

# Epstein–Barr virus latent membrane protein (LMP1) is not sufficient to maintain proliferation of B cells but both it and activated CD40 can prolong their survival

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Epstein–Barr virus (EBV) infects human primary B lymphocytes and induces and maintains proliferation of these cells efficiently *in vitro*. Mutants of Epstein–Barr virus which express EBV nuclear antigen 2 (EBNA2) in a conditional fashion allow dissection of individual contributions of viral genes to B cell immortalization. EBNA2 is a transcriptional activator of cellular and viral genes, including the viral latent membrane protein 1 (LMP1), which is essential for B cell immortalization and has oncogenic effects in non-lymphoid cells. To analyze the role of this gene in B cell immortalization, LMP1 was constitutively expressed in B cells infected with EBV carrying a conditional EBNA2 allele. In the absence of functional EBNA2, LMP1 was incapable of sustaining B cell proliferation in two independent assays but induced a phenotype consistent with prolonged cell viability. Activation of CD40 displayed a comparable phenotype. These data indicate that both CD40 activation and LMP1 expression may use a common pathway for B cell activation. Proliferation of human B cells, however, requires one or more additional signals triggered by EBNA2.

**Keywords:** B cell immortalization/CD40/Epstein–Barr virus/latent membrane protein 1

## Introduction

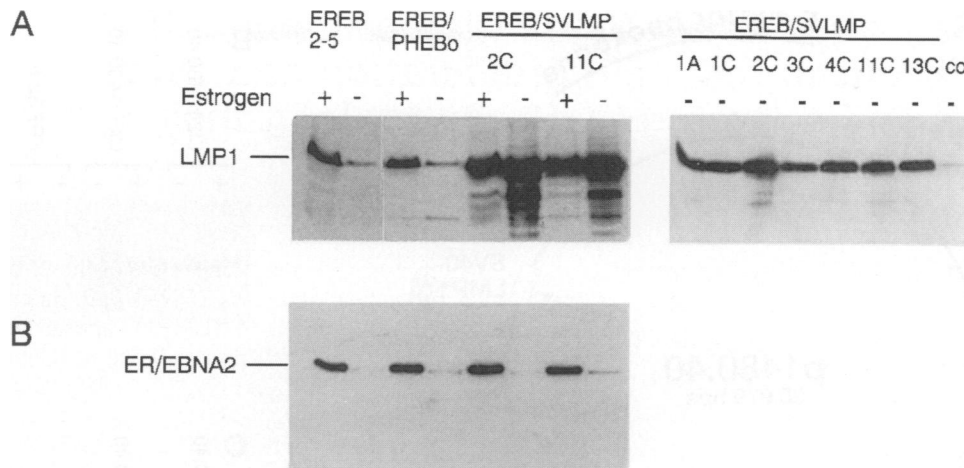
Epstein–Barr virus (EBV) is a widespread human herpesvirus. Primary infection in humans is usually asymptomatic or leads to infectious mononucleosis. EBV is also associated with a variety of malignancies that include Burkitt's lymphoma, B cell lymphomas in immunocompromised hosts, some T cell lymphomas, ~50% of Hodgkin's disease and nasopharyngeal carcinoma (Zeng *et al.*, 1985; Miller, 1990; Craig *et al.*, 1993; Pallesen *et al.*, 1993).

Experimental infection of resting B cells with EBV efficiently induces and maintains unlimited proliferation of the infected cells *in vitro*. This process is termed immortalization and is the *in vitro* model for this virus's contribution to B lymphoid diseases. EBV is a herpesvirus that encodes ~80 genes, 11 of which are found expressed

in *in vitro* infected B cells; five of these, the Epstein–Barr viral nuclear antigens (EBNA) 1, 2, 3a and 3c and latent membrane protein 1 (LMP1), appear to be absolutely required for B cell immortalization (Cohen *et al.*, 1989; Hammerschmidt and Sugden, 1989; Kaye *et al.*, 1993; Tomkinson *et al.*, 1993; for a recent review see Farrell, 1995). Two viral proteins, the EBNA leader protein (EBNA-LP) and latent membrane protein 2 (LMP2 or TP), seem to affect the efficiency of the process (Hammerschmidt and Sugden, 1989; Mannick *et al.*, 1991; Brielmeier *et al.*, 1996). The best characterized latent gene products are EBNA1, EBNA2 and LMP1. EBNA1 is necessary for episomal replication of the viral genome by binding to the viral origin of replication, *oriP* (Yates *et al.*, 1984). EBNA2 and LMP1 are essential for the immortalization potential of the virus.

EBNA2 acts as a transcriptional activator of cellular and viral genes. It modulates the expression of the B cell activation markers CD21 and CD23 and the tyrosine kinase *c-fgr* (Calender *et al.*, 1987; Wang, F. *et al.*, 1987; Cordier *et al.*, 1990; Knutson, 1990). In addition, EBNA2 transactivates the viral promoters of three latent membrane antigens (LMP1, LMP2A and LMP2B) and the Cp promoter, which regulates the expression of EBV nuclear antigens (Fahraeus *et al.*, 1990a; Sung *et al.*, 1991; Zimmer-Strobl *et al.*, 1991; Laux *et al.*, 1994b). EBNA2 does not bind to DNA directly (Ling *et al.*, 1993; Zimmer-Strobl *et al.*, 1993) but interacts with the cellular DNA binding proteins RBP- $\kappa$  and PU.1, which bind to EBNA2-responsive promoter elements (Grossman *et al.*, 1994; Henkel *et al.*, 1994; Laux *et al.*, 1994a; Waltzer *et al.*, 1994; Zimmer-Strobl *et al.*, 1994).

LMP1 is the only viral protein that is not only essential for B cell transformation but has also transforming effects in non-lymphoid cells. LMP1 acts as an oncogene in established rodent cells (Wang, D. *et al.*, 1985; Baichwal and Sugden, 1988; Moorthy and Thorley-Lawson, 1993) and blocks differentiation in epithelial cells (Dawson *et al.*, 1990; Fahraeus *et al.*, 1990b; Wilson *et al.*, 1990). In Burkitt's lymphoma cell lines LMP1 induces the expression of B cell activation markers CD23 and CD40 and cell adhesion molecules ICAM1, LFA1 and LFA3 and stimulates NF- $\kappa$ B activity (Wang, F. *et al.*, 1990; Hammarskjöld and Simurda, 1992; Laherty *et al.*, 1992; Huen *et al.*, 1995; Mitchell and Sugden, 1995). In primary human B cells LMP1 similarly stimulates some of the early events of B cell immortalization, like cellular enlargement, up-regulation of B cell markers and adhesion molecules and transient induction of DNA synthesis (Peng and Lundgren, 1992). LMP1 is an integral membrane protein with six transmembrane domains separated by short reverse turns. Both the 23 amino acid N-terminal and 200 amino acid C-terminal parts are cytoplasmatic (Fennewald *et al.*, 1984; Liebowitz *et al.*, 1986). Little is



**Fig. 1.** Constitutive expression of LMP1 in EREB2-5 cells. Different independent EREB2-5 cell clones transfected with LMP1 expressed from the SV40 early promoter/enhancer (EREB/SVLMPC), the parental line EREB2-5 and one clone transfected with the vector alone (EREB/pHEBo) were cultivated for 4 days in the presence or absence of estrogen and protein extracts were analyzed for the expression of LMP1 (**A**) and EBNA2 (**B**) by Western blotting. To compare the levels of LMP1 in different EREB/SVLMPC clones a shorter exposure is shown in the right part of (**A**). In the last lane, indicated by 'co', EREB2-5 extracts were analyzed as a control. All cell clones transfected with the SV40 promoter-driven LMP1 gene expressed LMP1 at levels comparable with or even exceeding those in EREB2-5 cells in the presence of estrogen. Estrogen withdrawal caused a slight increase in the expression levels of LMP1 in the transfectants, which is also noticeable in Figure 2B. (**B**) Estrogen-dependent expression of the ER-EBNA2 protein for EREB2-5 cells, one clone transfected with the vector alone (EREB/pHEBo) and two cell clones with a constitutively expressed LMP1 gene (EREB/LMP2C, EREB/LMP11C).

known about LMP1 function, but the recent finding by Mosialos and colleagues (Mosialos *et al.*, 1995) indicates that LMP1 interacts with TRAF1 and TRAF3 (also termed EBI6 and LAP1 or CRAF1 respectively). Both molecules are components of a signal transduction pathway which is used by members of the tumor necrosis factor receptor family. Binding of these TRAF signal mediators suggested that LMP1 could act as a receptor-like molecule which participates in CD40- or TNF-2 receptor-mediated signaling (Rothe *et al.*, 1994, 1995; Cheng *et al.*, 1995).

In this paper we address the question of whether LMP1 supports proliferation of EBV-immortalized B cells in the absence of functional EBNA2. Previously it was demonstrated that EBNA2 is necessary not only for initiation but also for maintenance of B cell immortalization (Kempkes *et al.*, 1995b). Since EBNA2 is one of the transactivators of the LMP1 gene it was possible that the contribution of EBNA2 to B cell proliferation is solely dedicated to up-regulation of the LMP1 gene. To address this hypothesis we analyzed the function of LMP1 in the absence of EBNA2 in EBV-immortalized B cells. We show that constitutive LMP1 expression alone is not sufficient to maintain B cell proliferation but LMP1 enhances the viability and survival of B cells and prolongs the period of DNA synthesis in the absence of EBNA2. Constitutive expression of LMP1 or stimulation of the CD40 receptor resulted in a comparable phenotype, supporting the assumption that both LMP1 and CD40 might engage the same signal transduction pathway to activate B cells.

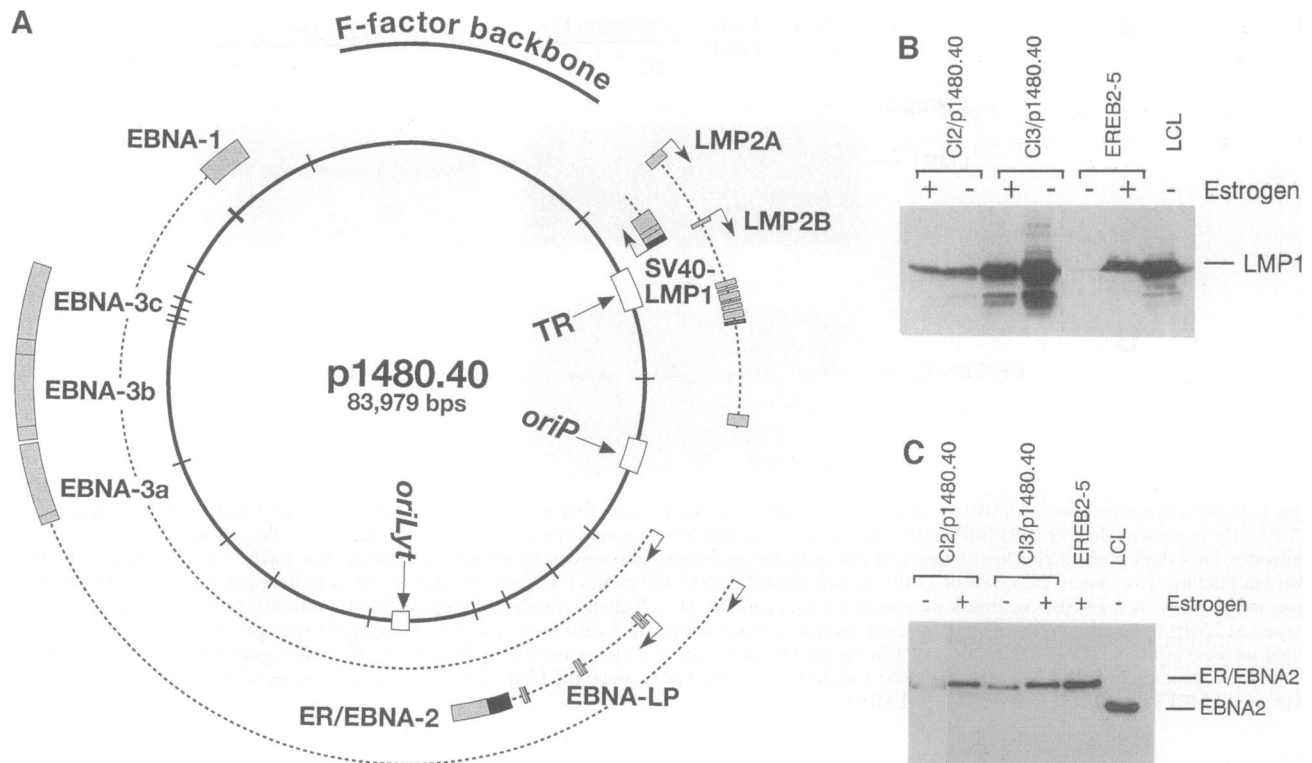
## Results

### Uncoupled LMP1 and EBNA2 expression in EBV-immortalized B cells

Proliferation of B cells which are infected with an EBV mutant encoding an EBNA2-estrogen receptor fusion

protein (ER-EBNA2) is dependent on estrogen (Kempkes *et al.*, 1995b). This experiment demonstrated that EBNA2 is necessary not only for initiation but also for maintenance of B cell proliferation *in vitro*. Since genetic and functional evidence indicates that LMP1 is likely to play a central role in B cell immortalization, we asked whether the sole function of EBNA2 required for maintenance of B cell proliferation is to up-regulate LMP1 expression.

To address the question of whether LMP1 maintains B cell proliferation independently of EBNA2 we established a system in which LMP1 is expressed constitutively in EBV-infected B cells. In a first approach, the LMP1 gene expressed from the SV40 early promoter/enhancer was cloned onto an episomal *oriP* vector encoding the gene for hygromycin phosphotransferase (Sugden *et al.*, 1985). This construct, termed p581, was stably introduced into EREB2-5 cells infected with the P3HR1 strain of EBV and a mini-EBV plasmid encoding the ER-EBNA2 chimeric gene (Kempkes *et al.*, 1995b, 1996). As negative controls, a p581-based plasmid with a truncated non-functional LMP1 gene or the parental *oriP* vector (pHEBo) were introduced in EREB2-5 cells. A number of independent hygromycin-resistant clones were isolated and tested for EBNA2 and LMP1 expression by Western blotting. In EREB2-5 cells transfected with the control plasmids, LMP1 expression was significantly down-regulated 4 days after removal of hormone, indicating that the LMP1 gene is under stringent control of EBNA2, as in the parental EREB2-5 cells (Figure 1A). In contrast, all 10 clones transfected with the wild-type LMP1 gene expressed from the SV40 promoter revealed a level of LMP1 which was not down-regulated after estrogen withdrawal. The LMP1 levels were comparable or even higher than in EREB2-5 cells in the presence of estrogen (Figure 1A; data not shown), which is not unexpected because in the transfectants there is probably expression of LMP1 both from the resident viral genome and from the introduced vector. In



**Fig. 2.** Generation of B cell clones with the p1480.40 mini-EBV plasmid encompassing the ER–EBNA2 fusion and a constitutive LMP1 gene. (A) The composition of the mini-EBV plasmid p1480.40 is shown, which was packaged into an EBV coat as described in Materials and methods to establish B cell lines encoding ER–EBNA2 and a constitutive LMP1 gene expressed from the SV40 early promoter/enhancer. The map shows the *cis*-acting elements (open boxes) *oriP*, *oriLyt* and TR, which constitute the plasmid origin, the lytic origin of replication and the terminal repeats respectively. Nine latent genes and their presumed primary transcripts are depicted and the exons are shown as gray boxes. Two black boxes indicate the genetic alterations of the EBNA2 gene (ER–EBNA2) and the promoter of the LMP1 gene (SV40–LMP1). The prokaryotic plasmid backbone of the F factor is annotated together with the location of *Bam*HI sites in the inner circle of the map. (B and C) Cells infected with p1480.40 were cultivated for 4 days in the presence or absence of estrogen and protein extracts were analyzed by Western blotting for the expression of LMP1 (B) and EBNA2 (C). As controls, EREB2-5 cells and a B cell line infected with wild-type EBV (LCL BPL2) (Kempkes *et al.* 1995) were tested. The ER–EBNA2 fusion protein and the wild-type EBNA2 protein migrated at sizes of ~120 and 80 kDa respectively.

all clones EBNA2 function was still estrogen dependent, as indicated by autoregulated expression of the ER–EBNA2 protein (Figure 1B) and regulated transactivation of EBNA2-responsive promoter constructs (data not shown).

In an alternative approach, the mini-EBV plasmid p1480.40 was constructed, which carries the ER–EBNA2 gene and the SV40 promoter-driven LMP1 gene in the context of an immortalization-competent mini-EBV construct (Figure 2A). The intention of this experiment was to establish a B cell clone in a single step which carried only the SV40 promoter/enhancer-expressed LMP1 allele but lacked LMP1 encoded from the P3HR1 strain of EBV. This experiment should also clarify whether EBNA2-regulated expression of LMP1 is essential for B cell immortalization. Virus stocks were produced containing p1480.40 genomes packaged into an EBV coat to infect and immortalize primary B cells as described (Kempkes *et al.*, 1995a). By PCR analyses, one B cell clone (clone 2 in Figure 2) was found to carry p1480.40 only, whereas other clones were doubly infected with p1480.40 and helper virus (clone 3 in Figure 2; data not shown). This result demonstrated that regulated expression of LMP1 is not mandatory for *in vitro* B cell immortalization. Two clones (clones 2 and 3) were analyzed in more detail for the expression of LMP1 and ER–EBNA2. As expected,

the ER–EBNA2 fusion protein was found to be expressed in a hormone-dependent manner (Figure 2C). In contrast, LMP1 expression levels were independent of EBNA2 and in the same range as in B cells immortalized by wild-type EBV or in EREB2-5 cells in the presence of hormone (Figure 2B).

#### **Constitutive LMP1 expression is not sufficient to maintain B cell proliferation in the absence of functional EBNA2**

In order to study the phenotype of constitutive LMP1 expression in B cells, proliferation of different B cell clones was analyzed in the presence and absence of estrogen. Two criteria, DNA content as detected by propidium iodide staining and incorporation of bromodeoxyuridine (BrdU), were measured by FACS analysis. In the presence of estrogen no significant differences in the cell cycle profiles were detected in parental EREB2-5 cells compared with EREB2-5 cells stably transfected with the SV40 promoter/enhancer-driven LMP1 plasmid (p581) or the vector control plasmid pHEBo (data not shown). After estrogen removal the number of BrdU-positive cells decreased in all clones, including those expressing high levels of LMP1, concomitant with a higher number of cells in G<sub>1</sub> (data not shown). This finding indicated that

LMP1 expression *per se* was not sufficient to maintain B cell proliferation in the absence of functional EBNA2.

### LMP1 expression delays the shut-off of DNA synthesis in the absence of EBNA2

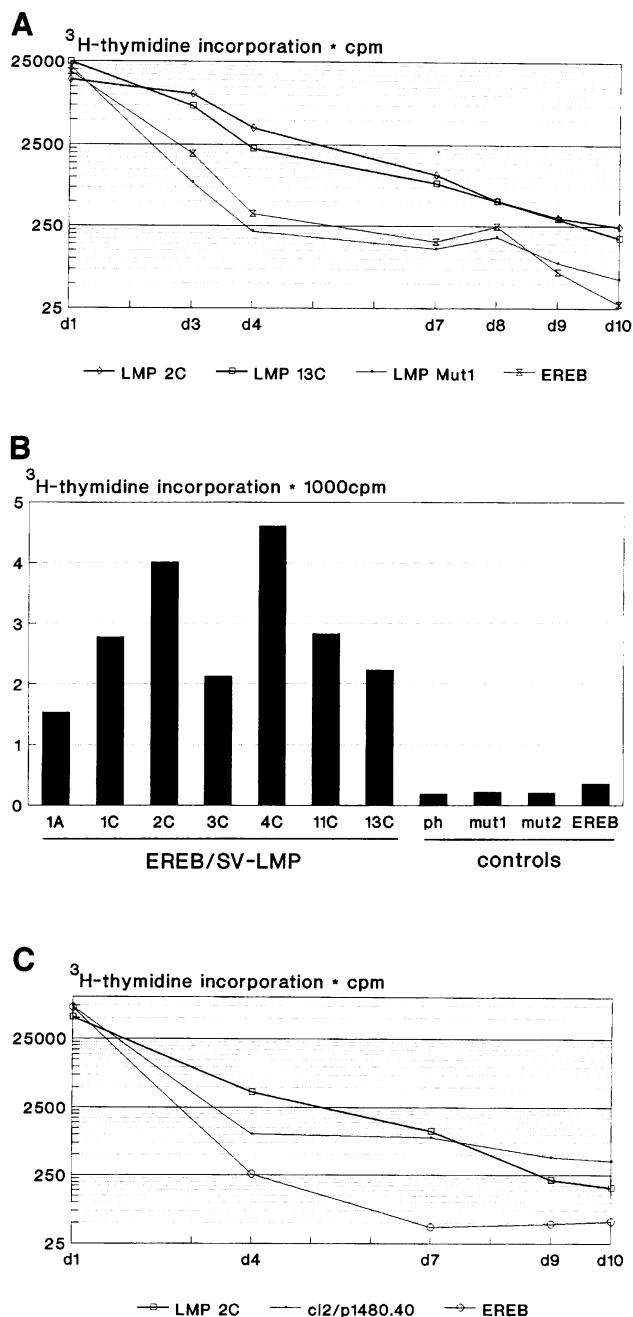
Cell cycle analyses revealed that the percentage of BrdU-positive cells was less dramatically reduced in LMP1 transfectants 4 days after withdrawal of estrogen compared with the parental or pHEBo-transfected EREB2-5 cells (data not shown). To confirm this observation, we measured the kinetics of [<sup>3</sup>H]thymidine uptake in the absence of estrogen in seven cell clones which constitutively expressed LMP1 for 10 days. The kinetics of [<sup>3</sup>H]thymidine incorporation during this period are shown in Figure 3A for two out of seven LMP1 transfectants (clones 2C and 13C), one clone transfected with mutated LMP (clone Mut1) and the untransfected parental cell line EREB2-5. [<sup>3</sup>H]Thymidine incorporation rates on day 4 after withdrawal of estrogen are summarized in Figure 3B for all clones tested. Cells which constitutively express LMP1 showed a significantly higher rate of [<sup>3</sup>H]thymidine incorporation in the absence of hormone than those cells with decreased levels of LMP1. Parental EREB2-5 cells and transfectants with the non-functional truncated LMP1 gene reached [<sup>3</sup>H]thymidine incorporation rates close to background levels 4 days after withdrawal of estrogen. In contrast, LMP1 transfectants clearly incorporated [<sup>3</sup>H]thymidine during the first 7 days and reached background levels finally ~10 days after removal of estrogen.

In a separate experiment, we analyzed the B cell clone infected with the mini-EBV plasmid p1480.40 (clone 2/p1480.40) for its kinetics of [<sup>3</sup>H]thymidine incorporation (Figure 3C). Again, constitutive expression of LMP1 had a similar effect on thymidine incorporation in this clone as in EREB2-5 cells transfected with the LMP1 expression vector p581. Immortalized B cells infected with p1480.40 mini-EBV were generated in a single step and carry only one LMP1 allele expressed from the constitutive SV40 promoter. It is very likely, therefore, that the increase in thymidine incorporation is a specific consequence of LMP1 expression and not an artifact of the selection procedure.

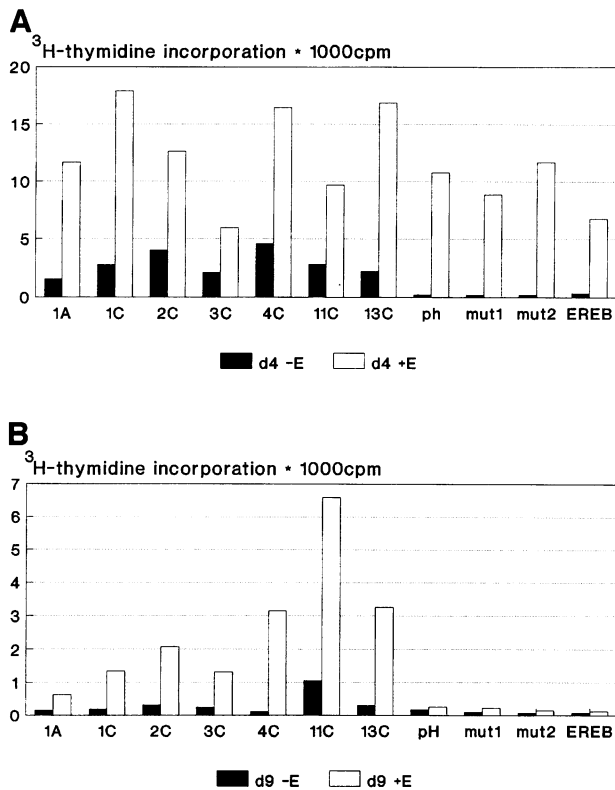
The results indicated that LMP1 expression in the absence of EBNA2 leads to a phenotype which is characterized by an increased and prolonged rate of thymidine incorporation, in contrast to the fast shut-off of DNA synthesis in EREB2-5 cells. Thymidine incorporation correlated with a low but detectable portion of cells in the S phase of the cell cycle (data not shown). Presumably, these cells supported DNA replication for a limited period of time only.

### Constitutive LMP1 expression permits re-induction of DNA synthesis 8 days after withdrawal of estrogen

In EREB2-5 cells growth arrest in the absence of hormone was reversible for up to 4 days, as indicated by re-induction of DNA synthesis and sequential accumulation and modification of cell cycle-regulating genes (Kempkes *et al.*, 1995b). In order to define the period in which DNA synthesis could be re-induced, seven clones of EREB2-5 cells transfected with the LMP1 expression vector p581 were cultivated in the absence of estrogen for different



**Fig. 3.** Prolonged incorporation of [<sup>3</sup>H]thymidine in cells which express LMP1 independently of EBNA2. As a measure of DNA synthesis, [<sup>3</sup>H]thymidine incorporation was determined in different clones derived from EREB2-5 cells as described in Materials and methods. On day 0 the cells were cultivated in the absence of estrogen for the indicated periods. (A) The time course of [<sup>3</sup>H]thymidine incorporation is shown for two cell clones transfected with wild-type LMP1 expressed from the SV40 promoter (LMP 2C, LMP 13C), one cell clone transfected with a LMP1 gene carrying a frameshift mutation (LMP Mut1) and the parental line EREB2-5 (EREB). (B) [<sup>3</sup>H]Thymidine incorporation 3 days after removal of estrogen is shown for all cell clones tested. EREB2-5 cells (EREB) were transfected with either wild-type LMP1 (EREB/SVLM), LMP1 carrying a reading frame mutation (mut1, mut2) or pHEBo (pH). (C) [<sup>3</sup>H]Thymidine uptake is shown for the CL2/p1480.40 cell clone established with the mini-EBV plasmid p1480.40 carrying the ER-EBNA2 fusion protein and a LMP1 gene expressed from the SV40 early promoter/enhancer as shown in Figure 2A. For comparison, the kinetics of [<sup>3</sup>H]thymidine uptake of the parental EREB2-5 cells (EREB) and EREB2-5 cells transfected with a constitutive LMP1 gene (LMP 2C) are shown.

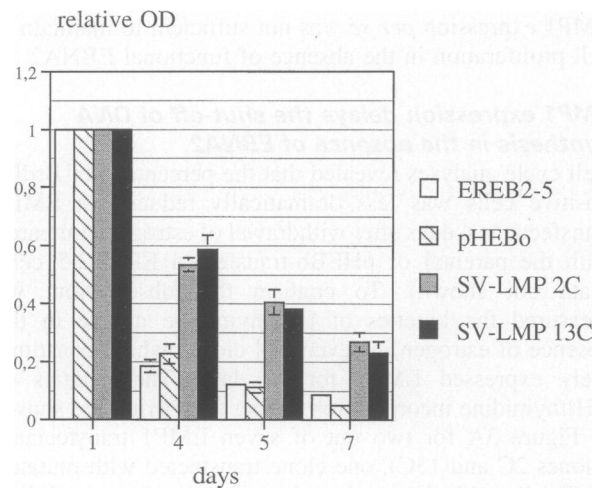


**Fig. 4.** DNA synthesis can be re-activated 8 days after estrogen deprivation only in cells with a constitutive LMP1 gene. Cell clones derived from EREB2-5 cells which were transfected either with the LMP1 gene expressed from the SV40 early promoter/enhancer (1A through 13C), the plasmid control pHEBo (pH), the LMP1 gene with a frameshift mutation (mut1, mut2) or the parental line EREB2-5 (EREB) were kept in the absence of estrogen. Estrogen was re-added to the culture medium (+E) after 3 (A) or 8 (B) days and [ $^3\text{H}$ ]thymidine incorporation was determined 24 h later (white columns in A and B). For comparison, [ $^3\text{H}$ ]thymidine incorporation was also determined for cells which had been kept in the absence of estrogen (-E) for the same period of time (black columns in A and B). Only cell clones which carried a constitutive LMP1 allele showed a marked rate of DNA synthesis after a period of 8 days in the absence of estrogen. Incorporation of [ $^3\text{H}$ ]thymidine was determined 4 h after addition of 0.5  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine to  $5 \times 10^4$  cells in 100  $\mu\text{l}$  as described in Material and methods.

time intervals and [ $^3\text{H}$ ]thymidine incorporation was measured 24 h after re-addition of hormone. As controls the parental EREB2-5 cells, cells transfected with the non-functional truncated LMP1 mutant or the pHEBo vector alone were tested in parallel. The rate of thymidine incorporation after 3 and 8 days of estrogen deprivation revealed remarkable differences. In the absence of estrogen, DNA synthesis could be re-induced in all cell lines tested, including the negative controls, after 3 days (Figure 4A). In contrast, all LMP1-expressing cell lines but not the negative controls showed clearly detectable [ $^3\text{H}$ ]thymidine incorporation even when estrogen had been omitted for a period of 8 days (Figure 4B).

#### LMP1 expression improves cell survival

Our results indicated that the expression of LMP1 extended the period during which DNA synthesis could be re-induced by estrogen in EREB2-5 cells. To determine whether this observation was the consequence of a prolonged survival of these cells, we determined the viability

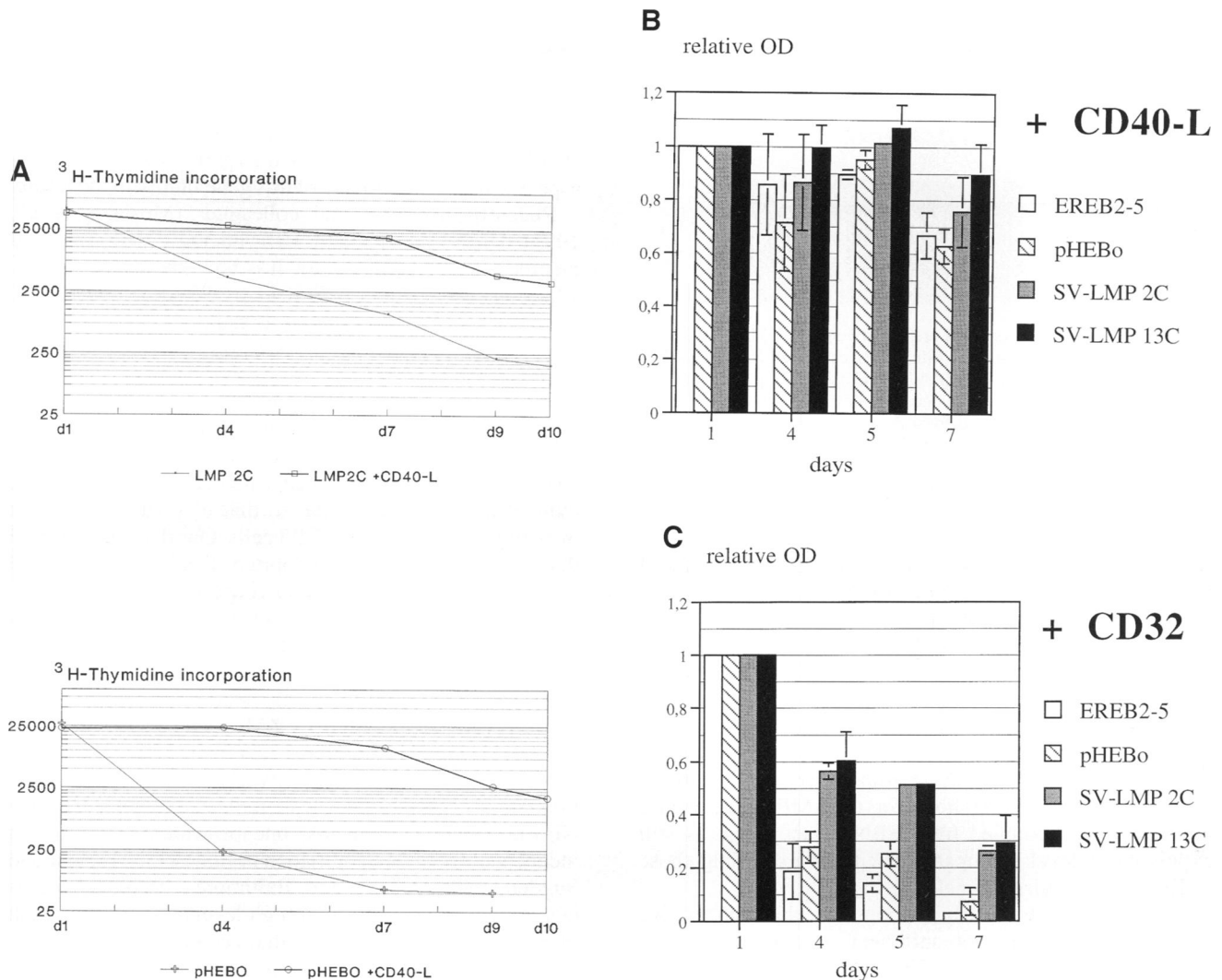


**Fig. 5.** A constitutive LMP1 allele improves cell viability. EREB2-5 cells transfected with the constitutive LMP1 construct (SV-LMP 2C, SV-LMP 13C) or the vector control plasmid pHEBo (pHEBo) and the parental cell line EREB2-5 were kept in the absence of estrogen for up to 7 days and cell viability was determined by MTT conversion on the days indicated. Mean values of three independent experiments are shown. To compare different experiments, the rate of MTT conversion at day 1 was set to 1 and OD values were normalized accordingly.

of the cells by MTT assay (Mosmann, 1983). Two LMP1 transfectants (clones 2C and 13C), the parental EREB2-5 cells and a pHEBo control transfectant were tested at different time points after removal of estrogen. In three independent experiments the viability was significantly higher only in cells expressing constitutive levels of LMP1 and not in the controls (Figure 5A and B). Withdrawal of estrogen leads to immediate cell death in a certain proportion of EREB2-5 cells, whereas the remaining cells enter a quiescent non-proliferative state (Kempkes *et al.*, 1995b). Therefore, our results suggested that LMP1 expression extends survival of the fraction of non-proliferating B cells in the absence of EBNA2 function.

#### CD40 stimulation and LMP1 have comparable effects in EREB2-5 cells lacking functional EBNA2

Previously it was shown that resting human B cells enter a finite state of sustained cell proliferation by CD40 stimulation through antibody-mediated cross-linking of either CD40 or its ligand, CD40-L (Banchereau *et al.*, 1991; Galibert *et al.*, 1996). CD40 stimulation acts as a survival factor and increases DNA synthesis in tonsillar B cells (Rousset *et al.*, 1991). Since LMP1 expression showed similar effects of B cell activation in EREB2-5 cells in the absence of functional EBNA2, we asked whether CD40 activation would mimic the phenotype mediated by constitutive LMP1 expression in these cells. EREB2-5 cells transfected with the LMP1 expression vector p581 (clone 2C and 11C), a control cell line transfected with pHEBo and the parental EREB2-5 cells were kept on CD40-L-expressing fibroblastic L cells in the absence of estrogen for up to 10 days. [ $^3\text{H}$ ]Thymidine incorporation was measured and the results indicated that CD40 activation stimulated DNA synthesis in EREB2-5 cells to a certain extent (Figure 6A). Surprisingly, not only the parental EREB2-5 cells and pHEBo-transfected cell line but also the LMP1-expressing clones 2C and 11C showed thymidine incorporation at a rate which was



**Fig. 6.** Stimulation of CD40 prolongs the survival of EREB2-5 clones in the absence of estrogen. (A) Two cell clones transfected with the control plasmid pHEBo or the LMP1 gene expressed from the SV40 early promoter/enhancer (LMP 2C) were cultivated in the absence of estrogen with or without CD40 stimulation. On the days indicated [ $^3\text{H}$ ]thymidine incorporation was determined as described in Materials and methods. (B and C) Two cell clones transfected with the constitutive LMP1 allele (SV-LMP 2C, SV-LMP 13C), one cell clone transfected with the control plasmid pHEBo A1(pHEBo) and the non-transfected parental EREB2-5 cells were kept on mouse L cells expressing either the Fc receptor as a control (C: CD32) or CD40 ligand (B: CD40-L). The viability of the different cell populations was determined by MTT conversion.

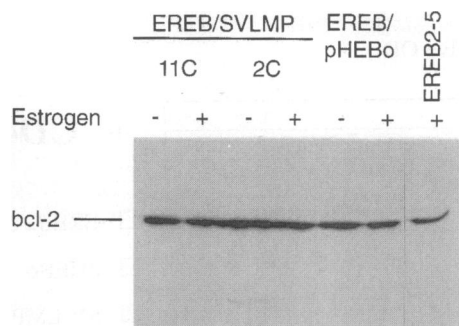
significantly enhanced by CD40 activation. In addition, we tested the survival of these cells in the absence of estrogen on CD40-L-expressing fibroblasts in MTT assays. The results clearly showed that CD40 activation markedly increased the survival rate of all EREB2-5 clones. Concomitant LMP1 expression did not grossly affect thymidine uptake (Figure 6A) or viability in the MTT read-outs (Figure 6B) in CD40-activated cells as compared with control cells (Figure 6C). It should be noted that irrespective of CD40 stimulation, neither the parental EREB2-5 cells nor their transfectants supported B cell proliferation in the absence of estrogen (compare Figures 5 and 6B) and CD40-activated cells eventually reached the same fate as LMP1 transfectants.

These data indicate that LMP1 expression and CD40 activation had comparable phenotypes in EREB2-5 cells. The differences appear to be quantitative in nature in that CD40 activation was superior to constitutive LMP1 expression. These quantitative differences could possibly be the result of differential expression of CD40 and LMP1.

Neither condition, however, supported B cell proliferation, but each prolonged cell survival with no apparent cooperative effect.

## Discussion

Nearly all B cell-specific effects of LMP1, like up-regulation of adhesion molecules and activation markers or activation of NF- $\kappa\text{B}$ , were determined in Burkitt's lymphoma cell lines after transfection of the LMP1 gene. Because Burkitt's lymphoma cells are immortalized by a mechanism not requiring expression of LMP1, it is difficult to assess the contribution of LMP1 to growth control of EBV-infected primary human B cells from experiments in Burkitt's lymphoma cells. To overcome this obstacle, we have established a cell system in which LMP1 function could be studied in the background of an EBV-infected, non-transformed B cell. Two complementing experimental approaches were used: (i) the LMP1 gene driven from the SV40 promoter was stably introduced into EREB2-5 cells



**Fig. 7.** Expression levels of *bcl-2* are not affected by LMP1. Two EREB2-5 cell clones transfected with LMP1 expressed from the SV40 early promoter/enhancer (EREBSVLMP 2C and 11C), the parental line EREB2-5 and one clone transfected with the vector alone (EREB/pHEBo) were cultivated for 4 days in the presence or absence of estrogen and protein extracts were analyzed for expression of *bcl-2* by Western blotting. No difference could be detected in the steady-state levels of the *bcl-2* gene product in the absence or presence of estrogen.

(Kempkes *et al.*, 1995b); (ii) normal resting human B cells were infected with a mini-EBV plasmid carrying an ER-EBNA2 gene fusion in conjunction with a LMP1 gene expressed from the SV40 promoter. In both systems, EBNA2 functions can be inactivated by omission of estrogen from the cell culture medium. As a consequence, EBNA2-dependent cellular and viral genes are down-regulated, which, under normal circumstances, includes the wild-type LMP1 allele (Kempkes *et al.*, 1995b). In this experimental setting, however, the LMP1 gene, whose expression is uncoupled from EBNA2 activation, is still expressed at levels comparable with or exceeding those in EBV-immortalized B cell lines.

Our experiments clearly demonstrate that constitutive LMP1 expression is not sufficient to maintain B cell proliferation in the absence of EBNA2. This finding means that EBNA2 acts beyond the up-regulation of LMP1 to maintain proliferation of EBV-infected immortalized cells, probably by cooperating with other viral and cellular genes. Constitutive LMP1 expression, however, revealed a reproducible phenotype in the absence of estrogen in all cell clones tested: (i) [<sup>3</sup>H]thymidine incorporation declined more slowly; (ii) cell survival was prolonged, as shown by MTT assays; (iii) cell proliferation could be re-activated even after a period of 8 days. These observations are in contrast to a paper by Peng and Lundgren (1992), which reported that expression of LMP1 in resting B cells is sufficient to induce limited DNA synthesis. Since we were studying EBV-positive B cells, the contrast between the two results might be more apparent than real. Our data demonstrate that LMP1 contributes to DNA synthesis in EBV-infected B cells but is not sufficient to promote productive B cell proliferation, presumably because the cells cannot progress through S phase (data not shown).

The main contribution of LMP1 is to prolong survival of EBV-infected cells, an effect which could be based on the ability of LMP1 to prevent apoptosis. In Burkitt's cells LMP1 has been reported to induce *bcl-2* expression (Henderson *et al.*, 1991), which protects cells from apoptosis. Whether *bcl-2* activation is due to clonal selection or mediated by LMP1 expression is still uncertain, because LMP1 expression in primary B cells does not induce *bcl-2* (Martin *et al.*, 1993; Rowe *et al.*, 1994).

Similarly, it was shown that LMP1 activates the A20 gene, which might also be involved in protection from apoptosis (Laherty *et al.*, 1992). Our experimental approach indicated that *bcl-2* expression is not affected by the level of LMP1 gene expression (Figure 7) but should permit exploration of this particularly interesting aspect with other members of this gene family in the future.

Recently, Mosialos and colleagues (Mosialos *et al.*, 1995) demonstrated that LMP1 interacts with LAP1, a member of the TRAF family. It is now known that LAP1, which is also called CRAF1 or TRAF3, specifically interacts with the intracellular domain of CD40, a TNF-R family member also expressed on activated B cells. Stimulation of CD40 by its ligand, CD40-L, activates proliferation and prevents apoptosis of tonsillar B cells. It was therefore tempting to speculate that LMP1 might mimic a constitutively active CD40-like receptor. To test this possibility, we asked whether CD40 stimulation induces a phenotype similar to that of LMP1 expression in estrogen-deprived EREB2-5 cells. Our data demonstrate that CD40 stimulation supported DNA synthesis and prolonged cell survival, as does constitutive LMP1 expression. The phenotype induced by CD40 stimulation was more pronounced than the effect induced by constitutive LMP1 expression but the differences appear to be quantitative. The reason for this phenomenon could be the result of differential expression of LMP1 and CD40 or could reflect differences in their cytoplasmic C-terminal domains, which are directly involved in signal transduction. In contrast to the CD40 molecule, LMP1 has two NF- $\kappa$ B activating domains, one of which seems to act independently of TRAF3 (Huen *et al.*, 1995; Mitchell and Sugden, 1995; Cheng and Baltimore, 1996). LMP1 is cytotoxic when expressed at high levels (Hammerschmidt *et al.*, 1989) and it is possible that parts of LMP1 resemble the signal transducing domains of FAS/APO-1 or TNF-R1, which induce apoptosis rather than cell survival and proliferation. As a consequence, constitutive LMP1 expression could cause a phenotype which results from interference of apoptotic and anti-apoptotic effects. LMP1 mutants carrying deletions in the C-terminal cytoplasmic part should be informative, as well as constitutive expression of different TRAF family members to elucidate whether LMP1 intersects with the CD40 signal pathway and mimics a constitutive receptor of the TNF-R family.

## Materials and methods

### Cell lines

All cell lines were grown in RPMI 1640 cell culture medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin in an atmosphere of 5% CO<sub>2</sub>. EREB2-5 is a lymphoblastoid cell line generated by infection of CD19-enriched human B cells with an Epstein-Barr virus mutant conditional for EBNA2 (Kempkes *et al.*, 1995b). The cell line was diluted 1:2 twice a week in standard RPMI 1640 medium supplemented with 1  $\mu$ M estrogen. HH514 is a single cell clone of the Burkitt's lymphoma cell line P3HR1 (Rabson *et al.*, 1982). MRC5, a human embryonic lung fibroblast cell line, was obtained from the American Type Culture Collection. Primary human B lymphocytes were isolated from routine tonsillectomies by generating single cell suspensions and T cell depletion by rosetting with whole sheep blood. The preparations were analyzed by FACS<sup>®</sup> analysis and were found to be >95% positive for the pan-B marker CD19 (DAKO, Hamburg, Germany). CD40L- and CD32-expressing L cells (Banchereau and Rousset, 1991) were diluted 1:4 twice a week.



### Plasmids

The expression plasmid for LMP1, p581, contains the *MspI*-*SmaI* fragment (nucleotide coordinates 169 476–167 920) of the EBV genome B95.8 under the control of the SV40 early promoter/enhancer in the *oriP* construct pHEBo, which also encodes for resistance against the drug hygromycin B (Sugden *et al.*, 1985). The construct which served as a negative control for LMP1 expression is a derivative of p581 in which the LMP1 gene was truncated after amino acid position 25 of LMP1 by an incorrect out-of-frame deletion between the *StuI* and *MscI* sites in LMP1. The mini-EBV plasmid p1480.40 is based on the plasmid p1244.8a-8.4, which can induce and maintain *in vitro* B cell immortalization (Kempkes *et al.*, 1995a). p1244.8a-8.4 was modified in *Escherichia coli* by allelic conversion (O'Connor *et al.*, 1989) to include an SV40 early promoter/enhancer-expressed LMP1 gene and the chimeric ER-EBNA2 gene, which consists of the ligand binding domain of the human estrogen receptor fused to the open reading frame of EBNA2 (Kempkes *et al.*, 1995b).

### Transformation of primary B cells

HH514 cells ( $1 \times 10^7$ ) were co-transfected with 10  $\mu$ g pCMV-BZLF1 and 10  $\mu$ g mini-EBV plasmid p1480.40. Electroporation was performed with a Bio-Rad gene pulser at 960  $\mu$ F and 250 V in a total volume of 250  $\mu$ l RPMI 1640. The harvest of the virus stocks and infection of purified B cells was carried out as described (Kempkes *et al.*, 1995b). The status of P3HR1 helper virus in the immortalized B cell clones was verified by PCR with appropriate primer pairs (R.Zeidler, G.Eißner, P.Meißner, S.Uebel, R.Tampé, S.Lazis and W.Hammerschmidt, submitted).

### Stable transfection of EREB2-5 cells

The EREB2-5 cell clone (Kempkes *et al.*, 1995b) was transfected by electroporation using a Bio-Rad gene pulser at 960  $\mu$ F and 230 V. After transfection the cells were seeded in 96-well flat bottom plates and selected in RPMI medium supplemented with  $\beta$ -estradiol and hygromycin B.

### Protein immunoblots

For Western blot analyses cellular extracts were prepared by sonification in H8 lysis buffer (20 mM Tris, pH 7.0, 2 mM EGTA, 2 mM EDTA, 6 mM  $\beta$ -mercaptoethanol, 50 mM NaF, 100 mM NaCl, 1% SDS). The protein concentration was determined and equal amounts of protein were separated on Laemmli 8 or 10% polyacrylamide-SDS gels. Proteins were transferred onto nitrocellulose filters (Amersham Hybond ECL) and protein expression was analyzed with monoclonal antibodies directed against LMP1 (S12; kindly provided by Dr David Thorley-Lawson), *bcl-2* (Santa Cruz Biotechnology Inc.) or EBNA2 (anti-EBNA2-R3). Immunoreactive proteins were detected by peroxidase-coupled secondary antibodies and enhanced chemoluminescence (ECL system; Amersham).

### Cell cycle analysis

For analysis of the cell cycle profile cells were kept in the presence or absence of estrogen for 4 days. After 4 days cells were incubated in the presence of BrdU for 4 h, fixed with ethanol and stained with anti-BrdU antibodies conjugated with fluorescein isothiocyanate and propidium iodide as described (Lowe *et al.*, 1993). The stained cells were analyzed on a Becton Dickinson FACS analyzer.

### [<sup>3</sup>H]Thymidine incorporation

For determination of DNA synthesis  $5 \times 10^4$  cells in 100  $\mu$ l/well were seeded into 96-well cluster plates in triplicate and kept in the absence or presence of estrogen for the time indicated. [<sup>3</sup>H]Thymidine at 0.5  $\mu$ Ci/well was added for 4 h and incorporation of radioactivity was determined in a scintillation counter after lysis of cells in water.

### MTT assays

Samples of  $3 \times 10^4$  cells were seeded in 100  $\mu$ l cell culture medium in triplicate in the absence or presence of estrogen for the indicated time. After incubation with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; 0.5 mg/ml] for 4 h, MTT conversion, which correlates with the number of living cells in the assay, was determined in an ELISA reader as described (Mosmann, 1983). Each experiment was repeated at least twice. To compare different experiments the OD on day 1 was set to 1 and normalized OD values are given for the following days.

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