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Clonal Propagation of Epstein-Barr Virus (EBV) Recombinants in EBV-Negative Akata Cells

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We lack a host cell supporting an efficient lytic replication of Epstein-Barr virus (EBV). Recently, we isolated EBV-negative cell clones from the Akata cell line (referred as Akata$^-$ [N. Shimizu, A. Tanabe-Tochikura, Y. Kuroiwa, and K. Takada, J. Virol. 68:6069–6073, 1994]). Since the parental Akata line is one of the highest EBV producers, we examined whether Akata$^-$ cells had become a good host for EBV propagation. The parental Akata cells have about 20 copies of EBV plasmid per cell. A drug resistance gene was inserted into one of them by homologous recombination. The resultant virus preparation, a mixture of wild-type and recombinant EBV, was used to infect Akata$^-$ cells. After incubation in the selective medium, drug-resistant Akata$^-$ cell clones were isolated and proved to be infected with recombinant EBV only. By treatment of the cells with anti-immunoglobulin antibodies, a large amount of recombinant EBV (i.e., more than 10 μg/1-liter culture) was produced. In contrast, three other B-lymphoma lines, BJAB, Ramos, and Louckes, were nonpermissive for virus replication. These results indicate that Akata$^-$ cells are suitable for propagation of recombinant EBV clonally, which becomes a powerful tool for determining EBV genetics and which makes it possible to use EBV as a vector for gene therapy.

Primary B lymphocytes are largely nonpermissive for Epstein-Barr virus (EBV) replication. In recent years, many attempts have been made to generate EBV recombinants (4, 7, 12, 13, 15, 22, 23, 25). However, since we lack a host cell capable of supporting an efficient lytic replication of EBV, it has been difficult to propagate EBV recombinants clonally and in large quantities. The Akata cell line is derived from an EBV-positive Burkitt's lymphoma of a Japanese patient (17, 19, 20). The Akata line is one of the highest EBV producers and has a unique property in that cells remain in a latent state under ordinary culture conditions and start to produce EBV efficiently after cross-linking of cell surface immunoglobulins (Igs) with anti-Ig antibodies. The Akata line is now commonly used not only as a virus source but also as an in vitro model for studying the reactivation cascade of latent EBV. Recently, we noticed that EBV is lost from some of the Akata cells during cultivation and isolated EBV-negative cell clones (referred as Akata$^-$) (16). The possibility has been proposed that EBV-negative Akata cell clones are reinfectable with EBV and permissive for virus replication upon treatment with anti-Ig antibodies.

We have investigated four EBV-negative B-lymphoma cell lines, Akata$^-$ (16), BJAB (10), Louckes (24), and Ramos (9), for their permissiveness for EBV replication. We show here that Akata$^-$ cells are reinfectable with EBV, are permissive for virus replication in response to anti-Ig antibody treatment, and make it possible to isolate recombinant EBV clonally and in large quantities.

Generation of an EBV recombinant with a selectable marker at the viral TK locus. Akata cells have about 20 copies of EBV plasmid per cell. The neomycin resistance gene (Neo^r) was inserted into an EBV plasmid of Akata cells by homologous recombination. The pUC-Xneo plasmid contained the Neo^r gene under control of the simian virus 40 early promoter inserted at the *Sma*I site of BXLF1, an open reading frame encoding the EBV thymidine kinase (TK) gene, which is a homolog of herpes simplex virus type 1 that is nonessential for infection and replication (Fig. 1A). The surrounding EBV DNA could target the marker into the reading frame in an EBV plasmid expected to be nonessential. The pUC-Xneo plasmid was transfected into Akata cells by the electroporation method. Cells (5×10^6) were suspended in 500 μl of ice-cold phosphate-buffered saline containing 40 μg of pUC-Xneo DNA that was digested with *Bam*HI to release the Neo^r DNA and the surrounding EBV DNA from the pUC vector. Cells were then electroporated with an Electro Cell Manipulator 600 (BTX) at room temperature in cuvettes having a 0.2-cm electrode gap. Transfected Akata cells were cultured in 5 ml of culture medium for 2 days and then transferred to 96-well, flat-bottom plates at 10,000 cells per well in complete culture medium containing 700 μg of G418 (Life Tech) per ml. Cultures were fed every 5 days by replacement of half of the medium until colonies emerged (3 weeks).

A total of 2,467 G418-resistant cell clones appeared from a total of 5,088 wells tested. Viruses released into the supernatant of anti-Ig antibody-treated cultures were analyzed by Southern blotting (25). Four of 2,467 resistant clones proved to contain targeted recombinant EBV. Figure 1B shows four clones that contained EBV recombinants with the Neo^r DNA recombined into the expected EBV BXLF1 site, as well as nonrecombinant Akata EBV. This was evidenced by the presence of the 2.1-kb *Bam*HI X fragment and the concurrent appearance of a novel 3.7-kb *Bam*HI fragment which hybridized to both *Bam*HI X and Neo^r probes.

Clonal isolation of EBV recombinants through infection of EBV-negative B-lymphoma cells. Akata$^-$ cells (5×10^6) were infected with 1 ml of diluted (1:50) culture supernatant from G418-resistant Akata cells, which contained a mixture of wild-type and recombinant EBV. Two days after infection, cells were plated in 96-well, flat-bottom plates at 5,000 cells per well with complete medium containing 700 μg of G418 per ml. After 3 weeks, 125 G418-resistant clones appeared from a total

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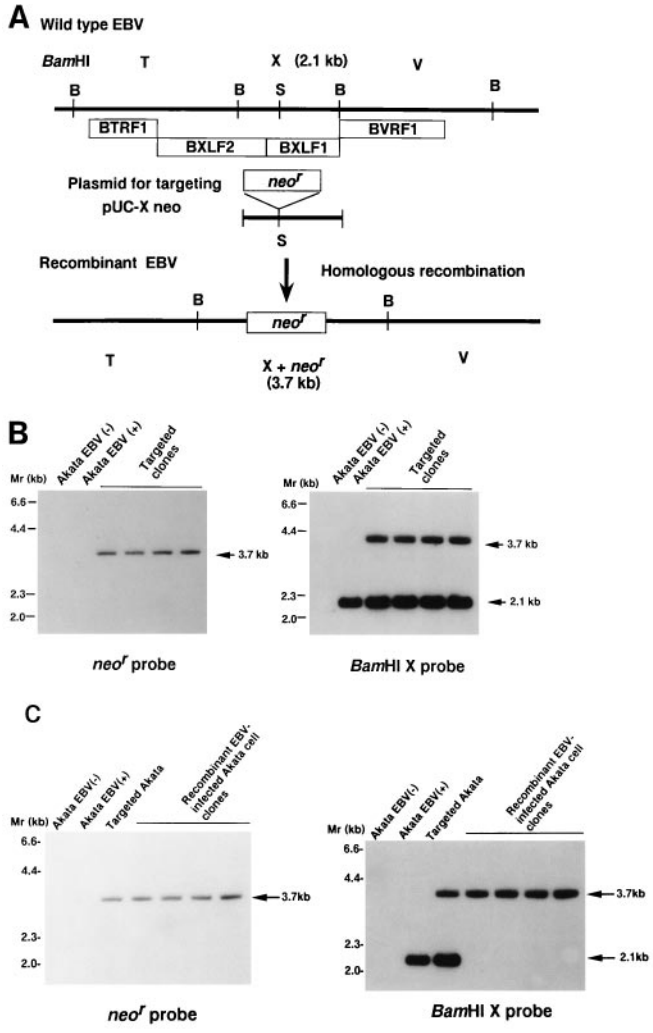

FIG. 1. (A) Schematic representation of plasmid constructs used to insert the Neo^r gene (*neo^r*) into the EBV genome. A map of the BXLF1 region (1) is shown at the top. To generate the pUC-Xneo plasmid (second line), the 2.1-kb *Bam*HI X fragment of Akata EBV DNA, which includes the EBV TK gene encoded by a leftward open reading frame, BXLF1, was cloned into the multicloning site of pUC19. The plasmid was digested with *Sma*I and treated with calf intestinal alkaline phosphatase, and then the 1,665-bp *Sal*I-*Eco*RI fragment from pDOL (11) containing the simian virus 40 promoter-driven Neo^r gene was inserted. When the pUC-Xneo plasmid had recombined into the BXLF1 site, a novel 3.7-kb *Bam*HI fragment appeared (bottom line). The cutting sites of the restriction enzymes are shown (B, *Bam*HI; S, *Sma*I). (B) Southern blot analysis of targeted Akata cell clones. Two micrograms of cellular DNAs was digested with *Bam*HI, blotted, and hybridized with the Neo^r or *Bam*HI X probe by the procedure described previously (25). Mr, molecular size markers. (C) Southern blot analysis of recombinant EBV-infected Akata$^-$ cell clones. Two micrograms of cellular DNAs was digested with *Bam*HI restriction enzymes, blotted, and hybridized with the Neo^r or *Bam*HI X probe.

of 480 wells tested. Twelve clones were examined and proved to be infected with only recombinant EBV. Examples of results showing evidence for infection with only a recombinant virus and not with a parental Akata virus are given in Fig. 1C. Southern analysis indicated that the EBV DNA in Akata$^-$ cells has the Neo^r DNA exactly recombined into the BXLF1 site. This was evidenced by the absence of a 2.1-kb *Bam*HI X fragment and the presence of a 3.7-kb *Bam*HI DNA fragment which hybridized to both *Bam*HI X and Neo^r probes.

Similarly, three other B-lymphoma cells, BJAB (10), Louckes (24), and Ramos (9), were infected with a mixture of wild-type and recombinant EBV and incubated in the selective media. G418-resistant cell clones were isolated and used for further analysis.

Permissiveness for EBV lytic infection of recombinant EBV-infected B-lymphoma cells. All recombinant EBV-infected B-lymphoma cell clones were virtually 100% positive for EBNA by anticomplement immunofluorescence with a polyvalent human antiserum (25). Attempts were made to induce EBV lytic infection in the recombinant EBV-infected cell clones by cross-linking of cell surface Ig or by transfection with the BZLF1 immediate-early transactivator gene (3, 5, 21) coupled with treatment with tetradecanoyl phorbol acetate (TPA) (26). The response to induction of lytic infection was assessed by expression of EBV early antigen (EA) and viral capsid antigen (VCA). The Western blot (immunoblot) analysis showed that Akata$^-$ cell clones were permissive for lytic infection, as were parental Akata cells (Fig. 2). The immunofluorescence assay showed that in Akata$^-$ cell clones, 47 and 40% of the cells expressed EA and VCA, respectively, after anti-Ig antibody treatment (Table 1). In contrast, the other three B-lymphoma

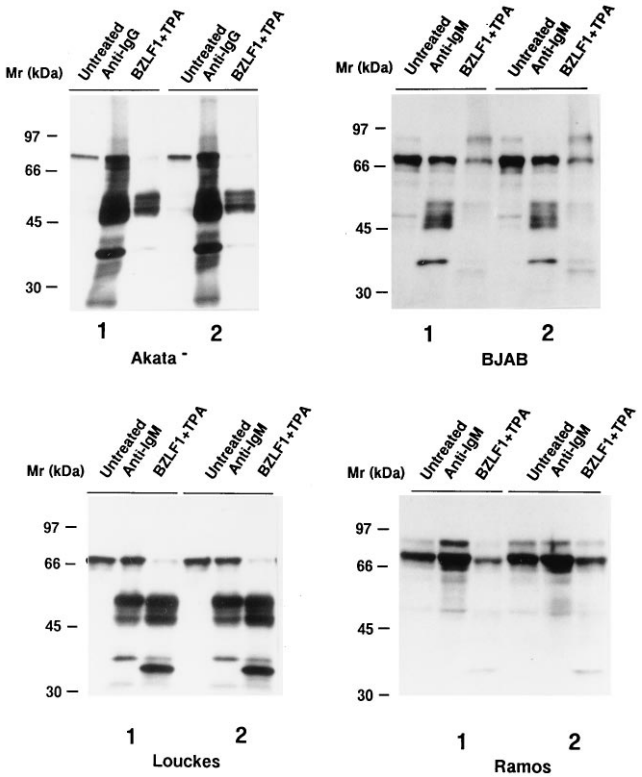

FIG. 2. Induction of lytic EBV infection in EBV-negative B-lymphoma cell clones infected with recombinant EBV. EBV replication was induced by cross-linking of cell surface Ig by anti-Ig antibodies (17, 20) or by transfection with pSG5-Z, an expression vector for the immediate-early lytic transactivator BZLF1 (3, 5, 21), coupled with TPA treatment (26). For anti-Ig antibody treatment, 5×10^6 cells were incubated in 5 ml of complete medium containing 1% (vol/vol) goat antibodies specific for human Ig (anti-IgG antibodies for Akata cells and anti-IgM antibodies for BJAB, Louckes, and Ramos cells [Cappel]) for 48 h. For transfection with the BZLF1 expression plasmid, 5×10^6 cells were suspended in 5 ml of phosphate-buffered saline containing 40 μg of closed circular pSG5-Z. Thereafter, cells were electroporated and incubated in 5 ml of complete medium containing 20 ng of TPA per ml for 3 to 5 days. Proteins from 2.5×10^5 cells were separated in 7.5% polyacrylamide gels and transferred to nitrocellulose membrane. The blots were treated with an EA- and VCA-positive serum from a nasopharyngeal carcinoma patient and peroxidase-labeled protein A. After immunostaining, the blots were developed by the enhanced chemiluminescence method (Amersham) according to the manufacturer's protocol. Mr, molecular size markers.

TABLE 1. Permissiveness for lytic infection of B-lymphoma cells infected with recombinant EBV

Cell line (no. of clones)[a]	% of:	
	EA-positive cells[b]	VCA-positive cells[c]
Akata	50.3 ± 10.5	45.8 ± 18.3
LCL	1.3 ± 1.1	0.7 ± 0.4
Akata$^-$ (12)	46.9 ± 16.2	40.3 ± 14.9
BJAB (12)	1.8 ± 0.5	<0.1
Louckes (12)	4.6 ± 4.5	1.2 ± 0.9
Ramos (12)	0.1 ± 0.1	<0.1

[a] Akata is an EBV-positive Akata cell clone. LCL is an EBV-transformed lymphoblastoid cell line. Akata$^-$, BJAB, Louckes, and Ramos are recombinant EBV-infected cell clones.

[b] Stained by the indirect immunofluorescence method with a serum sample from a patient with nasopharyngeal carcinoma (EA titer of 1:1,280).

[c] Stained by the direct immunofluorescence method with fluorescein isothiocyanate-conjugated IgG fraction obtained from mouse ascites containing monoclonal antibodies specific for VCA (gp110) (18). Values are means ± standard errors.

cell lines were refractory to inducing signals. EA was induced in 0.1 to ~5% of the cells, and VCA induction was only seen in Louckes cell clones in about 1% of the cells. EBV DNA amplification in recombinant EBV-infected B-lymphoma cell clones was examined by Southern analysis. As shown in Fig. 3, striking viral DNA amplification was induced in Akata$^-$ cells after anti-Ig treatment, while in other B-lymphoma cells, DNA amplification was not induced substantially.

To measure the amounts of virus produced by recombinant EBV-infected Akata$^-$ cell clones, virus was purified from the supernatants of anti-Ig-treated cells according to the method of Dolyniuk et al. (6). Viral DNA was obtained from purified virus preparations by lysis in 1% sodium dodecyl sulfate and phenol extraction and was cleaved with *Eco*RI. The DNA fragments were separated by electrophoresis in agarose gels (0.6%) and stained with ethidium bromide. The amount of viral DNA was estimated from the intensity of DNA bands. As a result, approximately 10 to 20 μg of viral DNA was obtained from 1 liter of culture supernatants.

Release of infectious EBV recombinants from Akata$^-$ clones was assayed by infection of EBV-negative Akata cells followed by G418 selection or by transformation of cord blood lymphocytes. The 50% tissue culture infectious dose for Akata$^-$ cells was $10^{3.8}$/ml, and the 50% transforming dose for cord blood lymphocytes was $10^{5.5}$/ml.

The present results show that EBV-negative Akata cells are good hosts for EBV replication. Virus yields in these clones reached 10 to ~20 μg/1-liter culture. In contrast, other B-lymphoma cells, BJAB, Louckes, and Ramos, were nonpermissive for viral replication. Some Louckes cell clones produced EA in about 5% of the cells after simultaneous BZLF1 transfection and TPA treatment but failed to produce infectious virus particles. These observations suggest the existence of a bottleneck between early lytic antigen expression and late lytic infection. Although Marchini et al. (13) reported that some EBV-negative B-lymphoma cell lines, including Louckes, could become good hosts for EBV replication, they did not show accurate EBV amounts produced in their Burkitt's lymphoma cells. Our results suggest that if some Burkitt's lymphoma cells are partially permissive for EBV replication, virus yields in these cells are extremely low compared with those in Akata$^-$ cells.

In the preceding paper (25), we generated an EBV recombinant in which the BXLF1 gene was replaced with a selectable marker. The replacement disrupted both BXLF1 and BXLF2 genes. This mutant EBV grown in Akata$^-$ cells was noninfectious, possibly because of deletion of the BXLF2 gene that is essential for membrane fusion after virus adsorption (14). Therefore, in this experiment, we generated an insertion mutant of the BXLF1 gene with the intact BXLF2 gene. The present results indicate that the BXLF1 gene is not necessary for infection, transformation, and replication and that disruption of the BXLF2 gene renders the virus noninfectious.

Together with the previous description of procedures for generating and isolating EBV recombinants (4, 7, 12, 13, 15, 22, 23, 25), these studies make possible the creation of EBV recombinants with specific mutations in any EBV gene, the cloning and passaging of these EBV recombinants, and analysis of the effects of the mutations on EBV genome structure and function. The procedure for producing recombinant EBV is summarized in Fig. 4. EBV-positive Akata cells have about 20 copies of EBV plasmid per cell. After insertion of the drug-resistant gene into an EBV plasmid of EBV-positive Akata cells by homologous recombination produced a virus preparation, a mixture of wild-type EBV and recombinant EBV was used to infect EBV-negative Akata cells. After 3 weeks of incubation in the selective media, many drug-resistant clones were isolated very easily, and most of them were infected with recombinant EBV only. By treatment of cells with anti-Ig antibodies a large amount of recombinant EBV, more than 10 μg/1-liter culture, was produced.

The Akata cell system also makes it possible to use EBV as a vector for human gene therapy. As a vector for gene therapy, EBV has several positive characteristics. EBV infects B lymphocytes with a high level of efficiency, nearly 100%. The entire viral genome is stably maintained in cells as plasmids. The genome size is 165 kb (2), and EBV has large capacities for added foreign sequences and is able to transfer and express large, intact genes. When considering the use of the EBV vector for application to humans, we need to develop an EBV

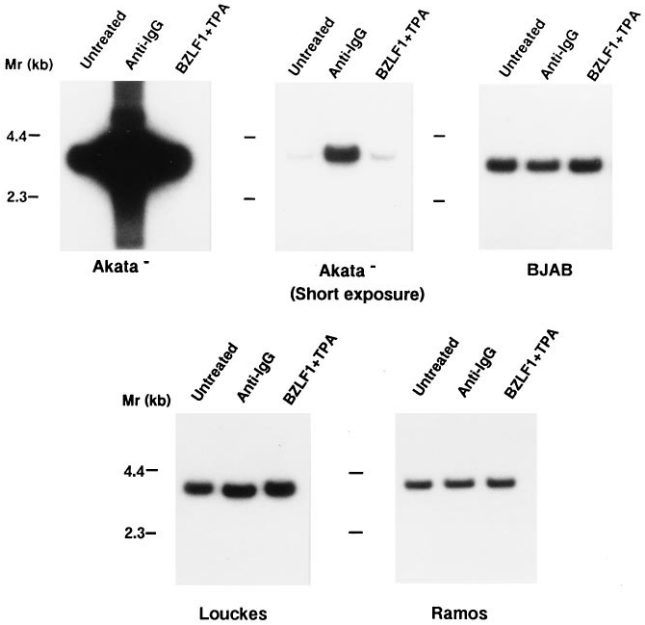

FIG. 3. Induction of EBV replication in EBV-negative B-lymphoma cell clones infected with recombinant EBV. Cells were treated to induce EBV replication with anti-Ig antibodies or TPA plus transfection of pSG-Z. Two micrograms of cellular DNAs was digested with *Bam*HI restriction enzyme, blotted, and hybridized with EBV *Bam*HI K probe. Mr, molecular size markers.

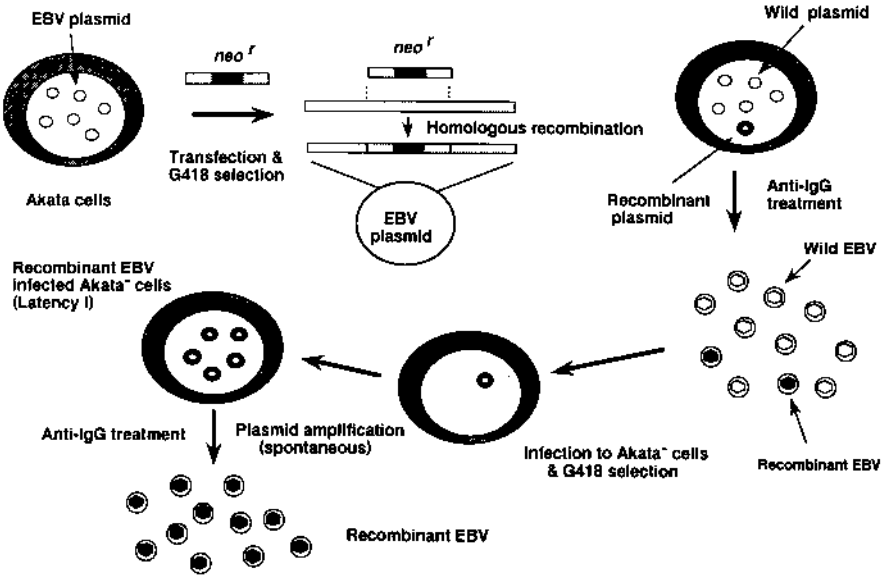

FIG. 4. Schematic representation of the Akata cell system, which allows propagation of EBV recombinants clonally and in large quantities. *neo*^{r}, Neo^r gene.

vector with deletions of the EBNA 2 and latent membrane protein 1 genes, which play crucial roles in lymphocyte transformation (8).

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