The latent membrane protein 2 gene of Epstein–Barr virus is important for efficient B cell immortalization

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The viral latent membrane proteins 2 (LMP2) of Epstein–Barr virus (EBV) were analysed genetically to evaluate their role in B cell immortalization. LMP2 is transcribed as two differently spliced mRNAs which code for the LMP2A and -B proteins, also called terminal protein-1 and -2. LMP2A and -B are found in latently infected, growth-transformed B lymphocytes in vitro, in different human tumours, and in latently infected B cells in vivo. Two different approaches were used to generate EBV mutants in which the second, third and part of the fourth exon of the LMP2 gene were deleted by insertion of a marker gene. Initially, conventional homologous recombination in a Burkitt's lymphoma cell line (P3HR1) between the endogenous EBV genome and an introduced plasmid was used to generate EBV mutants. This experiment identified LMP2 as dispensable for B cell immortalization as has been reported. In a second approach, the same LMP2 mutant gene was analysed in the context of a mini-EBV plasmid. These are E. coli constructs that are sufficient when packaged into an EBV coat both to initiate and to maintain proliferation of infected B cells. In comparison with a fully competent mini-EBV, LMP2- mini-EBVs were found to be greatly reduced in their capacity to yield immortalized B cell clones. This finding confirmed the initially observed bias against LMP2- B cell clones, most of which were found to be coinfected with complementing P3HR1 virus. These results indicate that LMP2 contributes to the efficiency of B cell immortalization and that the LMP2s phenotype is auxiliary in nature.

Introduction

Epstein–Barr virus (EBV) is a herpesvirus which is associated with a variety of malignancies that include B cell lymphomas in immunocompromised patients, Burkitt's lymphoma, certain T cell lymphomas, most cases of Hodgkin's disease and nasopharyngeal carcinoma (for review see Liebowitz & Kieff, 1993; Sugden, 1995). It was the first human tumour virus to be identified. EBV also infects resting human primary B lymphocytes and induces and maintains the proliferation of these B cells in vitro, a process called immortalization. Immortalized B cells are latently infected with EBV and only 11 out of ~ 80 viral genes are expressed in these cells. Some of the viral genes which are consistently found in immortalized B cells are also expressed in certain EBV-associated human tumours. Therefore, B cell immortalization by EBV is the in vitro model system for the contribution of this tumour virus to proliferative diseases.

Three of the 11 viral proteins which are expressed in EBV-immortalized B cells in vitro localize to the plasma membrane and are called latent membrane protein-1, -2A and -2B (LMP1, LMP2A and LMP2B). LMP2A and LMP2B, which are also called terminal protein-1 and -2 (TP1 and TP2), are expressed from mRNAs that are formed by transcription and splicing across the joined termini of the circularized EBV genome as it is present in latently infected cells (Laux et al., 1988, 1989; Sample et al., 1989). The gene structure of LMP2A and LMP2B consists of unique and common exons (Fig. 1). The common exons code for a highly hydrophobic protein which is predicted to form 12 transmembrane spanning domains reminiscent of transporter proteins (Laux et al., 1988). The additional first exon of LMP2A generates a presumable cytoplasmic hydrophilic domain which is missing in LMP2B. LMP2A but not LMP2B is detectable in B lymphocytes isolated from peripheral blood of EBV seropositives (Qu & Rowe, 1992) and LMP2 transcripts as well as antibodies against the proteins have been detected in patients with nasopharyngeal carcinoma (Brooks et al., 1992; Busson et al., 1992, 1995; Frech et al., 1990, 1993).
LMP2 associates with LMP1 and these proteins have a rapid turnover rate which suggests a role in receptor-mediated signal transduction (Baichwal & Sugden, 1988; Martin & Sugden, 1991a, b; Rowe et al., 1990). The amino-terminal part of LMP2A, which is encoded by its unique first exon, is associated with src family protein tyrosine kinases (Burkhardt et al., 1992; Longnecker et al., 1991). LMP2A serves as a substrate for these protein kinases and acts as a dominant negative regulator of lyn and fyn protein kinase activity (Miller et al., 1995). Thereby, LMP2A interferes with signal transduction in that it blocks calcium mobilization following cross-linking of cell-surface immunoglobulin molecules and impairs tyrosine phosphorylation of other cellular protein substrates and second messenger generation (Miller et al., 1993, 1994a). It is believed that these biochemical characteristics of LMP2A are indicative of a role in blocking induction of the lytic phase of the EBV life-cycle (Miller et al., 1994b, 1995).

Despite these important biological characteristics genetic analyses have not revealed any phenotype for several mutations in LMP2A or LMP2B (Kim & Yates, 1993; Longnecker et al., 1992, 1993a, b). The genetic data suggested that LMP2A and LMP2B are both dispensable for B cell immortalization and lytic functions of EBV. The technique which was used to generate mutant viruses, however, relies on fortuitous recombination between endogenous EBV DNA and plasmid DNA which is transiently introduced into latently EBV infected cells and carries the desired mutation. To promote recombination and release of virus, the lytic phase of the EBV life-cycle is concomitantly induced and the supernatant from these cells is harvested as a mixed virus stock that contains parental endogenous virus and a small proportion of recombinants whose structure is not known a priori. These stocks are used to immortalize B cells and the structure of the recombinant EBV DNA in them is analysed. This approach can only identify viral information sufficient to maintain proliferation of B cells (Cohen et al., 1989; Hammerschmidt & Sugden, 1989; Lee et al., 1992; Lee & Yates, 1992; Mannick et al., 1991; Marchini et al., 1991, 1992; Tomkinson & Kieff, 1992). This approach cannot identify information sufficient to initiate B cell immortalization since the initial infecting virus is not known and may be lost during propagation of the cell (Kempkes et al., 1995b).

To identify viral information crucial for initiation of B cell immortalization, the immortalized cells are induced to release recombinant virus which is subsequently used to infect primary B lymphocytes. If recombinant virus alone is able to immortalize B cells it is concluded that the recombinant virus is sufficient to induce proliferation as well as to maintain it. The disadvantage of this approach is that the amount of recombinant virus is so low that the efficiency of B cell immortalization cannot be determined.

To overcome this limitation we have developed a different approach which uses mini-EBV plasmids with the capacity to carry all the information needed to initiate and maintain B cell immortalization in vitro (Kempkes et al., 1995a, b). We used a single LMP2 mutation which affects both LMP2 proteins and tested this mutation in two different experimental settings for its effect on B cell immortalization. The mutation of the LMP2

![Fig. 1. Schematic representation of the region of EBV strain P3HR1 with the gene encoding LMP2A and LMP2B and the insertional mutation in p40. The genome of EBV strain P3HR1 is fused via the terminal repeats (TR) and the LMP2 gene is transcribed across TR such that LMP2A and LMP2B use two different first exons, 1A and 1B, respectively. The structure of the two mRNAs is indicated by lines connecting the numbered exons (grey boxes or open boxes for translated or non-translated exons, respectively). Arrows represent the two promoters expressing LMP2A and LMP2B. In p40 the fragment from an EcoRI site to the next SacI site (nucleotide coordinates #2 to #644 of the B95.8 prototype sequence) was replaced by the hygromycin expression cassette (HYG) to disrupt both LMP2 genes. The small arrows below the sketches of the P3HR1 and p40 structure represent the primers used for PCR analysis; the PCR products (double lines) and their sizes are given. Numbers indicate the nucleotide coordinates of selected restriction sites for SacI, EcoRI, SacI and SacI in the B95.8 genome. oriLy indicates the position of the lytic origin of EBV. IR represents the internal repeat units and EBNA2 indicates the EBNA2 gene. Single capital letters denote different BamHI fragments of the corresponding B95.8 strain and the deletion in the P3HR1 genome which removes the complete EBNA2 gene is depicted. The location of the three probes which were used in Southern blot hybridization is shown.](image-url)
gene was found to severely reduce the efficiency of B cell immortalization indicating that the phenotype of LMP2- EBV is quantitative.

Methods

Plasmid constructs. Most plasmids used in this study contain oriP, oriLy and the terminal repeats (TR) of the EBV strain B95.8. Such plasmids are also called mini-EBV plasmids since they replicate lytically and are packaged into infectious virus particles with the aid of an EBV helper virus. The packaged mini-EBV plasmids infect human primary B cells and replicate via oriP in proliferating cell lines.

Construction of p1189.19. The construction of p1189.19 required cloning and manipulation of more than 37 kb of EBV DNA on a prokaryotic E. coli vector; this was accomplished by the chromosomal building technique (O'Connor et al., 1989). The basic building strategy relies on an initial F-factor-based plasmid which is used as a recipient to add pieces of DNA by a combination of homologous and site-specific recombinations in E. coli. The sequential addition of partially overlapping DNA segments of about 10 to 20 kb in length, which are molecularly cloned on a so-called shuttle plasmid, results in a stepwise increase in size of the primary F-factor plasmid. The approach allows an F-factor construct of defined composition to be built and also permits the final plasmid to be modified at all positions by homologous recombination in E. coli. The modification technique is called 'crossing on mutations'.

Four partially overlapping fragments derived from the immortalization-competent B95.8 strain of EBV were cloned in E. coli, resulting in plasmid p1189.19 (Table 1), by the chromosomal building strategy (O'Connor et al., 1989). p931.12 was the first F-factor-based plasmid which served as recipient for the sequential addition of the consecutive plasmids p935.1, p1177.3 and p927.3 (Kempkes et al., 1995b). The joined parts of the plasmid p1189.19 constitute 37 kbp of genomic EBV DNA and span from nucleotide position #163477 to #56081 with a gap between #19359 and #43935. This gap removes eight of twelve complete internal repeats (IR).

Construction of p40. To introduce a mutated LMP2 locus into p1189.19 the 'crossing on mutations' method (O'Connor et al., 1989) was used. First, the shuttle plasmid p34 was constructed: this carries the LMP2- locus flanked by EBV sequences. p34 contains the BamHI hfr fragment from nucleotide coordinate #166614 to #3953 of the B95.8 sequence (Baer et al., 1984; Sugden et al., 1984) cloned into pMBO96 (O'Connor et al., 1989). The EcoRI–SalI fragment from nucleotide coordinate #2 to #644 of the B95.8 sequence was replaced in p34 by a BamHI–SalI fragment derived from plasmid pHEBo with the hygromycin phosphotransferase gene in the context of regulatory sequences from the thymidine kinase gene of herpes simplex virus (Yates et al., 1985) (Fig. 1). The p34 shuttle plasmid, which carried the desired LMP2- locus, was recombined with the parental p1189.19 F-plasmid to yield a cointegrate (Fig. 2). This construct was finally resolved to yield the LMP2- plasmid p40, which is identical to the wild-type plasmid p1189.19 except for the LMP2 gene (Fig. 2).

Construction of the mini-EBV plasmids p1478.A and p68. Starting with p1189.19 as F-factor recipient, the mini-EBV plasmid p1478.A was assembled in three additional basic building steps with the plasmids p948.27, p1470.1 and p1202.1 (Table 1). The p1478.A mini-EBV plasmid (Fig. 3) contains all EBV genes known to be required to initiate and maintain B cell immortalization by EBV in vitro (Farrell, 1995; Kempkes et al., 1995a, b). Starting with p40 as the recipient, plasmid p68 (Fig. 3) was assembled with the aid of the same three shuttle plasmids as were used for construction of the mini-EBV plasmid p1478.A. p68 carries the LMP2- mutation whereas p1478.A is wild-type. All intermediate plasmids and the final constructs were carefully checked by restriction enzyme analysis for the desired structures. With the exception of E. coli strain DH5α, which was purchased from Gibco BRL Life Technologies, all the cloning vectors and E. coli strains were obtained from M. O’Connor (O’Connor et al., 1989).

Table 1. Composition of plasmids used to assemble p1189.19, p40 (LMP2-), p1478.A and p68 (LMP2-) mini-EBV plasmids

The names of plasmids and the nucleotide coordinates of the EBV inserts are given together with the restriction enzyme sites used for subcloning B95.8 DNA. The genes and cis-acting elements pertinent to this study are indicated. The cloning vector pMBO96 was used for all shuttle plasmids. The first construct in the chromosomal building process was p931.12, which is based on pMBO132 (Kempkes et al., 1995a, b; O’Connor et al., 1989).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Plasmid</th>
<th>From # to # (cut)</th>
<th>Genes and cis-acting elements</th>
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</thead>
<tbody>
<tr>
<td>p1189.19 and p40</td>
<td>p931.12</td>
<td>163477 to 3994</td>
<td>LMP1, LMP2, TR</td>
</tr>
<tr>
<td></td>
<td>p935.1</td>
<td>644 to 8994</td>
<td>oriP, EBER1, 2</td>
</tr>
<tr>
<td></td>
<td>p1177.3</td>
<td>3994 to 13215</td>
<td>oriP, EBER1, 2</td>
</tr>
<tr>
<td></td>
<td>p927.3</td>
<td>7315 to 19359</td>
<td>EBNA-LP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43935 to 50081</td>
<td>EBNA-LP, EBNA2, oriLy</td>
</tr>
<tr>
<td>1478.A and p68</td>
<td>p948.27</td>
<td>52385 to 56081</td>
<td>EBNA3a, 3b, 3c</td>
</tr>
<tr>
<td></td>
<td>p1470.1</td>
<td>79658 to 89948</td>
<td>EBNA1</td>
</tr>
<tr>
<td></td>
<td>p1202.1</td>
<td>101426 to 113282</td>
<td></td>
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Cell line and packaging procedures. HH514 is a \textit{het}-free cell clone of the P3HR1 Burkitt’s lymphoma cell line (Heston et al., 1982; Rabson et al., 1982) and was grown in RPMI medium supplemented with 10% fetal calf serum. To generate recombinant virus or to package mini-EBV plasmids, $1 \times 10^7$ HH514 cells were transiently transfected by electroporation (Bio-Rad Electroporator; 0.4 cm wide cuvettes, 250 µl volume, 960 µF capacity, 240 V voltage) with 10 µg of an expression vector for the viral BZLF1 gene (pCMV-BZLF1) (Hammerschmidt & Sugden, 1988) and 10 µg of mini-EBV plasmid DNAs p40, p68, p1478.A or p1495.4. Cells were cultivated in 10 ml of medium and the supernatant was harvested after 4 to 6 days. Primary human B cells prepared from cord blood were infected with 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 (MRC 5 cells; ATCC). To determine the efficiency of B cell immortalization by different virus stocks, adult peripheral B lymphocytes were purified by magnetic cell sorting with anti-CD 19 microbeads according to the manufacturer’s protocol (Miltenyi Biotech Inc.). B cells ($8 \times 10^6$) were exposed to 8 ml of the virus stocks for 4 h at room temperature and subsequently plated in agarose as described (Sugden & Mark, 1977). Colonies with 50 or more cells were scored approximately 21 days after plating. As a positive control supernatants from B95.8 cells were used which yielded 30000 or 55000 colonies/ml in two independent experiments.

PCR analyses and Southern blot hybridization. Two PCR primer pairs (5' TGGCCGCTGCAAAAGGAAACTG and 5' CGTAAATGCCTGTAGTCCGCC or 5' TTCGCCAATGACAAGCCTG- G and 5' ACCACAGTTACAGCTCAAGGAGG) were used to dis-
Fig. 3. Maps of p1478.A wild-type mini-EBV plasmid and its p68 LMP2− derivative. The circularized map of both plasmids is shown with the BamHI sites schematically indicated on the inner circle. The 11 viral genes (EBNA1, EBNA-LP, EBNA2, EBNA3a to EBNA3c, LMP1, LMP2A and LMP2B, EBER1 and -2), generally expressed in immortalized B cells in vitro are either denoted as grey boxes together with the extension of the primary RNA transcripts (dashed lines) and promoters (→) or are too small to be presented (EBER1 and -2). The three cis-acting elements involved in DNA replication and maturation of EBV are indicated by black boxes: the latent origin of DNA replication (oriP) (Yates et al., 1984), the lytic origin of DNA replication (oriLyt) (Hammerschmidt & Sugden, 1988) and the cleavage and packaging signals (TR) (Hammerschmidt & Sugden, 1989). The prokaryotic plasmid backbone from the F-factor plasmid pMB0132 is indicated (O'Connor et al., 1989). The insertion of the gene encoding hygromycin phosphotransferase (hyg) in the p68 mini-EBV plasmid disrupts both LMP2A and LMP2B gene products.

Results

Homologous recombination yields immortalized LMP2− B cell clones at low frequency

The conventional strategy to produce recombinant EBV is based on homologous recombination events between the endogenous EBV genome of the Burkitt's cell line P3HR1 and introduced plasmid DNA. The P3HR1 cell line carries a viral DNA in which a portion of EBNA-LP and all of EBNA2 are deleted such that the endogenous DNA when amplified and packaged yields an immortalization-defective EBV also called P3HR1 (Bornkamm et al., 1980; Jeang & Hayward, 1983). Plasmid DNAs with homology to EBV can be introduced into P3HR1 cells, the lytic cycle of the endogenous EBV induced by cotransfection of an expression vector for the viral BZLF1 gene (Countryman & Miller, 1985; Takada et al., 1986), and recombination between the introduced DNAs and EBV DNA takes place. The virus stock harvested from these cells is mixed; it is composed of the parental endogenous virus, and recombinations between it and the introduced DNAs if they contain the terminal repeats (Hammerschmidt & Sugden, 1989). P3HR1 virus alone is deficient for B cell immortalization owing to a deletion which removed part of the EBNA-LP and all of EBNA2 gene (Bornkamm et al., 1980; Jeang & Hayward, 1983). This fact allows selection for recombinant virus by transfecting the sequences deleted in P3HR1 on one plasmid together with the DNA carrying the mutation to be introduced on a second plasmid and selecting for immortalizing virus after infection of susceptible B cells. Earlier studies have shown that about 5 to 10% of the immortalizing (EBNA2+) virus genomes also carry the mutation at the desired locus (Kim & Yates, 1993). This method was called 'second site homologous recombination'.
In order to increase the frequency of EBNA2+ and LMP2− EBV recombinants plasmid p40 was constructed; this contains the mutated LMP2 locus and the sequences deleted in P3HR1 virus on a single plasmid in their genomic context (Fig. 1). The gene for hygromycin phosphotransferase (hyg) was used as an insertional mutagen to evaluate whether the LMP2 gene is important for efficient immortalization of B cells. p40 contains sequence of the B95.8 EBV strain (Baer et al., 1984) from nucleotide coordinate #163477 to #56081 with the hyg gene replacing the second, third and part of the fourth exon of LMP2. The construction of such a plasmid required cloning of more than 37 kb of EBV DNA into a prokaryotic E. coli vector, which was accomplished by the chromosomal building technique (O’Connor et al., 1989) as described in Methods. The prokaryotic plasmid backbone and the introduced hyg gene bring the length of the plasmid DNA to 49.4 kbp. The size of the plasmid should allow it to be packaged into an EBV capsid as a trimer (Bloss & Sugden, 1994).

p40 was cotransfected with an expression plasmid for BZLF1 (pCMV-BZLF1) (Hammerschmidt & Sugden, 1988) into the HH514 cell line, a P3HR1 clone. pCMV-BZLF1 is able to induce the lytic cycle of the endogenous P3HR1 virus, which supports amplification of the transfected oriLyt-containing vector along with the endogenous EBV DNA. The supernatant of these cells contains a mixture consisting of P3HR1 helper virus, packaged p40 and different recombinants between P3HR1 virus and the transfected p40 DNA. This virus stock was used to infect primary B cells which were then seeded on irradiated fibroblast feeder layers in 96-well plates at limiting dilutions. Immortalized single-cell clones were analysed by PCR and Southern blot hybridizations to evaluate the infecting viruses retrospectively.

Four independent experiments yielded a total of 128 cell clones, 114 of which were further investigated by PCR analysis for the status of the LMP2 gene. About one-half of the clones were found to contain the insertional mutagen (data not shown). Seventy randomly selected B cell clones were analysed in Southern blot experiments for the presence of helper virus and for the characteristics of EBV recombinant virus. The results allowed the B cell clones to be grouped into five of six expected categories (Table 2). Thirty-seven clones were doubly infected with helper virus and p40 mini-EBVs (category E). In the remaining 33 clones various recombinants between both DNAs were detected. Three B cell clones (E109, E110 and E129 in category A) were found to contain only the desired recombinant EBV DNA with the mutated LMP2 locus, whereas ten clones carried the same recombinant viral genome in conjunction with P3HR1 helper virus DNA (category C). Clones in category B carried a wild-type LMP2 gene and were infected with a single EBV recombinant in which only the deletion in P3HR1 had been healed, presumably as a result of a double recombination event between P3HR1 and p40 DNA. No clones were found which resulted from coinfection with P3HR1 and LMP2−,EBNA2− recombinant virus (category D).

No attempt was made to characterize the mutant virus DNA in four clones which seemed to be infected with heavily rearranged EBV genomes.

The clones E109, E110 and E129 were further investigated in detail by Southern blot hybridization with EcoRI- and BamHI-digested cellular DNAs using various probes (Fig. 4). The desired composition could be confirmed in that all three clones showed the 3.7 kb EcoRI fragment specific for the mutated LMP2 locus and lacked the fragment specific for the wild-type gene (Fig. 4a, bottom panel). The BamHI H and Y fragments were clearly detectable, which is indicative of the EBNA2 locus in p40 or B95.8 virus (Fig. 4b), and the clones were free of helper virus DNA as indicated by the absence of the BamHI O' fragment (Fig. 4b). These clones also lacked the fragment indicative of a transcomplementing p40 genome (Fig. 4a, top panel). Once established the phenotype and behaviour of the LMP2− B cell clones were not grossly different from B cell lines immortalized by wild-type EBV with respect to growth kinetics and gene expression (data not shown).

Our data indicate that faithful recombination between P3HR1 virus DNA and p40 plasmid DNA could be observed in about 40% of the clones (13 out of 29) which were infected with recombinant virus. This finding supports the overall strategy of combining EBNA2 and the mutation to be introduced on one molecule. However, only three out of 13 clones (categories A and C) carried the desired mutation in the absence of helper virus. Very much in contrast, none of the LMP2−,EBNA2− clones in category B in which only the deletion in the P3HR1 gene was healed were coinfected with P3HR1 virus. This finding could mean that an LMP2− EBV is severely affected in its capacity to immortalize B cells efficiently since the majority of cells infected with LMP2− EBV carried a transcomplementing P3HR1 virus.

**Table 2. Summary of results of Southern blot hybridizations of single-cell B cell clones obtained with p40-containing virus stocks**

<table>
<thead>
<tr>
<th>Genotype of B cell clones</th>
<th>No. of clones</th>
<th>Category</th>
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<tbody>
<tr>
<td>EBV recombinants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMP2+,EBNA2+</td>
<td>3</td>
<td>A</td>
</tr>
<tr>
<td>LMP2−,EBNA2+</td>
<td>16</td>
<td>B</td>
</tr>
<tr>
<td>LMP2−,EBNA2+ plus P3HR1</td>
<td>10</td>
<td>C</td>
</tr>
<tr>
<td>LMP2+,EBNA2+ plus P3HR1</td>
<td>None</td>
<td>D</td>
</tr>
<tr>
<td>Transcomplementation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p40 plus P3HR1</td>
<td>37</td>
<td>E</td>
</tr>
<tr>
<td>Uncharacterized</td>
<td>4</td>
<td>‘Others’</td>
</tr>
</tbody>
</table>

The composition of 70 different B cell clones with respect to the genes LMP2 and EBNA2 in recombinant EBV, coinfected P3HR1 helper virus (LMP2−,EBNA2−) and p40 (LMP2+,EBNA2+) are summarized and grouped into categories.
Fig. 4. Analyses of virus DNA in B cells infected with p40-containing virus stocks. (a) Southern blot hybridization with cellular DNAs, cleaved with EcoRI, from different B cell lines infected with p40-containing virus stocks. Radioactive probe 1, which detected the prokaryotic plasmid backbone of the p40 construct only, was used in the upper panel. Probe 2 (lower panel) discriminates between a wild-type LMP2 fragment of 4.2 kb and an LMP2 fragment of 3.7 kb with hyg as an insertional mutagen as schematically shown in Fig. 1. The cell lines were grouped into categories indicated by capital letters according to the criteria listed in Table 2. The lane indicated ‘p40’ serves as a control for the mutated 3.7 kb EcoRI fragment and the 4.2 kb fragment detected in P3HR1 cellular DNA indicates the situation for the wild-type LMP genes. Three clones (E109, E110 and E129) carry a recombinant LMP2 genome as indicated by the lack of a signal indicative of the p40 plasmid backbone in the top panel and the presence of the mutated LMP2 gene in the lower panel. Cell lines E35, E39, E40 and E61 show the 26.9 kb fragment and the heterogeneous terminal fragment in the upper panel with probe 1 and therefore contain p40 DNA plus transcomplementing P3HR1 virus. (* Cell line E61 contains a very small amount of p40 DNA which is not visible on this exposure of the autoradiogram.) This finding was confirmed by the presence of two EcoRI fragments for the wild-type and mutant LMP2 allele detected by probe 2 in the lower panel. Cell line E128 contains additional non-characterized fragments as shown in (b) and is an example of an uncharacterized EBV recombinant ('others' in Table 2). (b) Probe 3, as shown in Fig. 1 (a cloned Drai fragment from nucleotide coordinate #44769 to #50305 of the B95.8 genome), was hybridized to BsmHI-cleaved cellular DNAs of individual B cell clones infected with p40 virus stocks. Lane p40 shows the position of the BsmHI fragments C, H, W and Y of the B95.8 genome present in p40. An additional fragment denoted ‘vector’ was also detected by the probe because the prokaryotic part of the radioactive probe hybridizes to pBR sequences present in the vector backbone of
Immortalization efficiency of LMP2- mini-EBV plasmid is greatly reduced

The initial approach to study LMP2 genetically relied on recombination between endogenous EBV DNA and a newly introduced plasmid to generate LMP2- EBV mutants. This approach is limited since recombination events yield a variety of progeny whose contributions to immortalization can only be assessed retrospectively. Moreover, this approach does not allow ready measurements of quantitatively distinct contributions to the overall process. To overcome these limitations mini-EBV plasmids were constructed which encompass all the EBV genes necessary and sufficient for efficient B cell immortalization (Kempkes et al., 1995a, b). When packaged by an endogenous P3HR1 helper virus (or transfected into B cells as plasmid DNA) these plasmids alone can initiate and maintain proliferation of infected primary human B lymphocytes.

To quantify the effect of LMP2 mutations, two mini-EBV plasmids were constructed, differing only in their LMP2 loci (Fig. 3). The wild-type plasmid and the LMP2- mutant plasmid, termed p1478.A and p68, respectively, encompass the latent and lytic origins of DNA replication, oriP and oriLy, the packaging signal sequences TR, and all the genes known to be expressed in EBV-immortalized B cells (Table 1). p1478.A is a parental construct of the mini-EBV plasmid p1495.4 which, once packaged, can infect primary B lymphocytes and initiate and maintain proliferation (Kempkes et al., 1995a). p1478.A and p68 are 81760 bp and 83014 bp in size including the prokaryotic plasmid backbone and are identical with the parental construct of the mini-EBV plasmid pi495.4 which, when packaged by an endogenous P3HR1 helper virus (or transfected into B cells as plasmid DNA) these plasmids alone can initiate and maintain proliferation of infected primary human B lymphocytes.

p1478.A and p68 were both packaged in the P3HR1 helper cell line as described (Kempkes et al., 1995b). Adult peripheral B lymphocytes were exposed to dilutions of the virus stocks and the infected cells were plated in semi-solid medium on irradiated fibroblast feeder cells. The number of proliferating colonies was counted after they had accumulated to more than about 50 cells. Virus stocks with packaged p1478.A mini-EBVs gave 48 and 113 clones, whereas p68 mini-EBV stocks generated only two and eleven clones in two independent experiments. Uninfected B cells or cells infected only with helper virus did not yield any clones.

B cell clones infected with virus stocks containing packaged p68 or p1478.A were obtained in additional experiments in which primary B cells were exposed to dilutions of virus stocks containing p68 or p1478.A mini-EBVs. Fifty-six single-cell B cell clones infected with p68 virus stocks were analysed in Southern blot hybridizations to investigate the status of their virus DNA. Total cellular DNAs were restricted with EcoRI and the resulting blots were probed with the prokaryotic vector backbone of the mini-EBV plasmids. Fifty-four out of 56 clones derived from a number of independent experiments were found to carry p68 mini-EBV DNA (data not shown). The two remaining clones presumably were recombinants between p68 and helper virus in which the P3HR1 deletion was healed. These two clones were not further analysed.

B cells infected with virus stocks containing wild-type mini-EBV-like p1478.A generate B cell clones readily with single-hit dose-response kinetics indicating that a single particle of virus is sufficient to induce and maintain proliferation (Kempkes et al., 1995b; data not shown). As a consequence, the majority of the cells are found to be free of P3HR1 helper virus and contain the mini-EBV genome only. To explore the status of P3HR1 helper virus in the cell clones obtained with p68 virus stocks, a probe was used which is able to distinguish between the two DNAs. Two clones (clones 29/1 and 22/14 in Fig. 5a) out of 52 contained p68 DNA only; the remaining cells were found to be coinfected with helper virus. To analyse the LMP2 gene for its specific hyg insertion in the two helper virus free cell lines, EcoRI-restricted cellular DNAs were hybridized to a radioactively labelled probe. To our surprise, EcoRI fragments were detected which are specific for both the mutant and the wild-type locus in the two cell lines 29/1 and 22/14 (Fig. 5b). Since the p68 mini-EBV plasmid is packaged as a dimer, the result indicated that recombination between P3HR1 and p68 DNAs in the packaging cell line had restored one of the two LMP2 alleles of the p68 dimeric genome. Alternatively, recombination between p68 and P3HR1 in coinfected B cells restored an LMP2 locus on p68 and the P3HR1 genome was subsequently lost. Together, these findings suggest that LMP2- mini-EBVs are severely crippled in their capacity to yield immortalized B cells in vitro.

Discussion

The results of this study indicate that the LMP2 gene plays a critical role in the efficiency of B cell immortalization. The LMP2 gene is not absolutely required for EBV to immortalize...
Fig. 5. Analyses of virus DNA in B cell clones infected with p68 mini-EBV plasmid-containing virus stocks. (a) The structure of a section of the P3HR1 virus genome and its corresponding part in p68 plasmid DNA are shown in the lower part of this figure. Denoted BamHI fragments are indicated by capital letters and the location of probe 4 is shown. The gap in the structure of p68 DNA is the joined F/M fragment of 5811 bp in length between the BamHI fragments M and F; this is indicative of the p68 genome since the F/M junction fragment is not present in any known EBV strain. The autoradiogram in the upper part shows a Southern blot of total cellular DNA of different B cell clones cleaved with BamHI and hybridized to probe 4. P3HR1 cellular DNA shows the S and M fragments, the lane with p68 plasmid DNA the S fragment together with the joined F/M fragment. The probe detected the ‘vector’ BamHI fragment of p68 because pBR sequences are present in the probe and in the vector backbone of p68. Cell lines 29/1 and 22/14 show the p68-specific fragments and lack the M fragment which is indicative of helper virus. Cell lines 22/13, 22/15 and 22/31 are coinfected with helper virus and p68 mini-EBV since they show the set of fragments specific for both DNAs. Cell lines 22/58 and 22/59 contain no vector fragment and seem to be recombinants between p68 and P3HR1. The nature of the recombination was not analysed further. Sizes of the HindIII-cleaved bacteriophage λ DNA fragments are indicated at the right. (b) The two B cell lines 22/14 and 22/19, which carry only p68 mini-EBV plasmid according to (a), were analysed after EcoRI digestion and Southern blot hybridization with probe 2 as shown in Fig. 4 (a) and in Fig. 1. Both cell lines contained fragments specific for wild-type as well as mutant LMP2 genes indicating that homologous recombinations between helper virus DNA and p68 mini-EBV plasmid lead to reversion of the mutant LMP2 allele in p68. P3HR1, B95-8, p1478.A and p68 are controls for the positions of the wild-type and mutant LMP2 EcoRI fragments. Sizes of HindIII-digested λ DNA marker are indicated on the left. Sizes and positions of the EcoRI fragments are indicated at the right.
B lymphocytes (Kim & Yates, 1993; Longnecker et al., 1992, 1993a, b) but our experiments suggest that LMP2 greatly influences the efficiency of this process.

The current approaches to studying immortalizing EBV genes are limited since they rely on recombination of an endogenous EBV with plasmid DNA to yield a mixed virus stock; the different members of this cannot be sorted other than through the successful immortalization of B lymphocytes. Clearly, such an approach does not allow ready measurement of quantitatively distinct contributions to the process of B cell immortalization in vitro as documented by our experiment with the LMP2- p40 construct. The analyses of the resulting immortalized B cell clones allow a statistical interpretation of numerical data which suggests a role of LMP2 in the process of B cell immortalization (Table 2). To solve this problem, we have developed mini-EBV plasmids to study the contributions of individual immortalizing genes to the efficiency of B cell immortalization.

Mini-EBV plasmids can carry all trans-acting functions required for immortalization and have been shown to initiate and maintain proliferation of B cells in the absence of any function provided by EBV helper virus (Kempkes et al., 1995a, b). p1478.A is such a mini-EBV plasmid which encompasses ~70 kb of EBV, and B cell clones infected with such mini-EBVs are usually free of P3HR1 helper virus. The mini-EBV construct p68, which differs from p1478.A only in a mutated LMP2 locus (Fig. 3), appears to be impaired in its capacity to initiate or maintain B cell immortalization efficiently. The majority of immortalized cells were found to be infected with p68 in conjunction with helper virus indicating that the mutation constitutes a disadvantage to B cell immortalization in vitro. Moreover, two cell clones which were infected with mini-EBV only had the defective LMP2 gene corrected by homologous recombination with the helper virus genome to compensate for the lack of a functional LMP2 gene.

The same LMP2 mutation was introduced into the context of the P3HR1 strain of EBV by single-site mutagenesis with p40 DNA. This attempt yielded three B cell lines which carried the desired mutation but the majority of the cell lines were wild-type with respect to LMP2 by trans-complementing P3HR1 virus (Table 2). This observation is not unique and had been noted previously (Longnecker et al., 1993a) but the aspect had not been pursued. It appears that functional LMP2 is advantageous, but not absolutely necessary, for the initiation or maintenance of B cell proliferation. Thus, the phenotype of the LMP2 gene is auxiliary and comparable with EBNA-LP (Hammerschmidt & Sugden, 1989).

Consistent with the phenotype of LMP2- mutants, LMP2A has been reported to function as a negative regulator of signal transduction in that it preserves the status of latency (Miller et al., 1994b, 1995). Conceivably, LMP2- mutant EBV would be expected to initiate and maintain B cell proliferation but these cells might be prone to enter the lytic phase of the EBV life-cycle. As a consequence, spontaneous activation of the lytic cycle would result in virus production accompanied by cell death and possible destruction of the immortalized cells. This model seems to be logical since the preservation of the latent state is a prerequisite for generation of stable B cell lines in vitro. However, B cell lines immortalized by p1478.A or p68 mini-EBVs cannot enter the complete lytic cycle since most genes involved in lytic phase gene expression are missing with the exception of BZLF1 and BRLF1. In addition, LMP2- EBV-infected B cell clones show a very much reduced level of spontaneous induction of the lytic phase of the EBV life-cycle compared with cells immortalized by wild-type EBV (Miller et al., 1994b) and B cell clones that stem from cord blood cells are completely refractory to this process (data not shown). It thus appears that the phenotype of LMP2- mutants in B cell immortalization relates to immortalizing functions yet to be defined.

We thank Georg Bornkamm for helpful discussions and ideas and Dagmar Pich for her expertise and advice in the chromosomal building strategy. We were supported by grants Fa138/6-1, Fa138/3-4 and Fa138/3-7 from the Deutsche Forschungsgemeinschaft, by grant AI-29988 from the National Institute of Health, and by institutional funds.

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Received 7 May 1996; Accepted 5 July 1996