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Epstein-Barr Virus Recombinants from Overlapping Cosmid Fragments

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Five overlapping type 1 Epstein-Barr virus (EBV) DNA fragments constituting a complete replication- and transformation-competent genome were cloned into cosmids and transfected together into P3HR-1 cells, along with a plasmid encoding the Z immediate-early activator of EBV replication. P3HR-1 cells harbor a type 2 EBV which is unable to transform primary B lymphocytes because of a deletion of DNA encoding EBNA LP and EBNA 2, but the P3HR-1 EBV can provide replication functions in *trans* and can recombine with the transfected cosmids. EBV recombinants which have the type 1 EBNA LP and 2 genes from the transfected *Eco*RI-A cosmid DNA were selectively and clonally recovered by exploiting the unique ability of the recombinants to transform primary B lymphocytes into lymphoblastoid cell lines. PCR and immunoblot analyses for seven distinguishing markers of the type 1 transfected DNAs identified cell lines infected with EBV recombinants which had incorporated EBV DNA fragments beyond the transformation marker-rescuing *Eco*RI-A fragment. Approximately 10% of the transforming virus recombinants had markers mapping at 7, 46 to 52, 93 to 100, 108 to 110, 122, and 152 kbp from the 172-kbp transfected genome. These recombinants probably result from recombination among the transfected cosmid-cloned EBV DNA fragments. The one recombinant virus examined in detail by Southern blot analysis has all the polymorphisms characteristic of the transfected type 1 cosmid DNA and none characteristic of the type 2 P3HR-1 EBV DNA. This recombinant was wild type in primary B-lymphocyte infection, growth transformation, and lytic replication. Overall, the type 1 EBNA 3A gene was incorporated into 26% of the transformation marker-rescued recombinants, a frequency which was considerably higher than that observed in previous experiments with two-cosmid EBV DNA cotransfections into P3HR-1 cells (B. Tomkinson and E. Kieff, J. Virol. 66:780–789, 1992). Of the recombinants which had incorporated the marker-rescuing cosmid DNA fragment and the fragment encoding the type 1 EBNA 3A gene, most had incorporated markers from at least two other transfected cosmid DNA fragments, indicating a propensity for multiple homologous recombinations. The frequency of incorporation of the nonselected transfected type 1 EBNA 3C gene, which is near the end of two of the transfected cosmids, was 26% overall, versus 3% in previous experiments using transfections with two EBV DNA cosmids. In contrast, the frequency of incorporation of a 12-kb EBV DNA deletion which was near the end of two of the transfected cosmids was only 13%. Other than through incorporation into recombinants arising from among the five cosmids, this marker was rarely incorporated into recombinants which had any marker from the P3HR-1 genome. Thus, the five-cosmid transfection strategy is particularly useful for incorporation of a nonselected marker mapping near the end of a transfected cosmid or near the site of a large deletion.

Epstein-Barr virus (EBV) is a human herpesvirus with tropism for B lymphocytes and some epithelial cells (12, 32, 39). EBV causes infectious mononucleosis and lymphoproliferative disease and is associated with several malignancies, including Burkitt's lymphoma, nasopharyngeal carcinoma, and Hodgkin's disease (4, 8, 11, 13, 38, 41, 50, 51). In vitro, EBV establishes a latent infection in B lymphocytes and causes indefinite cell proliferation (3, 12, 31–33). Eleven genes are expressed during latent infection, and five or six of these genes are responsible for the growth transformation (for a review, see reference 17). Most of the many other EBV genes function in lytic virus infection, which occurs spontaneously in a small fraction of infected B lymphocytes.

Recent advances have made possible the construction of EBV recombinants with specific mutations at any site in the genome (5, 6, 9, 16, 20–28, 43–46, 48). Most of these studies have utilized the P3HR-1 cell line. P3HR-1 cells are infected with a defective EBV which is lacking a DNA fragment which encodes EBNA 2 and part of EBNA LP and are unable to transform primary B lymphocytes (10, 14, 15, 18, 30, 35, 36, 40). Transfection of P3HR-1 cells with a wild-type cloned EBV DNA fragment which spans the P3HR-1 deletion and induction of virus replication results in homologous recombination between the transfected fragment and the P3HR-1 EBV genome and in EBV recombinants capable of transforming B lymphocytes (5, 6, 9, 16, 21, 22–24, 26, 43–46). When the progeny virus is used to infect primary B lymphocytes and the infected cells are plated under conditions in which each transformed cell gives rise to a separate long-term lymphoblastoid cell line (LCL), each LCL is infected with a single EBV recombinant.

When this strategy of marker rescue of transformation of primary B lymphocytes as a selection for EBV recombinants was used, a surprising finding was that transfection of a second, nonlinked, EBV DNA fragment into P3HR-1 cells resulted in 10% of the transformation marker-rescued recombinants also incorporating a marker from the nonlinked cotransfected

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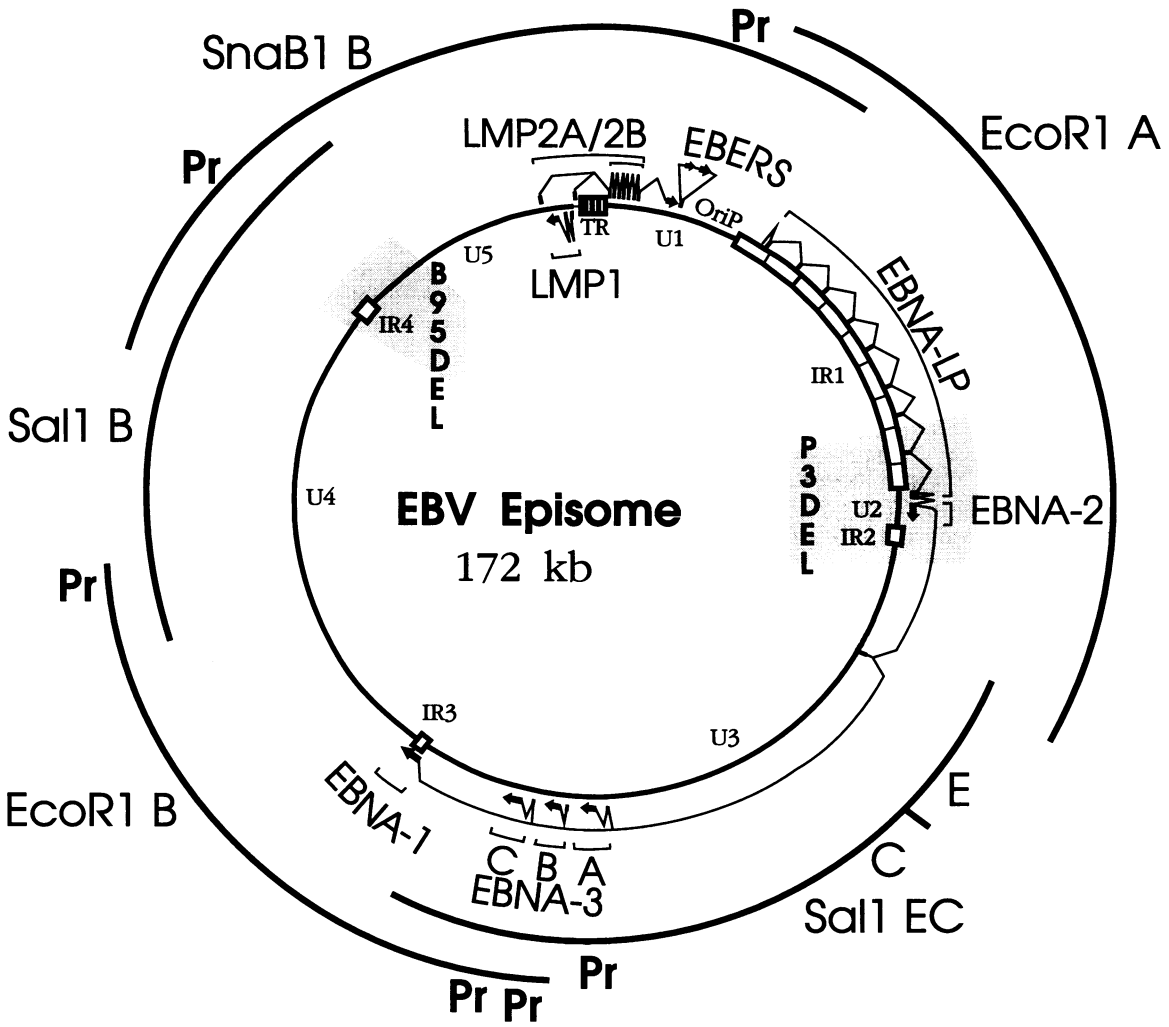

FIG. 1. Schematic diagram of the EBV genome, indicating the P3HR-1 and B95-8 deletions and the cosmid-cloned type 1 EBV DNA fragments. Pr, sites of the PCR primer pairs.

DNA (44–46). This latter strategy of marker rescue and second-site homologous recombination has been used to create and select for EBV recombinants with specific mutations at any site in the genome (16, 22, 28, 43–46). Because of the vast excess of nontransforming P3HR-1 EBV produced from the transfected P3HR-1 cell, some of the LCLs are initially coinfected with P3HR-1. Coinfection with P3HR-1 has been useful in *trans* complementing EBV recombinants with specific mutations in essential genes other than EBNA 2 or EBNA LP, since these are the only genes for which P3HR-1 is defective (16, 46).

The objective of the experiments reported here was to determine whether the efficiency of second-site homologous recombination could be improved by transfection with overlapping cosmid-cloned EBV DNA fragments that span the entire EBV genome. EBV recombinants could arise only from the transfected, *Escherichia coli*-cloned EBV DNA or as a result of recombination with the P3HR-1 EBV genome. Important precedents for this approach include the findings that (i) a cell which is competent for taking up exogenous DNA frequently incorporates many DNA molecules (49), (ii) homologous recombination readily occurs in cells permissively infected with herpesviruses (34, 42), (iii) pseudorabies virus (PRV) can be reconstructed by transfecting overlapping *E. coli*-cloned PRV DNA cosmids into cells permissive for PRV replication (47), and (iv) an EBV DNA molecule which has engaged in one homologous event is likely to engage in additional homologous recombinations (44–46).

MATERIALS AND METHODS

Plasmid and cosmid DNAs. Plasmid pSVNaeI Z was used to induce lytic EBV infection (7, 43–46). DNA fragments *Eco*RI-A, *Sal*I-E/C, *Eco*RI-B, and *Sal*I-B (Fig. 1) were cloned from virus produced from B95-8 cells (29, 31). The *Sna*BI-B DNA fragment, which overlaps *Eco*RI-A and *Sal*I-B and includes both ends of the linear EBV genome, was cloned from intracellular EBV episomes (10). B95-8 cells (4×10^9) were treated for 3 days with 20 ng of tetradecanoyl phorbol acetate per ml–3 mM *n*-butyrate to induce virus replication. Cells were suspended in 50 ml of 50 mM Tris–1 mM EDTA, pH 7.4, adjusted to 0.5% sodium dodecyl sulfate and 100 μg of proteinase K per ml, homogenized with a Dounce homogenizer, incubated at 60°C for 4 h, adjusted to 5% Sarkosyl and

an average CsCl density of 1.710 g/cm^3, and centrifuged at 40,000 rpm for 18 h in a VTi 50 rotor. The CsCl fraction with a density of 1.730 to 1.700 g/cm^3 was collected and centrifuged on a second CsCl gradient in 10 mM Tris–1 mM EDTA, pH 7.4. The EBV DNA fragments were cloned by incubating the purified DNA with the appropriate enzyme, heat inactivating the enzyme, and ligating the digested DNA into the cosmid vector pDVcosA2 (19). The cosmid *Sal*I-E/C was obtained following partial digestion of EBV DNA with *Sal*I and was cloned into pDVcosA2 in which the *Sal*I site was modified by the addition of an oligonucleotide which ablated the *Sal*I site and introduced a new *Sal*I site flanked by two unique *Sna*BI sites. Ligated pDVcosA2 was then packaged into lambda and used to infect PLK-A *E. coli* (Stratagene). Bacterial clones were screened by restriction endonuclease analysis to identify colonies with the correct cloned DNA fragment.

Cell cultures. The HH514-c16 subclone of P3HR-1 (a gift of George Miller, Yale University) is infected with a type 2 EBV genome lacking the EBNA 2 gene and part of the EBNA LP gene, rendering it nontransforming (14, 30). B95-8 is a marmoset LCL immortalized with a type 1 EBV (29). Human peripheral blood B lymphocytes were obtained from adult donors. T cells were removed with 2-aminoethyl isothiouronium bromide (Sigma)-treated sheep erythrocytes. Cell lines were maintained in complete medium, which consisted of RPMI 1640 supplemented with 10% inactivated fetal bovine serum, 2 mM glutamine, and 10 μg of gentamicin per ml.

Cosmid transfection. Cosmid DNA was digested with restriction endonucleases to release the EBV DNA from the vector. The *Sal*I-E/C DNA fragment was released from vector by digestion with *Sna*BI. Twenty micrograms of each cosmid DNA was mixed with 25 μg of pSVNaeI Z and used to transfect 10^7 P3HR-1 c16 cells in log-phase growth in 400 μl of complete medium (0.4-cm-gap cuvette [BioRad]). Following a 10-min incubation at 25°C, the culture was pulsed with 200 V at 960 μF. Cells were immediately diluted into 20 ml of complete medium.

Lytic infection was induced in LCLs by electroporation with 25 μg of pSVNaeI Z and addition of 20 ng of tetradecanoyl phorbol acetate per ml to the culture medium (44–46).

Infection of primary B lymphocytes. Primary human B lymphocytes were infected with filtered culture supernatant (0.45-μm filter) obtained from P3HR-1 c16 cells 6 days after transfection (44–46). For some initial experiments, virus was concentrated by centrifugation at 15,000 × *g* for 2 h before filtration. Virus was incubated for 2 h at 37°C with 10^7 T-cell-depleted human peripheral blood mononuclear cells. Infected cells were resuspended in complete medium and plated out at 5×10^4 cells per well in a total volume of 150 μl in 96-microwell plates. The cultures were fed at 14 days postplating with 100 μl of complete medium. LCLs were macroscopically visible 3 to 5 weeks after plating.

Polymerase chain reaction (PCR) analyses. (i) PCR primers. Oligonucleotides were synthesized on an Applied Biosystems model 391 synthesizer. The primers which amplify distinctive fragments for type 1 and type 2 EBNA 3A, 3B, and 3C have been described previously (37, 44–46). The EBNA 3A primers correspond to bases 93,596 to 93,665 and 93,871 to 93,852, and the EBNA 3C primers correspond to bases 99,742 to 99,961 and 100,095 to 100,076 in the prototype type 1 EBV DNA sequence (2). The *Bam*HI-C–*Alu*I primers correspond to bases 6861 to 6880 and 7017 to 6998 (2). These primers amplify a 157-bp fragment from both B95-8 and P3HR-1 EBV DNAs. Only B95-8 EBV DNA is digested by *Alu*I, into two smaller fragments of 52 and 105 bp (1). The B95-8 deletion primers correspond to bases 151,861 to 151,880 and 152,222 to 152,203 (2). These primers amplify a 362-bp fragment from B95-8 DNA and do not amplify any fragment from P3HR-1 DNA because of 12 kb of intervening DNA sequence. Primers which amplify an EBV DNA fragment which contains a *Bam*HI site in EBV B95-8 DNA and not in P3HR-1 DNA correspond to nucleotides 122,206 to 122,226 and 122,475 to 122,455 (2, 10).

(ii) PCR and restriction endonuclease digestion. DNA was prepared from 0.5×10^5 to 1×10^5 cells. Cells were resuspended in 0.2× isotonic phosphate-buffered saline, boiled for 10 min, and digested with 1/10 volume of 10-mg/ml proteinase K (Sigma) for 60 min at 55°C. Proteinase K was inactivated by incubation at 95°C for 20 min. DNA was amplified in a Perkin-Elmer thermal cycler machine, using 10 to 20 μl of DNA in a 50-μl reaction mixture. PCR-amplified DNA was analyzed by electrophoresis in 1% ME agarose–2% (wt/vol) NuSieve agarose (FMC) gels and visualized by staining with ethidium bromide. For restriction endonuclease digestion, 10 μl of DNA from PCRs was digested with a particular enzyme in 30 μl of buffer recommended by the manufacturer.

Immuno- and Southern blot analyses. Expression of EBV proteins was evaluated by electrophoresis of denatured infected-cell proteins in 7.0% denaturing polyacrylamide gels and immunoblotting with human EBV immune serum (44–46). For Southern blots, 15 μg of cell DNA was digested with appropriate restriction enzymes in 200 μl. The DNA was then precipitated, dried briefly, and resuspended in 40 μl of loading buffer. The DNA fragments were size fractionated by electrophoresis through a 0.8% agarose gel. DNA was then transferred to an activated nylon membrane (GeneScreen Plus; New England Nuclear). The filters were hybridized with ^{32}P-labeled EBV DNA probes.

RESULTS

Overlapping library of type 1 EBV DNA fragments in cosmids. Cosmid clones were constructed from linear and episomal type 1 EBV DNA of the B95-8 strain, to take advantage of the published DNA sequence and the smaller genome (2, 35). EBV B95-8 is lacking a 12-kb DNA segment, which does not adversely affect any known phenotypic characteristic. The entire genome could be included in five cosmids with 5- to 20-kb overlaps (Fig. 1).

Type 1 EBV recombinants following transfection of five overlapping EBV DNA fragments into cells containing a defective type 2 EBV. P3HR-1 cells are infected with a replication-competent type 2 EBV which lacks a DNA segment that encodes the last two exons of EBNA LP and the entire EBNA 2 gene (10, 15, 18, 35). As a result of the deletion, the P3HR-1 EBV is unable to transform cells (6, 9, 30, 40). P3HR-1 cells transfect well, with about 10% taking up plasmid and cosmid DNAs (5, 43–46). Transfected plasmid DNA expressing the Z immediate-early transactivator of EBV lytic replication induces replication of the endogenous P3HR-1 EBV genome, and 1 in 10^5 P3HR-1 EBV genomes undergoes recombination with the transfected EBV DNA *Eco*RI-A cosmid which spans the segment deleted from P3HR-1, rescuing transformation by restoring the complete EBNA LP and EBNA 2 genes (5, 6, 9, 22–24, 43–46). These recombinants can then be specifically identified and cloned because of their ability to transform primary B lymphocytes into LCLs. This marker rescue of transformation can be used to select for recombinants which have also recombined with a second, nonlinked, transfected cosmid EBV DNA fragment, since about 10% of the molecules which have recombined with the first transfected EBV DNA fragment will pick up most of the second EBV DNA (16, 22, 23, 44–46).

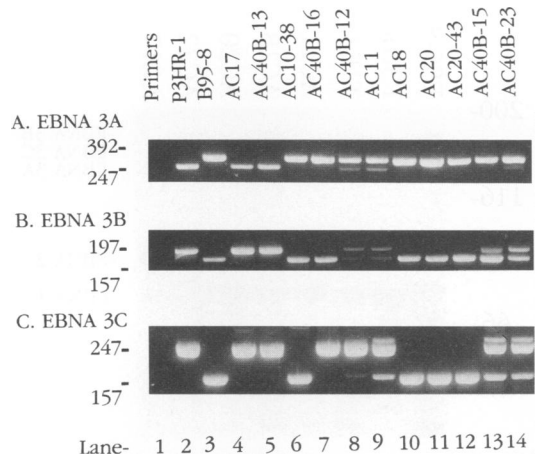

FIG. 2. PCR analysis of the EBV recombinants with primers which distinguish between type 1 and 2 EBNA 3 genes (37, 44–46). EBNA 3A primers amplify a 237- or 276-bp fragment from type 2 (P3HR-1) or type 1 (B95-8) EBV, respectively. EBNA 3B primers amplify a 218- or 183-bp fragment from type 2 or type 1 EBV, respectively. EBNA 3C primers amplify a 246- or 153-bp fragment from type 2 or type 1 EBV, respectively. Lanes: 1, control amplification with primers and no added DNA; 2 and 3, control amplifications of DNAs from type 2 P3HR-1 and type 1 B95-8 EBV, respectively; 4 and 5, amplifications of DNAs from LCLs infected with an EBV recombinant with type 2 EBNA 3 genes; 6 to 14, amplifications of DNA from LCLs infected with type 1 EBNA 3A EBV recombinants. Sizes (base pairs) of *Rsa*I fragments of ϕX174 are shown on the left. AC40B-15 in lane 13 yielded a barely visible type 2 237-bp DNA as well as the clearly visible type 1 276-bp DNA in the EBNA 3A panel.

In the experiments described here, transfection with five overlapping cosmids that encompass the entire EBV genome (Fig. 1) is now used to evaluate whether the frequency of incorporating the second cosmid would be increased by providing cosmids which could potentially provide linkage to the marker-rescuing *Eco*RI-A fragment and whether the overlapping cosmids would recombine among themselves to reconstitute a complete EBV genome. The P3HR-1 genome is type 2 and can be distinguished from the type 1 B95-8 EBV genome at the EBNA and other loci as a consequence of type- or strain-specific differences (37, 44–46).

Three independent series of experiments were done over a 3-year interval. In the first experiment 28 recombinant EBV-infected LCLs were derived, in the second experiment 105 were derived, and in the third experiment 152 were derived. Since the frequency of incorporating type 1 EBNA 3A into transformation marker-rescued recombinants following two-cosmid EBV DNA transfections into P3HR-1 cells had been previously established to be 10 to 11% (44–46), the new EBV recombinants were first analyzed for type 1 and type 2 EBNA 3A so that the relative frequency of EBNA 3A recombination after transfection with all five cosmid EBV DNA fragments could be compared with the previous results. Representative PCR analyses of LCLs infected with EBV recombinants and of cells lines infected with type 1 B95-8 and type 2 P3HR-1 EBVs are shown in Fig. 2. AC17 and AC40B-13 are infected with recombinants which have only the type 2 EBNA 3A, 3B, and 3C genes from P3HR-1. AC10-38, AC18, AC20, and AC20-43 are infected with recombinants which have only the type 1 EBNA 3A, 3B, and 3C genes. AC40B-16 is infected with one of the two recombinants which has incorporated type 1 EBNA 3A and 3B and is type 2 at EBNA 3C. AC40B12, AC11, AC40B-15, and AC40B-23 are each coinfected with a type 1 EBNA 3A, 3B, and 3C recombinant and with parental P3HR-1 EBV (the type 2 EBNA 3A is evident in other amplifications of AC40B-15 but not in those shown in Fig. 2). Such coinfected LCLs usually lose the coinfecting P3HR-1 EBV genome with continued passage of the cells, since it is not contributing to the ability of the cells to proliferate (16, 44–46).

With PCR primers for type 1 or type 2 EBNA 3A, of the 285 LCLs analyzed from the three series of experiments, 74 (or 26%) were infected with type 1 EBNA 3A recombinant virus. This recombination frequency, is more than double that observed in previous experiments in which P3HR-1 cells were transfected with *Eco*RI-A and *Sal*I-C (44–46). Even more startling was the finding that although the frequency of incorporation of type 1 EBNA 3C was only 3% in previous experiments with *Eco*RI-A and *Sal*I-C cotransfections (44–46), probably because of the proximity of EBNA 3C to an end of *Sal*I-C, the recombination frequency for type 1 EBNA 3C in these experiments with all five cosmids was 26%, identical to that for EBNA 3A. In almost every instance type 1 EBNA 3A-positive recombinants were also type 1 EBNA 3C positive. Two LCLs were infected with recombinants which were positive for type 1 EBNA 3A and negative for type 1 EBNA 3C, and two were infected with recombinants which were type 1 EBNA 3A negative and EBNA 3C positive. The higher frequency of incorporation of type 1 EBNA 3A could be partly due to the use of *Sal*I-E and -C as one fragment in these experiments and as two separate fragments in the previous experiments, thereby allowing *Sal*I-E to link *Sal*I-C to the marker-rescuing fragment, *Eco*RI-A, through the *Sal*I-E and *Eco*RI-A overlap (Fig. 1). The higher frequency of incorporation of EBNA 3C along with EBNA 3A could also be due in part to the presence of EBNA 3C in the overlap between *Sal*I-C and *Eco*RI-B (for which there was no selection, except through a possible linkage through *Sal*I-B and *Sna*BI-B to *Eco*RI-A [Fig. 1]). However, subsequent analyses revealed that most of the increased frequency of incorporation of EBNA 3A and 3C is due to virus genomes arising from recombination among the five transfected cosmids.

To investigate further the basis for the higher type 1 EBNA 3A and 3C incorporation into EBV recombinants, the recombinants were analyzed for other markers which distinguish between the transfected cosmid DNA fragments and the P3HR-1 EBV genome. From the first two series of experiments, 6 of the 14 type 1 EBNA 3A and 3C EBV recombinants which were also checked for the 12-kbp deletion at bases 152,012 to 152,013, which is uniquely characteristic of the B95-8 strain (35), were positive for the deletion. Five of these six also had the *Alu*I site at base 6912 which is in the B95-8 and not in the P3HR-1 EBV strain (1, 2). Representative PCR analyses for the B95-8 deletion or for the *Alu*I site are shown in Fig. 3. AC11, AC18, AC20, AC20-43, AC40B-15, and AC40B-23 are infected with recombinants which have the B95-8 deletion site, while AC17, AC40B-13, AC10-38, AC40B-16, and AC40B-12 are infected with recombinants which are not deleted at this site. The five recombinants in Fig. 3 which have the B95-8 deletion site also have the *Alu*I site characteristic of B95-8, while only one of the recombinants which lacks the B95-8 deletion site has the *Alu*I site characteristic of B95-8-derived DNA. To summarize the results from the first two experiments, 5 of 14 type 1 EBNA 3A recombinants had the B95-8 characteristic *Alu*I site at base 6912, type 1 EBNA 2 at bases 45,644 to 52,450, type 1 EBNA 3A and 3C at bases 93,596 to 100,095, and the B95-8 deletion site at bases 152,012 to 152,013. The *Alu*I site is unique to the transfected *Sna*BI-B fragment, type 1 EBNA 2 is unique to the *Eco*RI-A fragment,

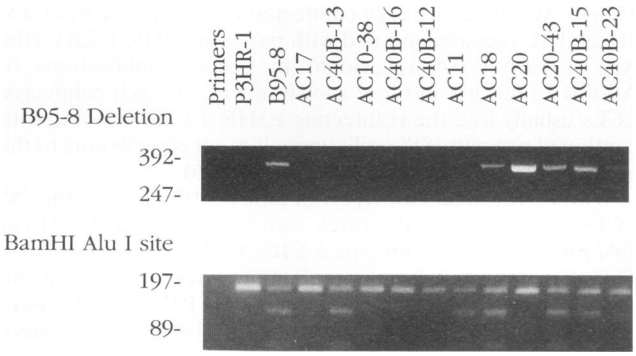

FIG. 3. PCR analysis of EBV recombinants with primers detecting differences between the type 1 B95-8 and type 2 P3HR-1 EBV strains at the site of the B95-8 deletion or at the site of a B95-8 unique *Alu*I site. The B95-8 deletion primers yield a 362-bp fragment from a B95-8-derived EBV DNA and no fragment from P3HR-1-derived EBV DNA because of failure to amplify across the 12 kbp of additional DNA in P3HR-1 (10, 35). The primers flanking the *Alu*I site at base 6912 in B95-8-derived EBV DNA amplify a 157-bp fragment from both B95-8 and P3HR-1-derived EBV DNAs, but only B95-8-derived DNA has an *Alu*I site which is partially digested into two smaller fragments (1). The primers lane shows a control amplification with primers and no added DNA. Sizes (base pairs) of *Rsa*I fragments of ϕX174 are shown on the left. The AC11 amplification in the B95-8 deletion panel yielded a fragment identical in size to the B95-8 fragment which was more obvious on the original photograph.

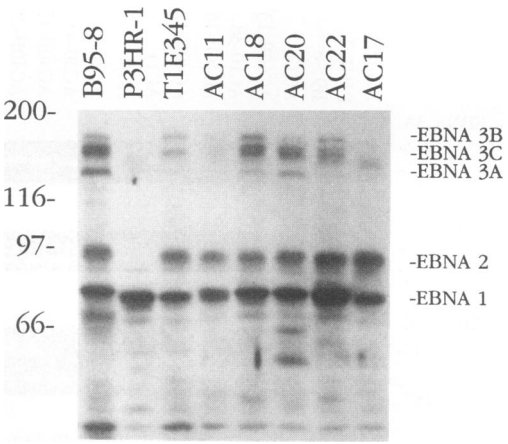

FIG. 4. Immunoblot analysis of proteins from representative LCLs. Immunoblots were incubated with a 1/10 dilution of a human immune serum which identifies EBNA 1 and type 1 EBV EBNA 2, 3A, 3B, and 3C and has only weak reactivity with the type 2 EBNA 3s. T1E345, AC11, AC17, AC18, AC20, and AC22 are cell lines infected with transformation marker-rescued virus from P3HR-1 cells and express type 1 EBNA 2, which is missing from P3HR-1 EBV (lane 2). AC indicates cell lines infected with recombinants resulting from transfection of P3HR-1 cells with all five cosmids. T1E345 and AC17 have the P3HR-1 EBNA 1, while AC11, AC18, AC20, and AC22 have the larger B95-8 EBNA 1. AC17 EBV has type 2 EBNA 3A, 3B, and 3C, while AC18, AC11, AC20, and AC22 EBVs have type 1 EBNA 3A, 3B, and 3C. T1E345 is an EBV recombinant from a previous series of experiments using second-site recombination and has type 1 EBNA 3A and 3B and type 2 EBNA 3C (44). Size markers (kilodaltons) of protein standards are shown on the left.

type 1 EBNA 3A is unique to the *Sal*I-E/C fragment, and the B95-8 deletion is in both the *Sal*I-B and *Sna*BI-B fragments (Fig. 1). These results suggest that a third of the recombinants which incorporate type 1 EBNA 3A are derived mostly or entirely from transfected cosmid DNA.

To evaluate further whether these five viruses are composed completely of transfected cosmid EBV DNA, immunoblot analyses were done with an EBV immune human serum (Fig. 4). The serum reacts with EBNA 1 and with type 1 EBNA 2, 3A, 3B, and 3C. As expected, all LCLs were infected with EBV recombinants that express a type 1 EBNA 2 identical in size to that of the B95-8 EBV. Since the B95-8 EBNA 1 is larger than the P3HR-1 EBNA 1, this immunoblot analysis also adds another marker at the EBNA 1 site (108 to 110 kbp in the EBV genome [2]). AC11, AC18, AC20, and AC22 have the B95-8 larger EBNA 1 and express type 1 EBNA 3A, 3B, and 3C (the individual type 1 EBNA 3s are not equally evident in Fig. 4). AC17 is infected with a recombinant which has the P3HR-1 EBNA 1, as is T1E345, which was derived from a previous cotransfection with *Eco*RI-A and *Sal*I-C (44).

Further genome analysis and passage of an EBV recombinant arising from the five transfected cosmid DNAs. Southern blot analysis of DNA from LCLs digested with *Bam*HI and probed with all five cosmid DNA fragments is shown in Fig. 5. AC18 has all the expected *Bam*HI fragments of B95-8 EBV DNA, including the *Bam*HI-B and -G fragments, which are fused into a single *Bam*HI-BG fragment in P3HR-1, T1E345, and AC17 EBV DNAs (Fig. 5). In most other experiments, the presence of the *Bam*HI site between *Bam*HI-B and -G was demonstrated by PCR of a DNA fragment including the site, incubation of the PCR product with *Bam*HI, and agarose gel electrophoresis.

AC18 was analyzed more extensively as the prototype of the five recombinants derived in experiments 1 and 2. The *Bgl*II, *Pvu*II, *Sac*I, and *Xho*I digests of AC18 were compared with the digests of B95-8 and P3HR-1 DNAs by Southern blot analysis with individual probes made from *Sal*I-E/C, *Sna*BI-B, or *Sal*I-B, -F, and -N EBV DNAs (Fig. 6 and data not shown). Overall, these probes reveal most of the EBV genome other than part of *Eco*RI-A, which must be derived at least in part from the transfected cosmid DNA in order to restore EBNA LP and EBNA 2. AC18 EBV DNA was identical to type 1

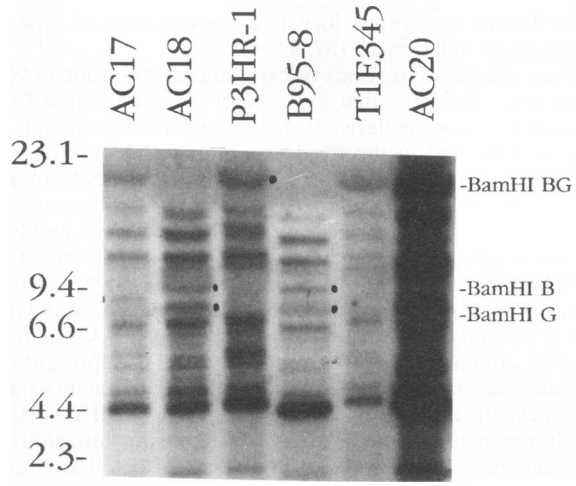

FIG. 5. Southern blot of DNA from LCLs infected with EBV recombinants or from cells infected with the parental type 2 P3HR1 or type 1 B95-8 EBV strain. DNA was digested with *Bam*HI, fragments were separated on a 0.8% agarose gel, and the probe was labeled EBV DNA prepared from the five cosmids. Sizes are in kilobase pairs.

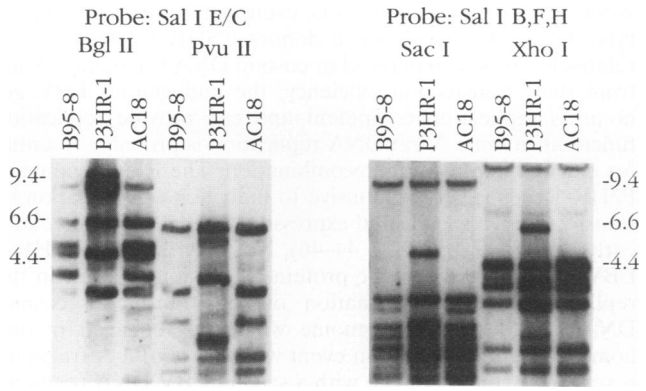

FIG. 6. Southern blots comparing the EBV DNA in an LCL (AC18) infected with an EBV recombinant with the DNA of the type 1 B95-8 or the type 2 P3HR-1 EBV strain. The DNA was cut with the indicated restriction endonuclease, and the probes are indicated. Sizes are in kilobase pairs.

B95-8 DNA and differed from P3HR-1 DNA in every polymorphism. AC18 and B95-8 differ from P3HR-1 in *Bgl*II sites at bases 81,230 and 105,414, in an *Xho*I site at base 148,225, in a *Pvu*II site at base 154,715, and in a *Sac*I site at base 158,295.

To test whether the AC18 recombinant virus was wild type in its ability to replicate and induce B-lymphocyte transformation, lytic virus infection was activated by electroporating the AC18-infected LCL in the presence of pSVNaeI Z and incubating the cells in tetradecanoyl phorbol acetate. Cell-free virus was harvested, filtered through a 0.45-μm filter, and used to infect and transform primary B lymphocytes. Multiple LCLs arose, indicating that the AC18 EBV was able to replicate in LCLs and to growth transform primary B lymphocytes into LCLs. The AC18-infected LCLs were identical to LCLs infected with other EBV recombinants or with B95-8 EBV in their initial appearance, outgrowth, and subsequent growth in culture (data not shown). PCR analysis of four AC18 progeny recombinant EBV-infected LCLs with EBNA 3A or B95-8 deletion-specific primers identified the B95-8 characteristic 276-bp type 1 EBNA 3A and the 362-bp deletion site DNA fragments, identical to the case of the parental AC18 EBV DNA (Fig. 7).

Differences in frequencies of incorporation of markers on transfected DNA fragments: incorporation of the B95-8 deletion into EBV recombinants rarely occurs other than as a result of recombination among the transfected cosmids. In the third experiment, 152 type 1 EBNA LP and EBNA 2 marker-rescued recombinants were derived, and, as in the first two experiments, 26% (40 of 152) had also incorporated the EBNA 3A and 3C genes. The *Bam*HI site characteristic of B95-8 DNA was incorporated into 21% (32 of 152) of the recombinants. The slightly lower frequency of incorporation of the *Bam*HI site, despite its presence in both the transfected *Eco*RI-B and *Sal*I-B fragments, is evidence that part of the slightly higher frequency of incorporation of EBNA 3A is due to the overlap between *Sal*I-E/C and the transformation marker-selected *Eco*RI-A DNA (Fig. 1).

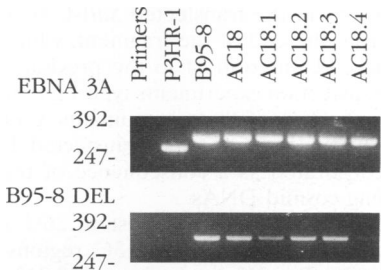

FIG. 7. PCR analysis of DNA from LCLs infected with the EBV recombinant from the AC18 LCL compared with that of DNA from the parental AC18 LCL or of DNA from the B95-8 or P3HR-1 EBV strain. The B95-8 deletion PCR is of particular significance, since that deletion is unique to virus derived from the B95-8 genome. Sizes (base pairs) of *Rsa*I fragments of ϕX174 are shown on the left.

In contrast to the high frequency of incorporation of type 1 EBNA 3A, EBNA 3C, or the B95-8 *Bam*HI-BG site, the frequency of incorporation of the B95-8 deletion site was only 12% (18 of 152), despite the presence of this site in both the *Sal*I-B and *Sna*BI-B fragments (Fig. 1). This lower frequency is probably due to an intrinsically low efficiency of recombination of the transfected *Sal*I-B and *Sna*BI-B fragments with the P3HR-1 genome near the site at bases 152,012 to 152,013 as a consequence of the 12-kbp DNA deletion from the transfected DNAs relative to P3HR-1 DNA. In fact, in parallel experiments with cotransfection of *Eco*RI-A and B95-8 *Sna*BI-B into P3HR-1 cells, the frequency of second-site incorporation of the B95-8 deletion site at bases 152,012 to 152,013 was nil (0 of 92).

Further analyses of the 18 recombinants from the third experiment which had incorporated the B95-8 deletion site provided evidence that recombination of this site with the P3HR-1 genome is inefficient except for incorporation of this site into recombinants as a consequence of recombination among the five transfected cosmids. Of the 18 recombinants, 15 had B95-8 markers of the *Alu*I site at base 6912, the type 1 EBNA LP and EBNA 2 sites at bases 45,644 to 52,450, the type 1 EBNA 3A and 3C sites at bases 93,596 to 100,095, the B95-8 size EBNA 1 at bases 108,000 to 110,000, the B95-8-specific *Bam*HI site between *Bam*HI-B and -G at base 122,313, and the B95-8 deletion site at bases 152,012 to 152,013 (Fig. 1). Thus, 15 of the 18 B95-8 deletion site-positive, marker-rescued recombinants from the transfection of five overlapping cosmids had markers at 30- to 40-kbp intervals throughout the genome characteristic of the transfected cosmid DNA and showed no evidence for recombination with the P3HR-1 genome. Only three marker-rescued recombinants which had incorporated the B95-8 deletion site did not incorporate all other markers from transfected DNA.

Several marker-rescued recombinant viruses also recovered in experiment 3 were recombinant for the marker-rescuing *Eco*RI-A fragment and only type 1 EBNA 3A and 3C, only the *Alu*I site, only the *Bam*HI site between *Bam*HI-B and -G, or only the B95-8 deletion, indicating an assortment of interactions between the transfected fragments and the endogenous P3HR-1 EBV genome.

An EBV recombinant which has incorporated type 1 EBNA 3A usually incorporates a third or fourth marker from the transfected cosmid DNAs and frequently has all seven markers characteristic of the transfected DNAs. The 40 of 152 EBV recombinants from the third experiment which had incorporated type 1 EBNA 3A were analyzed further for the *Alu*I site present only on the transfected *Sna*BI fragment, for the EBNA 3C site present on both the transfected *Sal*I-E/C and *Eco*RI-B fragments, for the B95-8 EBNA 1 (by immunoblot, which can distinguish the B95-8-encoded EBNA 1 from the smaller P3HR-1-encoded EBNA 1), for the *Bam*HI site polymorphism on both the transfected *Eco*RI-B and *Sal*I-B fragments, and for the B95-8 deletion on both the transfected *Sal*I-B and *Sna*BI-B fragments. All except one of the type 1 EBNA 3A-positive

recombinants also had type 1 EBNA 3C. Of the 40 EBNA 3A-positive recombinants, 33 (or 82%) had at least one marker from a transfected DNA fragment other than *Eco*RI-A or *Sal*I-E/C. Of the 40 type 1 EBNA 3A-positive recombinants, 24 (or 60%) had at least one marker characteristic of two additional transfected DNA fragments. Of the 40 EBNA 3A-positive recombinants, 15 (or 37%) had all seven markers characteristic of the transfected DNAs. These data indicate that a DNA which has participated in one homologous recombination event has a very high probability of participating in several subsequent homologous recombinations. Of the 152 recombinants, 15 (or 10%) have all seven markers characteristic of the transfected cosmid DNAs and no markers indicative of recombination with the P3HR-1 EBV genome.

DISCUSSION

These experiments demonstrate that infectious, replication-competent, and transformation-competent EBV recombinants can be generated by homologous recombination among five overlapping cosmid-cloned EBV DNA fragments when these fragments are transfected into cells harboring a lytic replication-competent EBV genome. Approximately 10% of all recombinants selected for a marker carried on one of the transfected DNA fragments arose from recombinations among the cosmids without any evidence for recombination between the transfected cosmids and the endogenous P3HR-1 genome. This is a new general strategy for constructing EBV recombinants which are specifically mutated at any site in the EBV genome.

In the specific experiments described, marker rescue of the phenotype of growth transformation is used to select for recombinants in cells harboring the transformation-defective P3HR-1 genome. Other selection strategies can be employed. For the derivation of recombinants by transfection into cells infected with other EBV strains, positive selection markers such as a hygromycin phosphotransferase expression cassette, cloned into one of the transfected EBV DNA fragments, could be used for selection and identification of recombinant EBV genomes after infection of primary B lymphocytes or of non-EBV-infected B lymphoma cells (20, 25–28, 48). The ability to use distant nonlinked markers simplifies the prokaryotic cloning and the experimental design. Once the phenotypic manifestations of insertion of a marker at a site in the genome are characterized, the marker can be used to select for mutations at any other site.

An important precedent for these experiments was the creation of infectious PRV from subgenomic DNA fragments by transfecting four or five overlapping DNA fragments into cells which are permissive for PRV replication (47). On the basis of those experiments and recent experiments with passage of EBV recombinants through non-EBV-infected Burkitt lymphoma cells (26, 27), non-EBV-infected B lymphoma cell lines could be used as hosts for creating EBV recombinants following transfection with the five overlapping EBV DNA cosmids and pSVNael Z to induce EBV replication. However, non-EBV-infected B lymphoma cells are at best only partially permissive for EBV replication. Our attempts to generate infectious EBV by transfecting overlapping DNA fragments and pSVNael Z into noninfected B lymphoma lines have not as yet been successful, most likely because of low permissivity for virus replication in most non-EBV-infected B lymphoma cells (25–27, 48).

Among B lymphoma cell lines, P3HR-1 cells offer several advantages. LCL outgrowth is a very powerful selection strategy since there is little or no background under the conditions employed in these experiments, even with primary B lymphocytes from EBV-seropositive donors. P3HR-1 cells transfect relatively easily with plasmid or cosmid DNA fragments. Aside from their transfection efficiency, the endogenous EBV genome is replication competent and can provide replication functions in *trans*. Viral DNA replication is probably essential for efficient homologous recombination. The EBV genome in P3HR-1 cells is very responsive to induction of replication by transfection with a plasmid expressing the EBV Z immediate-early gene (5, 7, 9, 22–24, 44–46). Not only does the P3HR-1 EBV genome provide lytic protein expression to assist in the replication and recombination of the transfected cosmid DNAs, but a P3HR-1 genome which has engaged in one homologous recombination event with the *Eco*RI-A fragment is very likely to recombine with a second EBV DNA fragment (44–46). Many of the EBV recombinants generated in the experiments reported here did not consist entirely of transfected cosmid DNA but had a marker(s) from P3HR-1 and from two, three, or four transfected cosmid DNAs. The vast excess of nonrecombinant P3HR-1 produced in the transfected cells is also useful in providing wild-type genes in *trans* when the EBV recombinant carries a specific mutation in an essential gene. By adjusting the number of cells being infected by the pooled virus from the transfection, many of the cells which are infected with one of the recombinants can also be infected with a parental P3HR-1 EBV (16, 46).

The results from this study have other important implications for the generation of EBV recombinants. First, the strategy of transfection with five overlapping cosmid EBV DNAs improves the efficiency of recovery of recombinants which have incorporated nonlinked, nonselected markers on cotransfected DNA fragments. The efficiency of incorporation of type 1 EBNA 3A increased from 11% in previous two-cosmid cotransfections (44–46) to 26% with all five cosmids. The *Bam*HI site at base 122,313, which maps 80 kbp from the positive selection site at bases 45,644 to 52,450, was incorporated into 21% of the recombinants. The increased incorporation efficiency compared with that seen in previous two-cosmid EBV DNA marker rescue experiments is largely due to recombination among the five transfected cosmid DNAs but is also probably facilitated by the overlap of the *Sal*I-E/C fragment with the marker-rescuing *Eco*RI-A fragment in these experiments.

Second, in previous experiments with the transfected *Sal*I-C fragment, the frequency of incorporation of the type 1 EBNA 3C was only 3%, far different from the 10 to 11% incorporation of type 1 EBNA 3A in those experiments (44–46). Since the type 1 EBNA 3A and 3C genes are both nearly 90% homologous to the corresponding type 2 genes in the P3HR-1 genome, the different incorporation efficiencies were attributed to their different locations in the transfected *Sal*I-C fragment. EBNA 3A is 12 kb from the end of the fragment; while EBNA 3C is 4 kb from the end. In contrast to the previous result, in the five-cosmid transfection experiments type 1 EBNA 3C was also incorporated into 26% of the recombinants. Clearly, a nonselected marker near the end of a transfected DNA is more efficiently incorporated as a consequence of transfecting the five overlapping cosmid DNAs.

Third, although there was a consistent 26% incorporation frequency for the EBNA 3A and 3C regions and a 21% frequency for the *Bam*HI site at base 122,313, this level of recombination was not observed for all regions of the EBV genome. Most notably, the B95-8 deletion site was incorporated with a very low frequency other than as a result of recombination among the five transfected cosmids. Overall, the incorporation frequency for the B95-8 deletion site in

experiment 3 was only 18 of 152, or 12%. Of these, 15 recombinants had only markers from the transfected cosmid DNAs. Exclusive of those recombinants, which probably result from recombination among the five cosmid DNAs, incorporation of the B95-8 deletion site into EBV recombinants which showed any evidence for interaction with the P3HR-1 genome occurred in only 2% of the 152 (3 of 152) recombinants. This frequency was unexpectedly low, considering that the B95-8 deletion site is contained in the overlapping segments of both the *Sal*I-B and *Sna*BI-B DNA fragments. In the case of type 1 EBNA 3C, presence on two overlapping fragments resulted in a 26% incorporation frequency. A similar low frequency of incorporation of the B95-8 deletion was observed with *Eco*RI-A and *Sna*BI-B cotransfection into P3HR-1 cells (0 of 92). One explanation for the decreased incorporation of the B95-8 deletion site is that the interaction between the *Sal*I-B or *Sna*BI-B fragment and the homologous regions in the endogenous P3HR-1 EBV genome is greatly influenced by the presence of 12 kb of DNA in P3HR-1 at that site. Alignment of the homologous sequences of the P3HR-1 EBV genome and the transfected B95-8 DNA fragments presumably requires P3HR-1 to loop out 12 kb of DNA, and this may result in decreased homologous recombination. Direct evidence in support of specific selection against incorporating the B95-8 deletion site is the finding that among the 25 type 1 EBNA 3A-positive recombinants which had incorporated at least one marker from the P3HR-1 genome in the third experiment, 11 had incorporated the *Alu*I site from the transfected *Sna*BI fragment and only 3 had incorporated the B95-8 deletion site even though the site was present not only on the *Sna*BI-B fragment but also on the *Sal*I-B fragments. These findings are in marked contrast to the efficient incorporation of EBNA 3C, which is at a similar distance from the selection marker and was incorporated into 16% of the recombinants which had at least one marker from P3HR-1 (Fig. 1).

Thus, the five-cosmid-based EBV genome reconstruction system described here is in general superior in efficiency to the previously used P3HR-1 second-site recombination system and has particular utility for the incorporation of markers near the ends of the transfected DNAs or for the incorporation of fragments having extensive deletions or regions of nonhomology. The B95-8 deletion markedly decreased the incorporation of the deletion site into genomes arising in part from P3HR-1 DNA but was readily recoverable in the recombinants which appear to have arisen entirely from transfected cosmid DNA. Other manipulations of EBV DNA sequences internal to the overlapping regions or additions of heterologous DNA should have little or no effect on the frequency of virus arising from recombination among the five transfected cosmids.

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