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Immortalization of human primary B lymphocytes *in vitro* with DNA

(Epstein–Barr virus/genetic analysis/transformation)

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ABSTRACT **Epstein–Barr virus (EBV) is a human DNA tumor virus that efficiently immortalizes human primary B lymphocytes *in vitro*. Although viral genes that are expressed in latently infected B lymphocytes have been shown to function in cellular growth control, their detailed genetic analysis has been cumbersome for two reasons. The viral genome is too large to permit genetic engineering and human primary B lymphocytes, the only targets for infection by EBV *in vitro*, are both intractable in culture and recalcitrant to DNA transfection. To overcome these obstacles, we have assembled all the essential genes of EBV on a single recombinant vector molecule in *Escherichia coli*. We show here that this mini-EBV plasmid can yield immortalized B cells upon transfer of its naked DNA into human primary B lymphocytes. Established cell lines carry recombinant vector DNA and cannot support virus production. Because this DNA can be easily manipulated in *E. coli*, mutant mini-EBVs as well as foreign genes can now be introduced and studied successfully in recipient B lymphocytes from any human donors. These mini-EBVs therefore are potentially useful for human gene therapy.**

Epstein–Barr virus (EBV) infects and efficiently immortalizes human B lymphocytes in cell culture. Similarly, B lymphocytes infected by EBV *in vivo* are found to be immortalized when cultivated *in vitro* as are EBV-infected Burkitt lymphoma biopsy cells (see refs. 1 and 2 for reviews). Eleven viral genes are consistently found expressed in B cells immortalized *in vitro* by EBV and these genes are likely candidates to be required for immortalization. However, only a subset of the 11 genes has been defined by genetic means to be required for this process. EBV nuclear antigen 2 (EBNA2) (3, 4), latent membrane protein 1 (LMP1) (5), EBNA3a, and EBNA3c (6) were found to be indispensable, whereas other genes can be omitted in the context of the EBV genome. No information has been obtained for EBNA1, which is necessary for viral plasmid replication (7) and presumably for EBV DNA replication in latently EBV-infected cells since the viral genome is usually maintained as plasmid DNA (8). This function of EBNA1 implies an evident contribution to the process of immortalization. EBNA2 is known to be required to initiate and to maintain immortalization (9), and it is likely that it acts to upregulate viral and cellular RNA synthesis by interacting with two cellular transcription factors (10–12). LMP1, which is also indispensable for B-cell immortalization, scores as an oncogene in established rodent cells (13, 14); can alter the state of differentiation of certain human epithelial cell lines (15, 16); and is associated with changes in the expression of cell surface differentiation markers under certain conditions (17, 18). It is likely that LMP1 acts via an unknown signaling pathway at the plasma membrane but its biochemical function remains to be elucidated. The contribution of other EBV genes is even less clear. EBNA3a is primarily involved in early events in B-cell immortalization but is not needed for maintenance of B-cell proliferation (19). Similarly, the function of the EBNA-LP gene appears to be auxiliary but not mandatory for the process of B-cell immortalization *in vitro* by EBV (3).

Previously, genetic analysis of EBV genes has relied on recombination events in lymphoblastoid cells between an endogenous EBV genome, usually the defective, nonimmortalizing P3HR1 strain, and a transiently introduced recombinant plasmid, which carries the mutation of interest. The induction of the lytic cycle triggers various homologous and illegitimate recombination events between both DNA molecules. Consequently, the definition of composition of the recombinant progeny viruses is difficult and usually uncertain. Moreover, such a strategy is limited in that only individual genes can be studied genetically and the minimal set of immortalizing genes cannot be defined. To overcome this obstacle, we have taken advantage of mini-EBV plasmids engineered in *Escherichia coli* that contain defined parts of the EBV genome cloned onto an F plasmid. Here we demonstrate that such a mini-EBV construct when introduced into human primary B lymphocytes of tonsillar origin contains sufficient genetic information to immortalize the transfected B cells. Since these mini-EBV plasmids can be easily manipulated in the prokaryotic host, it will be possible to delineate all the genes of EBV that are both sufficient and necessary for EBV-mediated B-cell immortalization *in vitro*.

MATERIALS AND METHODS

Construction of the Mini-EBV Plasmid p1495.4. The p1495.4 plasmid was constructed by the chromosomal building technique on the basis of the two cloning vectors pMBO132 and pMBO96 as described (20). The individual plasmids used for assembling the mini-EBV plasmid p1495.4 are shown in Fig. 1. In a first step, the plasmid p931.12 was constructed on the basis of the F-plasmid vector pMBO132 and all the remaining plasmids were sequentially added to this first construct in a clockwise fashion. All the other plasmid inserts were established on the basis of pMBO96 or a derivative of it (data not shown). The individual plasmids harbor the following inserts from the B95-8 strain (21) of EBV: p931.12 from nucleotides 163,477–3994; p935.1 from nucleotides 644–8994; p1177.3 from nucleotides 3994–13,215; p927.3 from nucleotides 7315–19,359 and 43,935–56,081; p948.27 from nucleotides 52,385–56,081 and 79,658–89,948; p1470.1 from nucleotides 88,248–102,891; p1202.1 from nucleotides 101,426–113,282; p1242.1 from nucleotides 110,942–113,282. In addition, this last plasmid carries the bacterial hygromycin phosphotransferase gene (*hyg*) in the context of the thymidine kinase regulatory sequences of herpes simplex virus (22).

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Abbreviations: EBV, Epstein–Barr virus; EBNA, EBV nuclear antigen; LMP, latent membrane protein.

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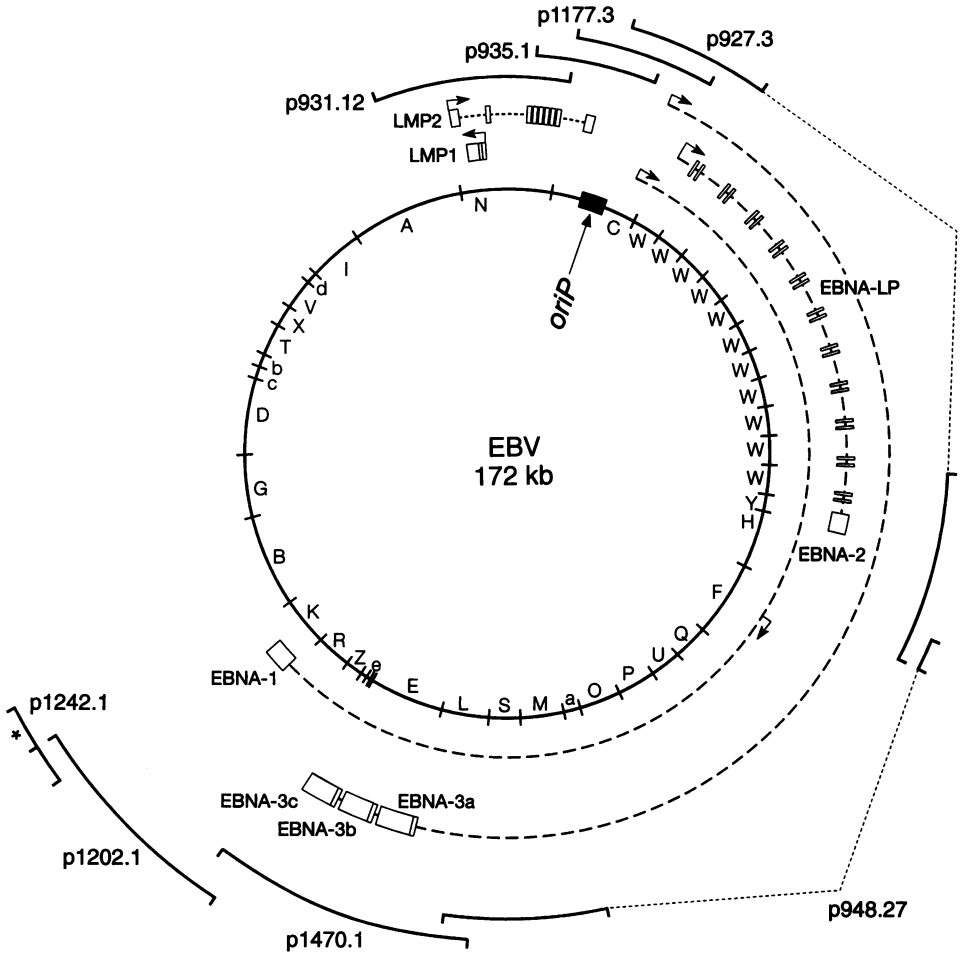

FIG. 1. Construction of mini-EBV plasmid p1495.4. The circularized genome of the B95-8 strain, which is 172 kbp, is shown as it is present in latently EBV-infected B cells. Letters within the inner circle indicate fragments found after digestion with *Bam*HI. The 11 viral genes (EBNA1, EBNA-LP, EBNA2, EBNA3a, -b, -c; LMP1, LMP2a, -b; EBER1, EBER2) generally expressed in the latent phase of the EBV life cycle are either denoted as open boxes together with the extension of their primary RNA transcripts (dashed lines) and promoters (→) or are too small to be represented (EBER1 and -2 are located in p935.1 and p1177.3). The cis-acting element involved in plasmid replication of EBV in latently infected cells, *oriP*, is indicated. The plasmid termed p1495.4 was constructed on the basis of eight partially overlapping plasmids. Their inserts are shown in the periphery along with the plasmid names. Dotted lines indicate parts omitted from the plasmids to exclude repetitive or other EBV sequences presumably not involved in B-cell immortalization. Asterisk indicates position of the added marker gene for hygromycin phosphotransferase. Prokaryotic plasmid backbone of p1495.4 is not shown.

Homologous recombinations were carried out in the *recA*$^+$ *E. coli* strain RVsmc [*ΔlacX74, rpsL41*, (r_k^+, m_k^+)] or in the *recA E. coli* strain CBTS carrying a *recA* amber allele and a temperature-sensitive amber suppressor [*leu(am), trp(am), lacZ2210(am), galK(am), galE?, sueC, rpsL, supD43,74, sueB, metB1, RecA99*(am)]. The combined plasmids were resolved with the aid of the *resD* expression plasmid pDCM111 via the two *rfsF* sites present in the cointegrate so that the F-factor-based prokaryotic backbone was retained together with the recombined EBV insert (20). In consecutive steps, the individual neighboring plasmids were added by homologous recombination and subsequent resolution. All intermediate plasmids and the final constructions were grown in *E. coli* strain DH5α [F$^-$, Φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR, recA1, endA1, hsdR17* (r_k^-, m_k^+), *supE44*, λ$^-$*thi-1, gyrA96, relA1*] and were carefully checked by restriction enzyme analysis for the desired structures. The assembled construct p1495.4 was calculated to be 83,851 bp.

Preparation and Transfection of Tonsillar B Lymphocytes. Human B lymphocytes were prepared and purified from routine tonsillectomies by generating single cell suspensions and depletion of T lymphocytes by rosetting with sheep red blood cells. The preparations were analyzed by fluorescence-activated cell sorter and were found to be >95% positive for the panB marker CD19. Ten micrograms of p1495.4 plasmid DNA was introduced into 1×10^7 tonsillar human B-lymphocyte preparations in a vol of 250 μl by electroporation (23) with a Bio-Rad electroporator at 320 V or at 340 V, 960 μF, in a 0.4-cm-wide cuvette. The electroporated cells were initially plated in 96-well cluster plates at a density of ≈5×10^4 cells per well on irradiated (5000 rad; 1 rad = 0.01 Gy) human fibroblast feeder layers (WI38 or MRC5 human fibroblast cells obtained from the American Type Culture Collection) in RPMI 1640 culture medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM pyruvate, 100 units of penicillin per ml, and 100 μg of streptomycin per ml. The clones that proliferated were expanded and total cell DNA was isolated from ≈1×10^7 cells as described (19).

Analysis of Immortalized B-Cell Clones. The p1495.4-related DNA was detected by Southern blot hybridization (24) with either pMBO132 plasmid DNA, which visualizes the plasmid backbone of p1495.4 (Fig. 2*b*), or with the cosmid probe cMB-14 (27), which spans DNA from nucleotides 48,040–87,237 of the B95-8 strain of EBV (Fig. 2 *c* and *d*). Other probes used to verify the predicted structure of p1495.4 and the absence of any wild-type EBV DNA included a mixture of different cloned EBV fragments (27) ranging from nucleotide 58,878 to 71,119, 116,208 to 137,222, 140,893 to

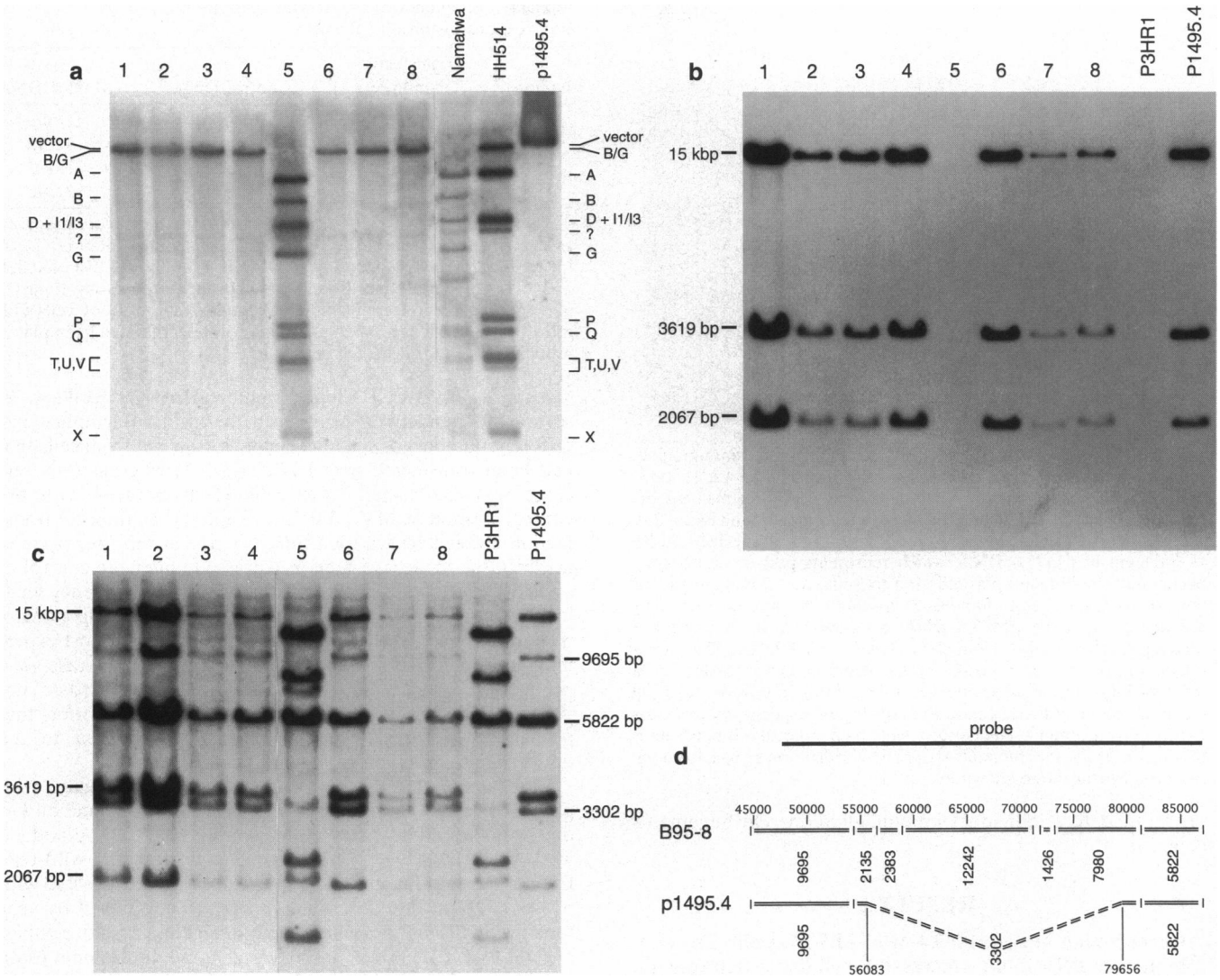

FIG. 2. Characterization of immortalized B-cell clones obtained by transfection of primary human B lymphocytes with p1495.4 mini-EBV DNA. (*a*) *Bam*HI-digested total DNAs (10 μg per lane) from B-cell clones established by transfection of p1495.4 mini-EBV DNA were analyzed by Southern blot hybridization. The blot was hybridized to a collection of probes that primarily detects EBV sequences present in all EBV strains but not in p1495.4 mini-EBV DNA. Only a very small part of the *Bam*HI A fragment present as a fusion fragment with the F-factor backbone is detected by this probe mixture (vector). Ten micrograms of Namalwa cell DNA carrying two integrated copies of EBV per genome (25) served as an internal standard to reconstruct the limit of detection. One clone (lane 5) was found to be infected with an EBV wild-type strain whereas 38 clones contained p1495.4 DNA (lanes 1–4 and 6–8). Letters indicate *Bam*HI fragments of the B95-8 prototype strain of EBV. Two *Bam*HI cleavage sites between fragments B and G and I1 and I3 are absent in our clone of P3HR1 virus and the fused fragments are indicated. Labeled mixture of several cloned EBV fragments encompassing ≈53 kbp weakly cross-hybridized to unidentified cellular sequences, one of which is clearly visible in the lane with P3HR1 DNA and is indicated with a question mark. (*b*) DNA isolated from individual cell lines from tonsillar B lymphocytes electroporated with p1495.4 DNA were cleaved with *Bgl* II and analyzed by Southern blot hybridization with the radioactively labeled pMBO132 probe, which detects the three *Bgl* II fragments characteristic of the pMBO132 F-factor plasmid backbone. Seven cell lines (lanes 1–4 and 6–8) carry p1495.4 DNA, whereas clone 5 and cellular DNA derived from the EBV-positive, *het*-free HH514 subclone (26) of Burkitt lymphoma cell line P3HR1 give no signal with the probe (lane 5). Lanes contained 10 μg of total cellular DNA; lane p1495.4 contained 2 ng of *Bgl* II-cleaved p1495.4 plasmid DNA prepared from *E. coli.* (*c*) Same blot as in *b* was rehybridized with the radioactively labeled probe in *d*. In addition to the three fragments detected in *b* with the p1495.4-containing cell clones, three fragments showed up whose sizes are indicated on the right. The same fragments were detected in p1495.4 plasmid DNA prepared from *E. coli.* Note that the probe detected several fragments in clone 5 and P3HR1 cell DNA that are different from those detected in the p1495.4-positive cells with the exception of the 5822-bp fragment. Signal profiles of clone 5 and P3HR1 were expected to be slightly different from each other since P3HR1 is a nonimmortalizing variant of EBV, which was visualized by the probe chosen here. (*d*) Structure of the linear form of B95-8 viral DNA [the immortalizing prototype EBV strain (21)] and the corresponding part in p1495.4 plasmid DNA is shown together with the nucleotide coordinates and locations of the *Bgl* II sites (small vertical bars). Location of the probe is shown as a thick line above the B95-8 structure. Perpendicular numbers indicate sizes of different fragments generated by *Bgl* II for the B95-8 strain of EBV (above) and the p1495.4 plasmid DNA (below). Gap in structure of p1495.4 DNA, flanked by nucleotides 56,083 and 79,656, is the junction between parts two and three in the p1495.4 structure (cf. Fig. 1). The resulting junction fragment of 3302 bp is characteristic for the p1495.4 mini-EBV DNA and is absent from any known EBV strain. The *Bgl* II fragment of 9695 bp (*c*) is detectable only in clones carrying p1495.4 mini-EBV DNA. Its corresponding fragment in P3HR1 is not visible here since it suffers from a substantial deletion compared to B95-8 virion DNA. In clone 5, a smaller fragment (third from the top in lane 5) is detected that is probably due to a size heterogeneity and might be equivalent to this *Bgl* II fragment.

146,916, and 10,593 of the Raji strain sequences (28) to 166,483 of the B95-8 strain of EBV (Fig. 2*a*). For PCR analysis, the primer sets used have been described (19). A 3.1-kbp *Eco*RI/*Hin*dIII fragment encompassing the human immunoglobulin heavy-chain locus from J_{H3} to the heavy-chain intron enhancer (29) cloned into pBluescript was used as a radioactively labeled

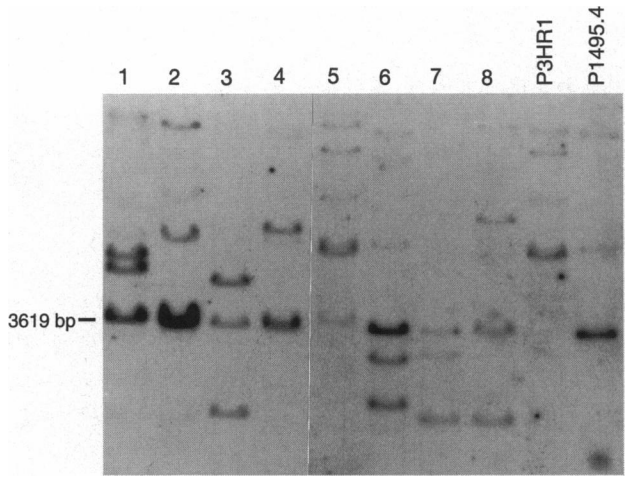

FIG. 3. p1495.4-transfected B cells carry individual immunoglobulin heavy-chain rearrangements. Blot shown in Fig. 2c was stripped and hybridized with a probe encompassing the J_H exons and the heavy-chain intron enhancer of the human immunoglobulin locus (29). The radioactively labeled plasmid probe hybridized to the 3619-bp *Bgl* II fragment of p1495.4 DNA, which represents part of the plasmid backbone of the F-factor plasmid. As a consequence, clones one to four and six to eight (lanes 1–4 and 6–8) contain this fragment since they are immortalized by p1495.4 DNA. As expected, this fragment is missing in cellular DNA from clone 5 and P3HR1 DNA. The immunoglobulin heavy-chain-specific probe would detect a 15-kbp and a 3.5-kbp *Bgl* II fragment in the germ-line configuration. Instead, all clones depict an individual pattern indicative of a specific *DJ* and class switch rearrangement. The faintly visible high molecular weight bands in some lanes are incompletely stripped hybridization signals from the previous hybridization shown in Fig. 2c.

probe to detect individual immunoglobulin rearrangements (Fig. 3).

RESULTS

Construction of the p1495.4 Mini-EBV Plasmid. The contributions of EBV to the process of B-cell immortalization (1, 2) have been especially difficult to study because the size of the viral genome [172 kbp in the B95-8 prototype strain (21)] prevents its manipulation by common recombinant DNA procedures. All current procedures are limited and rely on packaging of subgenomic plasmids into an EBV virion coat (3) or fortuitous homologous recombination events (4–6, 30–34). We have circumvented this problem by constructing a plasmid in *E. coli* that contains 71 kbp of EBV DNA cloned onto an F plasmid. This was achieved by a multistep chromosomal building technique that relies on an initial F-factor plasmid, which is used to add fragments of DNA by a combination of homologous and site-specific recombinations in *E. coli*. This approach allows the defined composition of the DNA and also permits the final plasmid to be altered or enlarged at any predetermined position (20). To assemble the recombinant DNA used in this study eight plasmids derived from the immortalizing B95-8 strain of EBV were sequentially joined into a mini-EBV plasmid (Fig. 1) that contains all the known genetic loci involved in B-cell immortalization. The mini-EBV plasmid termed p1495.4 also encompasses the EBV plasmid origin of DNA replication, *oriP*, which together with the viral gene product EBNA1 (Fig. 1) allows the extrachromosomal maintenance of the mini-EBV plasmids (7, 22, 35). Having established such a mini-EBV plasmid with the total size of 83,851 bp including the prokaryotic plasmid backbone, we tested its functionality by DNA transfection into human primary B lymphocytes. We prepared tonsillar B cells and transfected the cells by electroporation with the p1495.4 mini-EBV DNA.

Table 1. Frequency of outgrowing colonies after DNA transfections of tonsillar B cells

Donor	Transfection condition	Control DNA	p1495.4 DNA
A	320 V	0/96	11/192
B	320 V	0/96	4/192
C	320 V	1/96	21/192
C	340 V	0/84	12/192

Tonsillar B cells (1×10^7) of three different donors were prepared as described and transfected with 10 μg of control plasmid DNA [pCMV-LTR-Luc (36)] or p1495.4 DNA in a total vol of 250 μl at 320 or 340 V. About 5×10^4 cells were plated in each well of 96-well cluster plates on irradiated feeder cells. Numbers indicate ratios of wells with growth of B-cell clones vs. total number of wells that had been plated initially with the transfected cells.

Analysis of B-Cell Clones Immortalized by p1495.4. In several independent experiments with tonsillar B lymphocytes, different numbers of single-cell clones grew out from cells that had been transfected with p1495.4 DNA, whereas only one clone was established from cells electroporated with the control plasmid pCMV-LTR-Luc (Table 1). To monitor transfection efficiencies we used this very potent reporter plasmid in which the luciferase gene is expressed under the control of an artificial cytomegalovirus/human immunodeficiency virus long terminal repeat promoter. Under the conditions of electroporation used in this study, we were unable in most experiments to achieve detectable expression of the luciferase gene product in a highly sensitive luminometer assay (data not shown). This finding is in agreement with the notion that primary B lymphocytes are peculiarly recalcitrant to the uptake and expression of foreign DNA.

Thirty-nine of 48 clones that had been transfected with p1495.4 DNA were analyzed by Southern blotting and all but one were found to carry p1495.4 mini-EBV DNA and no wild-type EBV (Fig. 2). As expected, no trace of wild-type EBV DNA could be found in 38 B-cell clones transfected with p1495.4 DNA (Fig. 2a), which could be confirmed by very sensitive PCR analyses which easily detect less than five copies of wild-type EBV DNA in 20,000 genome equivalents (data not shown) (19). One of the clones analyzed was infected with a wild-type EBV strain derived from the patient's tonsils (clone 5 in Figs. 2 and 3), which presumably also gave rise to the single B-cell clone in the negative control (Table 1). The composition of the p1495.4 DNA was carefully checked by Southern blot hybridizations in the majority of proliferating clones and the mini-EBV DNA was found to be intact and unaltered in most of the clones analyzed (Fig. 2 b and c; data not shown). Long-term *in vitro* cultivation (>12 months) of the B-cell clones indicated that they are immortalized. Immunoglobulin heavy-chain rearrangements in the cell lines were analyzed by Southern blot hybridization, which confirmed the individual nature of the immortalized B-cell clones (Fig. 3). As expected, the transfected mini-EBV DNA was maintained as extrachromosomal plasmids as determined by the Gardella gel technique (37, 38) and all the cell clones were B lymphocytes as judged by fluorescence-activated cell sorter analysis with antibodies against differentiation antigens CD19 and CD3 (data not shown).

DISCUSSION

The mini-EBV p1495.4 contains ≈40% of the DNA of EBV and is sufficient to immortalize human B lymphocytes *in vitro*. Attempts to activate the lymphocytes by different means prior to DNA transfection were unsuccessful (data not shown), which might indicate that no contribution from the virus particle apart from its DNA is required for immortalization. The recombinant mini-EBV vector is of particular interest for

applications including expression of introduced foreign genes in virus-free human B lymphocytes. Because cells can be easily established from any human donor with this mini-EBV, it can also be used for human gene therapy. It is especially fortunate that the chromosomal building technique in *E. coli* has no real practical limitations (20); the mini-EBVs can readily accommodate several more genes of interest. It is also clear that the composition of a given mini-EBV can be altered at any desired position (20) so that individual EBV genes involved in the control of proliferation of human B lymphocytes can be dissected genetically. We have already established that much smaller mini-EBVs will support the immortalization of human B lymphocytes (unpublished data); therefore, we should be able define the minimal set of viral genes necessary to immortalize human primary B lymphocytes *in vitro*.

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