JOURNAL OF VIROLOGY, Jan. 1995, p. 231–238
0022-538X/95/$04.00+0

Vol. 69, No. 1

Immortalization of Human B Lymphocytes by a Plasmid Containing 71 Kilobase Pairs of Epstein-Barr Virus DNA

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Received 1 August 1994/Accepted 15 October 1994

We have assembled derivatives of Epstein-Barr Virus (EBV) that include 71 kbp of noncontiguous DNA sequences cloned into a prokaryotic F-factor plasmid. These mini-EBVs, when introduced into an EBV-containing lymphoblastoid cell, can be packaged by the endogenous helper virus. One such mini-EBV was found to have a single C residue deleted from its EBNA3a open reading frame. When packaged, this mini-EBV initiates proliferation of infected primary human B lymphocytes only in conjunction with a complementing helper virus. Proliferation of the infected cells, however, was maintained either alone by the mini-EBV containing the mutated EBNA3a open reading frame or alone by its derivative in which the EBNA3a open reading frame had been healed of its lesion by recombination with the helper virus. The mini-EBV with a wild-type EBNA3a open reading frame when packaged alone can both initiate and maintain proliferation upon infection of primary human B lymphocytes. These findings identify 41% of EBV DNA which is sufficient to immortalize primary human B lymphocytes and provide an assay to distinguish virus contributions to initiation or maintenance of cell proliferation or both. They also identify EBNA3a as a transforming gene, which contributes primarily to the initiation of cell proliferation.

Epstein-Barr virus (EBV) induces and maintains proliferation efficiently in human B lymphocytes which it infects. These infected, proliferating cells efficiently yield progeny capable of indefinite proliferation in cell culture (for reviews, see references 18 and 22). This process overall is referred to as immortalization, and it is likely to underlie EBV's pathogenicity in humans. It has been difficult to study EBV's contributions to immortalizing its host cell genetically because EBV lacks a tractable lytic cycle in which to generate and from which to select mutants. In addition, the target cell for immortalization by EBV, the primary, human B lymphocyte, is peculiarly recalcitrant to the uptake and expression of DNA. We have circumvented these problems by constructing a plasmid in *Escherichia coli* which contains 71 kbp of EBV DNA cloned onto an F-factor plasmid. This plasmid can be manipulated genetically in its prokaryotic host. We refer to these EBV DNA-containing plasmids as mini-EBVs, and when packaged, some are capable of infecting resting B lymphocytes and inducing and maintaining proliferation in the infected cell (12).

Previous strategies to study EBV genetically have relied largely on recombination in a lymphoblastoid cell between an endogenous EBV and a newly introduced vector to generate the mutants to be analyzed (4, 12, 16, 19, 21, 31, 32). Such recombination events yield a variety of progeny whose contributions to immortalization of subsequently infected cells can only be surmised retrospectively. Mini-EBVs can be introduced into lymphoblastoid cells in which they are packaged by products of an endogenous helper virus without recombining with the helper virus, and subsequently, they immortalize infected B lymphocytes (12).

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We have taken advantage of phenotypic differences between strains of EBV to gauge the contributions of specific virus genes to the maintenance of proliferation of infected cells. A strains of EBV yield immortalized cells that proliferate rapidly relative to cells immortalized by B strains of EBV. Our mini-EBVs were constructed from the prototypic A strain, B95-8 (2), while the helper virus, HH514 clone 16, is a B strain (15). Cells coinfected with a transformation-competent mini-EBV and the helper virus lose the helper virus during propagation. Cells coinfected with a transformation-incompetent mini-EBV and the helper virus either retain both species during propagation or recombine to yield a transformation-competent mini-EBV followed by loss of the helper virus. We have used these observations to identify the EBNA3a open reading frame (ORF) as encoding a gene that is required to initiate cell proliferation, that is not required to maintain cell proliferation, but that provides a selective advantage to the proliferating cell.

MATERIALS AND METHODS

Plasmid constructs. The plasmid p1244.8a was constructed with the set of plasmids described in Table 1 by the chromosomal building technique (23). This method allows the cloning of large and defined DNA regions into an F-factor-based plasmid in *E. coli*. Briefly, the initial F plasmid p931.12 (Fig. 1A and Table 1) served as a recipient for homologous recombination in *E. coli* with the next shuttle plasmid p935.1. This and the remaining recombinant plasmids were generated with the aid of a different cloning vector, pMBO96 (23). Homologous recombinations were carried out in *recA*$^+$ *E. coli* RVsmc (Δ*lacX74*, *rpsL41* [r_k^+, m_k^+]) or in *recA E. coli* 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 a *resD* expression plasmid pDCM111 via the two *rfsF* sites present in the cointegrate such that the F-factor-based prokaryotic backbone was retained together with the recombined EBV insert (23). 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* 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

TABLE 1. Composition of plasmids used to assemble p1244.8a mini-EBV plasmid[a]

Plasmid	Nucleotide coordinates of EBV insert	Restriction enzymes[b]	Gene and/or *cis*-acting element(s)
p931.12	163477-3994	*Sac*I, *Bam*HI	LMP1, LMP2a, LMP2b, TR
p935.1	644-8994	*Sal*I, *Eco*RV	*oriP*, EBER1, EBER2
p1177.3	3994-13215	*Bam*HI, *Bam*HI	*oriP*, EBER1, EBER2
p927.3	7315-19359	*Eco*RI, *Bam*HI	EBNA-LP
	43935-56081	*Bam*HI, *Sal*I	EBNA-LP, EBNA2, *oriLyt*
p948.27	52385-56081	*Sac*I, *Sal*I	
	79658-89948	*Pvu*I, *Xmn*I	
p1203.4	88248-93234	*Cla*I, *Spe*I	
p926.4	91821-102891	*Hin*dIII, *Hin*dIII	EBNA3a, EBNA3b, EBNA3c
p1202.1	101426-113282	*Bam*HI, *Sal*I	EBNA1
p1242.1	110942-113282	*Hin*dIII, *Sal*I	*hyg*

[a] The names of the plasmids and the nucleotide coordinates (2) of the EBV inserts are given together with the restriction enzyme sites used for subcloning of B95-8 DNA. The cloning vector pMBO96 was used for all constructs, except for p931.12, which was cloned into pMBO132 (23). The genes and *cis*-acting elements pertinent to this study are indicated; *hyg* is the hygromycin phosphotransferase gene, which was added to p1244.8a as a selectable marker not used in this study. Plasmid p1244.8a encompasses B95-8 EBV DNA from nucleotides 163477 to 56081 (minus the repeated sequences between 19359 and 43935) including the fused termini at positions 172272 and 1 and from coordinates 79656 to 113282 (Fig. 1A).

[b] Restriction enzymes correspond to the first and last nucleotides, respectively, of the EBV insert.

structures. The complete sequence of p1244.8a, including the prokaryotic vector backbone which was partially sequenced during this project, was calculated to be 83,851 bp. Mini-EBV plasmid DNA was rescued back into *E. coli* DH10B (F^-, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), Φ80d*lacZ*Δ*M15*, Δ*lacX74*, *deoR*, *recA1*, *endA1*, *araD139*, Δ(*ara*, *leu*)*7697*, *galU*, *galK*, λ^-, *rpsL*, *nupG*) from different proliferating B-cell clones which were found to carry mini-EBV DNA only (10). The plasmid derivatives p1244.8a-6.6 and p1244.8a-8.4 were isolated from the cell clones Be253.30 and Be253.33, respectively. With the exception of *E. coli* DH5α and DH10B, which were purchased from Gibco BRL Life Technologies, Inc., all the cloning vectors and *E. coli* strains were obtained from M. O'Connor (23).

Cell line and packaging procedures. HH514 is a *het*-free cell clone of the P3HR1 Burkitt's lymphoma cell line (15) which was grown in RPMI medium supplemented with 10% fetal calf serum. Ten micrograms of DNA of the plasmid p1244.8a was transiently introduced into HH514 cells by electroporation together with 10 μg of pCMV-BZLF1, an expression vector for the viral BZLF1 gene (11), and released virus was harvested 5 days later. B-cell preparations derived from cord blood or from buffy coat fractions of peripheral blood of adults were infected with sterile, filtered virus stocks from the transiently transfected HH514 cells and plated at limiting dilution in 96-well cluster plates on a lethally irradiated human fibroblast feeder cell layer. To determine the kinetics of B-cell immortalization by virus stocks derived from the supernatant of transfected HH514 cells, adult peripheral blood B lymphocytes (4×10^5 to 5×10^5 cells per point) were purified by panning (12) and exposed to serial twofold dilutions of the two stocks of virus, from undiluted to a 1:128-fold dilution, in a total volume of 2 ml for 4 h at room temperature. They were then plated in agarose as described previously (30), and colonies with 32 or more cells were scored approximately 21 days after plating.

Southern blotting and PCR analyses. Cellular DNA was prepared as described previously (12) and run on a 0.7% agarose gel. The radioactive probe used in Fig. 2A was a mixture of EBV DNA fragments derived from the M-ABA strain of EBV (25). The clones encompass certain parts of the B95-8 genome from nucleotides 58878 to 71119, from 116208 to 137222, from 140893 to 146916, and from 10593 of the Raji strain sequences (24) to 166483 of the B95-8 strain of EBV. In Fig. 2B, two PCR primers (5′-GTCAGCCGCCAGGGTCCGTTTA and 5′-AAGTTTCCTTGCCATCTAAAGC) were used to amplify 434 bp of EBV DNA from nucleotides 33 to 466 of the Raji strain sequence (24), which is absent in the prototype EBV B95-8. In each lane, 200 ng of total cellular DNA was used in the PCR; the EBV-negative DG75 cellular DNA was doped with H3-D2 plasmid DNA (25) to represent different copy numbers of the respective EBV sequence per cell genome. The marker lanes are composed of a 123-bp ladder DNA (Gibco BRL Life Technologies, Inc.). In Fig. 6B, two PCR primers (5′-TCGCCAGTGGTTGTATGTTCAG and 5′-TGTTCYCCTTCAGTTCTA AGCAAG) that hybridize to DNA stretches which are highly conserved between the A and B strains of EBV were used to amplify 347 bp from nucleotides 93008 to 93355 in the prototype EBV B95-8.

Western blotting. The monoclonal antibodies directed against EBNA1 and EBNA2 have been described previously (17, 35), and the antibody against LMP1 is commercially available (CS1-4 from Dako A/S, Denmark). The antibody directed against EBNA3a was made by a trpE-EBNA3a fusion protein which was partially purified to be used as an immunogen in a rabbit. The detection system for the Western blots (immunoblots) has been described previously (13).

RESULTS

Generation of mini-EBV plasmid in *E. coli* that contains 71 kbp of EBV DNA. Two nonconsecutive parts of the genome of the EBV from the B95-8 A strain (2) were cloned in a recombinant F-factor-based *E. coli* plasmid by a multistep chromosomal building technique (23). This technique relies on an initial F-factor plasmid, which is used to add pieces of DNA by a combination of homologous and site-specific recombinations in *E. coli*. The sequential addition of smaller, partially overlapping DNA segments which are molecularly cloned on a different shuttle vector plasmid results in a stepwise increase in size of the primary F-factor plasmid. The approach allows the definite composition of the final F-factor construct. This chromosomal building technique also permits the final plasmid to be enlarged or mutated at all positions.

Nine plasmids derived from the immortalization-competent B95-8 strain of EBV, an A strain (2), were established in *E. coli* (Fig. 1A and Table 1). p931.12 was the first F-factor-based plasmid which served as a recipient for the sequential addition of the remaining eight plasmids. The composition of the individual plasmids allowed the exclusion of repetitive sequence elements and intervening sequences, which presumably are not directly involved in the immortalization process by EBV. The joined parts together constitute 71 kbp of the genomic EBV DNA (Fig. 1A), which is itself approximately 172 kbp in size (2). The *E. coli* plasmid termed p1244.8a encompasses the latent and lytic origins of DNA replication, *oriP* and *oriLyt*, the packaging signal sequences TR, and all the genes known to be expressed in EBV-immortalized B-cell lines (Fig. 1A and Table 1). The prokaryotic plasmid backbone and a marker gene bring the length of the plasmid DNA (Fig. 1B) to 83,851 bp, which is about half the size of the prototypic EBV B95-8 plasmid. The *cis*-acting viral elements on p1244.8a should allow it to become amplified in an appropriate helper cell and packaged in an EBV capsid as a dimer. Similar packaged DNAs, also termed mini-EBVs, can infect human primary B lymphocytes and are useful vectors in these cells which are largely refractory to the uptake of DNA (12).

Immortalization of human primary B lymphocytes. The mini-EBV plasmid p1244.8a was introduced into a helper cell line derived from a Burkitt's lymphoma which was latently infected with the nonimmortalizing strain EBV P3HR1 (B

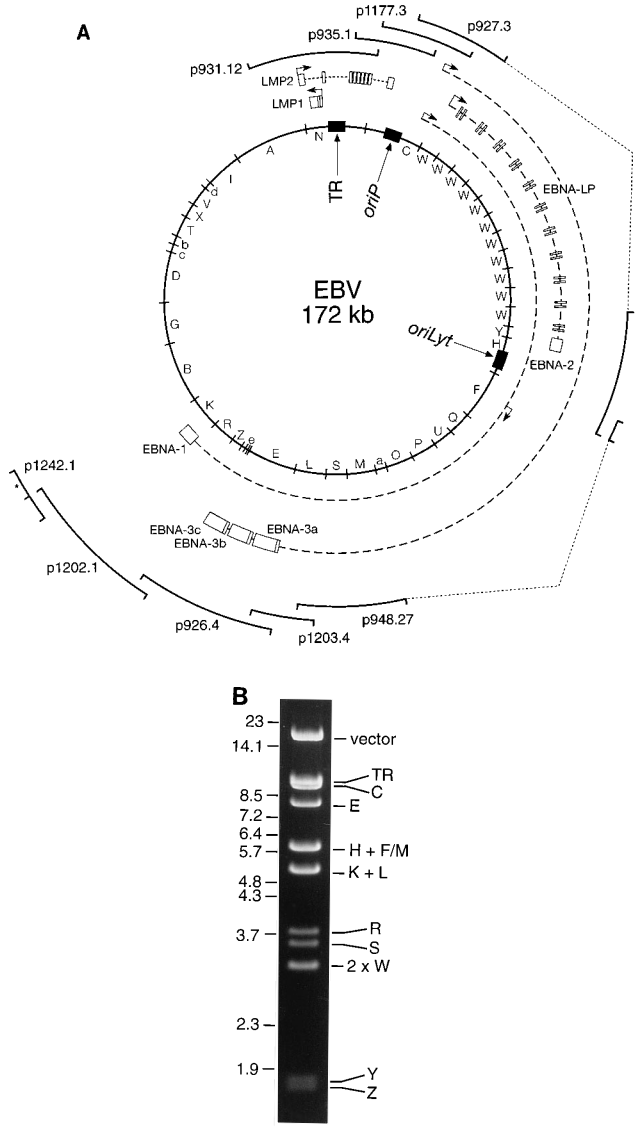

FIG. 1. Schematic diagram of genome of EBV B95-8 and mini-EBV plasmid p1244.8a. (A) The circularized genome of the B95-8 strain is shown as it is present in latently infected B cells. The letters within the inner circle indicate the fragments found after digestion with *Bam*HI. The 11 viral genes (EBNA1, EBNA-LP, EBNA2, EBNA3a to EBNA3c, LMP1, LMP2a, LMP2b, EBER1, and 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 EBER2 are located in p935.1). The three *cis*-acting elements involved in EBV DNA replication and maturation are indicated by closed boxes: the latent origin of DNA replication (*oriP*) (33), the lytic origin of DNA replication (*oriLyt*) (11), and the cleavage and packaging signals (TR) (12). The plasmid termed p1244.8a was constructed on the basis of nine partially overlapping plasmids (Fig. 1A and Table 1). Their inserts are shown on the periphery along with the plasmid names. The dotted lines indicate parts omitted from the plasmids to exclude repetitive or other EBV sequences presumably not involved in B-cell immortalization. The asterisk indicates the position of the added marker gene encoding hygromycin phosphotransferase. The prokaryotic plasmid backbone of p1244.8a is not shown. (B) The *Bam*HI-generated fragments of p1244.8a DNA isolated from *E. coli* were separated on an ethidium-bromide-stained agarose gel and denoted with the same capital letters as in panel A. One fragment (F/M) was generated by the fusion of the F and M *Bam*HI fragments; the vector fragment includes the truncated part from the *Bam*HI fragments A and B (Table 1). The smaller *Bam*HI fragments e to g are not visible on this gel. Size markers (in kilobase pairs) are indicated on the left.

strain) (15). Concomitantly, the lytic phase of the virus life cycle was induced by cotransfection with the virus *trans*-activating gene BZLF1 (5, 11). The viral particles released from the transfected cells contained either genomic P3HR1 virus DNA or encapsidated copies of the p1244.8a mini-EBV plasmid or possible recombinants between them (12). This virus stock was used to infect primary human B cells derived from cord blood of newborns or from peripheral blood of adults. Immortalized B-cell clones grew from infected but not from uninfected cells (and not from cells infected with P3HR1 virus alone). The mini-EBV plasmid p1244.8a replicated extrachromosomally as a plasmid in the infected cells as analyzed by the Gardella gel technique (6, 8) (data not shown) since the p1244.8a plasmid contains *oriP*, the plasmid origin of DNA replication (33), and encodes EBNA1 (Fig. 1A), which is the virus gene that supports plasmid replication (34). To determine whether clones of immortalized B cells contained the mini-EBV plasmid alone or in combination with P3HR1 DNA, we used Southern blot and PCR analyses to screen 47 clones randomly chosen from two independent sets of experiments. We found seven clones which did not contain any detectable P3HR1 helper virus (Fig. 2A and data not shown). The sensitivity of our detection assay was documented, in reconstruction experiments using PCR, to be fewer than 5 molecules of P3HR1 DNA in about 30,000 cell genomes (Fig. 2B). Forty clones, two of which are shown in Fig. 2, were doubly infected with p1244.8a and P3HR1 virus. Those immortalized cells that contained both DNAs had no detectable recombinant molecules, and no cell line was found which contained a P3HR1 molecule with its deletion repaired (data not shown), in contrast to the findings of previous experiments (12). All doubly infected cell clones, which were identified by Southern blot hybridizations with radioactive probes, as shown in Fig. 2, were not studied further, with the exception of the clone Be244.2. This cell clone showed a loss of P3HR1 helper virus over time; less than one molecule of P3HR1 DNA per cellular genome was detectable in the Southern blot shown in Fig. 2A, and the cell line was found to be free of P3HR1 DNA by PCR analysis after 7 months in culture (Fig. 2B). All cell lines except two which carried p1244.8a molecules and no helper virus DNA expressed the same set of virus latent proteins (EBNA1, EBNA2, EBNA3a to EBNA3c, and LMP1) as cells which were either doubly infected with both mini-EBVs and P3HR1 helper virus or infected with wild-type EBVs (Fig. 3).

p1244.8a mini-EBV is not sufficient to initiate immortalization of B lymphocytes. In our initial experiments, 40 of 47 clones of proliferating cells contained both p1244.8a and the helper virus. In addition, one clone which was initially infected with p1244.8a mini-EBV plus P3HR1 virus slowly lost the helper virus DNA over time (clone Be244.2; Fig. 2B). These observations prompted us to investigate whether p1244.8a is sufficient to initiate B-cell immortalization as is B95-8 virus (14, 30). Adult B lymphocytes were exposed to serial dilutions of virus stocks, the infected cells plated in semisolid medium, and the number of proliferating colonies were plotted as a function of serial dilutions of the virus stock. The results revealed that the number of proliferating B-cell clones followed two-hit kinetics, indicating that a single p1244.8a mini-EBV is not sufficient to yield efficient outgrowth of B lymphocytes as is the B95-8 virus (Fig. 4A). The P3HR1 virus along with p1244.8a appears to be needed to initiate proliferation of B lymphocytes.

Derivatives of p1244.8a mini-EBV are sufficient to initiate immortalization of B lymphocytes. Of the seven proliferating B-cell clones which contained mini-EBVs and no helper virus, two were studied further by rescuing the mini-EBV DNAs into

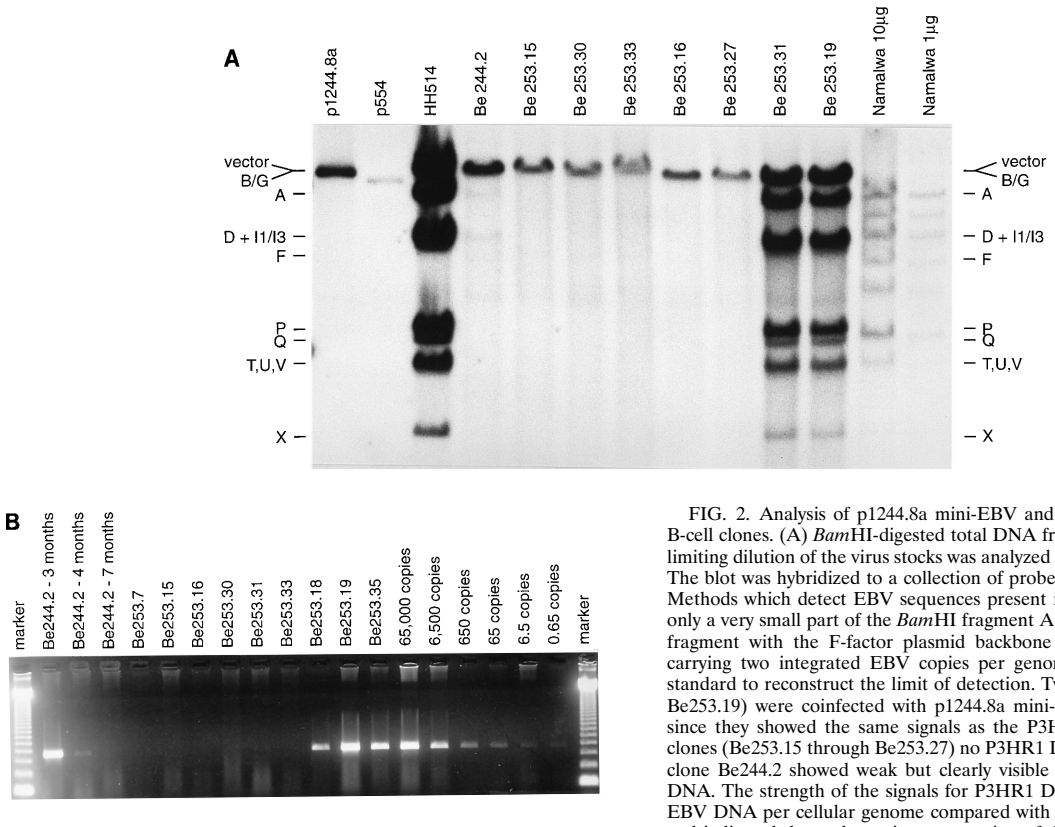

FIG. 2. Analysis of p1244.8a mini-EBV and P3HR1 helper virus DNAs in B-cell clones. (A) *Bam*HI-digested total DNA from B-cell clones established by limiting dilution of the virus stocks was analyzed by the Southern blot technique. The blot was hybridized to a collection of probes as described in Materials and Methods which detect EBV sequences present in the P3HR1 helper virus and only a very small part of the *Bam*HI fragment A present in p1244.8a as a fusion fragment with the F-factor plasmid backbone (Fig. 1). Namalwa cell DNA carrying two integrated EBV copies per genome (20) served as an internal standard to reconstruct the limit of detection. Two B-cell clones (Be253.31 and Be253.19) were coinfected with p1244.8a mini-EBV and P3HR1 helper virus since they showed the same signals as the P3HR1 clone HH514. In five cell clones (Be253.15 through Be253.27) no P3HR1 DNA was detectable. The B-cell clone Be244.2 showed weak but clearly visible signals of P3HR1 helper virus DNA. The strength of the signals for P3HR1 DNA was well below 0.2 copy of EBV DNA per cellular genome compared with that for Namalwa DNA (1 μg) and indicated that only a minor proportion of the cell population was infected with P3HR1 virus plus p1244.8a mini-EBV. The letters indicate the *Bam*HI fragments of the B95-8 prototype strain of EBV as shown in Fig. 1A. Two *Bam*HI cleavage sites between the fragments B and G (2) and between I1 and I3 (25) are absent in our clone of P3HR1 virus (data not shown). The fused fragments are indicated by B/G and I1/I3, respectively. The lanes contained plasmid DNA of p1244.8a (0.3 ng) and p554 (12) (0.1 ng), cellular DNA of strain HH514 clone 16 of P3HR1 (26) (10 μg), cellular DNA (10 μg) from eight clones (Be244.2, Be253.15, Be253.30, Be253.33, Be253.16, Be253.27, Be253.31, and Be253.19) derived from B cells infected with virus stocks containing p1244.8a mini-EBV and P3HR1 helper virus, and cellular DNA from the Burkitt's lymphoma cell line Namalwa (1) (10 and 1 μg). (B) PCR analysis of individual cell clones indicates that P3HR1 helper virus DNA was missing from several clones immortalized with virus stocks containing p1244.8a mini-EBV plus P3HR1 helper virus but was detectable in others. A specific 434-bp fragment was amplified in the cell lines Be244.2 (cultured for 3 and 4 months), Be253.18, Be253.19, and Be253.35. No helper virus-specific fragment amplified by PCR could be detected in clone Be244.2 after 7 months in culture nor in the cell lines Be253.7, Be253.15, Be253.16, Be253.30, Be253.31, and Be253.33. Reconstruction experiments with cellular DNA derived from an EBV-negative Burkitt's lymphoma cell line (DG75) (9) doped with different amounts of an appropriate target DNA (ranging from 65,000 to 0.65 copy per sample) indicated that the limit of detection was fewer than 5 EBV DNA molecules per 30,000 cellular genomes.

E. coli. These DNAs were purified and characterized subsequently by digestion with restriction endonucleases and DNA sequencing. Several plasmid DNAs were found to have acquired DNA from the helper virus in the region of the EBNA3a ORF, and two plasmid derivatives, termed p1244.8a-6.6 and p1244.8a-8.4, derived from the cell clones Be253.30 and Be253.33, respectively, were studied further (Fig. 5A and B). The region of the EBNA3a gene was the site for homologous recombination used to construct p1244.8a (Fig. 1A and 5B). EBNA3a has been found to be required for the efficient immortalization of B lymphocytes by EBV (31). The same region of the parental p1244.8a DNA was sequenced and found to lack a C residue, which, when expressed, would yield only the amino-terminal third of the protein (Fig. 6A and B). The two derivatives of p1244.8a, p1244.8a-6.6 and p1244.8a-8.4, had acquired that C residue upon recombining with the helper virus (Fig. 5A and B). Extensive analyses of the rescued 1244.8a-derived plasmid DNAs by digestions with a number of restriction enzymes indicated that their structure was unaltered compared with that of the parental p1244.8a plasmid, with the exception of the EBNA3 locus. It thus appears that restoration of a wild-type ORF for EBNA3a such that EBNA3a can be expressed is advantageous for the proliferating cells.

We tested whether the derivatives of p1244.8a (p1244.8a-6.6 and p1244.8a-8.4 in Fig. 5A), which contain a wild-type EBNA3a ORF, could initiate and maintain proliferation of infected cells by repeating experiments in which they were packaged and diluted serially prior to exposure to B lymphocytes. Proliferating clones arose subsequently with one-hit kinetics (Fig. 4B), indicating that these mini-EBVs are sufficient to induce proliferation. Eight clones were harvested, and their DNAs were analyzed by Southern blotting and PCR. All eight clones analyzed contained only mini-EBV DNAs and no detectable helper virus DNA (data not shown), confirming that these mini-EBVs are capable alone of initiating cell proliferation and indicating also that the EBNA3a gene is required for that initiation event.

Mini-EBVs that lack a functional EBNA3a ORF can maintain proliferation of B cells. The mini-EBV DNAs analyzed in two clones of B cells exposed originally to packaged p1244.8a plus P3HR1 virus were found to contain only p1244.8a DNAs with the EBNA3a frameshift mutation as in the parental *E. coli* plasmid. The cell lines Be253.15 and Be253.16 did not express EBNA3a in Western blot experiments with a monospecific serum directed against EBNA3a (Fig. 6C and data not shown). Extensive Southern blot hybridizations with cellular DNAs from these two clones indicated that they contained mini-EBV

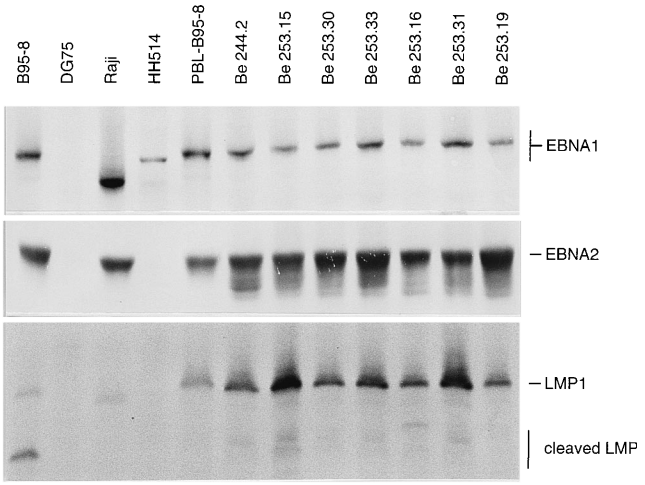

FIG. 3. Expression of latent genes in B-cell clones immortalized by virus stocks containing p1244.8a mini-EBV plus P3HR1 helper virus. Total cell extracts of different B-cell clones immortalized with virus stocks containing p1244.8a mini-EBV plus P3HR1 helper virus were investigated by the Western blotting technique. All B-cell clones carrying p1244.8a mini-EBV expressed EBNA1, EBNA2, and LMP1 as expected. Protein extracts of EBV-positive cell lines (B95-8, Raji, HH514, and PBL-B95-8 [a B-cell line obtained by infecting cord blood cells with B95-8 virus]) and an EBV-negative Burkitt's lymphoma cell line (DG75) served as controls. The cell lines Be253.31 and Be253.19 were doubly infected with p1244.8a mini-EBV plus P3HR1 virus; the remaining five B-cell clones were singly infected with p1244.8a mini-EBV.

plasmid DNA which was indistinguishable from the parental p1244.8a *E. coli* plasmid (Fig. 5C and data not shown). Apparently, the two clones have maintained the frameshift mutation originally present in p1244.8a. In addition, we sequenced the region spanning the frameshift mutation within the EBNA3a gene in the clones Be253.15 and Be253.16 by PCR-mediated DNA amplification. DNA sequencing confirmed the presence of the EBNA3a frameshift mutation in the two clones, indicating that no wild-type EBNA3a was present in the DNA of the cells (Fig. 6B). Only the region within the EBNA3a gene which contained the frameshift was sequenced, however, so that subtle mutations or products of recombination would not necessarily have been detected. Under standard culture conditions no apparent difference was observed between EBNA3a$^-$ and EBNA3a$^+$ cell lines.

Two EBNA3a$^-$ cell clones arose in our initial experiments in which 40 of 47 cell clones maintained helper virus DNA. We interpret these findings to indicate that these two cell lines were first infected with packaged p1244.8a plus P3HR1 helper virus DNA. That the cells continue to proliferate in the absence of a wild-type EBNA3a ORF (Fig. 6B and C) indicates that the carboxy-terminal two-thirds of this protein is not required by EBV to maintain proliferation of the infected B cell.

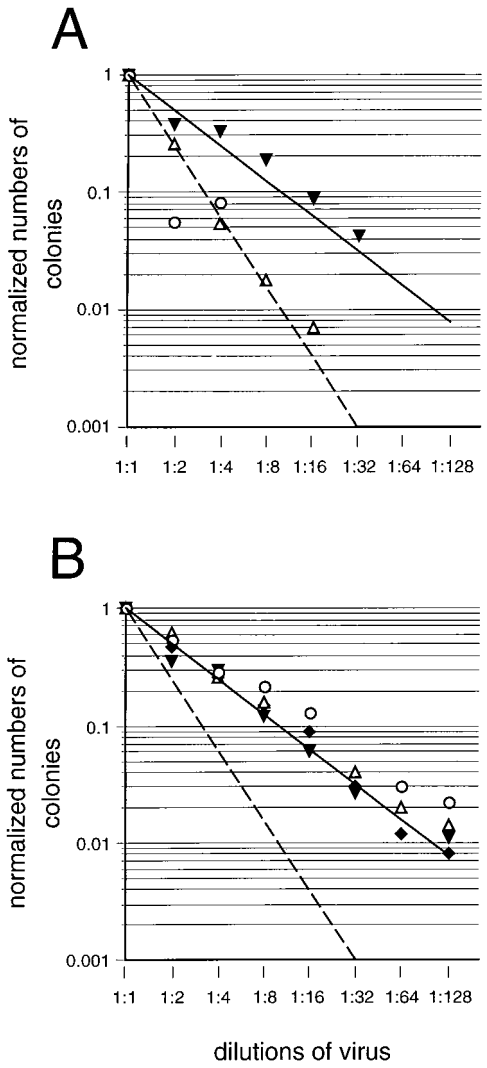

FIG. 4. Dose-response curves indicate which mini-EBVs are sufficient to induce and maintain proliferation of infected B lymphocytes. (A) Infection of purified human B lymphocytes with dilutions of stocks of p1244.8a mini-EBV plus P3HR1 helper virus yielded proliferating colonies in a dose-response curve indicative of a requirement for two particles for efficient induction of proliferation. The results of two experiments in which different stocks of virus were used to infect purified B lymphocytes from the same donor are depicted on a log/log plot. The numbers of colonies were normalized by setting the maximum value to 1 for each experiment. Infection with B95-8 virus yields a dose-response curve indicative of a requirement for one particle; the calculated dose-response curves for one (solid line) and two (broken line) particles are shown. (A) ○, experiment 1; △, experiment 2; ▼, B95-8 virus stock. (B) Four independent sets of experiments with two different p1244.8a-derived mini-EBVs with wild-type EBNA3a ORFs. Both p1244.8a derivatives (p1244.8a-6.6 [▼ and ◆, experiments 1 and 2, respectively] and p1244.8a-8.4 [○ and △, experiments 1 and 2, respectively] which were rescued from cell lines Be253.30 and Be253.33, respectively) yielded a dose-response curve indicative of a requirement for one particle; that is, these mini-EBVs are sufficient to induce and maintain proliferation of the infected B-lymphocyte.

DISCUSSION

We have constructed plasmids in *E. coli* that are composed of 71 kbp of EBV DNA and an F-factor plasmid, termed mini-EBVs. These mini-EBVs contain all of the virus *cis*-acting sequences required to participate in both the latent and lytic phases of the EBV life cycle. Consequently, they can be packaged when introduced into an EBV-positive cell in which the virus lytic cycle is induced. Some of the mini-EBVs once packaged can infect primary human B lymphocytes and alone initiate and maintain proliferation of their host cell and therefore carry the *trans*-acting functions required for immortalization. These observations indicate that the 59% of EBV DNA lacking the mini-EBVs is not necessary for immortalization of B lymphocytes. This finding excludes the multiply spliced transcripts in the *Bam*HI-A fragment (29) and an alternative Fp promoter presumably located within the *Bam*HI-F fragment (Fig. 1A), which is used to transcribe the EBNA1 gene under certain conditions (27, 28) even in in

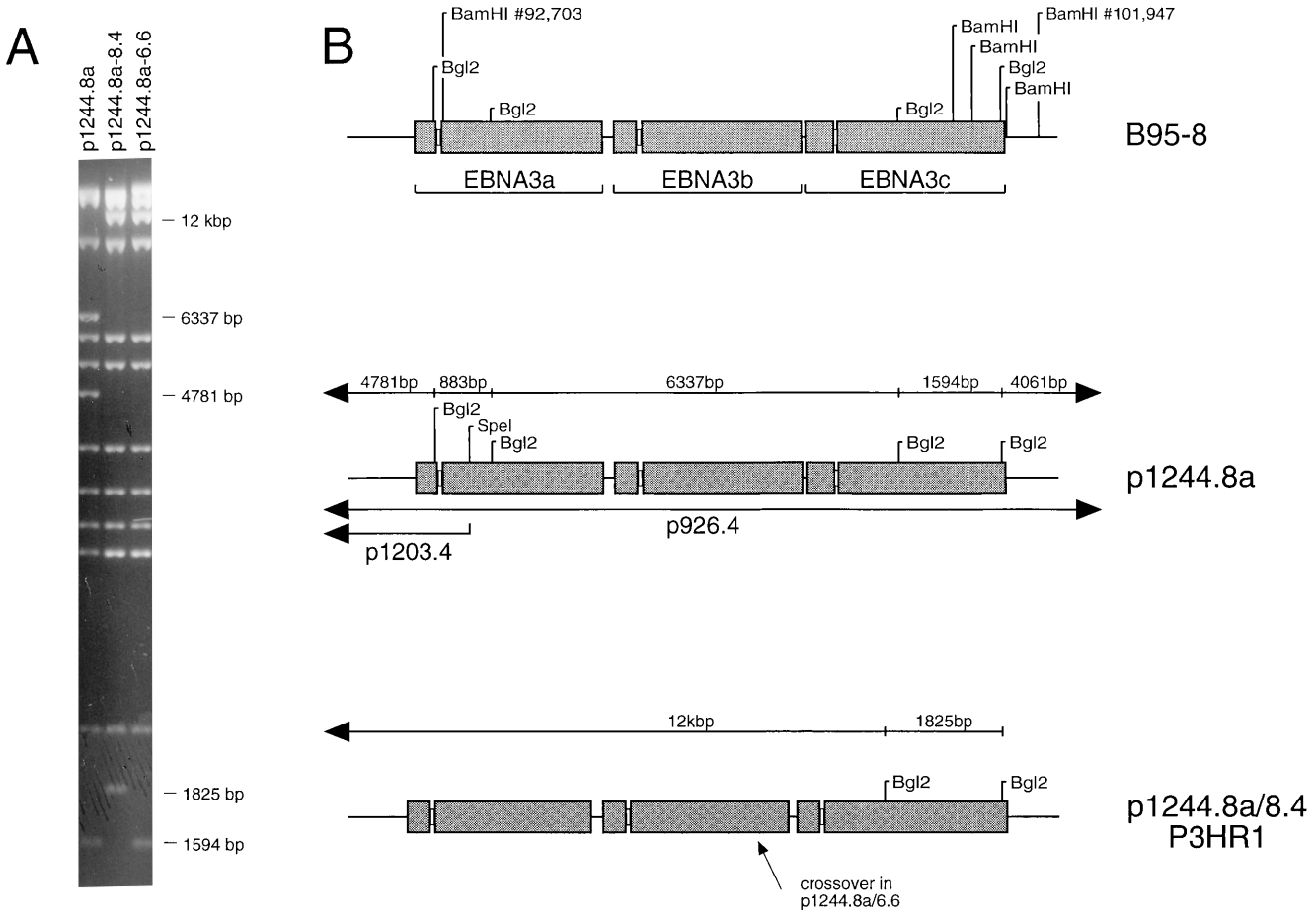

FIG. 5. Recombination of p1244.8a mini-EBV DNA with P3HR1 helper virus DNA at EBNA3a locus. (A) An ethidium bromide-stained agarose gel with the *Bgl*II-cleaved parental mini-EBV plasmid DNA prepared from *E. coli* is compared with that of its derivatives p1244.8a-6.6 and p1244.8a-8.4. The fragment sizes of certain *Bgl*II fragments, which refer to the fragments found at the EBNA3 locus in B95-8 (A strain) and P3HR1 (B strain) of EBV (schematically shown in panel B) are indicated on the right. The 883-bp fragment, which encompasses the first third of EBNA3a, is not shown. The loss of this *Bgl*II fragment together with the 6,337- and 4,781-bp fragments in p1244.8a-6.6 and p1244.8a-8.4 was accompanied by the gain of a 12-kbp fragment, which results from a recombination with P3HR1 helper DNA present in HH514 cells. This event was confirmed by restriction enzyme analyses with a number of different restriction enzymes (data not shown) which map the cross-over points during recombination 5′ to the small amino-terminal exon of EBNA3a and 3′ to the EBNA3c ORF in p1244.8a-8.4, as indicated by the presence of the 1,825-bp *Bgl*II fragment. In p1244.8a-6.6, the 3′ cross-over was mapped to the carboxy-terminal half of EBNA3b as indicated in panel B. (B) The 3′ end of the EBV insert in the prokaryotic shuttle plasmid p1203.4 cloned through a *Spe*I site at nucleotide 93234 of EBV (Fig. 1A and Table 1) is shown relative to the *Bgl*II sites in B95-8 and p1244.8a. (C) Autoradiogram of Southern blot with *Bgl*II-cleaved cellular DNAs from different cell lines immortalized with p1244.8a virus stocks probed with a plasmid encompassing the *Hin*dIII-E fragment (25). This probe detects the fragments depicted in panel B together with two fragments from the prokaryotic F-factor backbone. Note the absence of the 12-kbp *Bgl*II fragment in Be253.15 and Be253.16 DNA, indicating, together with data not shown, that these two cells lines carry the unmodified p1244.8a plasmid DNA exclusively. In contrast, other cell lines were doubly infected with either p1244.8a mini-EBVs and derivates of it with their EBNA3a loci repaired (Be244.2, Be253.30, Be253.33, and Be253.27) or P3HR1 virus (Be253.31 and Be253.19), as was also documented in Fig. 2A.

vitro-immortalized B-cell lines (3). The cells immortalized alone by these mini-EBVs lack most of the virus genes required for the lytic phase of the EBV life cycle and cannot support virus maturation (7). These mini-EBVs therefore may be useful vectors for human gene therapy.

The mini-EBVs are particularly informative virus derivatives with which to study virus contributions to immortalization of B lymphocytes. Stocks of the packaged derivatives can have up to 10^3 immortalizing units per ml in assays of 5×10^5 B lymphocytes. This number is high relative to that of potential recombinants of the mini-EBVs and the helper virus so that the parental mini-EBVs are usually found intact in immortalized cell clones. If the parental mini-EBVs were insufficient to mediate immortalization, as is the case for p1244.8a, then immortalized B-cell clones would usually maintain the complementing helper virus and the mini-EBVs. Because the mini-EBVs are defined by their construction in *E. coli* and usually remain intact during packaging, their contributions to the distinct phases of initiation and maintenance of proliferation of the host B cell can each be measured.

A mini-EBV with the C residue of the 304 codon of its

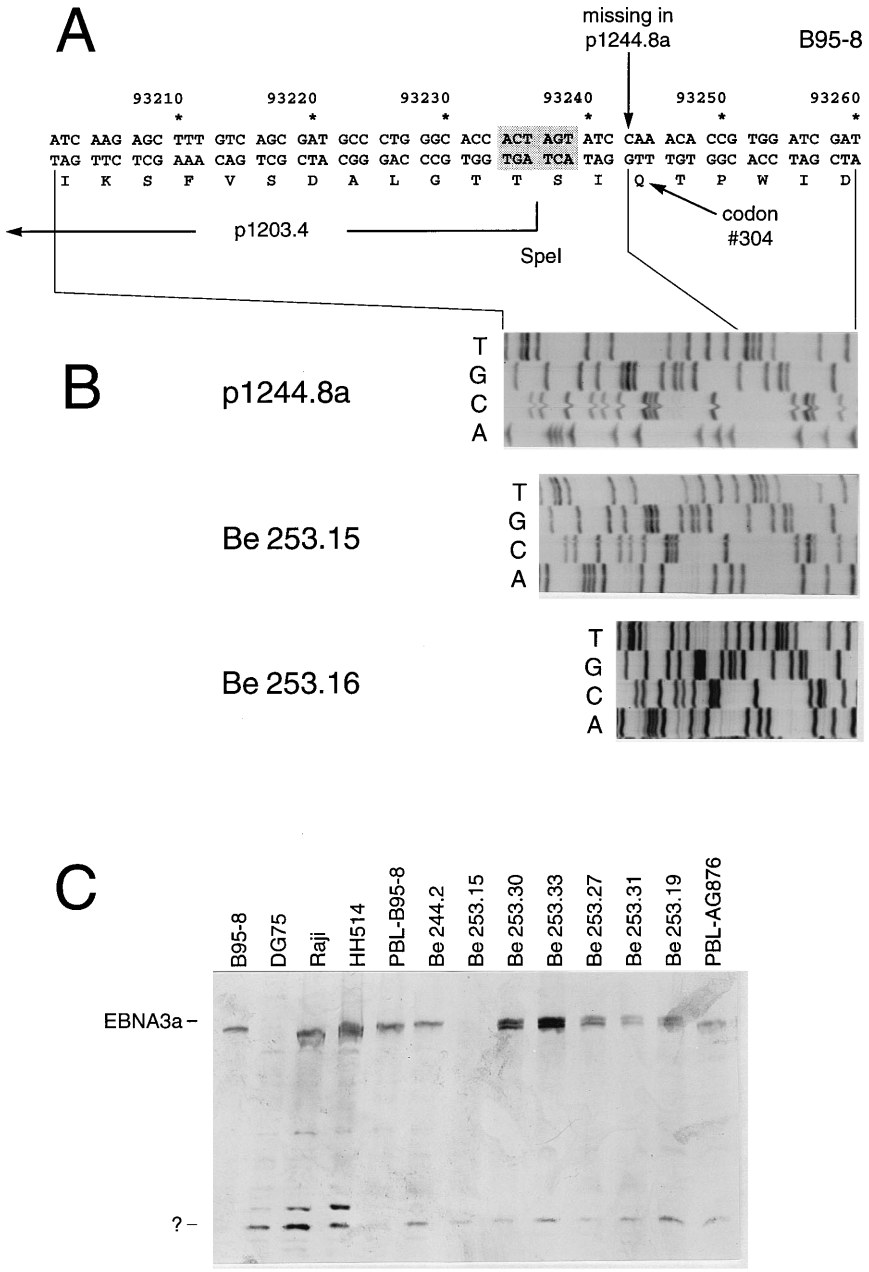

FIG. 6. Two B-cell clones from infection with p1244.8a mini-EBV and P3HR1 helper virus maintain only p1244.8a with its mutated EBNA3a. (A) The nucleotide sequence of a part of the EBNA3a gene as it is documented for the B95-8 strain of EBV (2) is shown together with the relevant nucleotide coordinates and the predicted codon usage of the EBNA3a ORF. The 3′ end of the EBV insert cloned with the aid of a *Spe*I site into p1203.4 (Fig. 1A and Table 1) is indicated. (B) The lower strand of this DNA sequence has been determined from p1244.8a plasmid DNA. This sequence points to a C nucleotide missing in p1244.8a DNA, resulting in a frameshift mutation in EBNA3a at codon 304. The same frameshift mutation is found in PCR-amplified DNA from the cell lines Be253.15 and Be253.16, which carry p1244.8a DNA only. As a consequence, EBNA3a cannot be detected in these cell lines in a Western blot (C) with rabbit antiserum directed against EBNA3a. This rabbit antiserum recognizes EBNA3a in B cells infected with the A or B strain of EBV, as is shown with cell protein extracts from B95-8 or HH514 cells, respectively. The question mark refers to background bands which are also recognized by this antibody. Cell lines with the prefix PBL are B-cell lines obtained by immortalizing cord blood cells from newborns with B95-8 or AG876 virus.

EBNA3a ORF deleted initiates proliferation of infected cells only in conjunction with a complementing helper virus. Mini-EBVs with that defect corrected both initiate and maintain proliferation of infected cells alone. We interpret these findings to mean that EBNA3a is an immortalizing gene whose contributions are essential early after infection; perhaps EBNA3a acts in the B lymphocyte prior to the cell's evolution into a blast. Two proliferating clones of cells exposed initially to the EBNA3a$^-$ mini-EBV and the helper virus were found to maintain only the EBNA3a$^-$ mini-EBV. We interpret this finding to mean that EBV-infected cells, once proliferating, can dispense with EBNA3a and continue to proliferate. This study of EBNA3a indicates that mini-EBVs derived from the A strain of EBV when used with a B strain helper virus allow the assignment of functions of immortalizing genes to distinct phases of the immortalizing process.

ACKNOWLEDGMENTS

We thank Noreen Warren for making the initial clones for chromosomal building, Josef Mautner for sequencing parts of the F factor plasmid pMBO132, and Toni Gahn for critically reading the manuscript. We are grateful to Michael O'Connor for providing plasmids, *E. coli* strains, and advice on the chromosomal building technique.

Our research was supported by grants AI-29988, CA-22443, and CA-07175 from the National Institutes of Health to B.S. and by grant Fa 138/3-7 from the Deutsche Forschungsgemeinschaft to W.H.

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