Epstein-Barr Virus Nuclear Antigen 2 Induces Expression of the Virus-Encoded Latent Membrane Protein

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Infection of Epstein-Barr virus-negative human B-lymphoma cell lines with the fully transforming B95.8 Epstein-Barr virus strain was associated with complete virus latent gene expression and a change in the cell surface and growth phenotype toward that of in vitro-transformed lymphoblastoid cell lines. In contrast, the cells infected with the P3HR1 Epstein-Barr virus strain, a deletion mutant that cannot encode Epstein-Barr nuclear antigen 2 (EBNA2) or a full-length EBNA-LP, expressed EBNA1, 3a, 3b, and 3c but were negative for the latent membrane protein (LMP) and showed no change in cellular phenotype. This suggests that EBNA2 and/or EBNA-LP may be required for subsequent expression of LMP in Epstein-Barr virus-infected B cells. Recombinant vectors capable of expressing the B95.8 EBNA2A protein were introduced by electroporation into two P3HR1-converted B-lymphoma cell lines, BL30/P3 and BL41/P3. In both cases, stable expression of EBNA2A was accompanied by activation of LMP expression from the resident P3HR1 genome; control transfectants that did not express the EBNA2A protein never showed induction of LMP. In further experiments, a recombinant vector capable of expressing the full-length B95.8 EBNA-LP was introduced into the same target lines. Strong EBNA-LP expression was consistently observed in the transfected clones but was never accompanied by induction of LMP. The EBNA2A gene transfectants expressing EBNA2A and LMP showed a dramatic change in cell surface and growth phenotype toward a pattern like that of lymphoblastoid cell lines; some but not all of these changes could be reproduced in the absence of EBNA2A by transfection of P3HR1-converted cell lines with a recombinant vector expressing LMP. These studies suggest that EBNA2 plays an important dual role in the process of B-cell activation to the lymphoblastoid phenotype; the protein can have a direct effect upon cellular gene expression and is also involved in activating the expression of a second virus-encoded effector protein, LMP.

Epstein-Barr virus (EBV) activates resting human B cells into the cell cycle and induces their in vitro outgrowth to permanent virus genome-positive lymphoblastoid cell lines (LCLs) (reviewed in reference 7). Such lines show a characteristic cell surface phenotype with high expression of several B-cell activation antigens and of cellular adhesion molecules, grow in tight clumps, and are capable of autocrine growth stimulation under conditions of limiting dilution (8, 11, 29, 38). Short-term antigen- or mitogen-stimulated B lymphoblasts show a similar phenotype, and thus EBV appears to achieve B-cell immortalization by constitutively driving a program of cellular gene expression that is used only transiently during physiological B-cell activation.

This process seems to involve the coordinate action of a limited number of viral proteins, the so-called latent gene products, which are consistently expressed in all LCLs. These include the following EBV nuclear antigens: EBNA1, encoded by the BKRF1 reading frame (2) of the EBV genome; EBNA2, encoded by BYRF1; EBNA-LP, encoded by multiple exons within the *Bam*HI WY region 5' of the EBNA2 sequence; and the EBNA3a, 3b, and 3c family of proteins encoded by the exon splices BLRF3/BERF1, BERF2a/2b, and BERF3/4 respectively (reviewed in references 5 and 17). All of the transcripts encoding the various EBNA species appear to map to one of two promoter regions in *Bam*HI-C or -W (36). A latent membrane protein (LMP) is

encoded by the BNLF1 reading frame (13, 19) under the control of a different promoter. Recently cDNA cloning has predicted the existence of a second potentially membrane-associated latent gene product, terminal protein or LMP2 (18, 34), whose expression requires circularization of the virus genome; this protein has not yet been identified unequivocally.

Apart from the well-documented role of EBNA1 in maintenance and replication of the episomal viral genome (43), little is known about the contributions that each of the individual EBV latent proteins makes to the process of growth transformation. There is, however, long-standing evidence implicating EBNA2 and/or EBNA-LP in the initial phase of B-cell activation. Thus the P3HR1 virus, a deletion mutant lacking the EBNA2-coding sequence and the last two exons of EBNA-LP (3, 16, 23), does not have a transforming function (20) and indeed cannot induce resting B cells into the S phase (10). Furthermore, P3HR1 virus infection of human B-lymphoma cell lines generates P3HR1-converted clones that remain phenotypically similar to the parental line, whereas infection of the same cells with the fully transforming B95.8 virus strain induces a dramatic change to the classic LCL phenotype (4). Therefore in B-lymphoma cells, just as in normal resting B cells, the inability to encode EBNA2 and full-length EBNA-LP is associated with an inability to initiate changes in cellular gene expression.

To investigate their effects upon the human B-cell phenotype, individual EBV latent genes have been introduced by

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transfection and expressed in human B-lymphoma cells of the kind described above. In this way EBNA2 has been shown to specifically up-regulate expression of a cellular activation antigen, CD23 (39), which is itself intimately involved in the pathway of autocrine growth stimulation (9, 37). Similarly a second latent gene product, LMP, already known to display growth transforming activity in rodent cell culture systems (40), was found to induce several facets of the LCL phenotype in human B cells, most notably high expression of the cellular adhesion molecules facilitating both homotypic and heterotypic cell aggregation (41). Thus in resting B cells EBNA2 and possibly EBNA-LP, which are the first viral gene products detectable after infection (1, 21; M. Rowe, unpublished observations), may initiate one set of changes in cellular gene expression, whereas LMP, which is expressed 2 to 3 days later (1), may initiate others.

The present experiments are concerned with the relationship between these two phases of the virus infection in B cells, in particular with the possibility that EBNA2 or EBNA-LP is capable of inducing the subsequent expression of LMP. This possibility was first raised when B95.8-converted and P3HR1-converted B-lymphoma cell lines were compared for their expression of virus-encoded proteins. Whereas B95.8 converts expressed the full spectrum of detectable latent gene products (EBNAs 1, 2, -LP, 3a, 3b, and 3c and LMP), P3HR1-converted clones not only lacked EBNA2 and full-length EBNA-LP but also failed to express detectable LMP (22). In the present work we have attempted to induce expression of the resident LMP gene in such P3HR1-converted clones by stably introducing plasmid vectors capable of encoding either EBNA2 or EBNA-LP.

MATERIALS AND METHODS

Cell lines. Initial experiments used four EBV-negative B-cell lines (IARC BL2, IARC BL30, IARC BL40, and IARC BL41, originally derived from rare cases of sporadic Burkitt's lymphoma) as well as EBV-converted clones derived from these cell lines by infection either with the transforming EBV strain B95.8 or with the nontransforming P3HR1 strain. These lines were kindly provided by G. Lenoir, International Agency for Research on Cancer Laboratories, Lyon, France; full details of their establishment are given elsewhere (4). Control cells included the virus-producing B95.8 cell line, a B95.8 virus-transformed nonproductive cord blood cell line (X50-7), the virus-producing P3HR1 cell line, and a nonproductive cord blood cell line (JC5) transformed with the Jijoye virus strain. Note that B95.8 virus is a type A isolate, whereas Jijoye virus is type B; P3HR1 is a deletion mutant originally derived from Jijoye virus (3). All cells were maintained by twice-weekly subculture in RPMI 1640 medium supplemented with 2 mM glutamine, 100 IU of penicillin per ml, 100 µg of streptomycin per ml, and 10% (vol/vol) selected fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.).

Recombinant plasmids. Expression of EBNA2 was obtained from two recombinant vectors, J144 and J289, in which EBV DNA sequences had been introduced into a modified pSV2gpt plasmid, pSV2gpt/BglII, in which the *PvuII* site had been exchanged for a *BglII* site (32). Details of the EBV DNA sequences present in J144 and in J289 are shown schematically in Fig. 1. The plasmid J144 contains the *BglII*-*NotI* subfragment of the *Bam*HI WYH region of the B95.8 virus genome (sequence coordinates 44664 to 50628); this plasmid was derived from the previously described plasmid PEΔA6 (25) by the upstream insertion of a simian

FIG. 1. Schematic representation of the B95.8 EBV DNA sequences present in the recombinant vectors J144 and J289. J144 is the *BglII*-*NotI* subfragment (sequence coordinates 44664 to 50628) of the *Bam*HI WYH region of the B95.8 virus genome; J289 contains the same sequence except for deletion of an internal *PvuII*-*XhoI* subfragment bracketed on the diagram. In the B95.8 virus strain, EBNA2 is encoded by the BYRF1 open reading frame (■) and EBNA-LP is encoded by repeated W1 and W2 exons (▨) and unique Y1 and Y2 exons (▨i) as shown. Note that both J144 and J289 contain the BYRF1 open reading frame and also one copy each of the W1 and W2 exons and the Y1 and Y2 exons. The P3HR1 virus strain has a deletion, bracketed on the diagram, that removes the BYRF1 reading frame and also the Y1 and Y2 exons. kb, Kilobases.

virus 40 *ori* sequence. Plasmid J289 (previously designated PEΔA8 [25]) contains the same EBV DNA sequences as J144, except for deletion of a *PvuII*-*XhoI* subfragment (Fig. 1). Both J144 and J289 contain the W1, W2, Y1, and Y2 exons of EBNA-LP and the complete BYRF1 EBNA2 open reading frame as well as most of the region of *Bam*HI-W (sequence coordinates 44547 through 45180) that was previously shown to give optimal expression in human B cells (24).

Expression of EBNA-LP was obtained from a recombinant vector, pSALP, constructed as follows. An *EcoRI*-*FnuDII* subfragment of the EBNA-LP cDNA clone T65 (33) was cloned into pUC18, a blunt-ended cassette containing the human immunoglobulin heavy-chain enhancer and simian virus 40 promoter sequence (kindly provided by M. Neuberger, MRC Laboratory for Molecular Biology, Cambridge, United Kingdom) was cloned upstream of the EBNA-LP cDNA, and the enhancer-promoter-EBNA-LP region was then recloned into the pUC18-based vector pUC18SVHygA (kindly provided by P. Berg, Stanford University of Medicine, Stanford, Calif.) containing the *hph* gene from *Escherichia coli* to confer hygromycin resistance. The vector pUC18SVHygA itself served as a control plasmid in transfection experiments with pSALP.

Expression of LMP was obtained by using recombinant vector pSV2gptMTLM, which was described fully elsewhere (40). This vector and the EBNA2 expression vectors described above carry the *gpt* gene from *E. coli* to confer mycophenolic acid resistance; the pSV2gpt plasmid served as a control in transfection experiments with these vectors.

Electroporation and isolation of transfected cells. Cells in the logarithmic phase of growth were washed in ice-cold Dulbecco phosphate-buffered saline (PBS). Then 6×10^6 cells in 0.6 ml of ice-cold PBS were pipetted into a sterile cuvette (Pulser Cuvette; Bio-Rad Laboratories, Richmond, Calif.) containing 10 to 20 µg of uncut plasmid DNA and

incubated for 10 min on ice. Electroporation was performed at 0.8 kV and 25 μ F settings on the Bio-Rad gene pulser apparatus, after which the cuvettes were returned to ice for a further 10 min. The cells were transferred to a tissue culture flask (Nunc, Roskilde, Denmark) containing 10 ml of warm growth medium and cultured at 37°C in 5% CO₂ for 3 days. The cells were then plated in sterile 96-well flat-bottomed microdilution plates (Nunc) at a density of 5×10^3 cells per well. Cells transfected with plasmid J144, J289, or pSV2gptMTLM, or with pSV2gpt as a control, were cultured in medium containing 75 μ g of xanthine (Sigma Chemical Co., St. Louis, Mo.) per ml and 0.5 μ g mycophenolic acid (Sigma) per ml. Cells transfected with pSALP, or with pUC18SVHygA as a control, were cultured in medium containing 300 μ g of hygromycin B (Sigma) per ml. Cells were fed with drug-containing medium on days 3 and 7. Thereafter the drug concentration was gradually reduced by feeding with normal medium but was increased to the initial levels as soon as clones of growing cells began to appear. The clones were subsequently expanded through 2-ml wells into tissue culture flasks and then passaged twice weekly in medium containing the appropriate drug.

Detection of EBV antigens. Protein samples from transfected clones and from reference cell lines were separated on Laemmli discontinuous polyacrylamide gels, transferred onto nitrocellulose, and subsequently analyzed for specific EBV latent proteins by antibodies and ¹²⁵I-labeled protein A as described previously (22). Briefly, protein from 10⁶ cells was applied to each gel track and separated routinely on a 7.5% polyacrylamide resolving gel or on a 12% gel when testing for low-molecular-weight forms of EBNA-LP. Nitrocellulose blots of the resolved proteins were incubated with either selected human sera (diluted 1/100), or with murine monoclonal antibodies (MAbs; culture supernatant, diluted 1/10), followed by a second step rabbit anti-mouse immunoglobulin G polyclonal antibody (Dakopatts; Z259 diluted 1/5,000). Human sera used in this work included RS22, which is reactive against EBNA1 and EBNA2 type A, and NZ, which is reactive against EBNA1, EBNA2 type B, and EBNA3a, 3b, and 3c type B (31). Antibodies against EBNA-LP were immunopurified as described elsewhere (31) on a β -galactosidase-EBNA-LP fusion protein (42) from human serum LF. MAbs used in this work included PE2 against EBNA2 A/B (44), JF186 against EBNA-LP (6), and CS.1-4 against LMP (28).

Expression of EBNA2, EBNA-LP, and LMP in transfected clones was also tested by immunofluorescence on cell smears that had been prepared on microscope slides, dried at room temperature, and fixed in methanol for 10 min at -20°C. The cell smears were rehydrated with PBS containing 20% heat-inactivated normal goat serum (PBS-NGS) for 30 mins at room temperature, followed by incubation with the relevant neat MAb supernatant for 2 h at 37°C. The slides were then washed twice for 10 min at room temperature in PBS and incubated at 37°C for 3 h with a fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Sigma; FO275) diluted 1/50 in PBS-NGS. After two 10-min washes in PBS, the slides were counterstained for 6 min with 0.01% Evans blue before mounting with DABCO-based antifading mountant (15) and examination under a fluorescence microscope.

Phenotypic analysis of transfectants. Cell surface markers were analyzed on viable cells by indirect immunofluorescence with specific MAbs to the B cell-associated antigens CD21 (MAb R3FC5 obtained from N. Ling, Department of Immunology, University of Birmingham, Birmingham,

FIG. 2. Immunoblot of protein extracts from EBV-negative BL cell lines (BL40, BL2, BL30, and BL41) and their corresponding P3HR1 and B95.8 converts probed with MAbs specific for EBNA2A/B (PE2) and LMP (CS.1-4). Reference tracks include B95.8 virus-transformed LCL X50-7 (EBNA2A positive, LMP positive) and Jijoye virus-transformed LCL JC5 (EBNA2B positive, LMP positive); note that P3HR1 virus is a deletion mutant of the Jijoye virus strain. EBNA2A is detected as a 85-kDa species, EBNA2B is detected as a 75-kDa species, and LMP appears as a major 60- to 63-kDa band with lower-molecular-mass species detectable in lines with high LMP expression. The doublet of cellular proteins at 43 to 44 kDa appears to be due to cross-reactivity of the CS.1-4 MAbs (28), and its presence in every track confirms equivalent protein loading.

United Kingdom) and CD23 (MAb MHM6 [29]) and to the cellular adhesion molecules LFA1 (MAb MHM23 [14]), ICAM1 (MAb RR1/1 [27]), and LFA3 (MAb TS2/9 [35]). Viable cells were washed in PBS containing 5% normal goat serum and incubated for 30 min at 4°C with the antibodies at a 1/100 dilution of ascitic fluid or a 1/20 dilution of culture supernatant in PBS-NGS. The cells were then washed twice in PBS-NGS and incubated for a further 30 min at 4°C with fluorescein-conjugated goat anti-mouse immunoglobulin G diluted 1/50 in PBS-NGS containing 10% normal human serum (from an EBV-negative donor) and 0.02% sodium azide. After washing, the cells were fixed in PBS containing 1% formaldehyde and 1% NGS and were analyzed for fluorescence on a FACS 440 cell sorter (Becton Dickinson Labware, Oxnard, Calif.). Results are expressed as mean fluorescence intensity values.

RESULTS

EBV latent protein expression in virus genome-converted BL cell lines. The first set of experiments reexamined the question of EBV latent protein expression in P3HR1- versus B95.8-converted BL cell lines. Figure 2 illustrates the most important differences between the two types of convert. Immunoblotting with pooled MAbs PE2 and CS.1-4 clearly showed that both EBNA2 and LMP were expressed in the B95.8-converted BL2, BL30, BL40, and BL41 cell lines, although levels of expression varied between lines. However, the corresponding P3HR1-converted lines were not only EBNA2 negative (because of the deletion) but also did not express detectable levels of LMP (Fig. 2). In further immunoblotting experiments with relevant human sera, these P3HR1 converts expressed the characteristic P3HR1-encoded 72-kilodalton (kDa) EBNA1 and type B EBNA3 protein family; expression of truncated EBNA-LP, which could be identified as a 28-kDa species in the P3HR1 cell line itself by using an affinity purified antibody preparation, was at best barely detectable in the P3HR1 converts (data not

FIG. 3. (A) Immunoblot of protein extracts from drug-resistant clones of BL30/P3 cells transfected either with the J289 or with the J144 recombinant vector. Expression of EBNA1 and EBNA2A was detected by using human serum RS22. All transfected clones expressed the 72-kDa P3HR1-encoded EBNA1 protein, as did the original BL30/P3 cells (reference track); several transfected clones expressed the recombinant vector-encoded EBNA2A protein as an 85-kDa species similar to that seen in B95.8-transformed LCL X50-7 (reference track). Longer exposure of this gel confirmed that BL30/P3 J289 clone 7 was weakly EBNA2A positive, whereas EBNA2A was not detectable in J289 clone 11. (B) Immunoblot of the same protein extracts as in A probed with the LMP-specific MAb CS.1-4. LMP is detected as a 60- to 63-kDa major band. Longer exposure of the gel confirmed that all transfected clones were LMP positive except for J289 clone 11.

shown). These findings, and those described in an earlier study (22), indicated that EBNA2 and/or full-length EBNA-LP may be required to achieve LMP expression in EBV-infected cells. The following experiments were therefore designed to investigate this possibility.

Expression of an introduced EBNA2 gene induces LMP expression. First, one particular clone of P3HR1-converted BL30 cells, BL30/P3 IIB1, was transfected with the J144 or J289 recombinant vector. Preliminary screening of drug-resistant clones by indirect immunofluorescence with the PE2 MAb showed that 8 of 24 J144 transfectants and 16 of 24 J289 transfectants gave detectable EBNA2 expression; thereafter, selected clones were expanded for further study. Figure 3A shows a representative immunoblot of protein extracts from such clones probed with the human serum RS22, which has specific reactivity against EBNA1 and EBNA2A. All clones expressed the characteristic P3HR1-encoded 72-kDa EBNA1 protein. In addition, many clones expressed the recombinant vector-encoded 85-kDa EBNA2A protein, in some cases (J289 clone 12 and J144 clone 4) at levels at least as high as that in a reference LCLs; other drug-resistant clones (J289 clone 11) were EBNA2 negative.

FIG. 4. Immunoblot of protein extracts from drug-resistant clones of BL41/P3 cells transfected either with the J289 or with the J144 recombinant vector. Expression of EBNA2 and LMP was detected by using the pooled MAbs PE2 and CS.1-4. Several transfected clones expressed the recombinant vector-encoded EBNA2A protein, and all such clones also expressed LMP; there was a direct correlation between the level of EBNA2A expression and the level of LMP expression induced. Longer exposure of this gel failed to detect either EBNA2A or LMP expression in J289 clone 18. Reference tracks include the BL41/P3, P3HR1, and B95.8 cell lines; as noted in earlier work (22) the P3HR1 line itself does express very low levels of LMP (see also Fig. 6B).

Further analysis showed that expression of the introduced EBNA2 gene was always accompanied by activation of LMP expression from the resident P3HR1 virus genome. Thus Fig. 3B shows an immunoblot of the same panel of BL30/P3 transfectants now probed with the anti-LMP MAb CS.1-4. All of the EBNA2-positive clones were demonstrably LMP positive, with the strongest EBNA2-expressing clones (J289 clone 12, J144 clone 4) showing the highest levels of LMP. In contrast, the EBNA2-negative J289 clone 11 was LMP negative (Fig. 3B), as were several transfectants produced with the pSV2gpt control plasmid (data not shown). Subsequent immunofluorescence staining with the CS.1-4 MAb preparation confirmed the induction of LMP expression in EBNA2-positive transfectants; the pattern of membrane and cytoplasmic staining was like that seen in conventional LCLs (28). A second series of transfection experiments with an independent clone of P3HR1-converted BL30 cells, BL30/P3 IB8, gave essentially similar results (data not shown).

To ensure that these observations were not peculiar to the BL30/P3 cell line, further experiments were conducted on P3HR1-converted BL41 cells. In this case 4 of 15 J144 transfectant clones and 14 of 55 J289 transfectant clones were EBNA2 positive in the initial screening by immunofluorescence. Figure 4 presents the results obtained when a representative selection of the BL41/P3 transfectants were simultaneously probed for EBNA2 and LMP by using a pool of the MAbs PE2 and CS.1-4. As with the BL30/P3 series of transfectants, EBNA2A expression was always accompanied by induced LMP expression. In clones expressing the highest levels of EBNA2A (J289 clones 2, 15, and 49) LMP was detected in amounts equivalent to that seen in LCLs, whereas there was no detectable LMP expression in EBNA2-negative clones (J289 clone 18).

In the above transfection experiments, immunoblotting analysis with human sera showed that all drug-resistant clones, like the BL30/P3 and BL41/P3 parent cells, expressed the P3HR1-encoded EBNA1 and EBNA3 family of proteins as well as trace levels of EBNA-LP. The relative amounts of these P3HR1 latent gene products were not noticeably influenced by the EBNA2/LMP status of the clones (Fig. 3A and data not shown).

FIG. 5. Immunoblot of protein extracts from drug-resistant clones of BL41/P3 cells transfected either with J289 or with the control pSV2 gpt vector, separated on a high-percentage (12% polyacrylamide) gel capable of resolving low-molecular-mass proteins down to at least 10 kDa. Probing with MAb JF186 revealed expression of 47- and 42-kDa species of B95.8 virus-encoded EBNA-LP in the reference X50-7 lymphoblastoid cell line and of a 24-kD EBNA-LP in the reference BL41/B95.8 cell line (6) but no related species encoded by the J289 vector. Note that the P3HR1-encoded EBNA-LP was not recognized by MAb JF186 (6).

Expression of an introduced EBNA-LP gene does not induce LMP expression. The J289 and J144 expression vectors containing B95.8-derived sequences could theoretically encode, in addition to EBNA2, a low-molecular-mass form of EBNA-LP with a predicted size of 13 kDa (from the single W1 and W2 and Y1 and Y2 exons; Fig. 1). To check this possibility, protein extracts from selected J289 and J144 transfectants of BL30/P3 and of BL41/P3 were run on higher-percentage acrylamide gels and probed for such an EBNA-LP species by using MAb JF186. This antibody specifically recognizes the B95.8 (but not the P3HR1) EBNA-LP through a peptide epitope encoded within the W1 exon (6). As illustrated by the representative results in Fig.

5, all transfectants, including those showing high EBNA 2A/LMP expression (e.g., J289 clones 2 and 49), did not contain any detectable vector-encoded EBNA-LP.

Whereas the above experiments indicated that the J289 and J144 vectors were mediating their effects through the expression of EBNA2, they did not rule out the possibility that a full-length EBNA-LP might also induce LMP expression in P3HR1-converted BL cells. In further experiments, therefore, the same target cells as used above were transfected with the EBNA-LP expression vector pSALP. Screening of drug-resistant clones by indirect immunofluorescence with MAb JF186 showed that 10 of 10 BL30/P3 clones and 6 of 6 BL41/P3 clones were EBNA-LP positive. Immunoblotting with JF186 confirmed expression of a predominant EBNA-LP band with an apparent molecular mass of 42 kDa in every transfectant. This is illustrated by representative results from three BL30/P3 transfectants (LP1, 2, and 5; Fig. 6A), in which the levels of EBNA-LP expression were similar to that seen in X50-7, a reference LCL which itself expressed unusually large amounts of this protein. When the various EBNA-LP transfectants were analyzed by immunoblotting with the LMP-specific MAb CS.1-4, there was no detectable induction of LMP expression in any clone (Fig. 6B).

Figure 6 also illustrates the pattern of protein expression observed in clones obtained from a parallel series of transfections involving the same target cells but using the LMP expression vector pSV2gptMTLM. Some 8 of 25 drug-resistant clones on the BL30/P3 background and 8 of 15 drug-resistant clones on the BL41/P3 background were LMP positive, although expression was usually below LCL levels (e.g., BL30/P3 MTLM clones 10, 12, and 15 in Fig. 6B). These EBNA2-negative, LMP-positive clones generated by pSV2gptMTLM transfection were used as a reference for comparison with the EBNA2/LMP-positive clones generated by J144 and J289 transfection.

Altered growth characteristics of EBNA2/LMP transfectants. The P3HR1-converted BL30 and BL41 cell lines grew predominantly as single cells like their original EBV-negative parent lines, whereas the corresponding B95.8 converts grew in large clumps like LCLs (4). We were therefore interested to see whether the above EBNA2-, EBNA-LP-,

FIG. 6. (A) Immunoblot of protein extracts from drug-resistant clones of BL30/P3 cells transfected either with the vector pSV2gptMTLM capable of expressing LMP (MTLM clones 10, 12, and 15) or with the vector pSALP capable of expressing EBNA-LP (LP clones 1, 2, and 5). The blot was probed with the EBNA-LP-specific MAb JF186. EBNA-LP was detected in all three pSALP-transfected clones at levels similar to that seen in the B95.8-transformed LCL X50-7 (reference track). Other reference tracks include BL30, BL30/P3, BL30/B95, and P3HR1 cell lines. Longer exposure of this gel indicated weak expression of EBNA-LP in BL30/B95 cells. (B) Immunoblot of the same protein extracts as in A probed with the LMP-specific MAb CS.1-4. The EBNA-LP-positive transfectants LP 1, 2, and 5 did not contain detectable levels of LMP. The clones MTLM 10, 12, and 15 expressed recombinant vector-encoded LMP at levels below that detected in the X50-7 LCL.

FIG. 7. Photomicrographs illustrating the growth pattern of (A) BL41/P3 cells, (B) a control transfectant BL41/P3 gpt clone 1, (C) a J289 transfectant BL41/P3 J289 clone 2 that is EBNA2A positive and LMP positive, and (D) a MTLM transfectant BL41/P3 MTLM clone 1 that is EBNA2 negative and LMP positive.

or LMP-transfected clones showed any alterations in growth pattern. Figure 7 illustrates photomicrographs of representative BL41/P3 transfectants. Control plasmid transfectants (Fig. 7B) and EBNA-LP transfectants (data not shown) retained the same single cell pattern of growth as the parental cells (Fig. 7A), whereas EBNA2 transfectants with high EBNA2/LMP expression tended to grow in clumps (Fig. 7C). This effect of an introduced EBNA2 gene may be mediated through its induction of LMP expression, since clones transfected with the recombinant vector pSV2gpt MTLM and expressing high levels of LMP in the absence of EBNA2 also displayed clumpy growth (Fig. 7D). Similar, although generally less dramatic, changes were also observed in the BL30/P3 series of transfectants.

Altered cell-surface phenotype of EBNA2/LMP transfectants.

Earlier work had shown that P3HR1-converted BL30 and BL41 cell lines resemble their EBV-negative parents in their low expression of certain B-cell activation antigens (4) and certain adhesion molecules (C. D. Gregory, personal communication). In contrast, these cell surface markers are upregulated in B95.8-converted BL30 and BL41 cell lines. We therefore analyzed a selected panel of BL41/P3 transfectants for their expression of two representative activation antigens, CD21 and CD23, and of the adhesion molecules LFA1, ICAM1, and LFA3.

Table 1 shows the results of immunofluorescence staining of viable cells with the relevant specific MAbs; the level of staining is expressed as a mean fluorescence intensity value obtained by analysis with a fluorescence-activated cell sorter. An EBNA2 transfectant with no detectable EBNA2/

TABLE 1. Cell surface phenotypic changes in transfected clones of BL41/P3 cells

Cell line	Viral gene expression ^a		Cell surface marker expression ^b				
	EBNA2	LMP	CD21	CD23	LFA1	ICAM1	LFA3
Parental line BL41/P3	—	—	8.0	26.0	5.4	107.2	5.8
EBNA2 transfectants							
J289/18	—	—	4.6	4.9	6.0	100.9	7.1
J89/42	++	+	59.3	39.8	27.2	105.0	11.2
J289/49	+++	+++	89.6	95.8	20.0	115.9	26.1
J289/2	+++	+++	67.5	75.0	47.6	166.4	20.0
LMP transfectants							
MTLM/10	—	—	4.1	5.8	16.5	123.8	8.4
MTLM/1	—	++	3.8	53.7	10.0	204.1	24.7
B95.8-converted BL41/B95	+++	+++	84.7	85.1	38.8	171.3	80.3

^a Levels of EBNA2 and LMP expression detected by immunoblotting with specific MAbs on a scale where +++ represents typical LCL levels.

^b Levels of the B-cell activation antigens CD21 and CD23, and of the cellular adhesion molecules LFA1, ICAM1, and LFA3, detected by specific MAb staining of viable cells and expressed as mean fluorescence intensity values obtained by analysis with a fluorescence-activated cell sorter.

LMP expression (J289/18), and control transfectants (data not shown), were phenotypically similar to the original BL41/P3 cell line in showing low levels of surface CD21, CD23, LFA1, and LFA3 and high levels of ICAM1. In contrast, EBNA2 transfectants with high EBNA2/LMP expression (J289/49 and J289/2) showed elevations of CD21, CD23, and LFA1 to levels comparable to those in B95.8-converted BL41 cells as well as a significant increase in LFA3. Interestingly, an LMP-transfected BL41/P3 clone (MTLM1) expressing reasonably high levels of LMP in the absence of EBNA2 showed a similar up-regulation of CD23 and LFA3 but did not reproduce the effects on CD21 and LFA1 observed in the EBNA2 transfectants.

DISCUSSION

The use of EBV-negative BL cell lines as *in vitro* targets for EBV infection has allowed the biological activity of the P3HR1 deletion mutant to be examined in a way that is not possible with freshly isolated normal B cells, in which P3HR1 infection does not progress beyond transient expression of a truncated EBNA-LP (26). Thus comparison of P3HR1-converted and B95.8-converted BL-cell lines has shown that the inability of the P3HR1 virus genome to encode EBNA2 and/or full-length EBNA-LP is associated with an inability to activate several features of the B-lymphoblastoid (*i.e.*, LCL-like) cellular phenotype (4). In monitoring expression of the resident EBV genome in these two types of converts, we noticed that B95.8 converts expressed the full range of known latent gene products, whereas P3HR1 converts were positive for EBNA1 and the EBNA3 family of proteins but were negative for LMP (22) (Fig. 2). The inference from this observation was that EBNA2 and/or full-length EBNA-LP was necessary for expression of LMP in an infected B cell. The present results (Fig. 3 through 6) in fact provide strong evidence that EBNA2, and not EBNA-LP, is required for induction of LMP expression. Thus transfection of BL30/P3 and of BL41/P3 cells with EBNA2A expression vectors produced a total of 42 EBNA2A-positive clones, and each of these clones showed activation of LMP expression from the resident P3HR1 virus genome. Indeed there appeared to be a direct quantitative correlation between the level of EBNA2A expressed from the recombinant vector and the level of induced LMP. In contrast, high expression of the full-length

B95.8 EBNA-LP in BL30/P3 and BL41/P3 cells was clearly unable to induce LMP.

The present study leaves open the question of the mechanism whereby EBNA2 induced subsequent LMP expression in infected cells. However, the observation that P3HR1-converted BL cell lines do not express detectable levels of LMP mRNA (F. Wang and E. Kieff, unpublished results) would suggest that the effect observed in this work is mediated at the level of LMP transcription. Very little is yet known about the control of LMP transcription, and there is no published evidence for any direct binding of EBNA2 to the LMP promoter region. The fact that EBNA2, when expressed alone in human B lymphoma cells, can induce selective changes in cellular gene expression, notably the induction of CD23 (39), suggests that its effect upon LMP expression may be mediated indirectly through changes in the availability of cellular transcription factors. One of the interesting features of experiments in which EBV latent genes have been expressed in virus-negative B cells (38a, 39, 41) is the dose-dependent nature of the induced changes in the cellular phenotype. Dose dependence of EBNA2-induced cellular changes necessary for LMP transcription might explain the marked correlation seen in the present work between levels of expressed EBNA2 and levels of induced LMP. There are rare cases of EBV-positive BL cell lines in which low LMP expression has been detected in the absence of EBNA2 (30), the best example being the P3HR1 cell line itself (22) (Fig. 4 and 6). These may reflect unusual situations in which the necessary cellular changes have been induced by another route. However, in the great majority of EBV-positive BL cell lines examined, EBNA2 status correlates with LMP status (30), consistent with the present suggestion that EBNA2 can facilitate LMP expression. Note also that the present findings do not preclude an ancillary role in LMP induction for any of the other EBV gene products present in P3HR1-converted cells, notably EBNA1 or EBNA3a, 3b, or 3c (22; this study) or indeed the small nonpolyadenylated EBER RNAs (26).

Virus-induced transformation of B cells to the lymphoblastoid phenotype is associated with the activation of specific sets of cellular genes. This leads, in particular, to increased expression of a variety of cell-surface proteins such as CD21, CD23, and the cellular adhesion molecules LFA1, ICAM1, and LFA3. The present experiments indi-

cate a key role for EBNA2 in the initiation of these changes. Thus expression of EBNA2 by gene transfection into P3HR1-converted BL cells produced a dramatic shift toward growth in clumps (Fig. 7) and toward a cell-surface phenotype (Table 1) like that seen in B95.8-converted BL cells and in conventional LCLs. In particular CD21, CD23, LFA1, and LFA3 were all up-regulated. This wide spectrum of changes reflects the fact that the EBNA2-transfected cells now express not just EBNA2 but also LMP. Independent experiments have shown that EBNA2 alone can activate CD23 and CD21 in B cells (38a, 39), whereas LMP alone can activate CD23 and the complete adhesion molecule family (38a, 41). In the present study we were able to compare the phenotypic changes induced by EBNA2 and LMP in the EBNA2-transfected clones with changes induced by LMP alone in the LMP-transfected clones (Table 1). This comparison led to conclusions that were broadly similar to those described above; namely, that some changes such as CD21 up-regulation were specific to EBNA2-expressing cells, whereas other changes such as LFA3 up-regulation appeared to be linked to LMP expression.

The present study suggests that the biological inactivity of P3HR1 virus in human B cells stems principally from its inability to encode EBNA2 rather than from its inability to encode a full-length EBNA-LP. It is interesting that recent studies with recombinant EBNA2/P3HR1 and EBNA-LP/P3HR1 viruses should point to a similar conclusion; only the EBNA2/P3HR1 recombinant was capable of immortalizing normal B cells *in vitro* (12). We infer that EBNA2 plays an important dual role in the early stages of an EBV infection in a resting B cell; it can have a direct effect upon cellular gene expression; and it is also involved in activating the expression of a second virus-encoded effector protein, LMP.

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