JOURNAL OF VIROLOGY, Mar. 1990, p. 1002–1013
0022-538X/90/031002-12$02.00/0

Vol. 64, No. 3

Stable Transfection of Epstein-Barr Virus (EBV) Nuclear Antigen 2 in Lymphoma Cells Containing the EBV P3HR1 Genome Induces Expression of B-Cell Activation Molecules CD21 and CD23

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Received 22 March 1989/Accepted 3 November 1989

A set of B-cell activation molecules, including the Epstein-Barr virus (EBV) receptor CR2 (CD21) and the B-cell activation antigen CD23 (Blast2/FcεRII), is turned on by infecting EBV-negative B-lymphoma cell lines with immortalizing strains of the viruslike B95-8 (BL/B95 cells). This up regulation may represent one of the mechanisms involved in EBV-mediated B-cell immortalization. The P3HR1 nonimmortalizing strain of the virus, which is deleted for the entire Epstein-Barr nuclear antigen 2 (EBNA2) protein open reading frame, is incapable of inducing the expression of CR2 and CD23, suggesting a crucial role for EBNA2 in the activation of these molecules. In addition, lymphoma cells containing the P3HR1 genome (BL/P_3HR1 cells) do not express the viral latent membrane protein (LMP), which is regularly expressed in cells infected with immortalizing viral strains. Using electroporation, we have transfected the EBNA2 gene cloned in an episomal vector into BL/P3HR1 cells and have obtained cell clones that stably express the EBNA2 protein. In these clones, EBNA2 expression was associated with an increased amount of CR2 and CD23 steady-state RNAs. Of the three species of CD23 mRNAs described, the FcεRIIa species was preferentially expressed in these EBNA2-expressing clones. An increased cell surface expression of CR2 but not of CD23 was observed, and the soluble form of CD23 molecule (SCD23) was released. We were, however, not able to detect any expression of LMP in these cell clones. These data demonstrate that EBNA2 gene is able to complement P3HR1 virus latent functions to induce the activation of CR2 and CD23 expression, and they emphasize the role of EBNA2 protein in the modulation of cellular gene implicated in B-cell proliferation and hence in EBV-mediated B-cell immortalization. Nevertheless, EBNA2 expression in BL/P3HR1 cells is not able to restore the level of CR2 and CD23 expression observed in BL/B95 cells, suggesting that other cellular or viral proteins may also have an important role in the activation of these molecules: the viral LMP seems to be a good candidate.

The Epstein-Barr human lymphotropic herpesvirus (EBV) is the etiologic agent of infectious mononucleosis and is associated with two human cancers, Burkitt's lymphoma (BL) and nasopharyngeal carcinoma. In vitro, EBV specifically infects normal B lymphocytes in which it persists in a latent form. The latently infected B lymphocytes become immortalized, such that they will grow continuously in culture, generating lymphoblastoid cell lines (LCLs) (8).

In the immortalized lymphocytes, the 175-kilobase (kb) linear EBV genome is circularized and maintained in an episomal form. Viral gene expression is then tightly regulated, and only a few of the approximately 100 genes are expressed. To date, fewer than 10 latent proteins have been identified and shown to be constituvely expressed in LCLs. These proteins include the Epstein-Barr nuclear antigens (EBNAs), the latent membrane protein (LMP), and a recently described terminal protein. EBNA1 is encoded by the BKRF1 reading frame of the viral genome (15, 42), EBNA2 by BYRF1 (6, 25), EBNA3 by a BLRF3-BERF1 spliced frame (16, 35), and EBNA-LP (EBNA5) by spliced exons from the *Bam*WY region (7, 40). LMP is encoded by the BNLF1 reading frame (14), and the terminal protein (21) is encoded by exons spliced through the terminal repeats of the viral genome after circularization. EBNA1 has been shown to bind to the origin of replication of the virus (oriP) and is implicated in maintenance of the episomal form of the viral genome (53). LMP has been demonstrated to transform rodent fibroblasts (49) and is part of the lymphocyte-defined membrane antigen, the target structure recognized by cytotoxic T cells during the EBV-specific cell-mediated immune reaction in vivo (26, 44). A role of EBNA2 in B-cell immortalization has long been suspected, since nonimmortalizing EBV strains have a deletion in the genomic region coding for EBNA2 (8). So far, however, no precise biological role of the latent proteins in relation to immortalization has been established, although their expression has been associated with B-cell phenotypic modifications: EBNA2 with the expression of the B-cell activation antigen CD23 (51) and LMP with the expression of CD23 and the adhesion molecules LFA1, LFA3, and ICAM1 (50).

To identify viral functions implicated in B-cell immortalization and to characterize the nature of virus-cell interactions occurring during this process, an in vitro system has been developed in our laboratory (4). A panel of EBV-negative Burkitt lymphoma cell lines (BL/EBV neg.) have been infected (or converted) with various viral strains. These conversions led to the observation that the expression of cellular molecules involved in B-lymphocyte proliferation

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was differently modulated, depending on the virus strain. Notably, conversion of BL/EBV neg. lines by the immortalizing strain B95-8 (BL/B95 cells) induces high-level expression of the EBV receptor-CR2 protein and of the B-cell activation antigen, CD23, at their surface. CR2 (CD21) (9, 10) and CD23 (Blast2/FcεRII) (2, 46, 55) are normal B-cell molecules expressed early during B-cell activation and are implicated in the regulation of the cell cycle. They are also regularly expressed on LCL cells. This supports the suggestion that EBV might activate the normal B-cell proliferation pathway and contribute to the establishment of an autocrine secretion loop of B-cell growth factors during B-cell immortalization (12, 45). Nevertheless, BL lines converted by the nonimmortalizing P3HR1 strain (BL/P3HR1 cells) which contains a deletion of 6.6 kb spanning the entire EBNA2 and part of the EBNA-LP protein reading frames do not express CR2 or CD23. It has also been demonstrated that the viral protein LMP is expressed only at the surface of cells infected with immortalizing strains. This suggests a direct role of the EBNA2 gene in the activation of CR2, CD23, and LMP observed in BL/B95 cells.

To evaluate the role of EBNA2 in the activation of CR2, CD23, and LMP observed in BL/B95 cells, we attempted to express an exogenous EBNA2 gene in BL/P3HR1 cells. For this purpose, we used electroporation to transfect an episomal vector containing an EBV genomic fragment spanning the EBNA2 gene in BL/P3HR1 cells. We now report that the stable expression of the EBNA2 gene in BL/P3HR1 cell clones is associated with the activation of the CR2 and CD23 expression, but not with that of the viral protein LMP.

MATERIALS AND METHODS

Cell lines and culture medium. BL41 is an EBV-negative BL cell line, spontaneously established at the International Agency for Research on Cancer (IARC), Lyon, France. IARC 171, IARC 167, and IARC 277 are LCL lines obtained through immortalization of normal B lymphocytes with B95-8 virus. BL41/P3HR1 and BL41/B95 are cell clones obtained after infection of BL41 cells by P3HR1 and B95-8 viruses, respectively, as previously described (4). Raji, BL74, and BL29 are EBV-positive BL lines, and HSB2 is a T-lymphoma cell line. These cell lines were routinely maintained by twice-weekly subculture in RPMI 1640 medium (GIBCO Laboratories) containing 18% (vol/vol) fetal calf serum and supplemented with 100 IU of penicillin per ml and 100 μg of streptomycin per ml.

Plasmids. Plasmid pU430/23, used to transfect the EBNA2 gene, was derived from the shuttle vector pHEBO (41) in which a large M.ABA genomic fragment of ca. 20 kb overlapping the BYRF1 reading frame has been cloned. The vector pHEBO was digested with *Hin*dIII and *Bam*HI and ligated with the 6.3-kb *Hin*dIII-*Bgl*II fragment derived from the M.ABA *Bgl*II C fragment cloned in p780-35 (31) and containing the left part of the large unique region of the EBV genome. The resulting construct was digested with *Hin*dIII, treated with calf intestine alkaline phosphatase, and ligated with the 14.6-kb *Hin*dIII fragment from the plasmid pM780-28 (31), carrying the tetracycline resistance gene of pBR322, three large internal repeats, and the border of the large unique region. Plasmid pU430/23 carries the 14.6-kb *Hin*dIII fragment in the proper orientation to reconstruct the EBV *Bgl*II C fragment. Plasmid p710, used as control in the transfection experiments, has been described elsewhere (34). It is similar to the pHEBO vector, except that only the repeats and dyad symmetry parts of oriP have been conserved.

Transfections of BL/P3HR1 cells. Transfection of BL/P3HR1 cells was performed by using electric field-mediated DNA transfer (electroporation) (32, 47). Cells from logarithmically growing cultures were collected by centrifugation, washed twice in phosphate-buffered saline (PBS) that was calcium and magnesium free, and resuspended in the same buffer to a final concentration of 2×10^7 to 5×10^7 cells per ml in the presence of 10 μg of plasmid DNA and 40 μg of sonicated salmon sperm DNA in a final volume of 0.8 ml. The resulting suspension was chilled on ice for 10 min, placed in a gene pulser cuvette, and subjected to a single electric pulse delivered by a Bio-Rad gene pulser. In our transfection experiments, we applied a pulse of 3,000 V/cm, with a capacitance of 25 μF. Under these conditions, the time constant (the time taken for the voltage to decline to about 37% of its initial value) was 0.5 ms. Immediately after the pulse, the cells were replaced on ice for 10 min. Culture medium (5 ml) was then added to the cell suspension, which was kept at room temperature for 10 min. The cells were then kept for 24 to 48 h at 37°C (under 5% CO_2) before selection.

The selection was performed by plating the cells in 96-well microplates (5×10^4 to 1×10^5 cells per well) in the presence of 250 μg of hygromycin B (Calbiochem-Behring) per ml of culture medium. The cells were then maintained under these conditions for 10 to 15 days, with the medium changed twice a week. Resistant cell populations were harvested from independent wells and tested by anticomplement immunofluorescence staining (ACIF) for EBNA2 expression. Independent transfected cell clones were immediately obtained by single-cell cloning in limited dilution.

ACIF. Cells were washed twice in PBS and dried on slides at room temperature before being fixed for 10 min in an acetone-methanol solution (1:1, vol/vol). EBNA expression was then tested by using the classic ACIF protocol (33). EBNA2 expression was detected by using a human serum (Ba) which gives no fluorescence on BL/P3HR1 cells but only on BL/B95 cells.

SDS-polyacrylamide gel electrophoresis and immunoblotting. Cells were washed once in PBS, lysed in sample buffer (10% sucrose, 60 mM Tris hydrochloride [pH 7], 2 mM EDTA, 2% sodium dodecyl sulfate SDS, 2 mM phenylmethylsulfonyl fluoride, 2% 2-mercaptoethanol), and boiled for 10 min. A protein sample (8 μl) with an optical density of 0.8 at 280 nm was size separated by discontinuous gel electrophoresis, with a stacking gel of 5% acrylamide and a resolving gel of 7.5 to 10% acrylamide, and transferred onto nitrocellulose (Hybond-C; Amersham Corp.) at 300 mA overnight. The blotted filters were incubated for 30 min at room temperature with PBS containing 10% skimmed milk and then for 2 h with the primary antibody diluted in PBS-milk; they were then washed in PBS containing 0.1% Tween 20. After being washed they were incubated for 2 h at room temperature either with anti-human immunoglobulin horseradish peroxidase-linked $F(ab')_2$ fragment (Amersham) diluted 1/250 in PBS-milk or with ^{125}I-protein A (Amersham) diluted to 0.1 μCi/ml in PBS-milk. In the experiments in which monoclonal antibodies (MAbs) were being assayed, the filters were first incubated for 1 h at room temperature with a rabbit anti-mouse immunoglobulin (Dako) diluted 1/5,000 in PBS-milk and washed again in PBS-Tween before being incubated with ^{125}I-protein A. The filters were then washed again in PBS-Tween 20. The specifically bound peroxidase-linked antibodies were revealed in the presence of diaminobenzidine and hydrogen peroxide. Following final

washes, the filters treated with ^{125}I-protein A were dried and subjected to autoradiography.

Antibodies. Tu 494 is an EBV polyspecific human serum from a patient with nasopharyngeal carcinoma and was used at a dilution of 1/50. CS1-4 is an anti-LMP MAb described elsewhere (36); it was provided by A. B. Rickinson. It was used at a dilution of 1/15. G643 is an anti-EBNA-LP rabbit serum directed against a fusion protein containing 22 amino acids of W1 exon of the *Bam*HI W viral fragment of the M.ABA strain; it is described elsewhere (39). It was provided by N. Mueller-Lantzsch and was used at a dilution of 1/50. OKB7 is a MAb specific for the human 140-kilodalton (kDa) EBV receptor-CR2 protein (Ortho Diagnostics). We used it at a dilution of 1/50. MAb 25 (or 9p25) is a MAb specific for the 44-kDa immunoglobulin E Fc receptor on human lymphocytes (CD23). It was kindly provided by J. Y. Bonnefoy (2); we used it at a dilution of 1/5. H107 and MHM6 anti-CD23 MAbs have been described elsewhere (30, 37). MHM6 was kindly provided by A. B. Rickinson.

FACS analysis. Cells were first incubated for 30 min at 4°C with MAb diluted in PBS containing 2% bovine serum albumin (BSA). After being washed in PBS-BSA, the cells were further incubated for 30 min at 4°C with fluorescein-conjugated F(ab')$_2$ antibodies to mouse immunoglobulin (BIOART) diluted to 1/200 in PBS-BSA and washed again in PBS-BSA. MAbs of the same isotype as the tested monoclonal antibodies were included as negative control for nonspecific binding. Immunofluorescence staining was then analyzed with a FACS-440 fluorescence-activated cell sorter (Becton Dickinson and Co.).

Sandwich enzyme-linked immunosorbent assay for soluble CD23 (SCD23). Lines tested for the production of SCD23 were cultured under the following conditions. Fresh culture medium (4 ml) was added to 10^5 cells in 1 ml of culture medium. After 72 h, the cell density was determined; it varied between 2.5×10^6 and 3×10^6 cells per ml. Cells were centrifuged, and the supernatant was collected, cleared on a membrane (pore size, 0.45 μm; Millipore Corp.), and stored at −20°C before being used in the enzyme-linked immunosorbent assay.

A 96-well plate (Interned) was first incubated with (per well) 50 μl of 2 μg of purified anti-CD23 MAb 25 (2) per ml in 0.1 M carbonate buffer (pH 9.6) at 4°C overnight. The wells were then washed five times with PBS containing 0.05% Tween-20 and coated with PBS containing 2% BSA for at least 4 h at room temperature. After five washes, 100 μl of samples (0.2-μm-filtered culture supernatant) was added to the wells, and the plates were incubated at 4°C overnight. The plates were then washed five times, and 50 μl of alkaline phosphatase-coupled anti-CD23 MAb H107 (30) (diluted to 2 μg/ml with 2% PBS–BSA) was added to each well. The plates were incubated overnight at 4°C, washed five times, and supplemented with 100 μl of *p*-nitrophenylphosphate (1 mg/ml; Sigma Chemical Co.) diluted with 9.7% diethanolamine, 3 mM NaN_3 and 0.5 mM $MgCl_2 \cdot 6H_2O$ (pH 9.8). After incubation at room temperature, A_{405} was determined by using the Titertek Multiscan (Flow Laboratories, Inc.).

RNA isolation and Northern blot. Total cellular RNA was prepared by using the guanidine isothiocyanate-cesium chloride centrifugation method (5). A 20- to 40-μg amount of each RNA sample was size separated on a 1% (wt/vol) agarose gel containing 2.2 M formaldehyde and was transferred in 20× SSC (3 M NaCl, 0.3 M sodium citrate [pH 7]) onto a GeneScreen Plus membrane (Du Pont Co.) (23).

Probes and hybridizations. Probes were labeled by random priming with [α-^{32}P]dCTP of the 1.6-kb *Eco*RI fragment from CR2-cDNA plasmid p1.6 (kindly provided by J. Weis [52]), of the 1.7-kb *Eco*RI fragment from CD23-cDNA plasmid pFcεR1 (kindly provided by H. Kikutani [20]), of total plasmid pGAPDH-13 (kindly provided by M. Piechaczyk, Université du Languedoc, Montpellier, France), and of a 3.7-kb EBV genomic fragment from plasmid pLMP (kindly provided by A. Sergeant, Ecole Normale Supérieure, Lyon, France).

Filters were prehybridized and hybridized overnight at 42°C in 0.05 M sodium phosphate buffer (pH 7)–1 M NaCl–50% (vol/vol) formamide–1% (wt/vol) SDS containing 100 μg of salmon sperm DNA per ml, 100 μg of yeast tRNA per ml, and 1×10^6 to 5×10^6 cpm of probe per ml of hybridization solution. After hybridization, the filters were washed at 65°C for 1 h in 2× SSC–1% (wt/vol) SDS and exposed to hyperfilm (Amersham) for autoradiography for 1 to 10 days.

RNase protection experiments. Plasmid construction and in vitro transcription. pG3Z/5′-CD23 was constructed by subcloning the 350-base-pair (bp) *Eco*RI-*Pvu*II fragment from pFcεR1 (54), corresponding to the 5′ extremity of the CD23 cDNA, into the *Sma*I site of the expression vector pGEM-3Z (Promega Biotec). pG3Z/5′-CD23 was linearized with *Hin*dIII, and radioactive RNA probe was synthetized by using T7 RNA polymerase as previously described (1); 85 μCi of [α-^{32}P]UTP (400 Ci/mmol) was used in a 25-μl reaction.

Hybridization and RNase digestion. Hybridization and RNase digestion were performed as previously described (1). Briefly, hybridizations were performed by mixing 5×10^5 cpm of [α-^{32}p]RNA probe with 20 to 40 μg of total RNA in hybridization solution [80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes; pH 6.7)] and incubating the mixture overnight at 45°C. The reactions were then incubated for 1 h at room temperature in the presence of RNase A and RNase T_1. The digestions were stopped by the addition of 20% SDS and proteinase K, and the mixtures were further incubated for 15 min at 37°C. After phenol-chloroform extraction and ethanol precipitation, the precipitated RNA was dissolved in formamide loading buffer (10% [wt/vol] xylene cyanol, 100% bromophenol blue, and 0.01 M EDTA in formamide) and fractionated on a 5% polyacrylamide–8 M urea sequencing gel. The gel was then exposed to X-ray film at −70°C.

RESULTS

Transfection of EBNA2 gene in BL41/P3HR1 cells. We have transfected the EBNA2 gene cloned in the episomal vector pHEBO (plasmid pU430/23 described in Materials and Methods) into BL41/P3HR1 cells by using the electroporation technique. The vector pHEBO possesses the oriP region of the EBV genome and is maintained in an episomal form and in many copies in cells expressing EBNA1, such as BL41/P3HR1 cells.

We performed two independent transfection experiments, with plasmid pU430/23 to transfect the EBNA2 gene and the plasmid p710 (34) as a control plasmid without the EBNA2 gene. These two plasmids confer resistance to hygromycin B. At 15 days after the beginning of selection, we selected independent populations resistant to hygromycin B. The resistant populations were independent, since they were recovered from independent wells of culture microplates. Because the genes conferring resistance to hygromycin B and EBNA2 were on the same vector, we expected the resistant populations transfected with pU430/23 to express EBNA2 protein. We first tested the expression of EBNA2

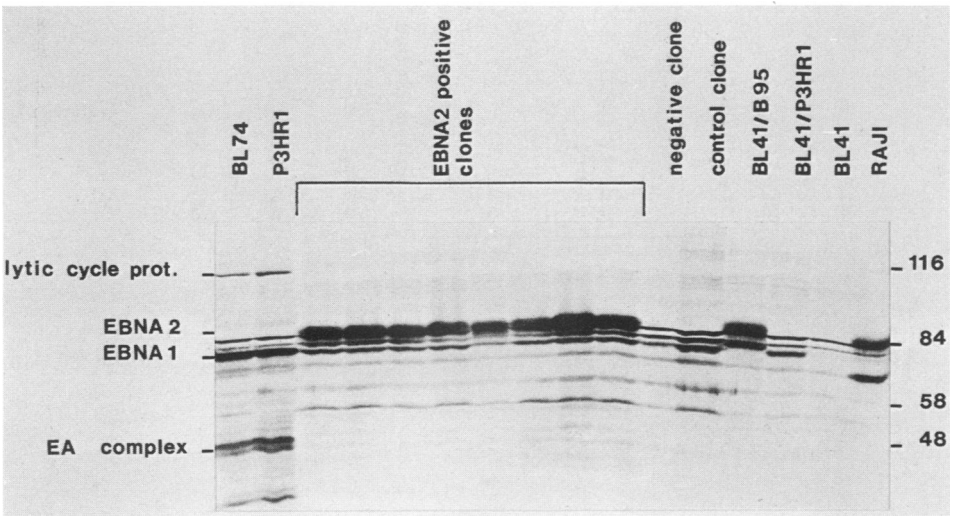

FIG. 1. Immunoblot analysis of the expression of EBNA2, EBNA1, and replicative-cycle proteins in the transfected clones, by using a human polyspecific serum Tu 494. Numbers on the right indicate the molecular size in kilodaltons. BL74 and P3HR1 are BL EBV-positive productive lines. Raji is an EBV-positive line. BL41/P3HR1 and BL41/B95 correspond to the BL41 EBV-negative BL line converted, respectively, with P3HR1 and B95 viruses. EBNA2-positive clones are clones expressing EBNA2: from left to right, B1-7, B1-9, B1-10, B1-13, B2-1, B2-2, B2-3, and B2-4. The lane labeled "negative clone" is clone B3-1 that was transfected with pU430/23 but did not express EBNA2, and the lane labeled "control clone" is clone N1-1 transfected with plasmid p710. The EBNA2 protein was detected at ca. 85 kDa, and the EBNA1 protein was detected at ca. 80 kDa. Lytic-cycle proteins of high molecular size are detected at ca. 110 kDa in the productive lines BL74 and P3HR1, and EA-complex antigens of low molecular size are detected at ca. 48 kDa.

protein in the hygromycin-resistant populations by using ACIF with human Ba serum, which gave no fluorescence on BL41/P3HR1 cells but only on BL41/B95 cells (data not shown) and which was therefore considered to detect EBNA2 expression. The percentage of cells expressing EBNA2 in the hygromycin-resistant populations varied considerably (from 20 to 80%) most of the time and was once about 100%. Hygromycin-resistant populations obtained after transfection were nonhomogeneous with respect to EBNA2 expression, and we have cloned them by using limited dilution to generate homogeneous EBNA2 expressing cell clones. We have cloned the following populations from the two independent transfection experiments described before. The first was a hygromycin-resistant population (B1) from the first experiment, transfected with pU430/23 and in which 70% of the cells expressed EBNA2 in ACIF. Of 24 clones harvested, 4 clones did not express EBNA2 at all in ACIF (B1-L, B1-M, B1-Q, and B1-S) and 16 clones expressed EBNA2 homogeneously; i.e., all cells were positive in ACIF with the same fluorescence intensity (B1-7, B1-9, B1-10, B1-13, B1-A, B1-B, etc.). The second was a hygromycin-resistant population (B2) from the second experiment, transfected with pU430/23 and in which more than 98% of the cells expressed EBNA2 in ACIF. We obtained only clones expressing EBNA2 homogeneously in ACIF, such as B2-1, B2-2, B2-3, and B2-4. The third was a hygromycin-resistant population (B3) transfected with pU430/23 but in which all the cells were negative for EBNA2 expression in ACIF; we obtained negative clones that did not express EBNA2 at all, such as B3-1. The fourth was a hygromycin-resistant population (N1) transfected with the control plasmid p710; we obtained control clones that did not express EBNA2 in ACIF, such as N1-1.

We have verified that the plasmids recovered from the transfected cell clones were in a whole episomal form. Analysis of the low-molecular-weight DNAs (Hirt DNAs) extracted from the cell clones showed that cell clones expressing EBNA2 protein were those in which no major rearrangement of the plasmid pU430/23 was detected after transfection (data not shown). There were two types of cell clones which did not express EBNA2: those named control clones, like N1-1, which transfected with the control plasmid p710, which did not carry the EBNA2 gene; and those named negative clones, like B3-1 and B1-L, B1-M, B1-Q, and B1-S, which transfected with pU430/23, but in which this plasmid was rearranged. Rearrangement of plasmid pU430/23 may have occurred very early during transfection, since the EBNA2-expressing clones have been maintained for several months in culture with stable expression of EBNA2 and no emergence of nonexpressing cells.

Expression of the transfected EBNA2 gene. By SDS-polyacrylamide gel electrophoresis and immunoblotting, we have analyzed the size and the level of expression of the EBNA2 protein expressed in the transfected clones and detected in ACIF, by using the human polyvalent serum Tu 494. Clones B1-7, B1-9, B1-10, B1-13, B2-1, B2-2, B2-3, and B2-4, which were positive in ACIF, expressed an EBNA-2 protein of around 85 kDa, the same size as the EBNA2 protein found in BL41/B95 cells and Raji cells (Fig. 1). The level of expression of EBNA2 in these clones was comparable to that in BL41/B95 cells and in Raji cells (comparing the intensity of the bands). We detected no expression of the EBNA2 protein in the control clone N1-1 or in the negative clone B3-1. Clone N1-1 was transfected with the control plasmid p710 without the EBNA2 gene, and clone B3-1 was transfected with the plasmid pU430/23 but which was deleted in the EBNA2 region after transfection. The results of immunoblot analysis of EBNA2 expression in the transfected clones are thus in good agreement with those obtained from the ACIF analysis of EBNA2 expression (see previous paragraph).

Expression of other EBV proteins. (i) EBNA1. We detected an EBNA1 protein of the same size (ca. 80 kDa) in BL41/P3HR1 cells and in all the transfected clones (Fig. 1). The EBNA1 protein was smaller in Raji cells and larger in BL41/B95 cells because of the heterogeneity of the EBV

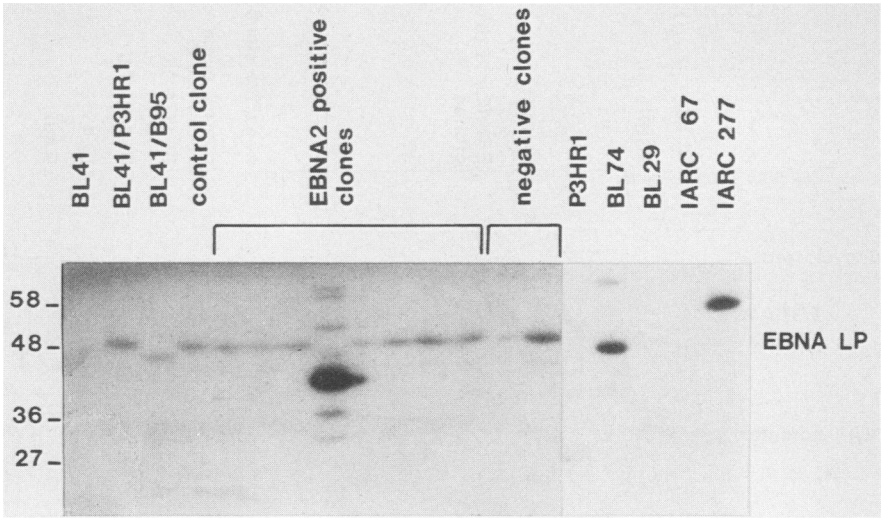

FIG. 2. Immunoblot analysis of EBNA-LP expression in the transfected clones by using the rabbit serum G643. Numbers on the left indicate molecular size in kilodaltons. IARC 277 and IARC 167 are LCLs. Raji, BL29, and BL74 are EBV-positive BL lines. BL41/P3HR1 and BL41/B95 correspond to the BL41 EBV-negative line converted, respectively, with P3HR1 and B95.8 viruses. EBNA2-positive clones are clones expressing EBNA2: from left to right, B1-7, B1-9, B1-10, B1-13, B2-1, B2-2, B2-3, and B2-4. Negative clones are clones that were transfected with pU430/23 but do not express EBNA2: from left to right, B3-1 and B3-2. The lane labeled "control clone" is clone N1-1 transfected with control plasmid p710. The size of the EBNA-LP protein varied from one line to another: 58 kDa in IARC 277, 27 kDa in P3HR1, 45 kDa in BL41/B95, 48 kDa in BL41/P3HR1, and 39, 45, 55, and 70 kDa in BL 74. The EBNA-LP protein detected in the transfected clones had the same molecular size as that in the BL41/P3HR1 parental cell. In clone B1-13 we found EBNA-LP at 30, 37, 40, 50, and 60 kDa.

genome in the region coding for EBNA1. No detectable changes in the level of expression of EBNA1 were observed between the BL41/P3HR1 parental cells and the transfected cell clones.

(ii) Lytic-cycle proteins-EA complex. Tu 494 serum also permits us to detect lytic cycle proteins of high molecular weights and EA-complex antigens (8) of low molecular weights. These proteins were detected in the productive lines BL74 and P3HR1, but were not observed in the nonproductive lines Raji, BL41/P3HR1, and BL41/B95, nor in the transfected clones. On the other hand, using a set of polyvalent human sera in the immunofluorescence analysis, we did not detect any expression of EA antigens (8) in these cell clones. We concluded that EBNA2 expression in the BL41/P3HR1 cells did not obviously modified the expression of antigens implicated in the lytic cycle of the virus.

(iii) EBNA3-EBNA4. Using anti-EBNA3- and anti-EBNA4-specific rabbit sera, we did not detect any modification in the expression of these proteins in the transfected clones (data not shown) compared with that observed in the parental lines and previously described (27).

(iv) EBNA-LP. The plasmid pU430/23 contained three entire W repeats in which transcriptional promoters have been found (48) and from where initiation of EBNA2 transcription occurs in our clones. The EBNA-LP protein has been shown to be encoded by small exons (W0, W1, and W2) spliced from the W repeats (7, 40) and may have been expressed in the clones transfected with pU430/23. Using a rabbit serum (G643) directed against 22 amino acids encoded by the W1 exon (39), we analyzed the expression of the EBNA-LP protein in these transfected clones. As previously described (39), we detected an EBNA-LP protein with a single size of ca. 58 kDa in IARC 277 cells, one of ca. 27 kDa in P3HR1 cells, and ones with different sizes (39, 45, 55, and 70 kDa) in BL74 cells (Fig. 2). We also detected an EBNA-LP protein of ca. 45 kDa in BL41/B95 cells and one of ca. 48 kDa in BL41/P3HR1 parental cells and in all the transfected cell clones, except in clone B1-13, in which EBNA-LP proteins of ca. 30, 37, 40, 50, and 60 kDa were found. We concluded that the expression of EBNA-LP protein in the transfected cell clones was not different from that in the parental BL41/P3HR1 line and that it consisted of a single protein form of ca. 48 kDa expressed at nearly the same level. There was, however, one exception with clone B1-13, in which the expression of EBNA-LP protein was completely different, and existed in many protein forms, perhaps generated from plasmid pU430/23.

(v) LMP. Using CS1-4 MAb, we have detected an LMP protein of ca. 66 kDa in Raji cells and BL41/B95 cells but not in BL41/P3HR1 cells (Fig. 3a). The LMP protein was also not detected in the transfected clones, even after a long exposure in autoradiography. We hybridized Northern blots of these clones, using as probe a 3.7-kb genomic EBV fragment overlapping the LMP open reading frame (pLMP). We did not detect any transcript corresponding to LMP in the transfected clones (even after a long exposure in autoradiography), although we detected a transcript of 2.9 kb in Raji and BL41/B95 cells (Fig. 3b). LMP RNA expression was compared with that of glyceraldehyde phosphatase dehydrogenase (GADPH), which was considered to be constant in all the cell lines studied (data not shown). We concluded from this series of results that LMP expression was not detectable at the protein or RNA levels in our EBNA2-transfected clones.

Activation of CR2 and CD23 in clones transfected with EBNA2. (i) Northern blot analysis of CR2 and CD23 RNA expression. With CR2-cDNA as a probe, a 5-kb CR2 transcript was detected in Raji, BL41/B95, and IARC 171 lines and in transfected clones expressing EBNA2 protein (B1-7, B1-9, B1-10, B1-13, B2-1, B2-2, B2-3, and B2-4) but not in the BL41/P3HR1 parental line, the negative clone B3-1, or the control clone N1-1 (Fig. 4a). In the same way, a 1.7-kb/CD23 transcript was detected in BL41/B95 and IARC 171 cells and also in all the transfected clones expressing

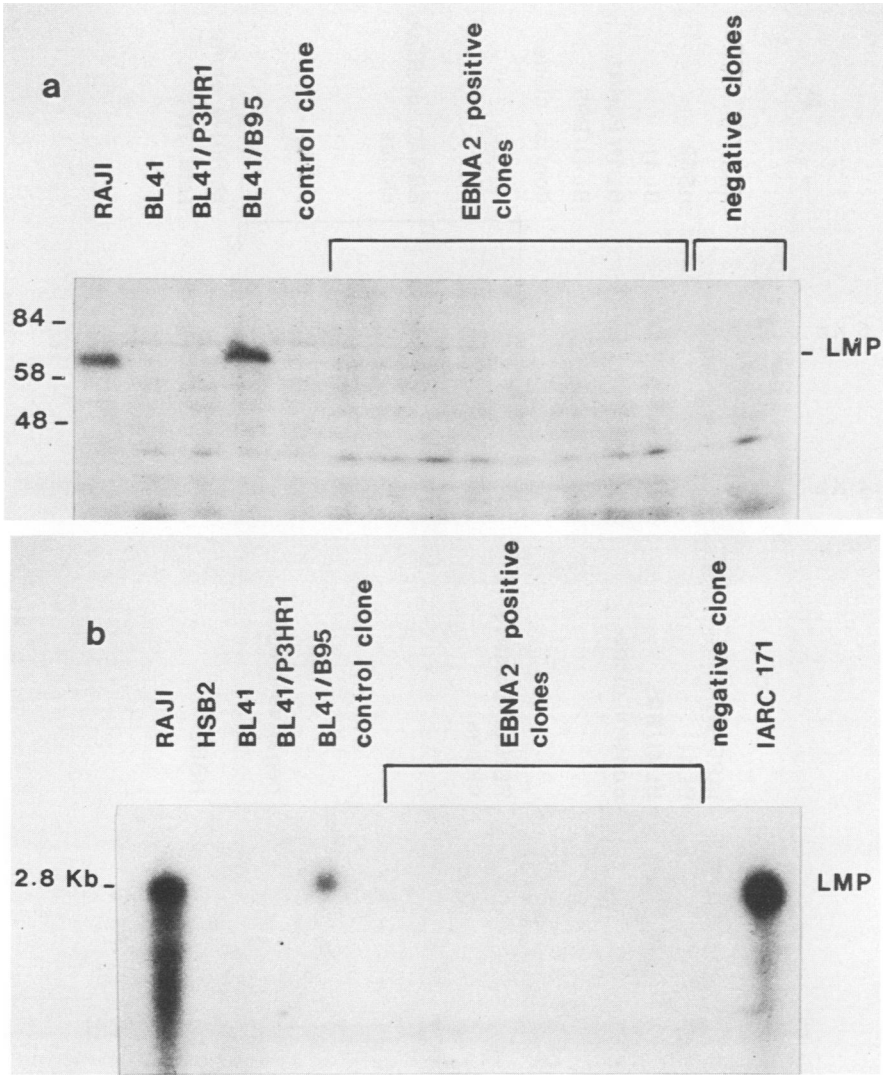

FIG. 3. LMP expression in the transfected clones. Raji is an EBV-positive BL line, and BL41 is an EBV-negative line. IARC 171 is an LCL, and BL41/P3HR1 and BL41/B95 correspond to the BL41 line converted, respectively, with P3HR1 and B95 viruses. HSB2 is a T lymphoma line. EBNA2-positive clones are clones expressing EBNA2: from left to right, B1-7, B1-9, B1-10, B1-13, B2-1, B2-2, B2-3, and B2-4. Negative clones are clones transfected with pU430/23 but that do not express EBNA2: from left to right, B3-1 and B3-2. The lane labeled "control clone" is clone N1-1 transfected with the control plasmid p710. (a) Immunoblot analysis of LMP expression with the CS1-4 MAb (36). The LMP was detected at around 66 kDa in Raji and BL41/95 lines but not in the transfected clone. (b) Northern blot analysis of LMP transcription. Total cellular RNA (20 μg per sample) was hybridized with probe pLMP corresponding to a 3.7-kb genomic fragment overlapping the LMP reading frame, and it detected a 2.8-kb transcript in Raji, BL41/95, and IARC 171 lines but not in the transfected clones. GAPDH expression analysis is not shown.

EBNA2, but not in the negative clones B1-L, B1-M, B1-Q, B1-S, and B3-1 or in the control clone N1-1 (Fig. 4b). These results demonstrate that there is an increase in the amount of CR2 and CD23 steady-state RNAs in clones expressing EBNA2 compared with the amount in BL41/P3HR1 parental cells or clones that do not express EBNA2, such as the control clone N1-1 transfected with the control plasmid p710 or the negative clones B1-L, B1-M, B1-Q, B1-S, and B3-1 transfected with pU430/23.

It seems that the level of CR2 and CD23 activation can vary a little from one clone expressing EBNA2 to another, but is still always lower than that observed in BL41/B95 cells. CD23 activation seems to be much weaker than that of CR2, since in the case of CR2 detection the quantities of RNAs for BL41/B95 and EBNA2-positive clones are the same, and in the case of CD23 detection the quantity of BL41/B95 RNA is half that in EBNA2-positive clones.

(ii) RNase protection analysis of CD23 mRNA expression. The existence of three species of CD23 mRNAs has been described previously (54), with differences in the region coding for the N-terminal cytoplasmic portion of the protein. These three mRNA species are generated by using different transcription initiation sites and differential RNA splicing. Two mRNA species coding for the FcεRIIa/CD23 form have been described whose sequences differ by only 4 nucleotides in their 5′ untranslated region (nucleotides 96 to 100 of the pFcεR1 cDNA clone [54]), possibly as a result of a differential splicing event. A third mRNA species coding for the

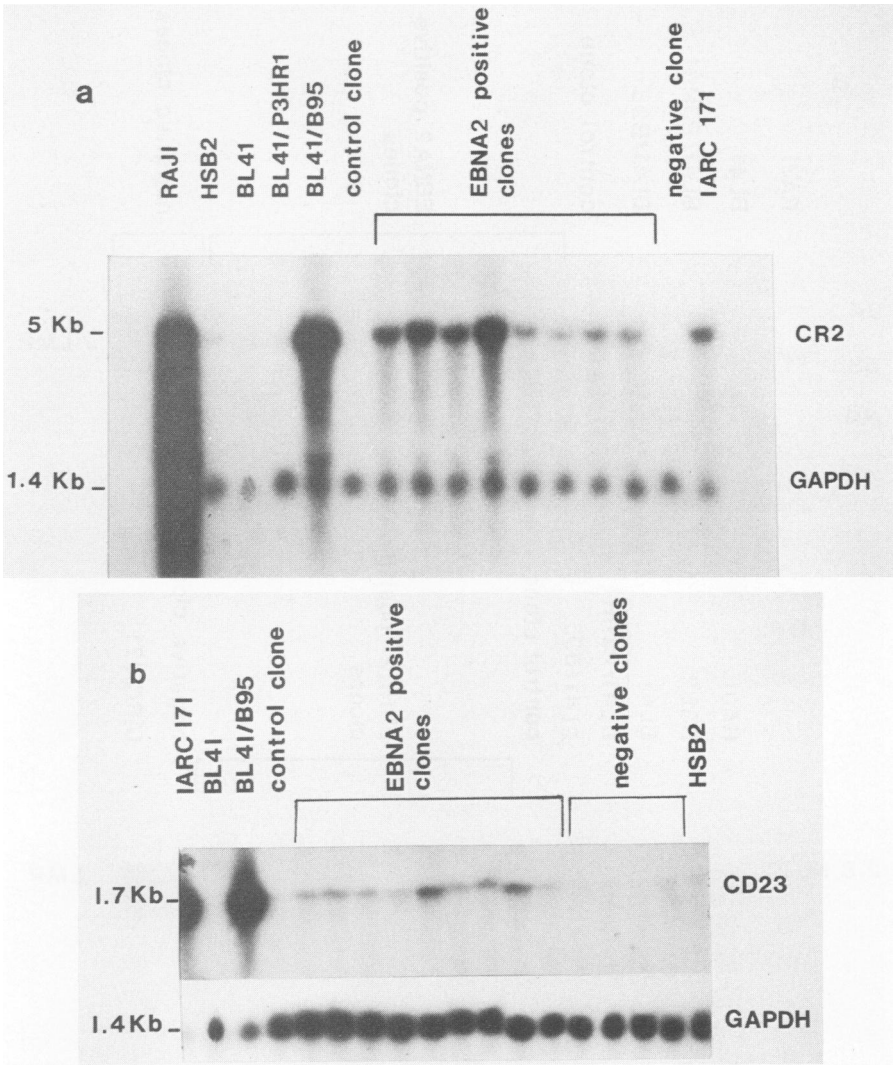

FIG. 4. CR2 and CD23 RNA expression in the transfected clones. (a) Northern blots with 20 μg of total cellular RNA per sample were hybridized with the p1.6 probe (52), which detects a 5-kb CR2 transcript. Raji is an EBV-positive line; IARC 171 is an LCL; and BL41/P3HR1 and BL41/B95 correspond to the BL41 EBV-negative line converted, respectively, with P3HR1 and B95 viruses. HSB2 is a T lymphoma line. EBNA2 positive clones are clones expressing EBNA2: from left to right, B1-7, B1-9, B1-10, B1-13, B2-1, B2-2, B2-3, and B2-4. Negative clones are clones that were transfected with pU430/23 but that do not express EBNA2: from left to right, B3-1 and B3-2. The lane labeled "control clone" is clone N1-1 transfected with the control plasmid p710. (b) Northern blot with 40 μg of total cellular RNA (20 μg for BL41/95 and IARC 171) were hybridized with the pFcεR1 probe (20), which detects a 1.7-kb CD23 transcript. HSB2, IARC 171, and BL41/95 lines are described in the legend to panel a. EBNA2-positive clones are, from left to right, B1-7, B1-9, B1-10, B1-13, B2-1, B2-2, B2-3, B2-4, and B1-A, and negative clones are, from left to right, B3-1, B1-M, B1-Q, and B1-S. CR2 and CD23 RNA expression has been compared with that of GAPDH (1.4-kb transcript) which was considered to be constant in all the cell lines studied.

FcεRIIb/CD23 form has been found to be completely different from the other two in the 5′ region up to nucleotide 208 of the pFcεR1 cDNA clone (54).

We have developed an RNase protection analysis experiment which permits us to discriminate between the FcεRIIa and FcεRIIb species. A 350-bp 5′ fragment of the pFcεR1 cDNA clone (54) has been subcloned in pGEM-3Z (see Materials and Methods). With this construct as a template, in vitro T7 RNA polymerase transcription permits us to obtain a 350-bp RNA fragment that can be used as a probe to detect FcεRII mRNA species. The 350-bp RNA probe protects either a 350-bp fragment or a 250-bp fragment plus a small (96-bp) fragment in the case of the FcεRIIa species (Fig. 5). A 142-bp fragment is protected in FcεRIIb species.

The FcεRIIa and FcεRIIb species were both detected in BL41/B95 and IARC 171 lines, and in equimolar quantities (Fig. 5). A small quantity of the FcεRIIa species was detected in BL41/P3HR1 cells and in negative or control clones. No CD23 RNA was detected in these clones by Northern blot analysis (Fig. 4b), showing that the RNase protection analysis test is more sensitive, perhaps because of greater RNA-RNA affinity than RNA-DNA affinity during hybridization. In clones expressing EBNA2, the two FcεRIIa species were detected in larger quantities than those

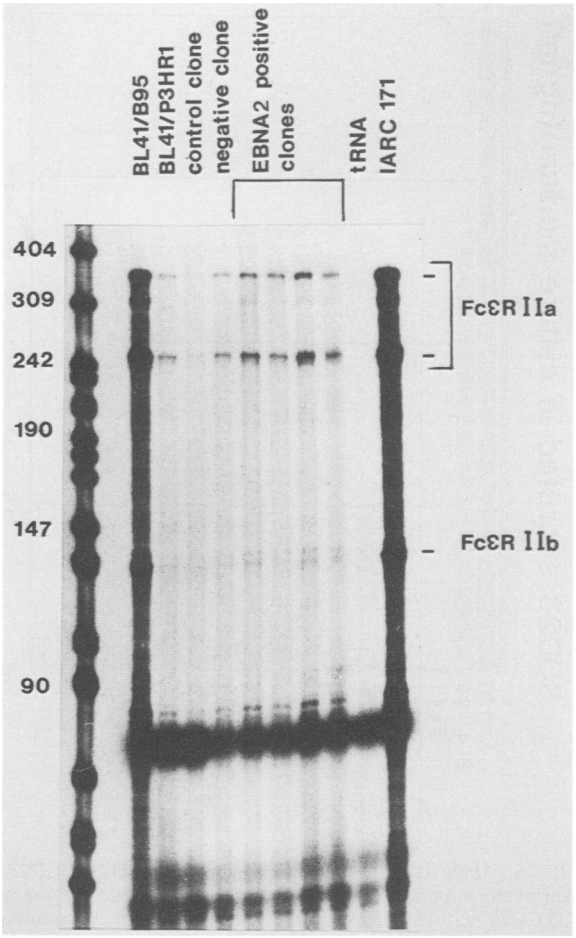

FIG. 5. RNase protection analysis of CD23 mRNAs expression. A 40-μg sample of total cellular RNA (20 μg for IARC 171 and BL41/B95 cells) was annealed to the RNA probe obtained from pG3Z/5'-CD23 (described in Materials and Methods) and corresponding to the 5' *Eco*RI-*Pvu*II fragment of pFcεR1 (54), and then digested with RNase A and RNase T_1. The size of protected bands was analyzed by polyacrylamide gel electrophoresis and autoradiography. tRNA is a negative control. IARC 171 is an LCL. BL41/P3HR1 and BL41/B95 correspond to the BL41 line converted, respectively, with P3HR1 and B95 viruses. The lane labeled "control clone" is clone N1-1 transfected with control plasmid p710. EBNA2-positive clones are clones expressing EBNA2: from left to right, B1-9, B1-13, B2-1, and B2-2. The lane labeled "negative clone" is clone B3-1 that was transfected with pU430/23 but that does not express EBNA2. Molecular sizes are given by pBR322 digested by *Msp*I (sizes in base pairs).

observed in the BL41/P3HR1 parental cells and in the negative or control clones. A small quantity of FcεRIIb species also appeared.

We conclude from these results that in agreement with Northern blot analysis, EBNA2 expression induces higher expression of CD23 RNA. The two FcεRIIa mRNA species seem to be preferentially induced in our EBNA2 transfected clones, compared with BL41/B95 cells in which equimolar quantities of the FcεRIIa and FcεRIIb species are expressed.

(iii) FACS analysis. Since transcriptional studies showed activated expression of CR2 and CD23 in the EBNA2-transfected clones, we analyzed the cell surface expression of these molecules by immunofluorescence staining and

TABLE 1. FACS analysis of CR2 and CD23 expression after membrane immunofluorescent staining with OKB7 (anti-CR2) and MAb 25 or MAb MHM6 (anti-CD23)[a]

Cell line[b]	EBNA2 expression	CD21 (CR2) with OKB7		CD23				Negative MAb control	
				MAb 25		MAb MHM6			
		%	MFI	%	MFI	%	MFI	%	MFI
BL41/B95	+	95.2	71	132.4	81	123.60	65	1.6	64
BL41/P3HR1	−	1.0	4	1.5	3	6.1	6	1.2	4
BL02/B95	+	87.0	10	6.1	5	5.0	8	1.5	5
IARC 167	+	28.1	15	98.0	188	98.2	314	7.5	9
N1-1	−	2.4	4	0.8	4	3.6	6	1.3	4
B3-1	−	1.5	5	1.0	4	5.3	6	0.8	4
B1-L	−	0.9	5	0.6	3	1.1	4	0.5	3
B1-M	−	1.3	6	0.8	3	1.7	5	1.2	3
B1-Q	−	1.9	6	0.7	4	1.1	5	0.9	4
B1-S	−	1.3	6	0.2	5	2.6	6	0.5	4
B1-7	+	97.0	13	0.7	4			0.5	4
B1-9	+	96.9	13	0.7	4	4.5	7	1.1	4
B1-10	+	98.3	9	1.0	4			0.7	4
B1-13	+	95.0	25	2.3	4	13.5	8	0.9	4
B1-A	+		10	2.3	4	4.3	6	1.1	5
B1-B	+	61.5	11	3.0	4	4.2	6	1.7	4
B1-C	+	93.9	11	4.8	4	6.7	7	0.7	5
B1-D	+	99.4	12	4.5	4	7.8	7	1.0	4
B1-E	+	98.6	11	2.1	5	6.1	6	0.8	4
B1-F	+	99.6	12	2.9	4	5.2	5	0.9	3
B1-G	+	98.8	10	2.1	5	5.4	6	0.2	4
B1-H	+	99.2	11	2.1	5	3.9	7	0.5	4
B1-I	+	98.8	11	4.8	5	4.4	7	0.4	3
B1-J	+	99.3	11	3.0	5	2.5	6	0.9	3
B1-K	+	99.6	12	1.3	4	3.7	6	0.9	3
B1-N	+	99.2	9	2.6	3	2.8	5	0.4	3
B1-O	+	93.3	11	0.8	3	3.4	5	0.6	3
B1-P	+		10	4.5	4	3.7	5	0.5	4
B1-R	+	98.7	13	4.5	5	4.1	7	1.1	4
B2-1	+	6.6	12	2.8	5	4.0		0.7	4
B2-2	+	33.8	14	8.8	6	58.9		2.4	5
B2-3	+	97.2	12	1.7	4			0.6	4
B2-4	+	99.7	11	2.4	3			0.8	4

[a] The percentage of fluorescent cells is given for the same time as the mean fluorescence intensity (MFI) in the linear schedule.

[b] IARC 167 is an LCL line. BL41/P3HR1 and BL41/B95 correspond to the BL41 line converted with P3HR1 and B95 viruses, respectively. BL2/B95 corresponds to the BL2 line converted with B95 virus. N1-1 is a control clone transfected with the control plasmid p710. B3-1, B1-L, B1-M, B1-Q, and B1-S are negative clones transfected with pU430/23 but that do not express EBNA2 protein. B1-7 through B1-13, B1-A through B1-R, and B2-1 through B2-4 are transfected clones that express EBNA2 protein.

FACS analyses. The increased amount of CR2 RNA was correlated with an increased cell surface expression of the CR2 molecule (Table 1). There was a clear difference in CR2 cell surface expression between the five negative clones tested that did not express EBNA2 (B3-1, B1-L, B1-M, B1-Q, and B1-S) or the control clone (N1-1) and the EBNA2-expressing clones. The negative and control clones did not express CR2 or expressed it at a very low level (percentage and mean fluorescence intensity comparable to those of BL41/P3HR1 parental cells). In contrast, in the EBNA2-expressing clones, all the cells were induced to express CR2 to a level approximately two to three times higher than that of the parental BL41/P3HR1 cells but to a lower level than that of BL41/B95 cells (mean fluorescence intensity was seven times lower). These results are in good agreement with those observed in Northern blot (see previous paragraph). Concerning CD23, no significant difference (Table 1) was detectable in the cell surface expression of the molecule

between BL41/P3HR1 cells and the clones expressing EBNA2, even though the CD23 RNA level was increased in the latter. This has been verified by using two different anti-CD23 MAbs, MAb25 and MHM6.

We also analyzed the cell surface expression of the histocompatibility leukocyte-associated antigens of class I (W6/32) and class II (DR, DQ), which did not vary between BL/P3HR1 cells and BL/B95 cells, as well as the expression of the lymphocyte adhesion molecules LFA1, LFA3, and ICAM1, which was differently modulated in BL/P3HR1 and BL/B95 cells (M. Billaud and A. Calender, unpublished results). We found that the expression of these molecules was not significantly modified in the transfected clones compared with the BL41/P3HR1 parental cells (data not shown).

(iv) Detection of soluble CD23 (SCD23) in the culture supernatants. Since CD23 was not expressed at the cell surface of the EBNA2-transfected clones but CD23 RNA was detected, we tested the presence of the soluble form of the molecule in the supernatant of the transfected cell clones. SCD23 was detected in large quantities in the supernatant of BL41/B95 and IARC 171 cells in quantities correlated to the level of cell surface expression of the molecule (Fig. 6). SCD23 was also detected in the supernatant of the clones expressing EBNA2 but not in that of BL41/P3HR1 parental cells or the transfected clones that did not express EBNA2. The quantity of SCD23 found in the supernatant of the clones expressing EBNA2 was approximately 7 to 10 times smaller than in that of BL41/B95 cells. The limit of detection of the test was about 0.02 ng/ml, and values above this limit were considered positive.

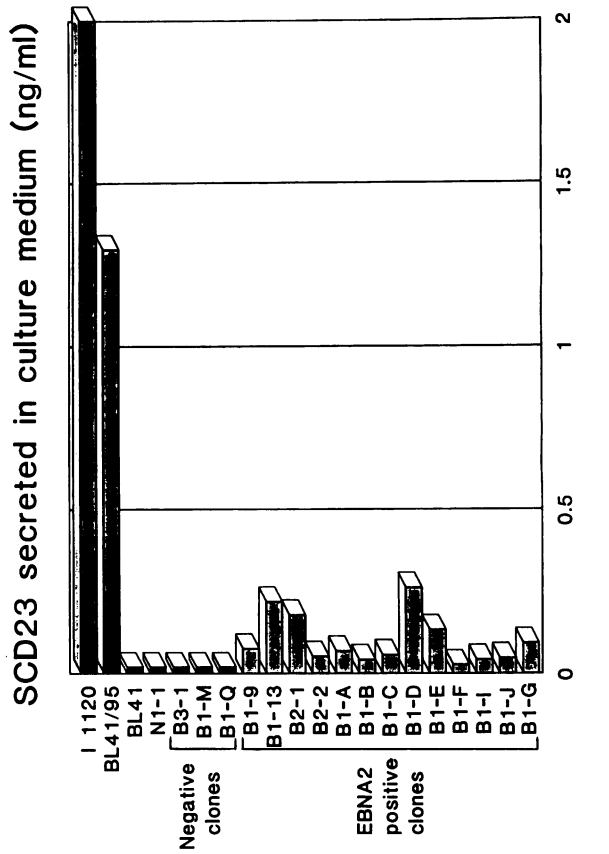

FIG. 6. Analysis of the quantity of soluble CD23 (SCD23) secreted in the supernatant of the transfected clones. The assay for SCD23 was performed by a sandwich enzyme-linked immunosorbent assay with MAbs 25 and H107. IARC 1120 is an LCL; BL41/P3HR1 and BL41/B95 corresponded to the BL41 EBV-negative line converted, respectively, with P3HR1 and B95 viruses. EBNA2-positive clones are clones expressing EBNA2. Negative clones are clones that were transfected with pU430/23 but that do not express EBNA2. The control clone is clone N1-1 transfected with control plasmid p710. Quantities are given in nanograms per milliliter; 0.02 ng/ml is the lowest level that can be detected by the test.

DISCUSSION

The stable transfection of the EBNA2 gene in B lymphoma cells has allowed us to demonstrate that the EBNA2 gene plays a crucial role in the activation of the expression of molecules such as CR2 and CD23 involved in B-cell proliferation. Our approach has been to use the in vitro system developed in our laboratory (4), consisting of BL lines converted with different EBV strains (BL/EBV lines), to study the effect of the expression of an exogenous EBNA2 gene in BL/P3HR1 cells which express neither EBNA2 nor the CR2 and CD23 molecules, and to compare this with BL/B95 cells which express EBNA2, CR2, and CD23.

Since classic chemical transfection protocols appeared inefficient in obtaining stable expression of EBV genes in BL cells, we developed a transfection system by using electric field-mediated DNA transfer of an episomal plasmid vector that does not require cellular genome integration for expression. This plasmid can be present in several copies and so leads to higher expression of the transfected gene. We have transfected such a plasmid, pU430/23 carrying the EBNA2 gene, into BL41/P3HR1 cells and obtained BL41/P3HR1 cell clones that stably express the EBNA2 protein. Immunoblotting analyses showed that the EBNA2 protein expressed in these transfected clones was similar in size and comparable in its level of expression to that in BL41/B95 and Raji cells. We were therefore able to study the effect of EBNA2 protein expression on the phenotype of BL41/P3HR1 cells and to compare it with the effect on BL41/B95. The expression of EBNA2 gene in BL41/P3HR1 cells is associated with an increased amount of the steady-state RNAs coding for CR2 and CD23, with greater cell surface expression of CR2 but not of CD23 and with the release of SCD23. We can ensure that the activation of CR2 and CD23 observed in these transfected clones is not due to the selection of clones already expressing CD23 and CR2. Activation is indeed observed in all EBNA2-expressing clones coming from two independent transfection, selection, and single-cell cloning experiments and in which pU430/23 is not rearranged. Moreover, control clones such as N1-1 transfected with the control plasmid without EBNA2 (p710) or negative clones like B3-1, B3-L, B3-M, B3-Q, and B3-S transfected with the plasmid pU430/23 but rearranged, do not showed activation of CR2 and CD23. On the other hand, since part of EBNA-LP open reading frame is present in the transfected plasmid but EBNA-LP expression does not differ between BL41/P3HR1 cells and the EBNA2-transfected clones (except for clone B1-13), the activation of CR2 and CD23 observed is very likely to be attributed to EBNA2 and not to the EBNA-LP protein. Our data thus demonstrate that the EBNA2 gene is necessary to complement the P3HR1 virus in BL41 cells to induce activation of CR2 and CD23 observed in BL41/B95 cells.

It is interesting that in our clones, EBNA2 induces CR2 and CD23 activation but always to a lower level than that

observed in BL41/B95 cells: the mean fluorescence intensity of CR2-positive cells in the EBNA2-transfected clones was nearly seven times lower than that in BL41/B95 cells, except for clone B1-13, in which it was only three times lower. In the same way, CD23 was not expressed at the membrane, but its soluble form, SCD23, released by the EBNA2-transfected clones was nearly 7 to 10 times less abundant than in the supernatant of BL41/B95 cells. A clear difference was also observed in Northern blot analysis between the intensity of CR2 and CD23 transcripts detected in BL41/95 cells and those detected in EBNA2-transfected cells. Since the level of EBNA2 expression is comparable in BL41/95 and EBNA2-transfected cells, we suggest that EBV latent proteins other than EBNA2 have an important role in the regulation of the level of expression of CD23 and CR2, either cooperating with EBNA2 to magnify CR2 and CD23 expression or regulating EBNA2 expression. The role of EBNA-LP and LMP proteins must therefore be investigated. In clone B1-13, modified EBNA-LP expression is associated with higher CR2 expression compared with the other clones. LMP is expressed in BL41/B95 cells, but not in the EBNA2-transfected clones, in which CR2 and CD23 expression is lower than in BL41/B95 cells. It is possible that EBNA-LP and LMP cooperate with EBNA2 to obtain higher CR2 and CD23 activation. In conclusion, the activation of CR2 and CD23 observed may occur via the direct modulation of cellular functions or via the modulation of different viral functions. Transfection of EBNA2 gene in BL/EBV neg. cells will permit us to elucidate whether P3HR1 functions are required to induce CR2 and CD23 activation in our transfected clones. Wang et al. (51) found that the expression of CD23 was increased in a BL/EBV neg. line (LOUCKES) expressing the EBNA2 protein following infection with a retrovirus vector carrying the EBNA2 gene. This has suggested that EBNA2 could be a transactivator of CD23, acting independently of other viral functions. We report here for the first time an EBNA2-mediated activation of CR2. This suggests that CR2 is a pivotal molecule responsible for EBV-mediated B-cell activation and that its expression is specifically related to EBNA2 expression.

EBV receptor-CR2 protein (CD21) and CD23 (Blast2/FcεRII) are molecules implicated at different stages of normal B cell activation and proliferation. CD23 is synthesized as a 45-kDa precursor and is expressed at the surface of B cells early after activation (46), before being shed into the culture medium in a 25- to 33-kDa soluble form (SCD23) (3, 43). It has been shown to be the low-affinity receptor for the Fc fragment of immunoglobulin E (2, 55). CD23 is involved in triggering the progression of activated B lymphocytes through the G_1 phase of the cell cycle (13). It has been suggested that CD23 may have a dual role in B-cell proliferation: the surface molecule might act as a growth factor receptor, and the processed molecule itself acts as a growth factor. Activation of CD23 expression could be implicated in the establishment of an autocrine loop of growth factors. CR2 is characterized as a 140-kDa glycoprotein which is the receptor on B lymphocytes for the C3d fraction of complement but also for EBV (9, 10). CR2 has been shown to transmit activation signals. Cross-linked C3d induces preactivated mouse B cells to enter the S phase (22, 24). Antibodies to the receptor or EBV-inactivated particles, together with B-cell growth factors, induced [^{3}H]deoxyribosylthymine incorporation in human B cells (11, 19, 28). The increased expression of CR2 may have several consequences for B-cell growth, especially more important fixation of viral particles or of B-cell activation factors (such as C3d) that will result in an amplified lymphocyte proliferation.

As in the case of BL41/B95 cells, the increased amount of CR2 RNA in the EBNA2-transfected clones was correlated with an increased cell surface expression of the CR2 molecule. This was not the case for CD23, which was not detected at the cell surface of the transfected clones, even though these clones had larger amounts of CD23 RNA. Nevertheless, SCD23 was found in the supernatant of transfected clones. This confirms the activation of CD23 in the EBNA2-transfected clones. A possible explanation is that CD23 is released directly into the medium, either because of a special conformation or because of the lack of expression of specific cell surface proteins. The expression of CD23 with no surface form, but only a soluble form, has also been observed in nasopharyngeal carcinoma epithelial cells (1) and may represent another functional behavior of the molecule. It has been recently described for the complement receptor CR1 (18) and for the T-cell antigen CD8 (29) that different splicings may generate different mRNAs from the same gene, which might encode the cell surface receptor or the secreted form of the molecule. It is possible that the same mechanism occurs in CD23 and that only the mRNA corresponding to SCD23 is generated in the EBNA2-transfected clones. Three species of CD23 mRNA have been described (54): the two FcεRIIa/CD23 species are specifically expressed in B cells at the same time as the third FcεRIIb/CD23 species. The FcεRIIb/CD23 species is also expressed by non-B cells. Interestingly, in our EBNA2-transfected clones, the FcεRIIa species are preferentially transcribed and the FcεRIIb/CD23 species is almost undetected. This suggests that EBNA2 preferentially turns on FcεRIIa species, but also that FcεRIIa species could correspond to the soluble form of the molecule or, referring to what happened in BL41/B95 and IARC 171 lines, the three FcεRIIa and FcεRIIb/CD23 species might have to be expressed in equimolar quantities to obtain stabilization of the CD23 molecule at the cell surface. Otherwise, the molecule is directly cleaved and secreted in the culture medium. Certainly, it seems that the physiological regulation of the expression of CD23 molecule is very complex.

Stable expression of the EBNA2 gene in BL41/P3HR1 cells provides a valuable model for studying the induced modulation not only of the expression of cellular genes, but also of other viral genes, including LMP. Indeed, BL41/B95 cells have been shown to express LMP (27), whereas BL41/P3HR1 cells do not, suggesting that the expression of the two viral proteins may be linked. Phenotypic studies on freshly established BL-negative lines have demonstrated that the expression of LMP is often associated with the expression of EBNA2 (38) in these cells. Surprisingly, our BL41/P3HR1 clones transfected with EBNA2 do not express LMP. We have not detected the protein by immunoblotting, and we were also unable to detect any corresponding transcript. This may suggest that the expression of these two proteins is not strictly coregulated, as previously described for BL29 and BL60 EBV-positive cell lines (36). It is also possible that the expression of LMP is induced via the modulation of cellular or viral proteins, which is not reproduced in the transfected clones. Since the EBNA-LP protein is largely expressed in clone B1-13 as the same time as EBNA2, it seems that the expression of this protein is not sufficient to induce the expression of LMP in our BL41/P3HR1 cells transfected with EBNA2. It is also possible that EBNA2 is unable to modulate the expression of LMP in P3HR1 virus. A definitive conclusion will require further

investigation. It has been found recently (50) that the expression of LMP in BL/EBV neg. cells is associated with the activation of the expression of the adhesion molecules LFA1, LFA3, and ICAM1 and of the activation antigen CD23. The fact that LMP is not expressed in our EBNA2-transfected clones may explain the lack of expression of LFA3 in these clones, although expression is observed in BL41/B95 cells (Calender and Billaud, unpublished).

In conclusion, the transfection of EBNA2 gene in BL41/P3HR1 cells further supports the hypothesis that EBNA2 protein plays a crucial role in the activation of the expression of CR2 and CD23 molecules. With regard to the importance of these molecules in B-cell activation and proliferation pathways, our study emphasizes the role of EBNA2 in EBV-induced B cell immortalization. The ability to obtain isolated and stable expression of EBV genes in B cells thus provides a valuable opportunity for elucidating the role of various EBV functions in B-cell infection.

ACKNOWLEDGMENTS

We thank M. Pawlita for valuable advice concerning the transfection experiments; M. Vuillaume, M. F. Lavoué, and S. Pauly for their assistance; I. Durand and J. P. Aubry for FACS manipulations; and B. S. Sylla for valuable suggestions. We thank A. Rickinson for the gift of CS1-4 and MHM6 MAbs, J. Yodoi for the H107 mAb, J. Y. Bonnefoy for mAb25, T. Kishimoto for CD23 pFcεRI probe, D. Fearon for CR2 p1.6 probe, M. Piechaczyk for pRGAPDH-13 probe, and A. Sergeant for pLMP probe. We thank A. Trochard for preparation of the manuscript, J. Cheney and S. Narod for editing, and G. Mollon for the photographs.

M. Cordier was supported by fellowships from the Ligue Nationale Française contre le Cancer and by the Association de Recherche contre le Cancer. A. Calender was supported by a special training fellowship from the International Agency for Research on Cancer. M. Billaud was supported by the Fondation Mérieux.

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