the dominant determinant of the type-specific growth effects (7). Although EBNA 2 is the dominant determinant of type-specific growth differences, the contribution of EBNA 3A, 3B, or 3C to the type-specific growth properties could not be previously determined. Isogeneic recombinants carrying type 1 or 2 EBNA 3A, 3B, or 3C would also be useful for dissecting the relative importance of type-specific differences in T-cell immune responses in EBV-infected people. Recent data indicate that EBNA 3A, 3C, and 2 contain important T-cell epitopes (4, 5, 23) and that the epitopes are frequently type specific (5, 23). These analyses would be facilitated by having identical cells infected with isogenic EBVs differing only in their EBNA 2 or EBNA 3 genes.

MATERIALS AND METHODS

Plasmid and cosmid construction. Plasmid pSVNaeI Z and the *Eco*RI A cosmid have been described previously (18, 32). The *Sal*I E and C fragments of the B95-8 EBV type 1 DNA were cloned from a partial *Sal*I digest into pDVcosA2 (14) by using an in vitro packaging extract and *Escherichia coli* PLK-A (Stratagene Corp.). pDVSalI-E/C (pDVSECWT) contains 42 kb of EBV DNA from positions 63201 to 105296 (3).

Cells and cell culture. The HH514-16 subclone of P3HR-1 (26a) (a gift from George Miller, Yale University) contains a type 2 EBV genome deleted for the EBNA 2 gene and part of the EBNA LP gene (13, 26), rendering it nontransforming (26). BJA-B is an EBV-negative B-lymphoma cell line (20). Louckes is an EBV-negative Burkitt's lymphoma cell line (35). Human mononuclear cells used for establishment of normal and mutant LCLs were obtained from normal seronegative and seropositive donors. Enriched B-cell populations were obtained by depleting T cells with *S*-(2-aminoethyl)isothiuronium bromide (Sigma)-treated sheep erythrocytes as described previously (18). All cultures were maintained in RPMI 1640 medium supplemented with 10% inactivated fetal bovine serum, glutamine, and 10 μg of gentamicin per ml.

Cosmid transfection. P3HR-1 c16 cells were transfected with EBV cosmid cloned DNA after release of the EBV DNA from vector DNA; pDVSECWT was digested with *Sal*I to liberate *Sal*I E and C DNA fragments. pSVNaeI *Bam*HI-Z was cotransfected to induce lytic replication (8, 21). P3HR-1 c16 cells (10^7 cells), harvested during log-phase growth, were resuspended in 400 μl of complete medium containing 25 mg of pSVNaeI Z plasmid or 25 mg of pSVNaeI Z plasmid plus 10 μg of *Eco*RI-A or 25 μg of pSVNaeI Z plus 10 μg of *Eco*RI-A plus 50 μg of *Sal*I-E/C. Resuspended cells were transferred to an electroporation cuvette (0.4-cm gap; Bio-Rad) and, following a 10-min incubation at 25°C, pulsed with 200 V at 960-μF capacitance. Cells were immediately diluted into 20 ml of complete medium supplemented with 10 μg of gentamicin per ml. Lytic EBV replication in LCLs was activated for virus passage by transfection with 25 μg of pSVNaeI Z.

Primary and secondary infections. Primary human B lymphocytes were infected with 0.45-μM filtered supernatants from transfected P3HR-1 c16 cells obtained 3 days after transfection. Intracellular virus was released into the medium by three cycles of freezing and thawing. The supernatant was filtered and used directly or first concentrated by centrifugation at 8,800 × *g* for 2 h. Virus was incubated with T-cell-depleted human mononuclear cells for 2 h at 37°C. Infected cells were resuspended in complete medium plus gentamicin to a concentration of 3.3×10^5 cells per ml, and 150 μl (5×10^4 cells) was distributed into wells of a 96-microwell plate. The cultures were fed at 14 days postplating with 100 μl of medium. LCLs were macroscopically visible 3 to 5 weeks after plating. LCL recombinant virus was passaged by cocultivation with human T-cell-depleted mononuclear cells. Three days after pSVNaeI Z transfection, 5×10^4 irradiated (8,800 rads) LCLs were cocultivated with 5×10^4 T-cell-depleted mononuclear cells in 150 μl of complete medium in a 96-microwell plate. Cultures were fed at 14 days with 100 μl of medium. Transformed-cell outgrowth was visible 3 to 5 weeks after plating. As controls, 5×10^4 irradiated LCLs were plated without B lymphocytes.

PCR analysis. (i) Primers. Oligonucleotide primers which distinguish among type 1 EBNA 3A (T1EBNA 3A), EBNA 3B (T1EBNA 3B), and type 2 EBNA 3A (T2EBNA 3A), EBNA 3B (T2EBNA 3B) have been described previously (29). A set of EBNA 3C (E3C-3′) primers was synthesized to a 3′ region in EBNA 3C which would amplify a distinct fragment for type 1 (158 bp) and type 2 (179 bp) EBV DNA as a result of a 21-bp deletion in type 1 EBNA 3C DNA (29). E3C-3′-PF primer (CACATCTGCAATCGGAGACA) corresponds to nucleotides 100996 to 101015, and E3C-3′-PR primer (GCTATCTTGTAACGGCATAG) corresponds to nucleotides 101134 to 101153 (see Fig. 1 for location of polymerase chain reaction [PCR] fragments) (3).

(ii) Reaction. Genomic DNA was prepared from approximately 5×10^4 to 1×10^5 cells. Following centrifugation to remove medium, cells were resuspended in 0.2× phosphate-buffered saline, boiled for 10 min, and mixed with 0.1 volume of 10-mg/ml proteinase K (Sigma), and the mixture was incubated for 30 min at 55°C. Proteinase K was inactivated by incubation at 95°C for 20 min.

PCR analysis was performed by using Perkin-Elmer thermal cycler with 10 to 20 μl of DNA in a 50-μl reaction. PCR-amplified DNA was analyzed by electrophoresis with 1% SeaKem ME–2% NuSieve agarose (FMC Corp.) gels and visualized by staining with ethidium bromide.

Immunoblotting and Southern hybridization. Latent or lytic EBV protein expression was analyzed by immunoblot following electrophoresis in 7% polyacrylamide gels with EBV immune human serum of previously characterized specificities (2, 25, 32). LMP 1 was detected with the monoclonal antibody S12 (16).

For Southern blot hybridization, 15 μg of cell DNA was digested with *Bcl*I restriction enzyme in a 200-μl volume (15). Following complete digestion, DNA was precipitated and resuspended in 40 μl of loading buffer. The DNA was size fractionated by electrophoresis in a 0.4% agarose gel and transferred to an activated nylon membrane (GeneScreen Plus; New England Nuclear). The filter was hybridized with a ^{32}P-labeled EBNA 3A cDNA probe. Southern blot hybridization with *Bam*HI was done similarly, except

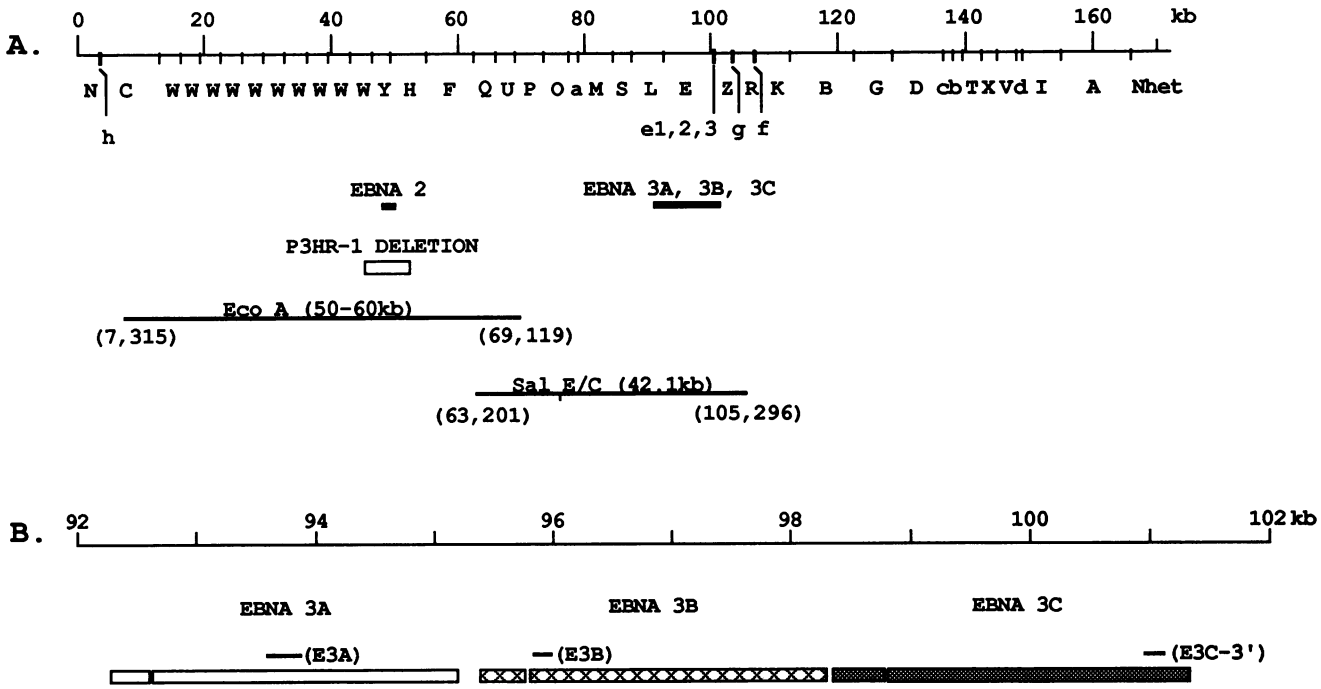

FIG. 1. (A) Schematic representation of the EBV genome with the location of EBNA 2 and the EBNA 3 genes and P3HR-1 deletion indicated. Also shown are the cloned EBV DNA fragments which were used to construct the pDVEcoRI A and pDVSEC cosmids. (B) Schematic representation of the *Bam*HI-E region indicating the exact location of the EBNA 3A, 3B, and 3C genes and the EBNA 3 PCR fragments.

that fragments were separated on a 0.8% agarose gel and hybridized with a ^{32}P-labeled *Bam*HI-H DNA probe.

In situ lysing-gel analysis (10) was performed by loading 2 $\times$ 10^6 cells into an in situ lysing-gel well containing agarose with 1% sodium dodecyl sulfate and proteinase K as previously described (37). In situ cell lysis was accomplished by running the gel at 0.5 V/cm (15 V) for 4 h, and then the episomal and linear EBV DNA was fractionated by increasing the voltage to 3.3 V/cm (100 V) for 18 h. The DNA was transferred to an activated-nylon membrane and probed with a ^{32}P-labeled EBV *Sal*I C DNA fragment.

RESULTS

Frequency of second-site recombination. To generate second-site recombinants containing the type 1 EBNA 3 genes, P3HR-1 cells were cotransfected with the EBV DNA *Eco*RI A fragment which spans the P3HR-1 deletion (Fig. 1), with the type 1 EBV DNA *Sal*I C fragment which contains the exons encoding EBNA 3A, 3B, and 3C (Fig. 1), and with the pSVNaeI Z expression plasmid to induce replication of the endogenous P3HR-1 genome. In these experiments, transfection with the Z expression plasmid resulted in EBV replication in approximately 1 to 10% of the P3HR-1 cells, as measured by expression of the major virion envelope glycoprotein, gp350 (data not shown). The resultant virus stocks from 10^7 transfected P3HR-1 cells consisting of approximately 10^8 (10^7 to 10^9) nontransforming parental P3HR-1 virus, based on a free-virus yield of 10^2 to 10^3 EBV per permissively infected cell (26a) and approximately 10^2 transforming P3HR-1 generated as a consequence of recombination with *Eco*RI-A (6), were used to infect 1.5 $\times$ 10^7 primary human B lymphocytes so that transforming recombinants could be isolated by their ability to cause long-term LCL outgrowth (6, 7, 11). The primary B lymphocytes were plated into 300 wells so that an average well would have less than 1.0 transforming recombinant virus. At this multiplicity of infection, the resulting LCLs would probably be infected with a single transforming recombinant virus, although there was a high probability that the LCLs would be initially coinfected with parental, nonrecombinant P3HR-1 virus. The resulting LCLs were analyzed by PCR with primers which would detect whether the viral genomes in the transformed cells had also undergone recombination with the transfected type 1 EBV DNA *Sal*I C fragment and now included type 1 EBNA 3A, 3B, or 3C genes. Since the parental P3HR-1 genome is type 2 and the transfected type 1 *Sal*I C fragment is approximately 10% divergent in nucleotide sequence within the EBNA 3 genes, recombination at these loci is readily detected by type-specific PCR for each of the EBNA 3 genes (29).

The results were remarkably consistent among experiments in which approximately 89% of the transformed LCLs had only P3HR-1-derived type 2 EBNA 3 genes, whereas approximately 11% were recombinant and had type 1 EBNA 3A, 3B, or 3C (Table 1). Of the LCLs infected with virus carrying type 1 EBNA 3A, 3B, or 3C, approximately 70% were coinfected with virus carrying type 2 EBNA 3 genes (Table 1). Examples of the results showing evidence for infection with only a type 1 EBNA 3A, 3B, or 3C recombinant virus and not with a parental P3HR-1 virus are given in Fig. 2, lanes T1E3-14, T1E3-28, T1E3-33, T1E3-35, and T1E3-45. In each of these examples, either type 1 or type 2 but not both genes are detected at the EBNA 3A, 3B, or 3C loci. An example of a result showing evidence for coinfection with an EBNA 3 recombinant virus and a parental P3HR-1 virus is given in Fig. 2, lane T1E3-107. This latter

TABLE 1. Frequency of second-site recombination

Expt	No. of type 1 EBNA 3 recombinants (no. of P3HR-1 coinfected)/total no.		
	EBNA 3A	EBNA 3B	EBNA 3C
1	10 (7)/107	1 (1)/25	1 (1)/25
2	3 (1)/39	1/17	1/17
3	9 (6)/68	9 (6)/68[a]	3 (NT)[b]/68[a]
4	11 (10)/91	11 (10)/91[c]	1 (NT)/88[c]
Total	33 (24)/305	20 (16)/159	4 (NT)/156
Total (% T1E3 recombinants)	11	12	3
Total (% P3HR-1 coinfected)	72	80	NT

[a] All nine clones were type 1 for EBNA 3A and 3B, and three were also type 1 for EBNA 3C.
[b] NT, not tested.
[c] All 11 clones were type 1 for EBNA 3A and 3B, and 1 was also type 1 for EBNA 3C.

LCL is coinfected with a type 1 EBNA 3A and 3B recombinant virus and with nonrecombinant P3HR-1 virus and therefore has type 1 EBNA 3A and 3B and type 2 EBNA 3C genes on the recombinant genome and type 2 EBNA 3A, 3B, and 3C genes on the parental P3HR-1 genome. Approximately 11% of the LCL clones were infected with recombinant virus which had type 1 EBNA 3A or 3B, and almost all had both type 1 EBNA 3A and 3B (Table 1). Examples of LCLs which were infected with an EBV that was recombinant for type 1 EBNA 3A or 3B but not both are given in Fig. 2, lanes T1E3-14, T1E3-28, and T1E3-33. Examples of LCLs which were infected with an EBV that was recombinant for type 1 EBNA 3A and 3B are given in Fig. 2, lanes T1E3-26, T1E3-35, T1E3-45, T1E3-100, and T1E3-107. One clone had type 1 EBNA 3A, 3B, and 3C (Table 1; Fig. 2, lane T1E3-35), and none had EBNA 3C only. The EBNA 3 genotypes of the recombinants are summarized in Table 2. The high frequency of recombination at the EBNA 3A and 3B loci and the low frequency of recombination at the EBNA 3C locus may be attributed to the termination of the EBNA 3C open reading frame only 4 kb from the right end of the type 1 *Sal*I C fragment which was used in these transfections, whereas the EBNA 3A and 3B open reading frames are more centrally located within *Sal*I C (Fig. 1).

Stochastic and empirical evidence indicates that the presence of both type 1 and type 2 EBNA 3A, 3B, or 3C genes, as was observed in 72% of the EBNA 3 recombinant LCLs, is almost always the result of coinfection with both a parental, nonrecombinant P3HR-1 virus and a doubly recombinant virus which is transforming and carries the type 1 EBNA 3 gene(s). In previous experiments with mutations within *Eco*RI-A, parental, nonrecombinant P3HR-1 virus was frequently present in such vast excess over recombinant viruses that many or most primary B lymphocytes were initially infected with the parental P3HR-1 virus in addition to transforming *Eco*RI-A recombinants (18, 32). This result was expected since the recombinant virus stocks are in P3HR-1 virus-infected cells and approximately 1 to 10% of the cells are induced to lytic virus infection, releasing approximately 10^8 parental P3HR-1 viruses and approxi-

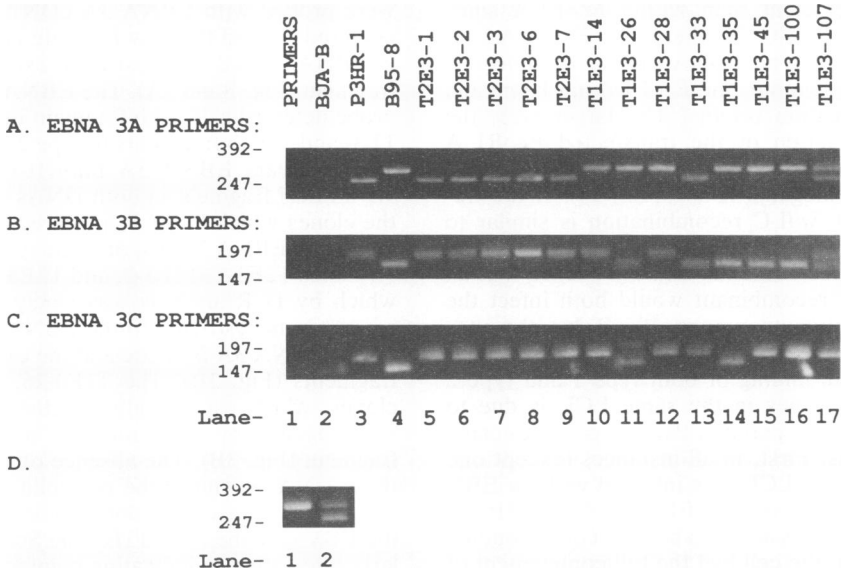

FIG. 2. Analysis of the EBNA 3 genotype of EBV-immortalized LCLs carrying wild-type type 2 (T2E3) and recombinant type 1 (T1E3) EBNA 3 genes by PCR. Panels A, B, and C illustrate the ethidium bromide staining of PCR-amplified fragments by using primers which distinguish between type 2 and type 1 EBNA 3A, 3B, and 3C genes, respectively. EBNA 3A primers amplify a 237-bp and a 276-bp fragment from type 2 and type 1 DNA, respectively (29). EBNA 3B primers amplify a 218-bp and a 183-bp fragment from type 2 and type 1 DNA, respectively (29). EBNA 3C-3′ primers amplify a 179-bp and a 158-bp fragment from type 2 and type 1 DNA, respectively. Lanes 1 and 2 are negative control amplifications with primers only and EBV-negative BJA-B cell DNA. Lanes 1 and 2 of EBNA 3C (panel C) show faint bands due to contamination with B95-8 DNA. Lanes 3 and 4 are positive control amplifications of P3HR-1 (type 2) and B95-8 (type 1) cell DNA. Lanes 5 to 9 are amplifications of LCL DNA transformed by viruses taken from P3HR-1 cells transfected with the *Eco*RI-A cosmid and pSZNaeI Z plasmid only and therefore should carry wild-type type 2 EBNA 3 genes (LCLs designated T2E3). Lanes 10 to 17 are amplifications of LCL DNA transformed by viruses taken from P3HR-1 cells transfected with the pSVNaeI Z plasmid, *Eco*RI-A cosmid, and *Sal*I-C cosmid and selected because of recombination in the EBNA 3 genes (LCLs designated T1E3). Panel D illustrates the ethidium bromide staining of EBNA 3A PCR fragment from DNA obtained from an early (lane 2) and a later (lane 1) passage of T1E3-100 demonstrating the loss of parental P3HR-1 virus over time. Size markers (kilobases) of *Rsa*I fragments of ϕX174 DNA are shown on the left.

TABLE 2. Genotypes and immunophenotypes of type 2 EBNA 3 recombinants

Clone	Genotype[a]	Immunophenotype[b]	Coinfection
T1E3-14	T1/T2/T2	T1/T2/T2	No
T1E3-26	T1/T1/T1-T2[c]	T1/T1/T1	No
T1E3-33	T2/T1/T2	T2/IND/T2[d]	No
T1E3-35	T1/T1/T1	T1/T1/T1	No
T1E3-45	T1/T1/T2	NT[e]	No
T1E3-100	T1/T1/T2	T1/T1/T2	Yes
T1E3-107	T1/T1/T2	NT	Yes
T2E3-1	T2/T2/T2	T2/T2/T2	NT
T2E3-2	T2/T2/T2	T2/T2/T2	NT
T2E3-3	T2/T2/T2	T2/T2/T2	NT
T2E3-6	T2/T2/T2	T2/T2/T2	NT
T2E3-7	T2/T2/T2	T2/T2/T2	NT

[a] Type for EBNA 3A/type for EBNA 3B/type for EBNA 3C.

[b] The type 1 immunophenotype was detected by a type 1-specific EBV immune serum. The type 2 immunophenotype was not directly tested, but was assumed to be type 2 since P3HR-1 virus is a type 2 EBV.

[c] T1-T2 indicates the presence of both type 1 and type 2 EBNA 3C owing to a nonhomologous recombination in EBNA 3C.

[d] IND, indeterminate. The EBNA 3B of T1E3-33 did not react with the type 1-specific serum, despite the PCR amplification of a type 1 EBNA 3B sequence. Type 2 EBNA 3B expression was not directly assayed. Therefore, T1E3-33 encodes a chimeric EBNA-3B with the type 1 sequence limited to a region surrounding the PCR primers which does not encode detectable type 1-specific epitopes; alternatively, the recombination has disrupted the EBNA 3B open reading frame and no EBNA 3B is expressed.

[e] NT, not tested.

mately 10^2 *Eco*RI-A recombinant, transforming P3HR-1 viruses. Independent recombination at the EBNA 3 loci is expected to be less efficient than within *Eco*RI-A since *Eco*RI-A is larger than *Sal*I-C. Furthermore, *Eco*RI-A contains 30 kb of long internal repeat DNA which is homologous to the parental P3HR-1 genome, and *Eco*RI-A has latent and lytic EBV DNA replication origins. The larger size, the repeats, and the replication of the transfected *Eco*RI A fragment within transfected cells is expected to make *Eco*RI-A more recombinogenic (24, 30, 33, 34). If the frequency of independent *Sal*I-C recombination is similar to that of *Eco*RI-A, there would be approximately 10^2 independent type 1 EBNA 3 recombinants, and the chance that an *Eco*RI-A and a *Sal*I-C recombinant would both infect the same primary B lymphocyte among 10^7 B lymphocytes which are used in one experiment would be very small. Direct evidence that the finding of both type 1 and type 2 EBNA 3A, 3B, or 3C genes in the same LCL is due to coinfecting nonrecombinant parental P3HR-1 genomes came from three observations. First, in all instances (except one described below) when an LCL was infected with an EBV carrying type 1 EBNA 3A or 3B, EBNA 3A and 3B, or EBNA 3A, 3B, and 3C genes and also had the corresponding type 2 EBNA 3 gene(s), the cell had the full complement of type 2 EBNA 3 genes, irrespective of whether one, two, or three type 1 EBNA 3 genes were present. Thus, these cells infected with EBV genomes with an insertion of type 1 EBNA 3A, 3B, or 3C genes also had genomes in which linkage was preserved through the entire type 2 EBNA 3 region. This could occur on the same genome only if the type 1 genes had inserted into a site 5′ or 3′ to the type 2 genes. Southern blot analysis of one putative coinfected and four singly infected type 1 recombinant LCL DNAs proved that both the type 1 and type 2 EBNA 3 genes were in the expected context in EBV genomes (see below). Second, although in initial PCR screening the P3HR-1-derived type 2 EBNA 3 genes were frequently equally represented (Fig. 2D, lane 2), subsequent PCR analyses of expanded LCL cultures revealed that the amplified fragments from type 2 EBNA 3 genes were now less evident than the type 1 fragments (Fig. 2D, lane 1). This is consistent with the expectation that parental coinfecting P3HR-1 genomes would be lost because they would be nontransforming genomes and therefore dispensable for LCL outgrowth. Third, when virus was passed from the putatively doubly infected LCLs to primary B lymphocytes, the resultant LCLs had whichever type 1 EBNA 3 genes were present in the original LCL (see below). The parental clone T1E3-100 in Fig. 2A to C has already lost most of its coinfecting nonrecombinant P3HR-1, and the progeny virus-transformed LCL (see Fig. 6) has only type 1 EBNA 3B. Thus, the type 1 EBNA 3A gene cosegregates with the transforming virus genome and the type 2 EBNA 3A gene does not.

Characterization of the type 1 EBNA 3 recombinants. Five type 1 EBNA 3 recombinants (resulting from recombination with transfected *Sal*I C and *Eco*RI A DNA fragments) and five type 2 recombinants (resulting from recombination with *Eco*RI-A only) which had been derived from two experiments were further characterized by restriction endonuclease digestion and Southern blot, by in situ lysis and Southern blot, by immunoblot for latent and lytic-cycle EBV gene expression, and by growth rate at low cell concentrations. LCL DNA was cut with *Bcl*I, which cleaves type 1 DNA at positions 79894 and 97110 or type 2 DNA at positions 79894, 93135, and 97052 (Fig. 3A). Thus, type 2 DNA has a distinctive *Bcl*I site near the center of EBNA 3A, which results in 13.3- and 3.9-kb fragments instead of the type 1 distinctive 17.2-kb fragment. Southern blots of LCL DNA were probed with EBNA 3A cDNA, which has two exons centered around the new *Bcl*I site characteristic of the type 2 EBNA 3 and an upstream exon from the *Bam*HI U fragment (Fig. 1 and 3A). The EBNA 3A open reading frame probe detected a 17.2-kb fragment in type 1 B95-8 DNA and 13.3- and 3.9-kb fragments in type 2 P3HR-1 DNA (Fig. 3B). The upstream EBNA 3A *Bam*HI-U exon also detected a 9.5-kb *Bcl*I fragment in both DNAs (Fig. 3B). As expected, the clones which by PCR appeared to be type 2 nonrecombinants at EBNA 3A had only the type 2 *Bcl*I fragments (Fig. 3B, lanes T2E3-1, T2E3-2, and T2E3-3). The T1E3-33 clone, which by PCR analysis was singly infected with a type 1 recombinant restricted to the EBNA 3B locus, had only the type 2 EBNA 3A *Bcl*I site and the expected 13.3- and 3.9-kb fragments (Fig. 3B). The T1E3-26, T1E3-28, and T1E3-35 clones, which were singly infected with type 1 EBNA 3A recombinants by PCR analysis, had only the type 1 *Bcl*I fragment (Fig. 3B). The absence of type 2 *Bcl*I fragments in these DNAs excludes the possibility that the type 1 EBNA 3A genes had inserted nonhomologously into another site in the EBV genome. The T1E3-100 Southern blot was particularly interesting in indicating coinfection with genomes that had both the type 1 and type 2 *Bcl*I fragments surrounding EBNA 3A (Fig. 3B; data not shown). This agrees with the early-passage PCR analysis of T1E3-100 shown in Fig. 2D, whereas PCR analysis of a later passage of T1E3-100 shown in Fig. 2 reveals that the coinfecting type 2 EBNA 3A gene has been lost, leaving a single virus genome which is recombinant for the type 1 EBNA 3A and 3B genes.

Southern blot analysis of the *Bcl*I and *Bam*HI (see below) digests of the LCL DNA probed with a *Sal*I-C DNA probe demonstrated the expected restriction fragments, indicating a single *Sal*I C fragment, further confirming the expected genome structures (data not shown). Thus, almost all PCR

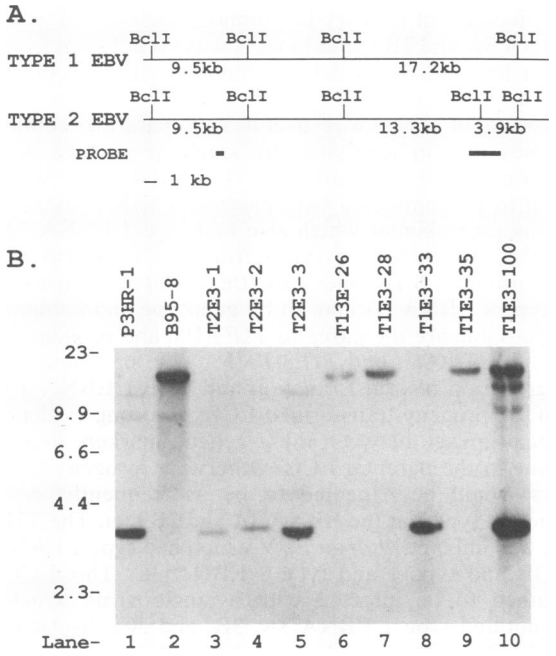

FIG. 3. Southern hybridization of genomic DNA from representative LCLs. Genomic DNA was digested with *Bcl*I restriction endonuclease and hybridized with ^{32}P-radiolabeled B95-8 EBNA 3A open reading frame cDNA probe containing genome sequence 67649 to 67535 (from the splice site in *Bam*HI-U) and 92238 to 95239 (EBNA 3A open reading frame) (panel A). (A) A schematic representation of the type 1 and type 2 EBNA 3A region and the expected *Bcl*I fragments. Type 2 DNA (P3HR-1) has an additional *Bcl*I site (position 93135) in the EBNA 3A open reading frame, resulting in a prominent 3.9-kb *Bcl*I fragment when hybridized with the EBNA 3A open reading frame probe. In contrast, type 1 (B95-8) DNA has no EBNA 3A *Bcl*I site and therefore has a 17.2-kb *Bcl*I fragment. (B) Southern hybridization results from representative LCLs. Digests of genomic DNA from P3HR-1 and B95-8 (lanes 1 and 2), three T2E3 LCLs (lanes 3 to 5), and five T1E3 LCLs (lanes 6 to 10) are shown. Size markers (kilobases) of *Hin*dIII fragments of lambda DNA are shown on the left.

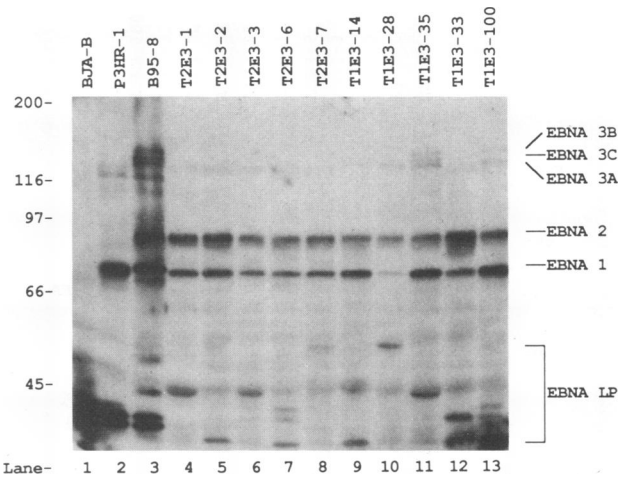

FIG. 4. Immunoblot analysis of total cellular proteins from representative LCLs. Protein blots were incubated with a serum specific for type 1 EBV latent proteins to examine recombination at the protein level (25). Type 1 EBNA 3A, 3B, and 3C are 145-, 165-, and 155-kDa proteins, respectively (11a, 25, 27a). Protein lysates from 5×10^5 cells were loaded and separated on a 7% polyacrylamide gel, transferred to nitrocellulose, incubated with a 1/10 dilution of the type 1 specific immune serum (previously absorbed with EBV-negative Louckes cells), and radiolabeled with ^{125}I-protein A. A negative control from the EBV-negative BJA-B is shown in lane 1. Protein lysates from P3HR-1 and B95-8 cells are shown in lanes 2 and 3. The specificity of the type 1-specific immune serum is shown by the strong reactivity to the B95-8 type 1 EBNA 3 proteins (lane 3) and the lack of reactivity to the P3HR-1 type 2 EBNA 3 proteins (lane 2). Protein lysates from five T2E3 (lanes 4 to 8) and five T1E3 (lanes 9 to 13) LCLs are shown. The location of the EBV latent proteins is shown on the right. The protein detected in the type 2 LCLs (lanes 2 and 4 to 8) and the T1E3-33 LCL (lane 12) which is migrating slightly faster than EBNA 3A, is a cross-reacting band and is not to be confused with the type 1 EBNA 3A band detected in lanes 3, 9 to 11, and 13. Size markers (kilodaltons) of protein molecular mass standards are shown on the left.

and Southern blot results were consistent with insertion of part of the type 1 *Sal*I C fragment into the P3HR-1 genome by homologous recombination. In addition, Southern blot analysis of the LCL genomic DNA digested with *Bam*HI and probed with a ^{32}P-labeled cloned *Bam*HI H DNA fragment (Fig. 1) demonstrated proper replacement of the P3HR-1 deletion by recombination with the cloned type 1 *Eco*RI A fragment (data not shown). All the LCLs examined had the B95-8 (type 1) size *Bam*HI H fragment, with no detectable difference between the type 2 EBNA 3 LCLs and the type 1 EBNA 3 LCLs (data not shown).

The single evidence for nonhomologous recombination among 33 clones which were analyzed was the T1E3-26 clone which by PCR and Southern analysis had only type 1 EBNA 3A and 3B (Fig. 2 and 3). The absence of type 2 EBNA 3A and 3B indicates that this LCL was not coinfected with another EBV genome and that the type 1 EBNA 3A and 3B genes had replaced the corresponding type 2 genes through site-specific integration into the EBV genome. However, the T1E3-26 genome is diploid for EBNA 3C (Fig. 2C). The triplet bands noted in the PCR analysis are due to type 1, type 2, and heterohybrid EBNA 3C bands. This suggests that recombination at the right side of *Sal*I-C for this recombinant was nonhomologous and that the recombination was 3′ to the type 1 EBNA 3C and 5′ to the type 2 EBNA 3C site. PCR analysis of this recombinant with an additional set of EBNA 3C primers (29) located approximately 900 bp 5′ to the first set of EBNA 3C primers (Fig. 1) also demonstrated amplification of both type 1 and type 2 EBNA 3C-specific fragments, further indicating that the EBNA 3C recombination was nonhomologous. The analysis with the second set of primers indicates that type 1 EBNA 3C is positioned 5′ to most or all of type 2 EBNA 3C in the recombinant genome (PCR data not shown). With virus replication in the T1E3-26 clone, progeny transforming viruses further recombined to delete the type 1 EBNA 3C gene, resulting in apparently fully homologously recombinant genomes carrying type 1 EBNA 3A and 3B genes and the type 2 EBNA 3C gene (see below).

Immunoblot analyses confirmed the genotypic analyses and indicated that there was no effect of type 1 EBNA 3A, 3B, or 3C substitution on the expression of other latent or lytic-cycle EBV genes. A largely type 1-specific human antiserum did not detect type 1 EBNA 3s in the fully type 2 cell lines T2E3-1-, T2E3-2-, T2E3-3-, T2E3-6-, and T2E3-7-infected LCLs (Fig. 4). The antiserum did detect type 1 EBNA 3A, 3B, or 3C in the T1E3-14-, T1E3-28-, T1E3-35-, T1E3-33-, and T1E3-100-infected LCLs (Fig. 4). EBNA 2

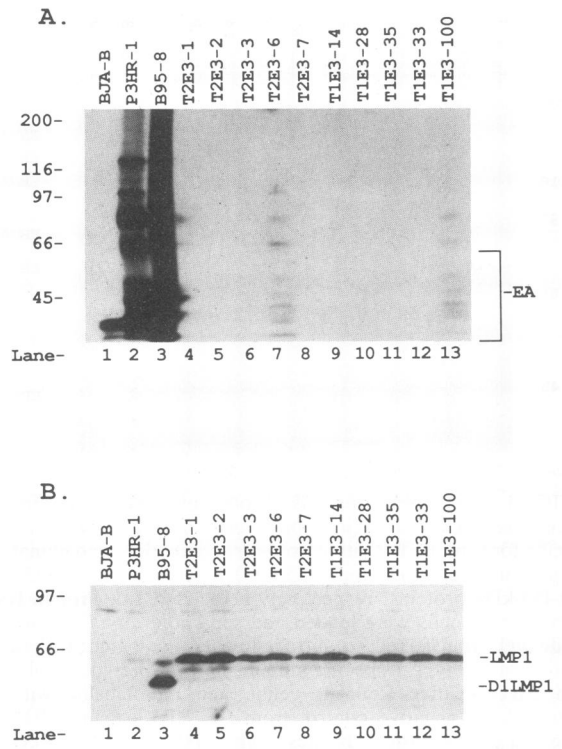

FIG. 5. Expression of lytic (A) and LMP 1 (B) proteins in representative LCLs. Polyacrylamide gels (7%) were loaded with protein lysate samples, and separated proteins were transferred to nitrocellulose. (A) Lytic-antigen expression was examined by incubation with 1/100 dilution of SP human serum and radiolabeling with ^{125}I-protein A. The location of the lytic antigens (early antigens [EA]) is shown on the right. SP also has reacts with the type 1 latent antigens. (B) LMP 1 expression was examined by incubating with the S12 LMP 1-specific monoclonal antibody (16), a rabbit anti-mouse secondary antibody and ^{125}I-protein A. LMP 1 and the lytic-cycle product of the LMP 1 gene, D1LMP1, are indicated. Both are expressed in B95-8 cells. EBV infection in the recombinant LCLs, unlike B95-8 cells, is primarily latent and D1LMP is not detected. A protein lysate from EBV-negative BJA-B cells is shown in lane 1. Protein lysates from induced P3HR-1 (lane 2), induced B95-8 (lane 3), and five T2E3 (lanes 4 to 8) and five T1E3 (lanes 9 to 13) LCLs are shown. Size markers (kilodaltons) of protein molecular mass standards are shown on the left.

and EBNA LP and LMP 1 expression was similar among all of the cell lines, including T1E3-35, which was type 1 at the EBNA 3A, 3B, and 3C loci (Fig. 4 and 5B). Most LCL clones exhibited little evidence of spontaneous lytic-cycle EBV gene expression irrespective of whether they were recombinant or nonrecombinant at an EBNA 3 locus (Fig. 5A). In situ lysis and Southern blot analysis of five LCLs infected with type 1 EBNA 3A and 3B or EBNA 3A, 3B, and 3C recombinants and of four LCLs infected with fully type 2 EBNA 3 genomes revealed no difference in EBV episome copy number or in spontaneous EBV replication as evidenced by linear EBV genomes (data not shown).

Replication and passage of the recombinant virus. Virus could be passaged from LCLs infected with recombinant virus whether or not the recombinant virus had also incorporated one or more type 1 EBNA 3 loci. As expected, the resultant infected LCLs had the EBNA 3 genotype and immunophenotype characteristics of the parental virus (Fig. 6 to 8; data not shown). The time to outgrowth and subsequent passage of primary B lymphocytes infected with type 1 EBNA 3A and 3B recombinants was no different from that of cells infected in parallel with an otherwise isogenic type 2 control.

Passage of virus was useful in separating coinfecting genomes and in resolving the single putatively partially homologous recombinant. The T1E3-100 LCL was presumed to be coinfected with parental P3HR-1 and a transforming recombinant which also had type 1 EBNA 3A and 3B genes. When progeny virus from T1E3-100 was used to infect primary B lymphocytes, the resultant transformants had type 1 EBNA 3A and 3B by genotype and immunophenotype (compare the parental T1E3-100 and progeny T1E3-100A, T1E3-100G, and T1E3-100J in Fig. 6, 7, and 8). The cosegregation of transformation and type 1 EBNA 3A and 3B in the progeny transformed LCLs is strong evidence for physical linkage between these genetic markers in a single genome in the parental LCL. Otherwise, progeny transformants would be expected to be as frequently or more frequently type 2 at the EBNA 3A and 3B loci. The T1E3-26 LCL was infected with an EBV which had type 1 EBNA 3A and 3B and type 1 and type 2 EBNA 3C. This LCL was presumed to be infected with a single virus which had incorporated type 1 EBNA 3A, 3B, and 3C, displacing the type 2 EBNA 3A and 3B genes, but with a nonhomologous recombination 3′ to the type 1 EBNA 3C gene, inserting the type 1 EBNA 3C 5′ to the type 2 EBNA 3C gene. The two progeny LCLs that arose following infection with virus from T1E3-26 were infected with genomes which had deleted the redundant type 1 EBNA 3C gene and were type 1 for EBNA 3A and 3B and type 2 for EBNA 3C (Fig. 6 and 7).

DISCUSSION

These experiments demonstrate important principles which will be useful for subsequent EBV recombinant molecular genetic experiments. First, a cotransfected EBV DNA fragment can be incorporated into replicating EBV genomes which had also recombined with a nonlinked positive selection marker. Thus, a replicating EBV DNA molecule which participates in one recombination event can participate in a second recombination event. Second, the recombination of the nonlinked EBV DNA fragments into the EBV genome is almost always fully homologous. Previous studies with transfected large EBV DNA fragments which included the DNA deleted from P3HR-1 and which used restoration of transformation as a positive selection revealed that the transformation-competent recombinants had incorporated the previously deleted DNA into the "correct" site (6, 7, 11, 17, 18, 31, 32). Our data indicate that the nonlinked cotransfected EBV DNA is also almost invariably targeted to the correct site, probably by reciprocal homologous recombination. PCR analysis of eight of nine LCLs singly infected with independently derived type 1 EBNA 3 recombinants demonstrated that the type 1 EBNA 3 gene(s) had replaced the type 2 gene(s). Most of the initial type 1 EBNA 3 recombinant-infected LCLs were coinfected with P3HR-1, complicating the interpretation of their initial PCR results. Even in those instances, passage of virus resulted in segregation of the transformation-competent type 1 EBNA 3 recombinant genomes from the nonrecombinant P3HR-1 genome. The single exception to complete replacement was a genome in which the three type 1 EBNA 3 genes had replaced the type 2 EBNA 3A and 3B genes but had not replaced the type 2 EBNA 3C gene. Passage of virus from this LCL to primary B lymphocytes resulted in an EBV

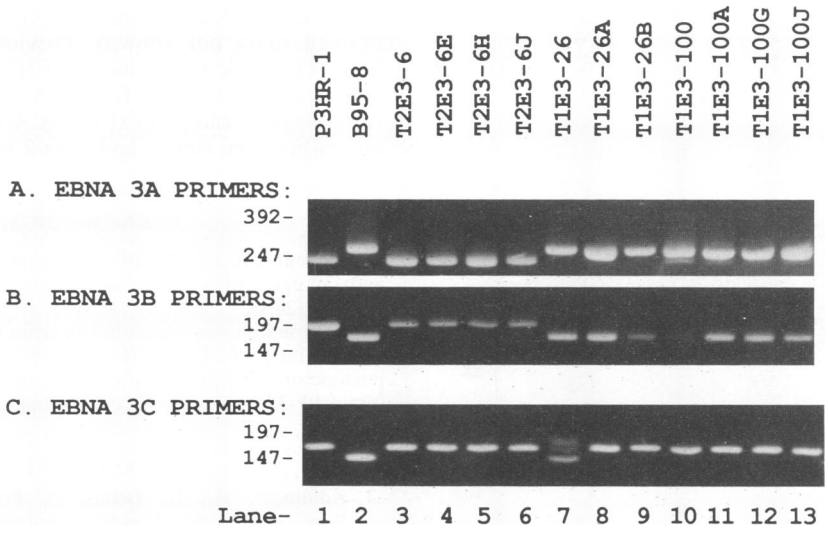

FIG. 6. PCR analysis of T2E3 and T1E3 progeny clones. Parental clones T2E3-6, T1E3-26, and T1E3-100 were transfected with pSVNaeI Z to induce virus replication, lethally irradiated (8,800 rads), and cocultured with human B cells. The PCR analysis of three progeny T2E3-6 LCLs (lanes 4 to 6) derived from the parental T2E3-6 LCL (lane 3), three progeny T1E3-100 LCLs (lanes 11 to 13) derived from the parental T1E3-100 LCL (lane 10), and two progeny T1E3-26 LCLs (lanes 8 and 9) derived from the parental T1E3-26 LCL (lane 7) analyzed with the EBNA 3A, 3B, and 3C-3′ primers is shown. The PCR analysis of P3HR-1 (lane 1) and B95-8 (lane 2) is also shown. Size markers (kilobases) of *Rsa*I fragments of ϕX174 are shown on the left.

genome which deleted the previously duplicated EBNA 3C gene, presumably resulting in a fully homologously recombined type 1 EBNA 3A and 3B and type 2 EBNA 3C genome. Third, the frequency of incorporation of the nonlinked EBV DNA fragment was 11% despite the absence of repeat DNA and latent or lytic-cycle replication origins. The lack of dependence on repeat DNA and on latent or lytic origins should make it possible to extend this approach to specifically introduce mutations into any site in the EBV genome.

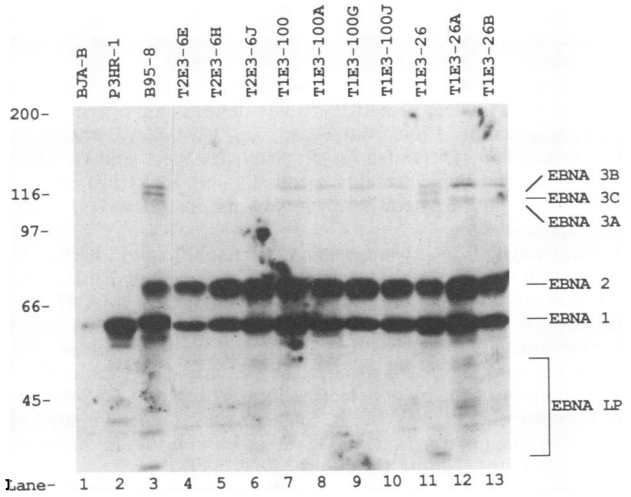

FIG. 7. Analysis of progeny LCLs for expression of latent proteins. Protein analysis of three progeny T2E3-6 LCLs (lanes 4 to 6) derived from the parental T2E3-6 LCL, three progeny T1E3-100 LCLs (lanes 8 to 10) derived from the parental T1E3-100 LCL (lane 7), and two progeny T1E3-26 LCLs (lanes 12 and 13) derived from the parental T1E3-26 LCL (lane 11) are shown. Also shown are the protein analyses from BJA-B (a negative control [lane 1]), P3HR-1 (lane 2), and B95-8 (lane 3). Protein lysates were separated and analyzed with the type 1 specific immune serum as discussed in the legend to Fig. 4.

The relatively high efficiency with which second-site homologous recombination occurred in these experiments suggests that second-site homologous recombination may be generally applicable to other herpesvirus or other virus or host cell molecular genetic experiments. Such an approach is particularly useful because it simplifies the strategy necessary to make mutations in any specific site in a genome which is linked to another site that is subject to a positive selection strategy. Indeed, the fact that successfully transfected cells take up and incorporate large quantities of DNA has been widely useful and is an important component of this procedure (28, 38). A high frequency of recombination among DNAs which participate in a single recombination event has also been noted in many systems (19, 22, 36).

These experiments used a 30-kb EBV DNA fragment, and the large fragment size probably contributed to the high efficiency of targeted homologous recombination. In a previous experiment with a positive selection marker in a 3-kb EBV DNA fragment, nonhomologous insertion was noted (37). The high frequency of insertion of both type 1 EBNA 3A and 3B and the low frequency of insertion of type 1 EBNA 3C are compatible with the working hypothesis that most of the 30-kb transfected DNA is inserted into the EBV genome and that the efficiency decreases within 4 kb of the ends. This occurred in these experiments despite type 1 and type 2 EBNA 3C having a similar level of homology to that between type 1 and type 2 EBNA 3A and transfection of *Sal*I-C as a free, linear DNA fragment. Although homologous recombination in other systems is facilitated by free DNA ends (5a), free ends may not be more recombinogenic in this system.

These experiments demonstrated no difference between EBV recombinants with type 1 or type 2 EBNA 3 genes in their ability to transform primary B lymphocytes or in the

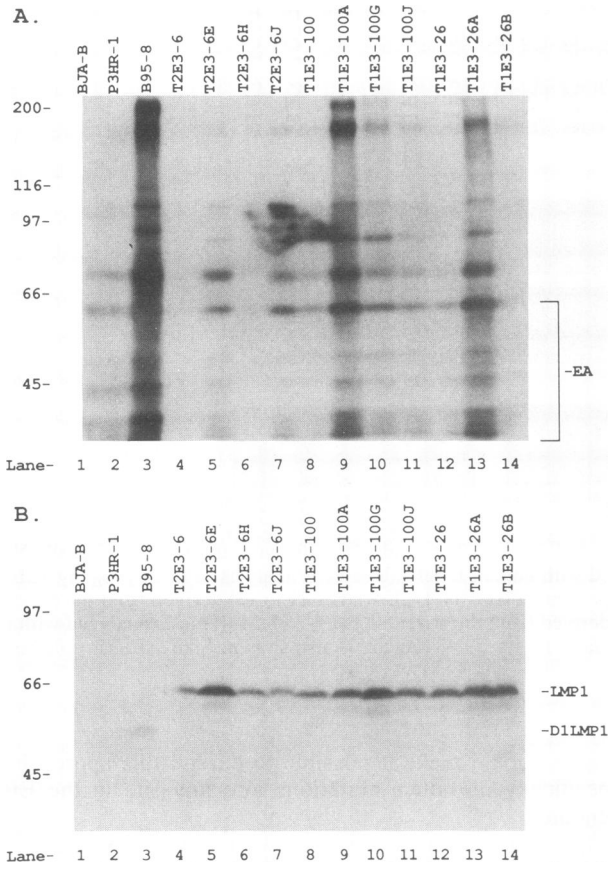

FIG. 8. Analysis of progeny LCLs for expression of lytic (A) and LMP1 (B) proteins. Three progeny T2E3-6 LCLs (lanes 5 to 7) derived from the parental T2E3-6 LCL (lane 4), three progeny T1E3-100 LCLs (lanes 9 to 11) derived from the parental T1E3-100 LCL (lane 8), and two progeny T1E3-26 LCLs (lanes 13 and 14) derived from the parental T1E3-26 LCL (lane 12) are shown. Also shown are the protein lysates from BJA-B (a negative control [lane 1]), P3HR-1 (lane 2), and B95-8 (lane 3). Protein lysates were separated and analyzed as discussed in the legend to Fig. 5.

growth of the resultant LCLs. Furthermore, there was no difference in latent or lytic-cycle EBV gene expression. These results are consistent with previous results which indicated that type-specific differences in EBNA 2 are primarily responsible for the different effects of type 1 or type 2 EBV in primary B-lymphocyte growth transformation (7, 27). Although no differences were observed in vitro, it is possible that EBV encoding type 1 or type 2 EBNA 3 differs in some aspect which may only be apparent in vivo. The functions of the EBNA 3 proteins are not known, except for the observation that EBNA 3C expression in Burkitt's lymphoma cells results in increased expression of CD21 (36a). Whether type 1 and type 2 EBNA 3C have the same effect on CD21 expression has not been examined. Furthermore, we have not examined or compared the level of CD21 or any other antigen expression in our EBNA 3 recombinant LCLs.

The type 1 EBNA 3A, 3A and 3B, and 3A, 3B, and 3C recombinants derived in these experiments will be useful in subsequent studies of the effect of type-specific differences on cell growth or immune T-cell cytotoxic killing of EBV-infected B lymphocytes. For these purposes, two additional type 1 EBNA 3A, 3B, and 3C recombinant EBVs have been derived (data not shown). Previous analyses of immune T-cell cytotoxic recognition of EBV-transformed B lymphocytes indicated that EBNA 3A has an epitope which is recognized in the context of HLA-B8 and that this epitope differs between type 1 and type 2 viruses (5, 23).

ACKNOWLEDGMENTS

Wonkeun Lee and Jennifer Lee contributed valuable assistance, and George Miller generously provided the HH514-16 clone of P3HR-1 (26A).

This research was supported by grant CA00449 from the National Cancer Institute, Public Health Service. B.T. was supported by postdoctoral research awards from the American Cancer Society (grant PF-3561) and the Baxter Foundation.

REFERENCES

1. **Adidinger, H., H. Delius, U. Freese, J. Clarke, and G. Bornkamm.** 1985. A putative transforming gene of the Jijoye virus differs from that of Epstein-Barr virus prototypes. Virology **141**:221–234.
2. **Alfieri, C., M. Birkenbach, and E. Kieff.** 1991. Early events in Epstein-Barr virus infection of human B lymphocytes. Virology **181**:595–608.
3. **Baer, R., A. Bankier, M. Biggin, P. Deininger, P. Farrell, T. Gibson, G. Hatfull, G. Hudson, S. Satchwell, C. Sequin, P. Tuggnell, and B. Barrell.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr genome. Nature (London) **310**:207–211.
4. **Burrows, S., I. Misko, T. B. Sculley, C. Schmidt, and D. J. Moss.** 1990. An Epstein-Barr virus-specific cytotoxic T-cell epitope present on A and B type transformants. J. Virol. **64**:3974–3976.
5. **Burrows, S., T. Sculley, S. Misko, C. Schmidt, and D. J. Moss.** 1990. An Epstein-Barr virus-specific cytotoxic T cell epitope in EBV nuclear antigen 3 (EBNA 3). J. Exp. Med. **171**:345–349.

5a. **Carroll, D., S. H. Wright, R. K. Wolff, E. Grzesiuk, and E. B. Maryon.** 1986. Efficient homologous recombination of linear DNA substrates after injection into *Xenopus* laevis oocytes. Mol. Cell. Biol. **6**:2053–2061.

6. **Cohen, J. I., F. Wang, and E. Kieff.** 1991. Epstein-Barr virus nuclear protein 2 mutations define essential domains for transformation and transactivation. J. Virol. **65**:2545–2554.
7. **Cohen, J. I., F. Wang, J. Mannick, and E. Kieff.** 1989. Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. Proc. Natl. Acad. Sci. USA **86**:9558–9562.
8. **Countryman, J., H. Jenson, R. Seibl, H. Wolf, and G. Miller.** 1987. Polymorphic proteins encoded within BZLF1 of defective and standard Epstein-Barr viruses disrupt latency. J. Virol. **61**:3672–3679.
9. **Dambaugh, T., K. Hennessy, L. Chamnankit, and E. Kieff.** 1984. U2 region of Epstein-Barr virus DNA may encode Epstein-Barr virus nuclear antigen 2. Proc. Natl. Acad. Sci. USA **81**:7632–7636.
10. **Gardella, T., P. Medveczky, T. Sairenji, and C. Mulder.** 1984. Detection of circular and linear herpesvirus DNA molecules in mammalian cells by gel electrophoresis. J. Virol. **50**:248–254.
11. **Hammerschmidt, W., and B. Sugden.** 1989. Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes. Nature (London) **340**:393–397.

11a. **Hennessy, K., F. Wang, E. Woodland-Bushman, and E. Kieff.** 1986. Definitive identification of a member of the Epstein-Barr virus nuclear protein 3 family. Proc. Natl. Acad. Sci. USA **79**:5916–5920.

12. **Heston, L., M. Rabson, N. Brown, and G. Miller.** 1982. New Epstein-Barr virus variants from cellular subclones of P3J-HR-1 Burkitt lymphoma. Nature (London) **295**:160–163.
13. **Jeang, K., and S. D. Hayward.** 1983. Organization of the Epstein-Barr virus molecule. III. Location of the P3HR1 deletion junction and characterization of the Not1 repeat units that form part of the template for an abundant 12-*O*-tetrade-

canoylphorbol-13-acetate-induced mRNA transcript. J. Virol. **48**:135–148.
14. **Knott, J., D. J. G. Rees, Z. Cheng, and G. G. Brownlee.** 1988. Randomly picked cosmid clones overlap the pyr B and oriC gap in the physical map of E. coli chromosome. Nucleic Acids Res. **16**:2601–2612.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Mann, K. P., D. Staunton, and D. A. Thorley-Lawson.** 1985. Epstein-Barr virus-encoded protein found in plasma membranes of transformed cells. J. Virol. **55**:710–720.
17. **Mannick, J. B., J. I. Cohen, M. Birkenbach, A. Marchini, and E. Kieff.** 1991. The Epstein-Barr virus nuclear protein encoded by the leader of the EBNA RNAs is important in B-lymphocyte transformation. J. Virol. **65**:6826–6837.
18. **Marchini, A., B. Tomkinson, J. I. Cohen, and E. Kieff.** 1991. BHRF1, the Epstein-Barr virus gene with homology to Bcl2, is dispensable for B-lymphocyte transformation and virus replication. J. Virol. **65**:5991–6000.
19. **Marsden, H. S., N. D. Stow, V. G. Preston, M. C. Timbury, and N. M. Wilkie.** 1978. Physical mapping of herpes simplex virus-induced polypeptides. J. Virol. **28**:624–642.
20. **Menezes, J., W. Leibold, G. Klein, and G. Clements.** 1975. Establishment and characterization of an Epstein-Barr virus (EBV)-negative lymphoblastoid B cell line (BJA-B) from an exceptional, EBV-genome-negative African Burkitt's lymphoma. Biomedicine **22**:276–284.
21. **Miller, G., M. Rabson, and L. Heston.** 1984. Epstein-Barr virus with heterogeneous DNA disrupts latency. J. Virol. **50**:174–182.
22. **Morse, L. S., T. G. Buchman, B. Roizman, and P. A. Schaffer.** 1977. Anatomy of herpes simplex virus DNA. IX. Apparent exclusion of some parental DNA arrangement in the generation of intertypic (HSV-1 × HSV-2) recombinants. J. Virol. **24**:231–248.
23. **Murray, R., M. Kurilla, H. M. Griffin, J. M. Brooks, M. Mackett, J. A. Arrand, S. R. Burrows, D. J. Moss, E. Kieff, and A. B. Rickinson.** 1990. Human cytotoxic T-cell responses against Epstein-Barr virus nuclear antigens demonstrated by using recombinant vaccinia virus. Proc. Natl. Acad. Sci. USA **87**:2906–2910.
24. **Nickoloff, J. A., and R. J. Reynolds.** 1990. Transcription stimulates homologous recombination in mammalian cells. Mol. Cell. Biol. **10**:4837–4845.
25. **Petti, L., J. Sample, F. Wang, and E. Kieff.** 1988. A fifth Epstein-Barr virus nuclear protein is expressed in latently infected growth-transformed lymphocytes. J. Virol. **62**:1330–1338.
26. **Rabson, M., L. Gradoville, L. Heston, and G. Miller.** 1982. Nonimmortalizing P3J-HR-1 Epstein-Barr virus: a deletion mutant of its transforming parent, Jijoye. J. Virol. **44**:834–844.
26a. **Rabson, M., L. Heston, and G. Miller.** 1983. Identification of a rare Epstein-Barr virus variant that enhances early antigen expression in Raji cells. Proc. Natl. Acad. Sci. USA **80**:2762–2766.
27. **Rickinson, A., L. S. Young, and M. Rowe.** 1987. Influence of the Epstein-Barr virus nuclear antigen EBNA 2 on the growth phenotype of virus-transformed B cells. J. Virol. **61**:1310–1317.
27a. **Ricksten, A., B. Kallin, H. Alexander, J. Dillner, R. Fahraeus, G. Klein, R. Lerner, and L. Ramos.** 1988. BamHI E region of the Epstein-Barr virus genome encodes three transformation associated nuclear proteins. Proc. Natl. Acad. Sci. USA **85**:995–999.
28. **Roizman, B., and F. J. Jenkins.** 1985. Genetic engineering of novel genomes of large DNA viruses. Science **229**:1208–1214.
29. **Sample, J., L. Young, B. Martin, T. Chatman, E. Kieff, A. Rickinson, and E. Kieff.** 1990. Epstein-Barr virus types 1 and 2 differ in their EBNA 3A, EBNA 3B, and EBNA 3C genes. J. Virol. **64**:4084–4092.
30. **Shulman, M. J., L. Nissen, and C. Collins.** 1990. Homologous recombination in hybridoma cells: dependence on time and fragment length. Mol. Cell. Biol. **10**:4466–4472.
31. **Skare, J., J. Farley, J. L. Strominger, K. O. Fresen, M. S. Cho, and H. Zur Hausen.** 1985. Transformation by Epstein-Barr virus requires sequences in the region of *Bam*HI fragments Y and H. J. Virol. **55**:286–297.
32. **Swaminathan, S., B. Tomkinson, and E. Kieff.** 1991. Recombinant Epstein-Barr virus with small RNA (EBER) genes deleted transforms lymphocytes and replicates *in vitro*. Proc. Natl. Acad. Sci. USA **88**:1546–1550.
33. **Thomas, B. J., and R. Rothstein.** 1989. Elevated recombination rates in transcriptionally active DNA. Cell **56**:619–630.
34. **Thomas, K. R., and M. R. Capecchi.** 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell **51**:503–512.
35. **van Santen, V., A. Cheung, and E. Kieff.** 1981. Epstein-Barr virus RNA: size and direction of transcription of virus specific cytoplasmic RNAs in a transformed cell line. Proc. Natl. Acad. Sci. USA **78**:1930–1934.
36. **van Ziji, M., W. Quint, J. Briaire, T. de Rover, A. Gielkens, and A. Berns.** 1988. Regeneration of herpesviruses from molecularly cloned subgenomic fragments. J. Virol. **62**:2191–2195.
36a. **Wang, F., C. Gregory, C. Sample, M. Rowe, D. Liebowitz, R. Murray, A. Rickinson, and E. Kieff.** 1990. Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. J. Virol. **64**:2309–2318.
37. **Wang, F., A. Marchini, and E. Kieff.** 1991. Epstein-Barr virus (EBV) recombinants: use of positive selection markers to rescue mutants in EBV-negative B-lymphoma cells. J. Virol. **65**:1701–1709.
38. **Wigler, M., A. Pellicer, S. Silverstein, and R. Axel.** 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. Cell **14**:725–731.