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An Epstein–Barr virus (EBV)-determined nuclear antigen (EBNA5) partly encoded by the transformation-associated *Bam* WYH region of EBV DNA: Preferential expression in lymphoblastoid cell lines

(viral transformation/synthetic peptide)

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ABSTRACT Four peptides were synthesized on the basis of amino acid sequences deduced from a highly spliced transcript encoded by the *Bam* W, Y, and H fragments of the Epstein–Barr virus (EBV) genome [Bodescot, M., Chambraud, J. B., Farrell, P. J. & Perricaudet, M. (1984) *EMBO J.* 3, 1913–1917]. Rabbit antisera against three of the four peptides identified a nuclear polypeptide that varied between 22 and 70 kDa in molecular size. Four of 20 EBV-positive human sera contained antibodies against this polypeptide. Since this is the fifth EBV-determined nuclear antigen (EBNA) discovered in growth-transformed cells, it is designated EBNA5. The antigen was detected in virus nonproducer lines (<0.01% EBV early antigen expression) and is thus not dependent on the viral cycle. It was differentially expressed depending on the origin of the lines. All 10 lymphoblastoid cell lines tested expressed EBNA5, but it could not be detected in 10 of 11 EBV-carrying Burkitt lymphoma lines. Infection of tonsillar lymphocytes with the B95-8 strain of EBV induced six EBNA5-specific polypeptides that varied between 41 and 70 kDa in molecular size with regular increments of 6 kDa. This may be due to the fact that the EBNA5 coding sequence includes the *Bam* W internal repeat. Parallel infection of the EBV-negative Burkitt lymphoma line Ramos with the same viral substrain did not induce detectable levels of EBNA5, nor was this antigen present in permanently EBV-converted Ramos sublines. These findings imply that the expression of the viral genome varies among B cells having different phenotypes.

Epstein–Barr virus (EBV) can convert normal B cells into permanently growing lymphoblastoid cell lines (LCLs) (1). The EBV-determined nuclear antigen (EBNA) can be detected by anticomplement immunofluorescence in all transformed cells (2). EBNA appears at an early stage of primary B-cell infection, prior to the onset of cellular DNA replication (3). Recent studies have shown that EBNA consists of a family of EBV-determined nuclear antigens. Four EBNAs have been identified previously: EBNA1 (4), EBNA2 (4–7), EBNA3 (8–10), and EBNA4 (10). EBNA1 is encoded by the *Bam* HIK fragment (11) and EBNA2, by the *Bam* Y and H fragments (12, 13). The sequences coding for EBNA3 and EBNA4 have not been identified. All four EBNAs bind to DNA *in vitro* (10). EBNA1 has high-affinity binding sites for DNA sequences in the EBV origin of plasmid maintenance (14).

The *Bam* WYH region is deleted in the nontransforming P3HR-1 strain of EBV (15, 16). Superinfection of EBV-carrying, virus nonproducer cells with P3HR-1 virus induces the release of recombinant viruses with regained transforming ability (17, 18). All such recombinants have been shown to have acquired the *Bam* WYH region (19, 20). It has been suggested that the apparently essential role of the *Bam* WYH region in transformation is due to the expression of EBNA2 (6, 12).

A cDNA clone has been isolated from the EBV-carrying, virus nonproducer Burkitt lymphoma line Raji (21). The transcript contains two exons from each internal repeat (*Bam* W), three exons from the *Bam* Y fragment, and one exon from the *Bam* H fragment. A 784-base-pair open reading frame extends through the *Bam* W exons and ends in the second exon of the *Bam* Y fragment. The transcript shares its 3′ part with the EBNA2 transcript and both transcripts may end at the same poly(A) site. However, the predicted protein has no homology to the EBNA2 amino acid sequence.

The purpose of this study was to investigate whether a protein encoded by the transcript corresponding to the cDNA clone isolated by Bodescot *et al.* (21) is expressed in EBV-transformed cells. This was approached by synthesizing peptides on the basis of the deduced amino acid sequences. The corresponding peptide-specific rabbit antibodies were used to identify a previously unknown EBV-encoded nuclear antigen, expressed in EBV-transformed LCLs but not in EBV-carrying Burkitt lymphoma lines.

MATERIALS AND METHODS

Methods. Subcellular fractionation and immunoblotting were performed as described (13). Molecular weight markers (Bio-Rad) were visualized by staining with ponceau S (Sigma). Peptide synthesis, ELISA, and affinity purification of peptide-specific antibodies were performed as described (22). Purification of tonsillar B-cells and EBV infection were performed as described (10, 23).

Cell Lines. Ramos-HR1K, BJA/B95-8, and E95-D-Ramos are sublines of the originally EBV-negative BJAB and Ramos lines converted to EBV positivity by P3HR-1 or B95-8 virus, respectively. Fleb 14-14, Fleb 14-16, and Fleb 14Δ3tet are clones of precursor B cells transformed by EBV prior to the immunoglobulin gene rearrangement (24). FEBM 15 is a fetal bone marrow-derived line transformed by B95-8 virus; it has rearranged IgH genes but makes no immunoglobulins. PJ is a LCL that grew out spontaneously from a tonsil of a mononucleosis patient. CBC-SEB-M106 and CBC-SEB-M108 are cord blood lymphocytes that have been transformed with the saliva of different mononucleosis patients. CBC-E95-C139 and CB-M3-JIJ-STO are LCLs transformed

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Abbreviations: EBV, Epstein–Barr virus; LCL, lymphoblastoid cell line; EBNA, EBV-determined nuclear antigen.
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by B95-8 or Jijoye viruses, respectively (I.E., unpublished work).

RESULTS

Subcellular Fractionation. Washed, latently infected (0.05% EBV early antigen at the time of harvest) IB4 cells were disrupted in nuclei isolation buffer (13). The nuclei were sequentially extracted with the same buffer containing increasing amounts of NaCl (0.15 M, 0.4 M, and 2 M). After the wash with 2 M NaCl, the nuclear residual pellet was dissolved in electrophoresis sample buffer (25). Affinity-purified antipeptide 186 antibodies (Fig. 1) detected a 46-kDa polypeptide in the nuclear residual pellet (Fig. 2). A faint 46-kDa polypeptide could also be detected in whole nuclei on overexposure (data not shown). The nuclear residual pellet was used as the target antigen in all subsequent immunoblotting experiments.

Reactivity of Antipeptide Antibodies. The affinity-purified antipeptide 186, 188, and 189 antibodies were allowed to react with immunoblots of nuclear residues prepared from a panel of EBV-carrying lymphoid lines. All three antisera were reactive with a 46-kDa polypeptide in IB4 cells, not present in EBV-negative BJAB cells. Both antipeptide 186 and antipeptide 188 antibodies reacted with a 52-kDa antigen in FEBM 15 cells and with 58- and 64-kDa polypeptides in PJ cells (Fig. 3). Antipeptide 188 and antipeptide 189 antibodies also reacted with non-EBV-related proteins present in BJAB cells (Fig. 3 *B* and *C*). The antipeptide 187 serum was tested only in its unpurified form and was nonreactive.

EBNA5 Expression in EBV-Carrying Cell Lines of Different Origins. The most-reactive antiserum (antipeptide 186) was selected to assay EBNA5 expression in a panel of 14 Burkitt lymphoma-derived lines and 10 LCLs. All 10 LCLs contained EBNA5 proteins of different molecular sizes and several expressed multiple EBNA5 polypeptides (Figs. 3*A* and 4). Thirteen of 14 Burkitt lymphoma-derived lines contained no detectable EBNA5 protein (Fig. 3*A* and Table 1). Namalwa cells gave a smeared band of 75–95 kDa (Fig. 3*a*).

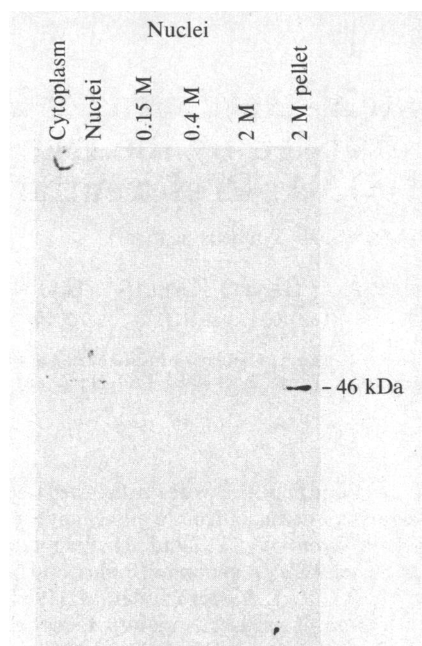

FIG. 2. Subcellular localization of the EBNA5 polypeptide. An immunoblot was allowed to react with affinity-purified synthetic peptide 186 antibodies, diluted 1:2. IB4 cells were fractionated into cytoplasm and nuclei. Nuclei were sequentially extracted with different salt concentrations as indicated. Extracts corresponding to 5×10^6 cells were applied to each lane of a 10% polyacrylamide gel.

Differential Expression of EBNA5 After Primary Infection of Tonsillar B Cells Compared to EBV-Negative Burkitt Lymphoma Cells. Normal tonsillar B cells and the EBV-

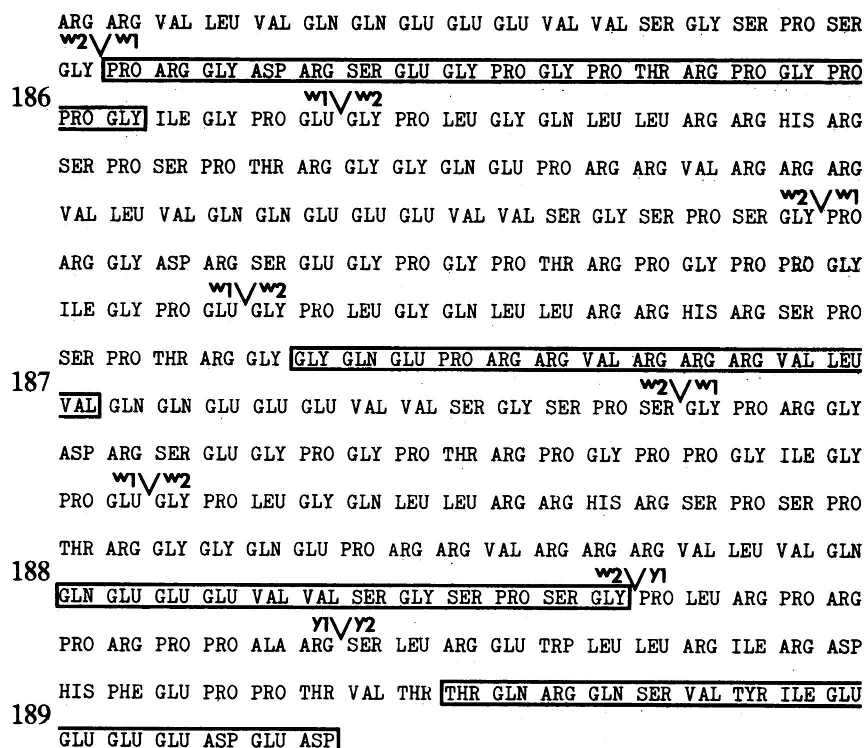

FIG. 1. Predicted carboxyl-terminal amino acid sequence of EBNA5, deduced from the cDNA clone of Bodescot *et al.* (21). The boxed amino acid sequences correspond to the numbered peptide sequences. ∨, Position of a splice junction; Y_1, Y_2 and W_1, W_2, first and second exons in the *Bam* Y and *Bam* W fragments, respectively.

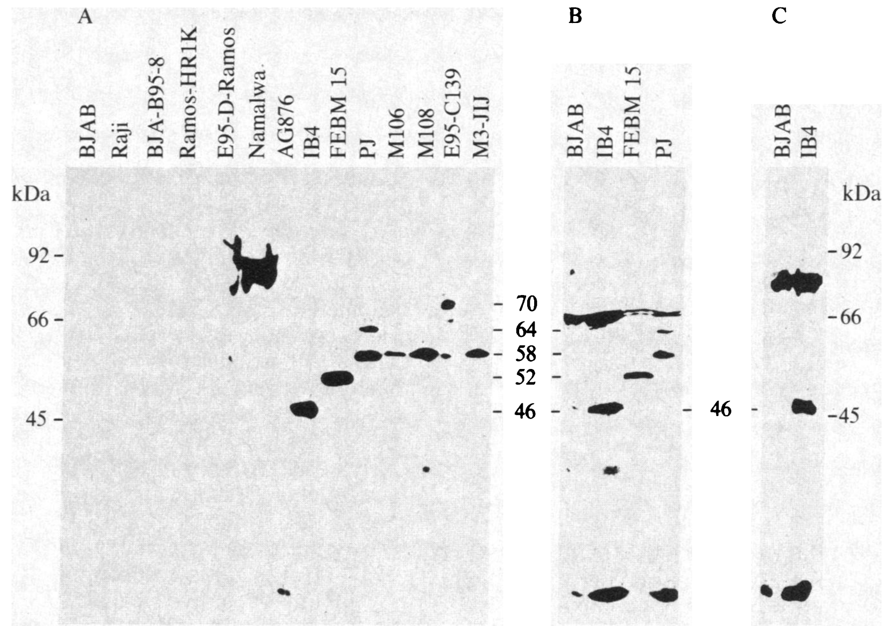

FIG. 3. Identification of EBNA5 in Burkitt lymphomas and LCLs by three different antipeptide sera. Immunoblots of a 10% polyacrylamide gel were stained with affinity-purified antipeptide 186 antibodies (*A*), affinity-purified antipeptide 188 antibodies (*B*), or affinity-purified antipeptide 189 antibodies (*C*). A nuclear residue corresponding to 2×10^7 cells was applied to each lane. Three types of cell line were used: EBV-negative Burkitt lymphoma—BJAB; EBV-carrying Burkitt lymphoma—Raji, BJA/B95-8, Ramos-HR1K, E95-D-Ramos, Namalwa, and AG 876; LCL—IB4, FEBM 15, PJ, CBC-SEB-M106 (M106), CBC-SEB-M108 (M108), CBC-E95-C139 (E95-C139), and CB-M3-JIJ-STO (M3-JIJ). The data are summarized in Table 1

negative Burkitt lymphoma line Ramos were infected with B95-8 virus. Cells were harvested at 24-hr intervals and analyzed by immunoblotting with the antipeptide 186 serum (Figs. 4 and 5). Six polypeptides were induced in the tonsillar B cells, beginning to appear 1 day after infection. They became more abundant on the 2nd day and remained stable thereafter. The second-smallest species comigrated with the 46-kDa EBNA5 polypeptide in IB4 cells. The different

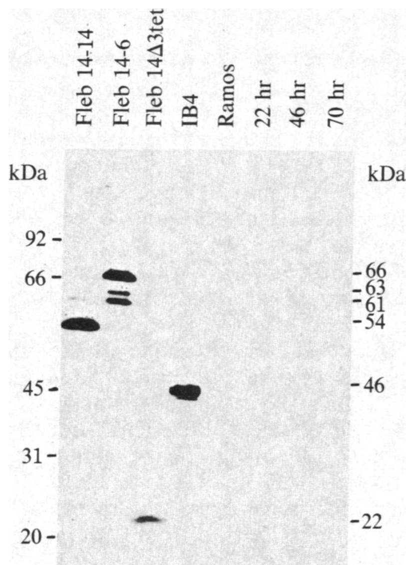

FIG. 4. Infection of the EBV-negative Burkitt lymphoma line Ramos with B95-8 virus. An immunoblot of a 10% polyacrylamide gel was stained with a 1:2 dilution of affinity-purified antipeptide 186 antibodies. Ramos cells were harvested 22, 46, and 70 hr after EBV infection, and an extract corresponding to 1×10^6 cells was applied to each lane. For LCLs IB4, Fleb 14-14, Fleb 14-6, and Fleb 14Δ3tet, extracts corresponding to 2×10^7 cells were applied to each lane.

species increased regularly in size, by 6-kDa intervals, with 70 kDa as the largest (Fig. 5). On a parallel blot stained with

Table 1. Expression of EBNA5 in Burkitt lymphoma-derived lines and in LCLs

	EBV carrier state	EBNA5 expression
Burkitt lymphoma-derived lines		
Seraphine	Positive	No
Raji	Positive	No
Ramos/HRIk	Positive	No
E95-D-Ramos	Positive	No
Namalwa	Positive	75–95 kDa?
Ag876	Positive	No
BJA/B95-8	Positive	No
Jijoye nude	Positive	No
Jijoye M13	Positive	No
Rael	Positive	No
P3HR-1	Positive	No
BJAB	Negative	No
Ramos	Negative	No
Loukes	Negative	No
LCLs		
Fleb 14-14	Positive	*54 kDa*, 61 kDa
Fleb 14-6	Positive	*66 kDa*, 61 and 63 kDa
Fleb 14Δ3tet	Positive	22 kDa
IB4	Positive	46 kDa
FEBM 15	Positive	52 kDa
PJ	Positive	*58 kDa*, 64 kDa
CBC-SEB-M106	Positive	58 kDa
CBC-SEB-M108	Positive	58 kDa
CBC-E95-C139	Positive	*70 kDa*, 58 kDa
CB-M3-JIJ-STO	Positive	58 kDa
Marmoset LCL-like line		
B95-8	Positive	No

Figures in italics denote the major species.

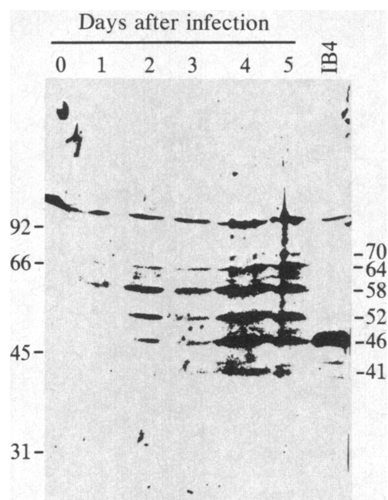

FIG. 5. Primary infection of tonsillar B cells with B95-8 virus, harvested at different times after EBV infection. An immunoblot of a 10% polyacrylamide gel was stained with affinity-purified antipeptide 186 antibodies. Extracts corresponding to 5×10^5 cells were applied to each lane. For the EBNA5-positive LCL IB4, an extract corresponding to 2×10^7 cells was applied to the lane.

an EBNA-positive human serum, EBNA2 was detected 1 day after infection, EBNA1 and EBNA3, after 2 days, and EBNA4, after 3 days (10). On the 3rd day after EBV infection, 10% EBNA (anticomplement immunofluorescence)-positive cells were present in the Ramos line, as expected. However, no new polypeptides could be detected with the antipeptide 186 antibodies. Four EBNA5-positive LCLs were included in the same blot as positive controls (Fig. 4).

Reactivity of Human EBV Antibody-Positive Sera with EBNA5 and with Corresponding Synthetic Peptides. Twenty human sera from EBV-positive healthy donors or Burkitt lymphoma patients were tested for reactivity with EBNA5 on immunoblot strips containing nuclear residual material from EBNA5-positive IB4 and EBNA5-negative Raji cells. Four EBNA5-positive sera were found (data not shown). Thirty-two EBV antibody-positive sera were tested for reactivity with synthetic peptides 186, 188, and 189 in a direct-binding ELISA. None of them reacted with peptides 188 and 189. Four sera reacted with peptide 186, at a 1:80 titer (data not shown). The K. F. serum reacted both with peptide 186 in ELISA and with EBNA5 on immunoblots.

DISCUSSION

Using three different antipeptide sera directed against amino acid sequences deduced from the nucleotide sequence of a cDNA clone transcribed from the *Bam* W, Y, and H fragments of EBV (21), we have shown that the virus encodes a previously unknown 22- to 70-kDa nuclear protein, designated EBNA5. Subcellular fractionation of IB4 cells localized EBNA5 to the nucleus. The protein was not solubilized by NaCl concentrations up to 2 M. In contrast, the four previously known EBNA proteins are largely solubilized by 0.4 M NaCl and almost completely extractable by 2 M NaCl (10). The tight association of EBNA5 to the insoluble fraction of nuclei is reminiscent of nuclear matrix proteins.

EBNA5 is associated with growth-transformed cells. Its expression is not dependent on induction of the lytic cycle, since it was present in virus nonproducer lines, such as IB4 and Fleb 14-6 (0.05% and <0.01% EBV early antigen-positive cells, respectively). In contrast, some EBV producer lines, such as B95-8, were negative.

A cDNA clone has been isolated that corresponds to the EBNA1-encoding message (25). It contains seven exons separated by almost 70 kilobases. The first exons of the EBNA1 message map to the *Bam* W and Y fragments and are identical with two of the exons in the cDNA clone of Bodescot *et al.* (21). The sequences that encode the carboxyl terminus and the repetitive element of EBNA5 are also present in EBNA1 mRNA. It is thus possible that EBNA5 may be encoded by the same mRNA as EBNA1. However, all EBV-positive lines express EBNA1, irrespective of their EBNA5 expression. Raji cells contain unusually large amounts of EBNA1 (26, 27) but are EBNA5 negative. Since the expression levels of EBNA1 and EBNA5 do not correlate, possible translation from the same mRNA would require host-cell-dependent translational regulation. It is noteworthy, in this context, that the cDNA clone of Bodescot *et al.* was isolated from Raji cells, which we find EBNA5 negative. Neither the cDNA clone of Bodescot *et al.* nor the EBNA1 cDNA clone include the 5′ end of the message and the N-terminal structure of EBNA5 is therefore unknown. As shown in Fig. 1, the carboxyl-terminal amino acid sequence of EBNA5 features a 66-amino acid proline, arginine, and glycine-rich repetitive element. A high content of proline and basic amino acids is in line with the finding that EBNA5 is a nuclear antigen. The EBNA5 carboxyl terminus contains seven consecutive aspartate and glutamate residues. Interestingly, EBNA1 and EBNA2 also have highly charged, acidic carboxyl termini (28).

The size variation of EBNA5 is based on a regular increment of 6 kDa, in agreement with the prediction that the polypeptide has repeated 66-amino acid domains, corresponding to each internal repeat (*Bam* W) (21). It is known that B95-8 virus is heterogeneous with respect to the number of internal repeats contained in the viral particles (29). EBV infection was found to follow "one-hit" kinetics (30). Ordinary primary infection with virus-rich B95-8 supernatants may be expected to hit many B cells. This may account for the fact that six different EBNA5 species were detected after primary infection, whereas clonal cell lines contain only one or a few EBNA5 polypeptides. Several of the LCLs analyzed were established by infection with B95-8 virus: namely, IB4 (46-kDa EBNA5), FEBM 15 (52-kDa EBNA5), and CBC-E95-C139 (70- and 58-kDa EBNA5). It is noteworthy that species of 46, 52, 58, and 70 kDa were induced by B95-8 virus during primary infection.

Only 4 of 20 EBV-antibody-positive human sera contained antibodies to EBNA5. Two synthetic peptides were not recognized by human sera at all and one peptide reacted with only 4 of 32 EBV-antibody-positive sera. This is in contrast to the normal reactivity of EBV-antibody-positive sera with synthetic peptides deduced from the EBNA1 and EBNA2 amino acid sequences (13, 22, 31) and indicates a poor humoral immune response to EBNA5.

The level of EBNA5 expression was very variable, even among cell lines that carry the same EBV strain. The B95-8 line was EBNA5 negative, but primary infection of B cells with the derived B95-8 virus induced unusually high EBNA5 expression, as indicated by the small number of tonsillar B cells that gave positive signals on the immunoblots. The most abundant protein was detected in FEBM 15, a B95-8-transformed fetal bone marrow-derived line. The three "Fleb" lines (24) are derived from pre-B-cells, prior to the rearrangement of the *IgH* loci, and thus represent an even earlier stage of differentiation. Fleb 14-14 and Fleb 14-6 were strongly positive, but Fleb 14Δ3tet was only weakly positive. Thus, the level of EBNA5 expression does not vary with the stage of differentiation, as far as LCLs are concerned. In addition, the expression could not be related to the number of EBV genome copies, since Fleb 14Δ3tet contains 150 and Fleb 14-14, only 3 copies (M.U., unpublished data).

Burkitt lymphoma-derived lines were strikingly different from the LCLs, since 10 of 11 EBV-carrying Burkitt lymphoma lines did not express detectable levels of EBNA5, in contrast to the regular detectability of EBNA5 in all 10 human LCLs tested. One exceptional Burkitt lymphoma line, Namalwa, gave a smeary 75- to 95-kDa band while the marmoset LCL B95-8 was EBNA5 negative. Since the marmoset is not the natural host of EBV, the virus–host cell interaction may be different in this system. The apparent lack of EBNA5 expression in Burkitt lymphoma-derived lines cannot be attributed to genetic variation between viral substrains, since both the EBNA5-negative Burkitt lymphoma lines BJA/B95-8 and E95-D-Ramos and the EBNA5-positive LCLs IB4, FEBM 15, and CBC-E95-C139 were generated by infection with the same viral substrain (B95-8). The fact that both the originally EBV-positive Burkitt lymphoma lines and the *in vitro*-converted EBV-carrying sublines of EBV-negative Burkitt lymphoma lines are EBNA5 negative suggests that cells with a Burkitt lymphoma phenotype may down-regulate the expression of EBNA5. This is also indicated by the appearance of EBNA5 in primarily infected normal B cells but not in infected, EBNA (anticomplement immunofluorescence)-positive Burkitt lymphoma cells of the EBV-negative Ramos line. The presence of putative EBNA5-encoding messages in the Raji line (21, 25) in the absence of any detecatable EBNA5 protein suggests that Burkitt lymphoma cells down-regulate EBNA5 at a posttranscriptional level.

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