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Epstein-Barr Virus Nuclear Antigen EBNA3C/6 Expression Maintains the Level of Latent Membrane Protein 1 in G_1-Arrested Cells

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The Epstein-Barr virus in the Burkitt lymphoma-derived cell line Raji has a deletion in the EBNA3C gene. When Raji cells are allowed to grow to high density and most of the cells become growth arrested in the G_1 phase of the cell cycle, the level of detectable latent membrane protein 1 (LMP1) is substantially reduced. After dilution of the cells with fresh growth medium, within 8 h, there is a large increase in LMP1 mRNA, and by 12 h, LMP1 is expressed to a high level (H. Boos, M. Stoehr, M. Sauter, and N. Mueller-Lantzch, J. Gen. Virol. 71:1811–1815, 1990). Here we show that in Raji cells which constitutively express a transfected EBNA3C gene, the down-regulation of LMP1 in growth-arrested cells does not take place. Furthermore, we show that in wild-type Raji cells, low-level LMP1 expression occurs when most of the cells are arrested at a point(s) early in G_1 (or G_0) when the product of the retinoblastoma gene, pRb, is hypophosphorylated. The dramatic synthesis of LMP1 coincides with the progression of these cells to late G_1 when pRb becomes hyperphosphorylated. Thus, in Raji cells, the LMP1 gene is apparently regulated in a cell cycle- or proliferation-dependent manner, but when EBNA3C is present, sustained LMP1 expression occurs as it does in a lymphoblastoid cell line. EBNA3C appears to either relieve the apparent repression of LMP1 in cells progressing through early G_1 or possibly alter the stage at which the cells growth arrest to one where they are permissive for LMP1 expression.

Latent membrane protein 1 (LMP1) and EBNA3C (also known as EBNA6) are expressed in all Epstein-Barr virus (EBV)-immortalized B-lymphoblastoid cell lines (LCLs) and also in some EBV-associated tumors and cell lines derived from them (reviewed in reference 25). They are two of the nine so-called latent proteins expressed in LCLs. The others are the other five nuclear antigens EBNAs1, -2, -3A, -3B, and -LP (also known as EBNA1 through -5, respectively) and two further membrane proteins, LMP2A and -2B (also known as terminal proteins TP1 and -2) (25). It is generally assumed that together these proteins are responsible for the activation of resting B cells, induction of their continuous proliferation, and maintenance of the EBV genome in its episomal latent form (37). EBNA1 is necessary for the replication of the episomal DNA (45), and recently, by using mutant viruses, it has been shown that EBNA2, -3A, and -3C and LMP1 are absolutely necessary for the immortalization of resting human B cells in vitro (12, 18, 24, 38). However, the precise biochemical function of any of the latent gene products in the establishment and maintenance of immortalization is unknown. The timing and sequence of viral and cellular gene expression immediately after infection of resting human B cells suggest that an orderly cascade of gene expression is necessary for cellular activation and immortalization to occur. EBV gene expression is initiated with the synthesis of EBNA2 and -LP, which is soon followed by synthesis of the remaining EBNAs. There is then a delay during which the cells progress toward S phase of the cell cycle; LMP1 expression appears to just precede or coincide with the first sign of DNA synthesis (2, 4, 27, 35).

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Although the precise function of LMP1 is unknown, it can induce cellular changes consistent with B-cell activation (42), when overexpressed can be toxic (19), and will induce oncogenic transformation in immortal rodent cells (8, 41). Its expression appears to be tightly regulated and involves a number of positively and negatively acting factors (15, 16, 43). There is a considerable body of evidence that at least one latent viral protein, EBNA2, is necessary for the efficient expression of LMP1 in B cells. For instance, infection of EBV-negative Burkitt lymphoma cells with the B95-8 strain of EBV results in LMP1 expression; however, infection with the EBNA2-negative P3HR1 strain does not (30). Similarly, infection of resting B cells with P3HR1 virus fails to produce LMP1 synthesis (4). Transfection of a recombinant EBNA2 gene into P3HR1 virus-converted B-lymphoma lines results in LMP1 expression (1), and it has now been shown by transient transfection analysis that the 5′-flanking region of the LMP1 gene contains negative *cis*-acting regulatory sequences which suppress the activity of the LMP1 promoter in B cells and that EBNA2 overrides this suppression (15, 16, 43). However, the regulation of LMP1 is very complex. For instance, there are various cell types in which EBNA2 is unnecessary for LMP1 expression; these include the parental P3HR1 Burkitt lymphoma cell line (4, 30), many nasopharyngeal carcinomas (14, 46), and the Reed-Sternberg cells which carry EBV in Hodgkin's disease (21, 31). Also, in some B-cell settings, although EBNA2 is presumably necessary, it is not sufficient for activation of LMP1. Early after the infection of resting B cells, for example, there is a window of many hours when EBNA2 is present but LMP1 is not expressed (2, 4, 27). Similarly, in EBV-infected chronic lymphocytic leukemic cells, EBNA2 is synthesized but LMP1 is not (40), and if normal B cells are separated early after infection with EBV according to whether or not they express the B-cell antigen CD23, then the

CD23-negative population, which is EBNA2 positive, remains LMP1 negative (7). Little is known about the relative contributions of cellular factors and/or viral proteins other than EBNA2 in the regulation of LMP1.

Raji is a cell line derived from an African Burkitt lymphoma which carries EBV genomes with several unusual features. A large deletion removes most of the main exon encoding EBNA3C (20); thus, although Raji cells express the remainder of the EBNAs, they are EBNA3C negative (3, 33). A second deletion, which affects early lytic cycle viral gene expression, means that the cells cannot be induced to produce infectious virus (20); it has not therefore been possible to examine directly whether EBNA3C-negative Raji virus immortalizes B cells. Another unusual feature of Raji concerns the expression of its LMP1 gene: the levels of both LMP1-specific mRNA and protein in these cells appear to be highly dependent on their proliferative state. Thus, there is a transient increase in expression shortly after feeding of a culture with fresh growth medium containing 10% fetal calf serum (FCS), and approximately 96 h after this, when the majority of the cells arrest in G_1 of the cell cycle, LMP1 expression becomes almost undetectable (11). We previously described clones of Raji which constitutively express a transfected EBNA3C gene; these and further clones that we have established appear to sustain a higher level of LMP1 expression than wild-type Raji (5). In this study, we have examined in detail the relationship between proliferation, the distribution of cells in the cell cycle, and the expression of LMP1 in Raji and in EBNA3C-negative and EBNA3C-positive clones of Raji. This has revealed that in Raji (and 3C-negative clones), the expression of LMP1 is repressed in cells which are growth arrested at a point(s) early in G_1 (or G_0) of the cell cycle when the product of the cellular retinoblastoma gene, pRb, is hypophosphorylated. The induction of LMP1 after dilution of these cells with fresh growth medium coincides with their progression to late G_1 when the pRb protein becomes hyperphosphorylated (22). In the EBNA3C-expressing cells, including an LCL, LMP1 expression is constitutive; although the cells also appear to arrest early in G_1, EBNA3C expression relieves the repression seen previously. Finally, we show that both the cell cycle progression and the increased synthesis of LMP1 can be induced not only by the addition of complete growth medium but also by dilution with RPMI medium containing no FCS. This finding shows that the induction of LMP1 in Raji in these circumstances cannot, as was originally proposed (10, 11), be due to serum stimulation.

MATERIALS AND METHODS

Cell culture. All cell lines, Raji and subclones (5, 34), the LCL X50-7 (44), and the marmoset B-cell line B95-8 (28), were grown routinely at 37°C in RPMI 1640 medium supplemented with 10% FCS.

Immunoblotting and quantitation of proteins. Protein extracts were made by sonicating washed cell pellets in sodium dodecyl sulfate (SDS) sample buffer; after boiling for 5 min, the pellets were separated on SDS–7.5% polyacrylamide gels and transferred to nitrocellulose filters as previously described (3, 4). Protein from 10^6 cells was loaded in each track for the EBNA blot (Fig. 1), and 2×10^5 cells were loaded for the LMP1 and pRb blots. The human serum RT (3, 4) was used to show EBNA1, -2, -3A, -3B, and -3C, serum GM (6) was used for EBNA1 alone, monoclonal antibody S12 (27) was used for LMP1, and monoclonal antibody PMG3-245 (Pharmingen) was used for pRb. In all cases, the final antibody was the relevant immunoperoxidase-antibody conjugate diluted 1/10,000. For the LMP1 and pRb blots, a second layer of rabbit anti-mouse immunoglobulin (diluted 1/2,000) was used to amplify the signal.

Reactive protein bands were visualized by using an enhanced chemiluminescence system (ECL; Amersham) as instructed by the manufacturer. Protein bands were quantified by scanning the autoradiographs with a GS300 scanning densitometer (Hoefer) and integrating the area under each peak recorded.

Immunofluorescence and flow cytometry. Cells were washed in phosphate-buffered saline (PBS), fixed, and permeabilized in 80% ethanol which had been prechilled to −20°C. The fixed cells were stored in suspension for up to a week at 4°C. They were then stained with a saturating concentration of S12 (27) for LMP1 followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin as previously described (23). Cells stained with only the second antibody were used as a negative control. A total of 5×10^5 to 1×10^6 cells were washed and stained, and quantitative analysis was performed on at least 10,000 cells, using an EPICS profile analyzer (Coulter).

[^{3}H]thymidine incorporation. Approximately 4×10^5 cells were labeled with 5 μCi of [^{3}H]thymidine per ml for either 3 or 40 h. Samples were harvested from 96-well round-bottom tissue culture plates, using a semiautomatic cell harvester (Skatron), and analyzed in an LS 3801 beta counter (Beckman). Each experiment was performed in triplicate at least twice.

Cell cycle analysis by flow cytometry. Cells were washed in PBS and fixed in 80% ethanol which had been prechilled to −20°C. The fixed cells were stored in suspension for up to 2 weeks at 4°C. They were then repelleted and resuspended at approximately 10^6/ml in PBS containing 20 μg of propidium iodide per ml and 10 μg of RNase A per ml. After incubation in the dark for at least 30 min, quantitive analysis was performed on 5,000 to 10,000 cells on an EPICS profile analyzer (Coulter).

RESULTS

Expression of EBNA3C in Raji cells. The establishment of EBNA3C-positive and control-transfected Raji cells has been described previously (5). Before and during the course of the present study, the cells were analyzed periodically by immunoblotting with a human serum (RT) which recognizes EBNA1, -2, -3A, -3B, and -3C. The EBNA3C-positive clones, which have been both continuously passaged for many months and also frozen and thawed on several occasions, all continue to express EBNA3C as they did when they were first established, even in the absence of hygromycin selection. Figure 1 shows the three EBNA3C-positive clones used throughout this study, 11.1.1 (track 3), 11.1.9 (track 4), and 11.5.8 (track 5). These were run in parallel with B95-8 (track 1), which expresses all three of the EBNA3 proteins, and the LCL X50-7 (track 2), which expresses EBNA3A and -3C but not EBNA3B. The EBNA3C-negative clones 13.6.7 and 13.6.20 were run in tracks 6 and 7, respectively. In the tracks containing extracts from the positive clones, a second EBNA3C-related species can be seen migrating just below EBNA3A as described previously (5).

Expression of LMP1 in Raji cells after feeding with fresh medium and then after 4 days in culture. We showed previously that stable constitutive expression of EBNA3C leads to an increase in detectable LMP1 in Raji cells (5). Mueller-Lantzsch and colleagues reported that when a growth-arrested culture of Raji cells was fed with medium including 10% FCS, within 8 h, there was a substantial increase in LMP1 mRNA, and by 12 h, levels of the protein were elevated (11).

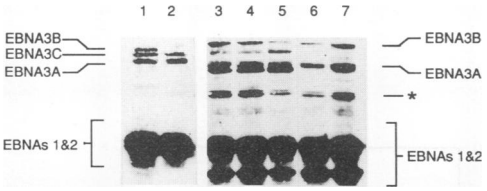

FIG. 1. EBNA3C expression in Raji. Shown is a Western blot (immunoblot) (probed with human serum RT) of total protein extracts from EBNA3C-positive controls B95-8 and the LCL X50-7 (tracks 1 and 2, respectively) and Raji clones expressing EBNA3C (11.1.1 [track 3], 11.1.9 [track 4], and 11.5.8 [track 5]). Extracts from clones transfected with the SV2Hyg vector alone, 13.6.7 and 13.6.20, are shown in tracks 6 and 7. All of the EBNA species recognized by RT are indicated, and a cross-reacting cellular protein is marked (*).

To determine whether the oscillation in LMP1 expression seen after diluting Raji cells occurs in EBNA3C-expressing Raji cells and can be detected at a single-cell level, immunofluorescence staining and flow cytometry (fluorescence-activated cell sorting [FACS] analysis) were performed. An EBNA3C-negative clone, an EBNA3C-positive clone, and, for comparison, the LCL X50-7 were grown to high density ($>3 \times 10^6$ cells per ml) over 4 days, and samples were taken at the times shown in Fig. 2. The number of LMP1-positive cells in the EBNA3C-negative clone reaches a peak at 12 h after feeding and declines to about 5% in the 4-day-old, saturated culture (an approximately 12-fold reduction). This finding is consistent with previous observations (10, 11). The EBNA3C-positive clone, on the other hand, behaves similarly to the LCL in that although feeding induces a modest increase in the number of LMP1-positive cells, the number does not decline significantly when the cells approach their saturation density (see below). It is not possible to say whether the cells which remain negative (even in the LCL, this is >25% of the cells) are genuinely expressing no LMP1 or are expressing it at a level which is below the threshold of detection in this particular assay.

LMP1 expression in quiescent cells. To establish more precisely how LMP1 expression in Raji cells relates to growth

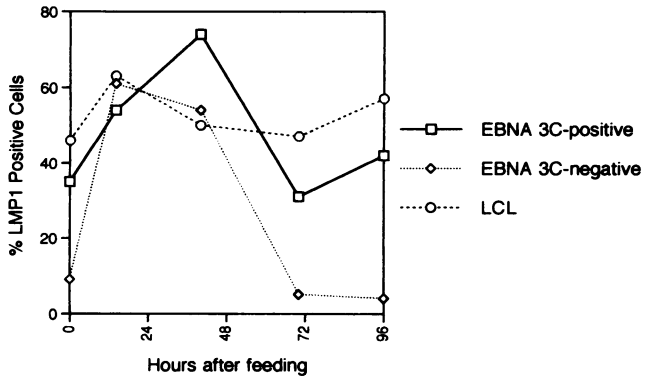

FIG. 2. Time course of LMP1 immunofluorescent staining after feeding. The number of LMP1-positive cells was determined by FACS analysis of cells stained with monoclonal antibody S12 and a fluorescein isothiocyanate-conjugated second antibody. Samples of the EBNA3C-positive clone 11.5.8, EBNA3C-negative clone 13.6.7, and the LCL X50-7 were taken for staining 14, 40, 70, and 96 h after dilution of a saturated culture to 2×10^5 cells per ml with fresh growth medium.

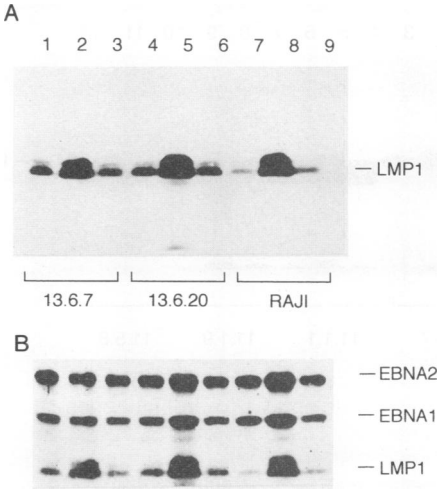

FIG. 3. LMP1 expression in EBNA3C-negative cells growth arrested in G_1 of the cell cycle. (A) Western immunoblot with the anti-LMP1 monoclonal antibody S12. Analysis of protein extracts from Raji cells and EBNA3C-negative clones 13.6.7 and 13.6.20 4 days after feeding cultures at saturation density is shown in tracks 1, 4, and 7. Samples from the same cultures 14 h after dilution back to 2×10^5 cells per ml with fresh medium are shown in tracks 2, 5, and 8. Samples from the unfed 5-day-old cultures are shown in tracks 3, 6, and 9. (B) The same filter reprobed with serum RT shows EBNA1 and EBNA2. The bands corresponding to LMP1 can again be seen reacting weakly on the reprobed blot because of residual activity from the first probing.

arrest and subsequent cell cycle progression, all of the lines under study were diluted to 2×10^5 cells per ml then grown for 4 days. An aliquot of cells was taken from each culture, and protein extracts were made. The remaining cells were split into two aliquots; one aliquot was diluted back to 2×10^5 cells per ml in fresh medium containing 10% FCS, and the other was returned to 37°C. Both were then incubated for a further 12 to 14 h, and protein extracts were made. The immunoblots performed with these extracts are shown in Fig. 3 (EBNA3C-negative cells) and Fig. 4 (EBNA3C-positive cells). It is clear from this analysis that in Raji and the negative subclones of Raji, LMP1 expression is greatly reduced both 4 and 5 days after feeding (Fig. 3A), tracks 1, 3, 4, 6, 7, and 9). However, approximately 12 h after dilution with fresh medium the levels have dramatically increased (tracks 2, 5, and 8). This result shows that when cells are quiescent and largely arrested in G_1 (see below), there is a down-regulation of LMP1 expression. Reprobing the filter with serum RT revealed in some cases (Fig. 3, tracks 5 and 8), small changes in the levels of EBNA1 and EBNA2 after feeding, but these were extremely modest in comparison with the induction of LMP1. Quantitation of protein bands by scanning the autoradiographs showed a seven- to eightfold greater induction of LMP1 after the addition of medium to the EBNA3C-negative cells. However, quantitative analysis is shown better in Fig. 8, where the levels of LMP1 could be normalized to an internal control (see below).

The immunoblot analysis of the LCL and EBNA3C-positive Raji clones (Fig. 4) confirms the data shown above (Fig. 2) in clearly showing sustained LMP1 expression in these cells even when they are quiescent. The blots in Fig. 3 and 4 also confirm our previous observation (5) that when LMP1 is expressed at a high level in Raji, it is resolved in an SDS–7.5% polyacrylamide gel as a protein doublet. The upper band corresponds in its

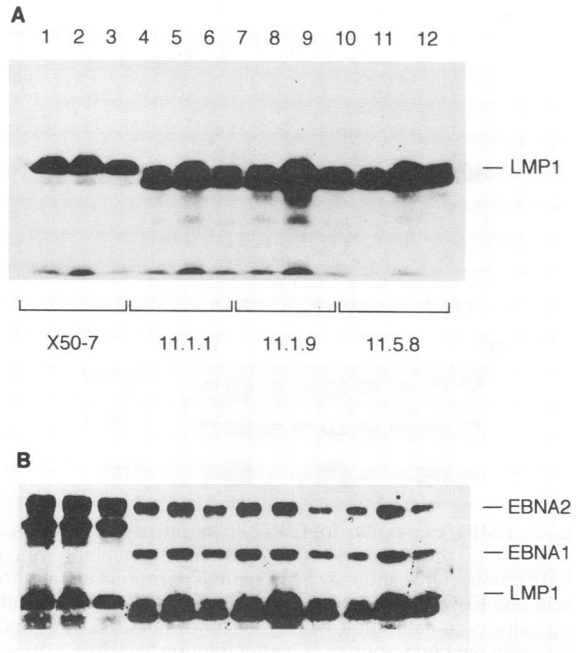

FIG. 4. LMP1 expression in EBNA3C-positive cells growth arrested in G_1 of the cell cycle. (A) Western immunoblot with the anti-LMP1 monoclonal antibody S12. Analysis of protein extracts from the LCL X50-7 and the EBNA3C-positive clones 11.1.1, 11.1.9, and 11.5.8 4 days after feeding cultures at saturation density is shown in tracks 1, 4, 7, and 10. Samples from the same cultures 14 h after dilution back to 2×10^5 cells per ml with fresh medium are shown in tracks 2, 5, 8, and 11. Samples from the unfed 5-day-old cultures are shown in tracks 3, 6, 9, and 12. (B) The same filter reprobed with serum RT shows EBNA1 and EBNA2. The bands corresponding to LMP1 can again be seen reacting weakly on the reprobed blot because of residual activity from the first probing.

electrophoretic mobility to that extracted from the LCL (compare tracks 3 and 4 in Fig. 4A).

Kinetics of cell proliferation, [^{3}H]thymidine incorporation, and cell cycle analysis. To understand more clearly when and how EBNA3C might be influencing LMP1 expression, the growth characteristics of a wild-type Raji culture and of two EBNA3C-negative and three EBNA3C-positive cultures were analyzed. Viable cells were counted periodically over a 6-day time course, the level of [^{3}H]thymidine incorporation in quiescent and stimulated cells was determined, and samples were taken for cell cycle analysis by flow cytometry. All of the growth curves in Fig. 5A and B have very similar sigmoid forms with the characteristic lag, exponential, and stationary phases. There are no significant differences between the LCL, wild-type Raji, or any of the Raji clones. Although the particular experiment shown suggests that two of the EBNA3C-positive clones (11.1.1 and 11.1.9) might have a slightly lower saturation density, counts of similar 4- to 6-day-old cultures on numerous (>10) occasions have shown that all of the lines become saturated at densities of 2.5×10^6 to 4.5×10^6 cells per ml, depending on the individual experiment. No consistent variations between EBNA3C-positive and -negative Raji cells were observed. The LCL X50-7, however, generally grew slightly more slowly and became stationary between days 5 and 6.

Cell cultures which had reached saturation density (4 to 5 days after dilution with fresh medium) were labeled with [^{3}H]thymidine in order to determine whether the apparently

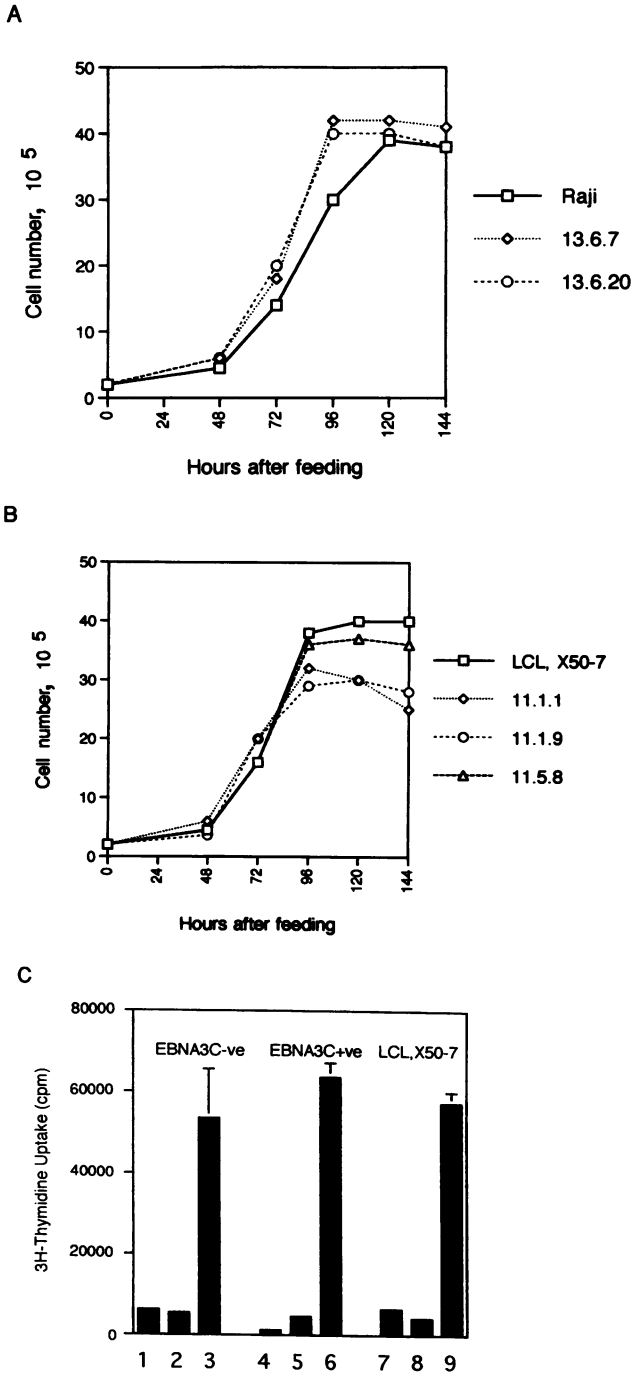

FIG. 5. Kinetics of proliferation and [^{3}H]thymidine incorporation. (A) Raji and the EBNA3C-negative clones 13.6.7 and 13.6.20. (B) EBNA3C-positive clones 11.1.1, 11.1.9, and 11.5.8 and the LCL X50-7. Only cells which excluded trypan blue were counted. (C) [^{3}H]thymidine incorporation by 4×10^5 quiescent cells after labeling for 3 h (columns 1, 4, and 7) or 40 h (columns 2, 5, and 8). Samples from the same cultures labeled for 40 h after dilution in fresh medium are shown as columns 3, 6, and 9.

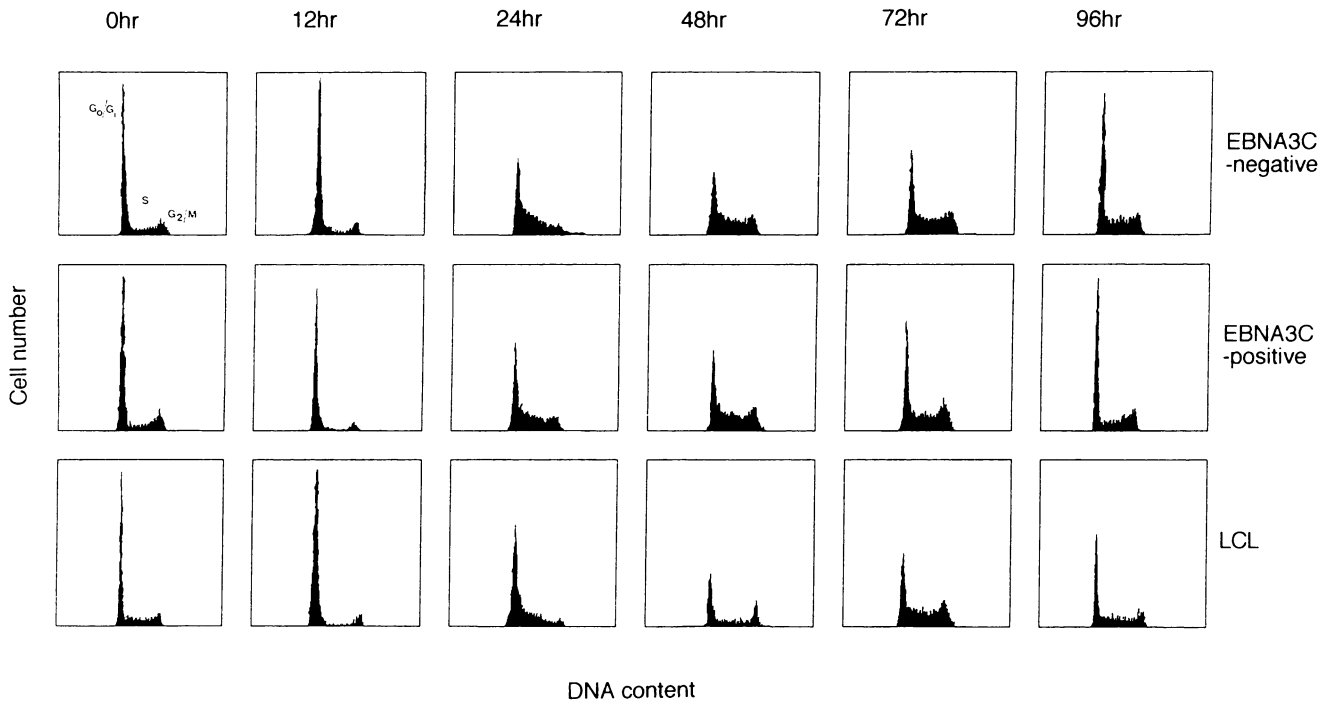

FIG. 6. Cell cycle analysis by FACS. Samples were taken and fixed in 80% ethanol at 0, 12, 24, 48, 72, and 96 h after dilution of a 4- to 5-day-old saturated culture to 2×10^5 cells per ml. After RNase treatment and propidium iodide staining, the samples were analyzed on an EPICS profile analyzer. The DNA content, which indicates the phase of the cell cycle, is plotted against the number of cells analyzed. The G_0/G_1 peak represents the cells with a 2N DNA content, and the G_2/M peak represents those with 4N DNA content. The area under the profile between these peaks is taken to represent cells in the S phase of the cell cycle. The EBNA3C-negative clone is 13.6.7, the EBNA3C-positive clone is 11.5.8, and the LCL is X50-7. The number of cells analyzed at 48 h from the LCL culture is slightly low because of a sampling error.

quiescent cells were synthesizing DNA. Figure 5C shows the level of incorporation by quiescent EBNA3C-negative Raji, the EBNA3C-positive clone 11.5.8, and the LCL X50-7 during 3 h (columns 1, 4, and 7) and 40 h (columns 2, 5, and 8) of labeling. There was no significant increase in uptake of [^{3}H]thymidine between 3 and 40 h, showing that the cells were largely growth arrested during this period. Samples of the same cultures taken 40 h after dilution with fresh medium showed a 10- to 15-fold increase in [^{3}H]thymidine uptake (columns 3, 6, and 9); this time point coincides with entry into the logarithmic phase of growth (see Fig. 5A and B). An equivalent sample from actively cycling culture of X50-7 incorporated more than 200,000 cpm (data not shown).

The results of the cell cycle analyses (Fig. 6) were consistent with both the cell counts and labeling experiments; prior to and 96 h after feeding, the majority of the cells in all the cultures are arrested in either G_0 or the G_1 phase of the cell cycle. Twelve hours later (seen as part of a lag period in the growth curves), most of these cells are still in G_0/G_1; virtually no cells are seen in S phase. At 24 h, cells start progressing into S phase, and then between 48 and 72 h, when the cell counts show exponential growth, the profiles show that all phases of the cell cycle are well represented and are consistent with an actively cycling population. The majority of cells start to become growth arrested again between 72 and 96 h. Only three complete sets of profiles are shown, but all of the clones and wild-type Raji behaved in essentially the same way.

pRb and cell cycle progression. The experiments described above showed that when Raji, Raji subclones, and an LCL are growth arrested at their saturation densities, they can be induced to start proliferating again by dilution with fresh growth medium. However, the cell cycle profiles show that 12 h after receiving medium, when LMP1 is induced, the vast majority of cells still have a diploid (2N) DNA content and therefore must still be in the G_1 phase of the cell cycle. This finding suggests that the cells were initially growth arrested early in G_1 (or G_0) and that G_1 must be longer than 12 h in these B cells. To determine if this interpretation of events is correct, aliquots of the samples used for the LMP1 analysis (Fig. 3 and 4) were immunoblotted for pRb. It is widely accepted that this protein is hypophosphorylated when cells are quiescent in early G_1 (or G_0) and becomes phosphorylated late in G_1 as the cells are about to progress to S phase (22, 36). After 4 days in culture (Fig. 7, tracks 2, 6, 10, and 14), it can be seen that a hypophosphorylated Rb species (Rb in Fig. 7) is present in each of four representative cell lines, although in some cases, hyperphosphorylated pRb ($pRbPO_4$) is still visible. This finding is consistent with many cells being arrested in early G_1 (or, more precisely, not in late G_1). The hypophosphorylated form of the protein is even more pronounced in extracts from the 5-day-old cultures (tracks 4, 8, 12, and 16). In all extracts from the cultures which had been diluted in fresh medium, there was significant phosphorylation of pRb, resulting in a predominant $pRbPO_4$ band (tracks 3, 7, 11, and 15).

Extracts from actively cycling cell cultures run as controls show only $pRbPO_4$ (tracks 1, 5, 9, and 13); this finding is consistent with previous reports (22, 36). All of the samples were analyzed in this way, and the LCL, wild-type Raji, an EBNA3C-negative clone, and an EBNA3C-positive clone were found to be entirely representative.

Cell cycle progression, pRb phosphorylation, and LMP1 expression can be induced by serum-free medium. When it was

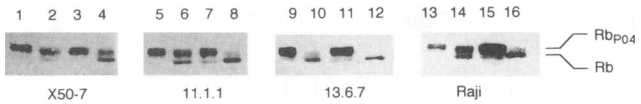

FIG. 7. pRb expression in cells arrested in G_1 of the cell cycle. Aliquots from the samples used in the experiments shown in Fig. 3 and 4 were subjected to Western immunoblot analysis using the anti-pRb monoclonal antibody PMG3-245. Tracks: 2, 6, 10, and 14, 4-day-old cultures; 3, 7, 11, and 15, cultures 14 h after dilution with fresh medium; 4, 8, 12, and 16, 5-day-old cultures with no additional medium. Samples from actively cycling cells (taken 48 h after feeding) were run in tracks 1, 5, 9, and 13. Clones 11.1.1 and 13.6.7 were taken as representative of EBNA3C-positive and -negative Raji clones, respectively. The hypophosphorylated (Rb) and hyperphosphorylated ($pRbPO_4$) forms of retinoblastoma protein are indicated.

originally observed that LMP1 is induced in Raji after the dilution of a dense culture with fresh medium, it was concluded that this induction and the accompanying cell cycle progression were in response to the serum included in the medium (10, 11). We performed experiments to determine whether 0.1% serum (data not shown) or serum-free medium would have any effect on growth-arrested Raji.

Figure 8 shows an experiment in which the level of detectable LMP1 and the phosphorylation status of pRb were determined by immunoblotting. The cell extracts from 5-day-old cultures of Raji and an EBNA3C-positive clone are shown in tracks 1 and 4, respectively. Extracts from the same cultures which had been diluted approximately 1/20 on day 4 in medium including 10% FCS and reincubated overnight (tracks 2 and 5) and from a culture diluted and reincubated in a similar manner with serum-free medium (tracks 3 and 6) are also shown. The results of this experiment are consistent with the results of FACS analysis performed on similar samples (data not included) showing cell cycle progression, as judged by the phosphorylation of pRb and also the induction of LMP1, occurred irrespective of whether the medium used to dilute the cells contained serum. In this experiment, LMP1 expression was quantified by scanning densitometry, normalized to the total pRb detected in the extracts, and expressed as the fold induction over the level in quiescent cells.

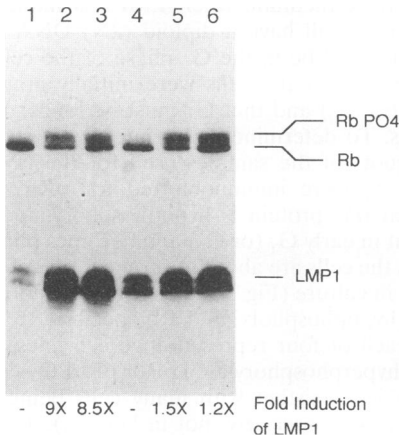

FIG. 8. Western immunoblot of extracts from Raji cells and the EBNA3C-positive clone 11.1.1 probed with a mixture of anti-LMP1 (S12) and anti-pRb (PMG3-245). Cultures were split into three 96 h after dilution to 2×10^5 with fresh medium. One sample of each line was reincubated for a further 14 h without the addition of fresh medium (tracks 1 and 4). One was diluted to 2×10^5 with fresh medium including 10% FCS and incubated for 14 h (tracks 2 and 5), and one was diluted and incubated in a similar manner with medium containing no FCS (tracks 3 and 6). The hypophosphorylated (Rb) and hyperphosphorylated ($pRbPO_4$) forms of pRb and also LMP1 are indicated. Protein levels were determined by scanning densitometry of the autoradiographs, and the induction or reduction of LMP1 is shown after normalization of the values to the total level of pRb detected in each track.

DISCUSSION

This study extends and links two previous observations: (i) in Raji cells, the amount of LMP1 expressed is dependent on the proliferative state of the cell (11); and (ii) constitutive expression of a transfected EBNA3C gene in Raji increases LMP1 expression and the degree of activation the cells display (5). Here we have shown that in cells expressing EBNA3C, the repression of LMP1 seen in quiescent wild-type Raji cells does not occur. In the 3C-negative Raji cells, there is an 8- to 9-fold reduction in LMP1, whereas in the converted lines, the reduction is hardly significant (<1.5-fold). Furthermore, we have mapped more precisely the stage of the cell cycle when these cells arrest, thus indicating when EBNA3C is likely to be biologically active. However, several questions have been raised by the results.

The nature of the growth arrest (and activation) in Raji cells. By a variety of criteria, we have shown that quiescent Raji cells 4 to 5 days after feeding are largely arrested in G_1 or G_0. The growth curves show that the populations were stationary, with no net increase in the number of cells and no cell death (as judged by trypan blue staining). The cell cycle analysis shows that the quiescent cells are largely (~80%) in G_1 and that 12 h after dilution, virtually no cells are seen in S phase. To establish whether the quiescent cultures were actually arrested, they were allowed to incorporate [^{3}H]thymidine for up to 40 h; no significant increase in incorporation (signifying entry into S phase) was seen between 3 and 40 h of labeling. Finally, the detection of hypophosphorylated pRb is consistent with all or most of the cells arresting early in G_1; this isoform of the protein is found almost exclusively in G_0/G_1 in a variety of cell types, including human B cells growth arrested by alpha interferon (22, 36).

The point at which Raji cells arrest when they reach saturation density is characterized by a 2N DNA content; it is therefore after mitosis and before the next S phase. As pRb is hypophosphorylated in these cells, they must be either in early G_1 or out of cycle in the G_0 (resting) state (22, 36). However, Raji, being derived from a Burkitt lymphoma, has a chromosomal translocation which results in the deregulation of the c-*myc* gene (17) and high-level constitutive expression of Myc protein (23). Myc is sufficient to drive serum-starved fibroblasts from G_0 into the cell cycle (9, 13), and it has been argued that in Burkitt lymphoma cells, it is the constitutive expression of Myc which prevents them from reentering G_0 and so contributes to their tumorigenicity (26). We therefore think it unlikely that Raji cells at saturation density growth arrest in G_0. Furthermore, the observation that cell cycle progression is induced by serum-free medium is consistent with this interpretation. If a stimulatory factor in serum is not required for progression, then the cells probably arrest as a result of either the accumulation of a negative-acting factor like alpha interferon or a suboptimal supply of a critical nutrient such as an essential amino acid. Arrest by either of these mechanisms would almost certainly be early or in the middle part of G_1, prior to the cell cycle restriction point described by Pardee (32) and beyond which pRb is thought to become hyperphosphorylated (22, 36).

Role of EBNA3C in the regulation of LMP1 and in the immortalization of B cells. We suggested previously that EBNA3C/6 is likely to be a regulator of gene expression; it is a nuclear protein with a heptad repeat of leucine residues, similar to the leucine zipper motif, adjacent to a basic region that might act as a DNA-binding domain (5). It has two highly negatively charged regions, one of which includes two consensus recognition sites for casein kinase II. In these various features, it resembles several viral and cellular *trans* regulators of gene expression (9, 29, 39). We also showed previously that in Raji cells, EBNA3C increases the expression of LMP1; it is now clear that this is particularly apparent in dense cultures of Raji cells. Here we have focused attention on the early G_1 phase of the cell cycle, when pRb is hypophosphorylated, as the window when EBNA3C can exert a particularly significant effect on gene expression. The result of EBNA3C expression is the derepression of LMP1 in early G_1, leading to the type of expression found in LCLs rather than the cell cycle-regulated or proliferation-associated expression found in wild-type Raji. However, it is not yet possible to say whether this is because EBNA3C subtly alters the stage of growth arrest to one permissive for LMP1 expression or whether the arrest is the same and EBNA3C directly or indirectly derepresses LMP1. In either case, the presence of a significant detectable level EBNA2 in growth-arrested Raji is clearly inadequate to maintain high-level expression of LMP1.

The action of EBNA3C in G_1 would be consistent with it having an important role in the activation of resting B cells as they enter the cell division cycle. Its essential role in immortalization could be because it is a crucial factor in ensuring LMP1 expression before entry into S phase and/or in the progression of the newly infected cells through G_1. These results provide a framework for understanding the biochemical mechanism by which EBNA3C operates in the life cycle of EBV and in the immortalization of B cells.

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