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A Subpopulation of Normal B Cells Latently Infected with Epstein-Barr Virus Resembles Burkitt Lymphoma Cells in Expressing EBNA-1 but Not EBNA-2 or LMP1

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Using reverse transcription of whole cellular RNA and nested PCR, we have performed experiments mixing different proportions of Epstein-Barr virus (EBV)-carrying and EBV-negative cells. Based on the results, a method that detects viral transcripts for EBNA-1, EBNA-2, LMP1, and LMP2a from less than one positive cell among 10^5 negative cells was developed. With this method we have shown that the EBV DNA positive cells among small, high-density peripheral blood B-lymphocytes of normal healthy persons express EBNA-1-mRNA but not EBNA-2 or LMP1. A similar EBV expression pattern is found in type I Burkitt lymphoma cells. We suggest that the expression pattern in the lymphoma cells reflects the viral strategy in normal resting B cells and meets the requirements of latent persistence.

B lymphocytes and epithelial cells are the best known host cells for Epstein-Barr virus (EBV) infection (see, e.g., reference 21). Also, rarely, T lymphocytes and T-cell-derived leukemia lines have been found infected (16, 28, 31, 42, 43). Two phenotypically distinct EBV-carrying B-cell types can be grown in vitro as continuous lines: lymphoblastoid cell lines (LCL) and type I Burkitt's lymphoma (BL) cells. They show different patterns of EBV gene expression and regulation. Type I BL cells have a similar phenotype as the tumor in vivo. On prolonged in vitro cultivation, they often drift towards a more LCL-like (type III) phenotype (13, 34, 36). Virally encoded proteins compatible with cell survival and latent infection can be expressed according to three different, host cell phenotype-dependent patterns: (i) EBNA 1 to 6 and LMP1 and -2 in LCL and LCL-like type III BL lines; (ii) EBNA-1 and LMP1 and -2 in the majority of nasopharyngeal carcinomas, EBV-carrying T-cell lymphomas, and Hodgkin's disease; and (iii) only EBNA-1 and LMP2a in type I BLs (7, 8, 14, 15, 20, 23, 35). These are designated latencies I to III (35).

EBNA-1 transcripts can be generated either alone or together with EBNA-2 to -6, according to two different viral programs, activated or silenced, depending on the cell phenotype. These programs can be referred to, depending on promoter usage and designation, as the WpCp program and the Fp program (now Qp program [see below]) (1, 3, 24, 37, 39, 44). The WpCp program has been identified only for immunoblasts and leads to full expression of all six EBNAs. EBV-carrying cells of all other phenotypes were suggested to express EBNA-1 from a promoter designated Fp but not EBNA-2 to -6. Their expression of LMP1 was variable. Cells in this category include type I BLs, nasopharyngeal carcinomas, and somatic hybrids with non-B phenotypes (5, 6, 17, 20, 40). Recently, it was reported at a meeting that these EBNA-1-coding transcripts are rather initiated downstream of the Fp, in the adjacent Q fragment (38a), while Fp is a lytic cycle promoter (24). It may thus be more appropriate to designate this element the Q promoter (Qp). The ability of the virus to switch its program in B cells of different subtypes is particularly interesting. We have recently shown (1, 17) that the silent WpCp program can be activated in BL type I cells by 5-azacytidine treatment and also by the spontaneous drift of the cells to a more immunoblastic, type III phenotype. The converse, WpCp-to-Qp shift can be achieved when the immunoblastic phenotype is induced to eclipse, as in somatic hybrids between B cells and non-B cells. This suggests that the cell phenotype-dependent differences in viral genome expression reflect the strategy of the virus in relation to latent persistence. If so, the EBNA-1-only program, as used in BL type I cells, would reflect the corresponding program in some normal B-cell subtype. This may be viewed against the fact that none of the laboratories involved in the production and analysis of EBV-specific cytotoxic T lymphocytes against EBV-carrying autologous lines has been able to generate the cytotoxic T lymphocytes against EBNA-1 so far, in contrast to EBNA-2 to -6 and the LMPs that have all been found to be immunogenic towards appropriate major histocompatibility complex class I allotype positive cells (9, 10, 21, 29). EBNA-1 may secure the latent maintenance of the virus, unrecognized by immunosurveillance and without undue expansion of the virus-carrying cell population (since blast expansion would lead to the expression of EBNA-2 to -6 plus LMP and, thereby, the elimination of the cell) (9, 21, 29). A normal B cell that expresses only EBNA-1 may thus perform an important function in maintaining the latent virus, unrecognized by immune surveillance.

The purpose of our present study was to search for the postulated EBNA-1-only cells within the peripheral B-cell population of healthy EBV-seropositive individuals.

Earlier outgrowth experiments have suggested that, on average, normal seropositive individuals carry one EBV-carrying cell per 10^6 lymphocytes or per 10^5 enriched B lymphocytes, with a variation of 1 log unit (2, 30, 33). These figures have been estimated from limiting-dilution–outgrowth experiments.

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TABLE 1. PCR conditions[a]

Primer[b]	Mg^{2+} (mM)	Annealing temp[c] (°C)	No. of cycles	Product (bp)
EBNA-1				
Common U-K				
Outer	1.5	60	35	421
Inner	1.5	67	25	293
Q-U-K (Qp)				
Outer	1	63	35	421
Inner	1.5	66	35	293
Y3-U-K (Cp/Wp)				
Outer	2	63	35	256
Inner	1.5	66	30	293
Cp				
Outer	1	66	30	365
Inner	0.8	68	30	150
Wp				
Outer	0.65	63	35	216
Inner	1.5	63	30	97
EBNA-2				
Outer	2.5	60	35	338
Inner	2.5	63	30	271
LMP-1				
Outer	3.5	45	30	469
Inner	3.0	65	25	129
LMP2a				
Outer	1	45	30	421
Inner	1.5	66	25	411
BZLF1	1.0	67	40	430

[a] Denaturing, 94°C for 2 min except for Wp outer (45 s), Wp inner (30 s), and BZLF-1 (30 s) primers; extension, 72°C for 2 min.
[b] See Table 2 for sequences.
[c] For 1 min.

Between 10 and 30% of seropositive donors yield such lines after in vitro culturing of blood cells (11). The true number of latently EBV-infected B cells in blood is unknown.

We have used reverse transcription (RT) followed by a highly sensitive and specific nested-PCR method to study the expression of EBV genes in normal B cells of healthy seropositive donors. LMP2a expression has earlier been demonstrated by RT-PCR in peripheral blood (32, 42a).

We have now identified a population of small, high-density B cells which express EBNA-1 but not EBNA-2 or LMP1. This expression pattern resembles that of the phenotypically representative type I BLs.

MATERIALS AND METHODS

Cells. The BL cell lines Rael and Namalva, the EBV-positive marmoset cell line B95-8, the LCLs CBM1-Ral and BL72 (transformed with Rael and B95-8 viruses, respectively), the EBV-negative B-cell line BJAB, and the EBV-negative leukemia cell lines K562 and HL 60 were used as references and controls (for detailed descriptions, see reference 1). They were propagated in RPMI 1640 with 10% fetal calf serum, supplemented with penicillin and streptomycin in 5% CO_2 with humidified air.

Preparation of blood cells. Lymphocytes were obtained by separation from erythrocytes on a cushion of Lymphoprep (Nycomed, Oslo, Norway). The interphase cells were collected with a Pasteur pipette and washed twice with phosphate-buffered saline. B cells were purified by adherence to a plastic layer for 90 min at 37°C, and the cells were mixed with 5% sheep erythrocytes in 20% newborn calf serum to form rosettes. After 15 min on ice the rosetting and nonrosetting cells were separated on a Lymphoprep gradient. Subsequently, an isotonic stock of Percoll (Pharmacia) was prepared by mixing nine parts of Percoll with one part of a 10-fold concentrated balanced salt solution and adjusting osmolarity with 9% NaCl or distilled water. This stock solution was mixed with balanced salt solution or RPMI 1640 (GIBCO) to 65 and 45% Percoll. Three-milliliter portions of the two solutions were layered over each other with decreasing density in a 15-ml Falcon tube to form noncontinuous two-step gradients. The cells were suspended in 2 ml of RPMI 1640–10% fetal calf serum (GIBCO) and layered on top of the gradient. The gradient was centrifuged for 30 min at 800 × *g* in a swing-out rotor, and the cell bands were carefully collected with a Pasteur pipette. The cells were washed immediately with balanced salt solution and frozen as dry pellets for PCR. The procedure yielded 80 to 85% CD19/CD20-positive B cells as determined by fluorescence-activated cell sorter analysis.

Extraction of RNA. Total RNA was extracted from small, high-density peripheral blood B lymphocytes by the modified guanidium-thiocyanate method (4).

Amplification and analysis of RNA transcripts by PCR. First-strand cDNA was synthesized from 1 to 2 μg of total RNA by using avian myeloblastosis virus (AMV) reverse transcriptase. Briefly, the extracted RNA was incubated with 25 μl of reaction buffer containing 50 mM Tris HCl (pH 8.5), 145 mM KCl, 10 mM $MgCl_2$, 10 mM dithiotreitol, 1 mM deoxynucleoside triphosphate, 4 μM 14-mer random primer, 30 U of RNase inhibitor (Promega), and 15 U of avian myeloblastosis virus reverse transcriptase (Promega) for 60 min at 42°C and then for 5 min at 95°C to denature the reverse transcriptase. The reaction was performed in an automated thermal cycler (Techne PHC-2). The cDNA was stored at −20°C until PCR amplification.

Three microliters of cDNA was used as a template for nested PCR. The reaction was performed in 50 μl containing 20 mM Tris (pH 8.5), 50 mM KCl, $MgCl_2$ (concentration according to titrated optimum [1 to 4 mM]), 1 μM primers, and 200 μM each dATP, dGTP, dCTP, and dTTP. One unit of *Taq* polymerase (Perkin-Elmer) was added per 50 μl of the reaction mixture. The samples were overlaid with 2 or 3 drops of mineral oil and then subjected to 30 to 35

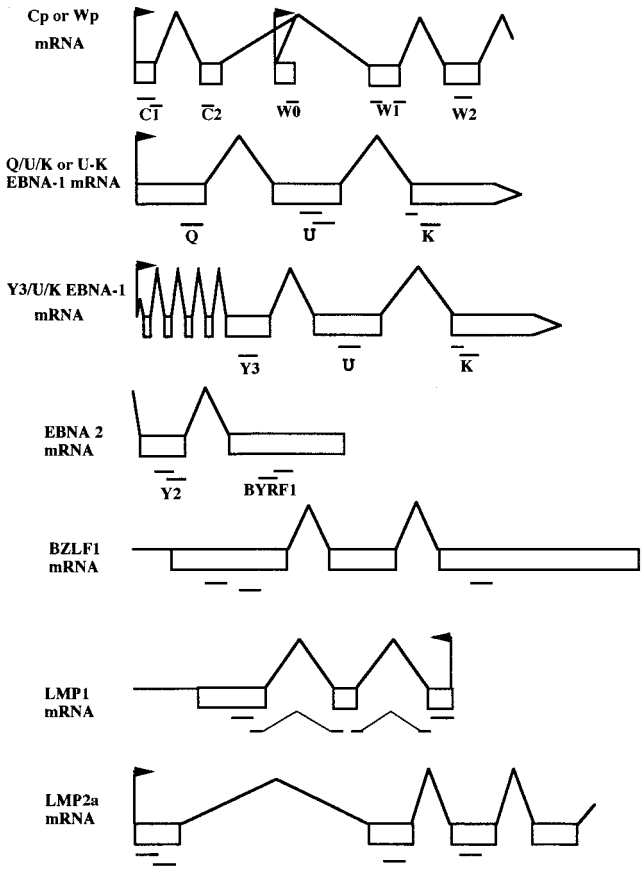

FIG. 1. Schematic drawing of transcripts and location of seven different primer combinations used for detection of C-promoter-generated splice products (Cp), W-promoter-generated mRNA (Wp), Q-promoter generated products (Q/U/K EBNA-1 or U-K EBNA-1), C- or W-promoter-derived transcripts detected by the common Y exon (Y3/U/K EBNA-1), EBNA-2 mRNA (BYRF-1 exon), and LMP1, LMP2a, and BZLF1 transcripts. Bars, exons; angles, spliced introns; arrows, sites of promoters. The locations of primers are indicated below each diagram. For LMP2a, only the first four exons are shown.

TABLE 2. Primers[a]

Primer	Sequence
EBNA-1	
Common U-K	
5′, outer, U (67497)	5′-GTAACTTAGGAAGCGTTTCT-3′
3′, outer, K (108171)	5′-CAATGCAACTTGGACGTTTT-3′
5′, inner, U (67519)	5′-AGCTTCCCTGGGATGAGCGT-3′
5′, inner, K (108065)	5′-TCTTCCCCGTCCTCGTCCAT-3′
Q-U-K (Qp)	
5′, outer, Q (62458)	5′-GTGCGCTACCGGATGGCG-3′
3′, outer, K (108148)	5′-GGTCTCCGGACACCATCTCT-3′
5′, inner, U (67519)	5′-AGCTTCCCTGGGATGAGCGT-3′
5′, inner, K (108065)	5′-TCTTCCCCGTCCTCGTCCAT-3′
Y3-U-K (Cp/Wp)	
5′, outer, Y3 (48400)	5′-CGTGTGACGTGGTGTAAAGT-3′
3′, outer, K (108148)	5′-GGTCTCCGGACACCATCTCT-3′
5′, inner, U (67519)	5′-AGCTTCCCTGGGATGAGCGT-3′
5′, inner, K (108065)	5′-TCTTCCCCGTCCTCGTCCAT-3′
EBNA-2	
5′, outer (47911)	5′-ACTTTGAGCCACCCACAGTAACCA-3′
3′, outer (48613)	5′-TGGAGTGTCTGACAGTTGTTCCTG-3′
5′, inner (47954)	5′-CATAGAAGAAGAAGAGGATGAAGA-3′
3′, inner (48589)	5′-GTAGGGATTCGAGGGAATTACTGA-3′
LMP1	
5′, outer (169456)	5′-CTGAGGATGGAACACGACCTTGAGA-3′
3′, outer (168858)	5′-TGAGCAGGATGAGGTCTAGG-3′
5′, inner (169124)	5′-TT(T/G)GT(A/G)TACTCCTACTGATGATCACC-3′
3′, inner (169045)	5′-AGTAGATCCAGA(G/T)ACC(G/T)AAGACAAGT-3′
Cp	
5′, outer (11337)	5′-GATCAGATGGCATAGAGACAAGGAC-3′
3′, outer (14829)	5′-TGAAGGTGAACCGCTTACCA-3′
5′, inner (11360)	5′-ACACCGAAGACCCCCAGAGC-3′
3′, inner (11651)	5′-ACGTGCAGGAGGCTGTTTCTTCAG-3′
Wp	
5′, outer (14391)	5′-CGCCAGGAGTCCACACAAAT-3′
3′, outer (14829)	5′-TGAAGGTGAACCGCTTACCA-3′
BZLF1	
5′, outer (102871)	5′-CACCTCAACCTGGAGACAAT-3′
3′, outer (102201)	5′-TGAAGCAGGCGTGGTTTCAA-3′
3′, probe (102100)	5′-GCACATCTGCTTCAACAGGA-3′
LMP-2a	
5′, outer (166672)	5′-TCCATCTGCTTCTGGCTCTT-3′
3′, outer (399)	5′-AAACAGAAGAGAATTAAAA-3′
5′, inner (166694)	5′-CGAACGATGAGGAACCTGAA-3′
3′, inner (207)	5′-TTTGTGCAGCGGCATATGAG-3′

[a] See Table 1 for PCR conditions.

cycles of amplification in the thermal cycler for the outer primers and 25 to 35 cycles per reaction for the inner primers. The conditions for the specific PCRs are given in Table 1.

All the primers were synthesized as single-stranded DNA 17- to 25-nucleotide oligonucleotides by Scandinavian Gene Synthesis Systems (Enköping, Sweden). The PCR products were visualized by electrophoresis in 1 to 2% agarose gels, ethidium bromide staining, and photography with a Polaroid camera. Alternatively, the PCR products were blotted onto nylon filters (Hybond N; Amersham) and detected by Southern hybridization using a specific oligonucleotide probe end labelled with [γ-^{32}P]ATP (19).

RESULTS

B lymphocytes from healthy blood donors express EBNA-1. Nine combinations of PCR primers were applied to cDNA prepared from whole-cell RNA isolated from human purified B lymphocytes and cell lines used as EBV-negative controls, EBV-positive type I BL cells, and LCL (Fig. 1; Table 2). These were common EBNA-1 (primers localized in exons U and K), EBNA-1 from Qp (Q, U, and K), the common splice product from WpCp-initiated EBNA-1 (Y3, U, and K), Cp (exons C1 and C2), Wp (exons W1 and W2), EBNA-2, LMP1 (exons BNLA a to c), LMP2a (first three exons), and BZLF1 (two outer exons). The positive-control cell lines were selected on the basis of earlier data obtained by us or by others, showing that they either make EBNA-1 only or produce all six EBNAs and the LMPs (1, 25, 37, 39, 41, 44).

The method of nested PCR provides both a high specificity and sensitivity. Instead of hybridizing to demonstrate the specific products, two inner primer pairs increase the specificity by only amplifying the correct sequence in the second PCR cycle. To demonstrate the sensitivity of the PCR, the LCL CBM1-

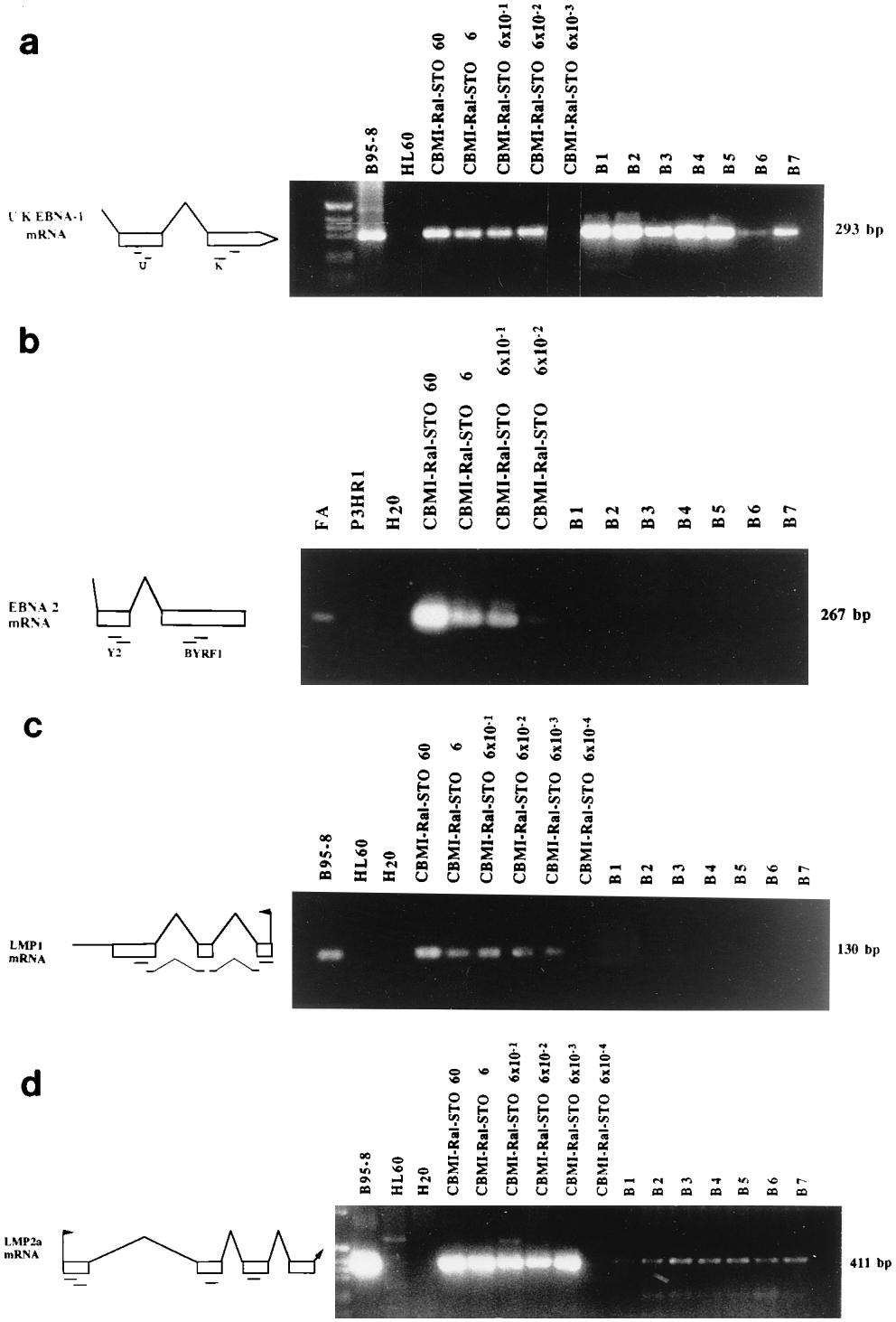

FIG. 2. RT-PCR for EBV gene expression in high-density B cells from peripheral blood from seven healthy donors, B1 to B7. Primers and conditions are indicated in Tables 1 and 2 and Fig. 1. B95-8 and EBNA-2-transfected FA cells were used as positive controls, and HL 60 and P3HR-1 were used as negative controls. Dilutions of CBM1-Ral-STO were used to establish sensitivity. EBNA-1, EBNA-2, LMP1, and LMP2a could be detected in RNA derived from less than one positive cell (0.06 to 0.006). A 1-kb ladder from Bethesda Research Laboratories was used as the molecular weight marker. (a) EBNA-1 with U-K primers. The expected product has the size of 293 bp. (b) EBNA-2. Cell line FA, transfected with the EBNA-2 gene was used as a positive control, and P3HR-1 with a deletion of the EBNA-2 gene was used as a negative control. The expected product has the size of 267 bp. (c) LMP1. The expected product has the size of 130 bp. (d) LMP2a. The expected product has the size of 411 bp.

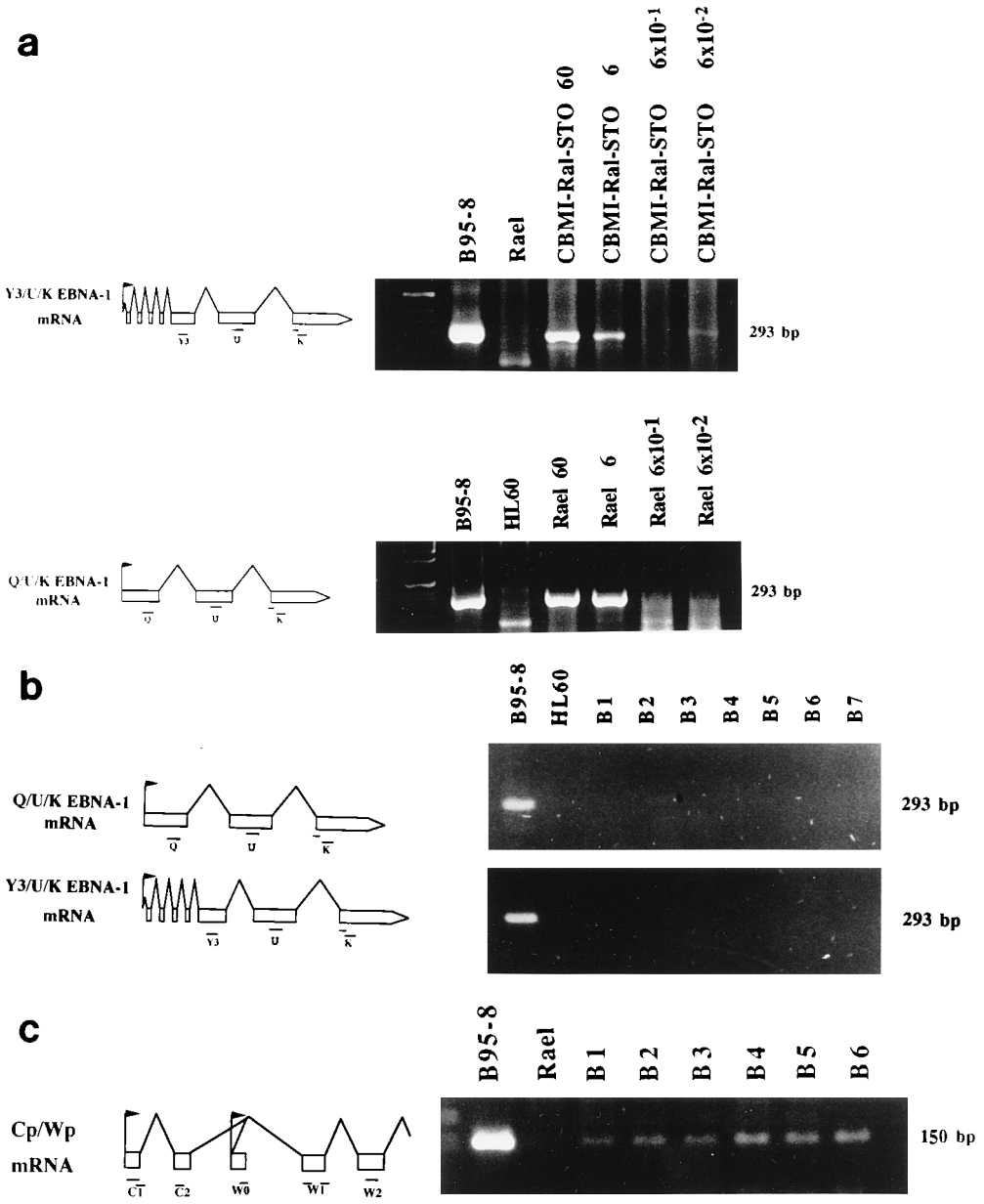

FIG. 3. RT-PCR for Cp/Wp- or Qp-generated EBNA-1 transcripts in RNA from high-density B cells from seven healthy donors, B1 to B7. B95-8 was used as a positive control and HL 60 or Rael was used as a negative control. One-kilobase ladder from Bethesda Research Laboratories was used as the molecular weight marker. (a) Sensitivity test for the Y3/U/K primer and for Q/U/K RT-PCR combinations (Fig. 1) using dilutions of LCL CBM1-Ral-STO and type I Rael cells as a negative control. (b) Q/U/K transcript in high-density B cells from donors B1 to B7 and Y3/U/K transcript in the same B lymphocytes. The expected products have the size of 293 bp for both Q/U/K and Y3/U/K. (c) Cp-initiated transcript in the seven donors, using primer W0 and a primer in C1 as outer primers and primers in C1 and C2 as inner primers.

Ral was diluted stepwise with EBV-negative cell RNA as carrier and cDNA was prepared. With primer combinations for mRNAs from EBNA-1, EBNA-2, LMP1, LMP2a, and Cp, between 0.006 and 0.06 positive cells could be detected among 10,000 negative cells (Fig. 2a to c). The sensitivity was lower for Qp-initiated mRNA (Q-U-K), the Y3-U-K common for Cp/Wp-initiated mRNA, Wp, and BZLF-1, requiring approximately 6 positive cells in 10^4 for a positive signal (Fig. 3).

The purified high-density B cells from seven normal healthy blood donors expressed EBNA-1, while they did not express EBNA-2 or LMP1 (Fig. 2a to c; Table 3). The negative controls gave no product with any of the primer combinations. BZLF1 was negative (Table 3), indicating that lytic viral cycle was not switched on at a detectable level. We could also confirm expression of LMP2a and the exons C1 and C2 immediately downstream of the C promoter, as also reported by Qu and Rowe (Table 3) (32).

Isolation and sequencing of cDNA clones and earlier PCR data have indicated the existence of three distinct EBNA transcript splice patterns (3, 17, 35). Primers were chosen so as to detect one of these patterns, namely, Q-U-K or Y3-U-K (Fig. 1 and Table 2). The Y3-U-K primer pair can detect the common splice pattern for transcripts initiated at the Cp or Wp promoters but not Qp (17, 35, 42a). Neither of these splice

TABLE 3. EBV gene expression in high-density peripheral blood B lymphocytes[a]

Gene	Expression
EBNA-1	
U-K	+
Y3-U-K	−
Q-U-K	−
Cp	+
Wp	−
EBNA-2	−
LMP1	−
LMP2a	+
BZLF1	−

[a] Of seven healthy donors.

patterns was detected in the B cells, although they could readily be demonstrated in the appropriate control cell lines. However, the sensitivity of the assay is lower for these transcripts, which may impair detection in the B-cell population.

DISCUSSION

Latent EBV genomes are differentially expressed in different cell types. Apparently the virus uses the regulatory proteins of its host cell to suit its strategic requirement for latent persistence, avoidance of immune rejection, and the needs for occasional viral replication. All current information is based on studies of EBV-carrying cell lines in vitro and tumors in vivo. Three forms of latent expression could be defined on the basis of such studies, as detailed in the introduction. In order to elucidate the significance of these patterns in the viral life cycle in vivo and to better understand the normal host-virus interaction, it is now imperative to perform corresponding studies on the normal host cells of the virus. Latent EBV infection can now be studied in different hematopoietic compartments by RT-PCR. This is a major step forward, compared with the earlier indirect methods, based on rescue of virus-infected cells. Previous studies have suggested that the virus persists in the hematopoietic compartment, including, if not exclusive to, the B-cell fraction (11, 12, 25, 45).

The different forms of latent infection have been linked to promoter switches between the Wp/Cp promoter and the Qp (Fp) promoter in in vitro-growing cell lines. Our findings show that resting B lymphocytes that express only EBNA-1 do not use the Qp (Fp) program like type I BL cells. They appear to use Cp, with a nonconventional splice pattern, excluding the Y-3 exon. This suggests yet another form of transcriptional control in normal B cells. The difference may be related to the fact that these are G_0 cells, in which requirements for renewal of the protein pool and transcription may be different than those in proliferating cells. One factor involved may be the negative feedback autoregulation of the Qp (Fp) (38), which can result in downregulation of EBNA-1 transcription. EBNA-1 mRNA synthesis may even be at rest as long as the binding sites for EBNA-1 in oriP and downstream of Qp (Fp) are saturated (18, 38). Normal B lymphocytes switch between proliferating and resting states, in response to specific signals. Unless activated by an appropriate signal, they are short-lived. B lymphocytes that are not legitimately activated run a high risk of cell death by apoptosis. The vast majority of B lymphocytes (more than 99%) are thus continuously eliminated (26, 27). Antigenic stimulation leads to massive clonal proliferation in the follicular centers. From the known information on EBV–B-cell interactions, with the added evidence presented in this article, it is evident that the virus–B-cell interaction can follow one of two main alternatives, driving an immunoblastic expansion or maintaining a balanced state of latency, probably in resting B cells. The established fact that the virus utilizes two different programs may reflect the dual need for an initial rapid rise in the number of infected B cells after primary infection and the steady maintenance of virally infected, nonexpanding cells, unrecognized by immune rejection, after convalescence.

EBV-carrying resting B cells that express only EBNA-1 may undergo activation in vivo, following exposure to specific or nonspecific stimuli. When activated, they would express the full EBNA-1 to -6–LMP program, as indicated by the fact that explanation of normal resting B cells can give rise to immunoblastic lines, in the presence of viral inhibitors that express the full set of EBNAs and LMPs (25). The occurrence of a similar event in vivo is suggested by the fact that both antibodies to and CTL memory of EBNA-2 to -6 are maintained in normal healthy seropositive individuals over a lifetime.

In the course of such events the cells may acquire the LCL-like phenotype to expand the population of virus-infected cells and protect against apoptotic death of EBV-carrying cells. The LCL phenotype may therefore also play a vital role for virus survival strategy in vivo, together with the small, resting, EBV-positive B cells.

Why does the virus use the EBNA-1-only program, postulated to have evolved for the latently infected, resting B cell, in the proliferating BL cell? This may be related to the fact that the BL cell is not an immunoblast but, rather, an illegitimately proliferating cell that corresponds, phenotypically, more to a resting cell than to an immunoblast. Because of the constitutive activation of c-*myc* by the chromosomal translocation, leading to the juxtaposition of *myc* to constitutively active immunoglobulin sequences, the cell cannot leave the cycling compartment. Unlike the immunoblast, this proliferating cell is not driven by EBV. Rather, EBV is carried along just like in the normal, nonproliferating EBV-harboring B cell. It may be noted that type I BL cells can be activated to type III, with full EBNA and LMP expression, but this cell is encountered only in vitro.

Our findings indicate that latently infected B cells in normal seropositive individuals express only EBNA-1 like EBV-carrying BL cells, in the absence of the BL cell-associated c-*myc* activation by chromosomal translocation. The absence of EBNA-2 to -6 and LMP expression in the BL cells may thus reflect the viral strategy in normal resting B cells rather than any tumor-associated change.

Since EBNA-1 has not been found to induce cytotoxic T-cell responses, in contrast to the other EBV encoded growth transformation-associated proteins, its exemption from downregulation in the latently infected B cell may satisfy the dual need for viral episome maintenance (for which EBNA-1 is essential) and escape from immune rejection.

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ADDENDUM IN PROOF

After the manuscript was submitted for publication, Tierney et al. reported on Qp-derived transcripts in mononuclear cells from three healthy donors (42a).

REFERENCES

1. **Altiok, E., J. Minarovits, L.-F. Hu, B. Contreras-Brodin, G. Klein, and I. Ernberg.** 1992. Host cell phenotype dependent switch on/off of the BCR2/BWR1 promoter complex regulates the Epstein-Barr virus nuclear antigen (EBNA) 2-6 expression. Proc. Natl. Acad. Sci. USA **89:**905–909.
2. **Birx, D. L., R. Redfield, and G. Tosato.** 1986. Defective regulation of Epstein-Barr virus infection in patients with acquired immunodeficiency syndrome (AIDS) or AIDS-related disorders. N. Engl. J. Med. **314:**874–879.
3. **Bodescot, M., M. Perricaudet, and P. Farrell.** 1987. A promoter for the highly spliced EBNA family of RNAs of Epstein-Barr virus. J. Virol. **61:** 3424–3430.
4. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
5. **Contreras-Brodin, B. A., M. Anvret, S. Imreh, E. Altiok, G. Klein, and M. G. Masucci.** 1992. B cell phenotype-dependent expression of the Epstein-Barr virus nuclear antigens EBNA-2 to EBNA-6: studies with somatic cell hybrids. J. Gen. Virol. **72:**3025–3033.
6. **Contreras-Salazar, B., G. Klein, and M. G. Masucci.** 1989. Host cell-dependent regulation of growth transformation-associated Epstein-Barr virus antigens in somatic cell hybrids. J. Virol. **63:**2768–2772.
7. **Dillner, J., and B. Kallin.** 1987. The Epstein-Barr virus proteins. Adv. Cancer Res. **50:**95–158.
8. **Fåhraeus, R., L.-F. Hu, I. Ernberg, J. Finke, M. Rowe, G. Klein, K. Falk, E. Nilsson, M. Yadav, P. Busson, T. Tursz, and B. Kallin.** 1988. Expression of EBV-encoded proteins in nasopharyngeal carcinoma. Int. J. Cancer **42:**329–338.
9. **Gavioli, R., P. O. DeCampos-Lima, M. G. Kurilla, E. Kieff, G. Klein, and M. G. Masucci.** 1992. Recognition of the Epstein-Barr virus-encoded nuclear antigens EBNA-4 and EBNA-6 by HLA-A11-restricted cytotoxic T lymphocytes: implications for down-regulation of HLA-A11 in Burkitt lymphoma. Proc. Natl. Acad. Sci. USA **89:**5862–5868.
10. **Gavioli, R., M. G. Kurilla, P. O. de Campos-Lima, L. E. Wallace, R. Dolcetti, R. J. Murray, A. B. Rickinson, and M. G. Masucci.** 1993. Multiple HLA A11-restricted cytotoxic T-lymphocyte epitopes of different immunogenicities in the Epstein-Barr virus-encoded nuclear antigen 4. J. Virol. **67:**1572–1578.
11. **Gratama, J. W., M. A. P. Oosterveer, W. Weimar, K. Sintnicolaas, W. Sizoo, R. L. H. Bolhuis, and I. Ernberg.** 1994. Detection of multiple 'Ebnotypes' in individual Epstein-Barr virus (EBV) carriers following lymphocyte transformation by EBV derived from peripheral blood and oropharynx. J. Gen. Virol. **75:**85–94.
12. **Gratama, J. W., M. A. P. Oosterveer, F. E. Zwaan, J. Lepoutre, G. Klein, and I. Ernberg.** 1988. Eradication of Epstein-Barr virus by allogenic bone marrow transplantation: implications for sites of viral latency. Proc. Natl. Acad. Sci. USA **85:**8693–8696.
13. **Gregory, C. D., M. Rowe, and A. B. Rickinson.** 1990. Different Epstein-Barr virus (EBV) B cell interactions in phenotypically distinct clones of a Burkitt lymphoma cell line. J. Gen. Virol. **71:**1481–1495.
14. **Hennessy, K., S. Fennewald, M. Hummel, T. Cole, and E. Kieff.** 1984. A membrane protein encoded by Epstein-Barr virus latent growth-transforming infection. Proc. Natl. Acad. Sci. USA **81:**7207–7211.
15. **Hennessy, K., and E. Kieff.** 1983. One of two Epstein-Barr virus nuclear antigens contains a glycine-alanine copolymer domain. Proc. Natl. Acad. Sci. USA **80:**5665–5669.
16. **Herbst, H., M. Dallenbach, G. Hummel, G. Niedobitek, N. Pileri, N. Muller-Lantzsch, and H. Stein.** 1991. Epstein-Barr virus latent membrane protein expression in Hodgkin and Reed-Sternberg cells. Proc. Natl. Acad. Sci. USA **88:**4766–4770.
17. **Hu, L.-F., F. Chen, E. Altiok, G. Winberg, G. Klein, and I. Ernberg.** Cell phenotype dependent alternative splicing of EBNA mRNAs in EBV-carrying cells. J. Gen. Virol., in press.
18. **Jones, C. H., S. D. Hayward, and D. R. Rawlins.** 1989. Interaction of lymphocyte-derived Epstein-Barr virus nuclear antigen (EBNA-1) with its DNA-binding sites. J. Virol. **63:**101–110.
19. **Kawasaki, E. S.** 1990. Amplification of RNA, p. 21–27. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc., San Diego, Calif.
20. **Kerr, B. M., A. L. Lear, M. Rowe, D. Croom-Carter, L. S. Young, S. M. Rookes, P. H. Gallimore, and A. B. Rickinson.** 1992. Three transcriptionally distinct forms of Epstein-Barr virus latency in somatic cell hybrids: cell phenotype dependence of virus promoter usage. Virology **187:**189–201.
21. **Khanna, R., S. Borrows, M. Kurilla, C. A. Jacob, I. S. Misko, T. B. Sculley, E. Kieff, and D. J. Moss.** 1992. Localization of Epstein-Barr virus cytotoxic T cell epitopes using recombinant vaccinia: implications for vaccine development. J. Exp. Med. **176:**169–176.
22. **Klein, G.** 1989. Viral latency and transformation: the strategy of Epstein-Barr virus. Cell **58:**5–8.
23. **Laux, G., M. Perricaudet, and P. J. Farrell.** 1988. A spliced Epstein-Barr virus gene expressed in immortalized lymphocytes is created by circularization of the linear viral genome. EMBO J. **7:**769–774.
24. **Lear, A. L., M. Rowe, M. G. Kurilla, S. Lee, S. Henderson, E. Kieff, and A. B. Rickinson.** 1992. The Epstein-Barr virus (EBV) nuclear antigen 1 *Bam*HI F promoter is activated on entry of EBV-transformed B cells into the lytic cycle. J. Virol. **66:**7461–7468.
25. **Lewin, N., P. Åman, M. G. Masucci, E. Klein, G. Klein, B. Öberg, H. Strander, W. Henle, and G. Henle.** 1987. Characterization of EBV-carrying B-cell populations in healthy seropositive individuals with regard to density, release of transforming virus and spontaneous outgrowth. Int. J. Cancer **39:**472–476.
26. **Liu, Y. J., D. E. Joshua, G. T. Williams, C. A. Smith, J. Gordon, and I. C. M. MacLennan.** 1989. Mechanism of antigen-driven selection in germinal centres. Nature (London) **342:**929–931.
27. **MacLennan, I. C. M., and D. Gray.** 1986. Antigen-driven selection of virgin and memory B cells. Immunol. Rev. **91:**61–85.
28. **Minarovits, J., L.-F. Hu, S. Imai, Y. Harabuchi, A. Kataura, S. Minarovits-Kormuta, T. Osato, and G. Klein.** 1994. Clonality, expression and methylation patterns of the Epstein-Barr virus genomes in lethal midline granulomas classified as peripheral angiocentric T cell lymphomas. J. Gen. Virol. **75:**77–84.
29. **Murray, R. J., M. G. Kurilla, J. M. Brooks, W. A. Thomas, M. Rowe, E. Kieff, and A. B. Rickinson.** 1992. Identification of target antigens for the human cytotoxic T cell response to Epstein-barr virus (EBV): implications for the immune control of EBV-positive malignancies. J. Exp. Med. **176:**157–158.
30. **Nilsson, K., G. Klein, W. Henle, and G. Henle.** 1971. The establishment of lymphoblastoid lines from adult and fetal human tissue and its dependence on EBV. Int. J. Cancer **8:**443–450.
31. **Pallesen, G., S. J. Hamilton-Dutoit, and X. Zhou.** The association of Epstein-Barr virus (EBV) with T cell lymphoproliferations and Hodgkin's disease: two new developments in the EBV field. Adv. Cancer Res., in press.
32. **Qu, L., and D. Rowe.** 1992. Epstein-Barr virus latent gene expression in uncultured peripheral blood lymphocytes. J. Virol. **66:**3715–3724.
33. **Rocchi, G., A. DeFelici, G. Ragona, and A. Heinz.** 1977. Quantitative evaluation of Epstein-Barr virus-infected peripheral blood leukocytes in infectious mononucleosis. N. Engl. J. Med. **296:**132–139.
34. **Rowe, M., and C. Gregory.** 1989. Epstein-Barr virus and Burkitt's lymphoma. Adv. Viral Oncol. **8:**237–259.
35. **Rowe, M., A. L. Lear, D. Croom-Carter, A. H. Davies, and A. B. Rickinson.** 1992. Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes. J. Virol. **66:**122–131.
36. **Rowe, M., D. T. Rowe, C. D. Gregory, L. S. Young, P. Farrell, H. Rupani, and A. B. Rickinson.** 1987. Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitts lymhoma cells. EMBO J. **6:**2743–2751.
37. **Sample, J., L. Brooks, C. Sample, L. Young, M. Rowe, C. Gregory, A. Rickinson, and E. Kieff.** 1991. Restricted Epstein-Barr virus protein expression in Burkitt lymphoma is reflected in a novel, EBNA 1 mRNA and transcriptional initiation site. Proc. Natl. Acad. Sci. USA **88:**6343–6347.
38. **Sample, J., E. B. D. Henson, and C. Sample.** 1992. The Epstein-Barr virus nuclear protein 1 promoter active in type I latency is autoregulated. J. Virol. **66:**4654–4661.

38a. **Schaefer, B. C., and S. H. Speck.** Unpublished data.

39. **Schaefer, B. C., M. Woisetschläger, J. L. Strominger, and S. H. Speck.** 1991. Exclusive expression of Epstein-Barr virus nuclear antigen 1 in Burkitts lymphoma arises from a third promoter, distinct from the promoters utilized in latently infected lymphocytes. Proc. Natl. Acad. Sci. USA **88:**6550–6554.
40. **Smith, P., and B. Griffin.** 1991. Differential expression of Epstein-Barr viral transcripts for two proteins (TP1 and LMP) in lymphocyte and epithelial cells. Nucleic Acids Res. **19:**2435–2440.
41. **Speck, S., and J. Strominger.** 1989. Transcription of Epstein-Barr in latently infected, growth transformed B-lymphocytes. Adv. Viral Oncol. **8:**133–150.
42. **Su, I.-J., K.-H. Lin, C.-J. Chen, H.-F. Tien, H.-C. Hsieh, D.-T. Lin, and J.-Y. Chen.** 1990. Epstein-Barr virus-associated peripheral T-cell lymphoma of activated CD8 phenotype. Cancer **66:**2557–2562.

42a. **Tierney, R. J., N. Steven, L. S. Young, and A. B. Rickinson.** 1994. Epstein-Barr virus latency in blood mononuclear cells: analysis of viral gene transcription during primary infection and in the carrier state. J. Virol. **68:**7374–7385.

43. **Watry, D., J. A. Hedrick, S. Siervo, G. Rhodes, J. J. Lamberti, J. D. Lambris, and C. D. Tsoukas.** 1991. Infection of human thymocytes by Epstein-Barr virus. J. Exp. Med. **173:**971–980.
44. **Woisetschlaeger, M., J. L. Strominger, and S. H. Speck.** 1989. Mutually exclusive use of viral promoters in Epstein-Barr virus latently infected lymphocytes. Proc. Natl. Acad. Sci. USA **86:**6498–6502.
45. **Yao, Q. Y., P. Ogan, M. Rowe, M. Wood, and A. B. Rickinson.** 1988. Epstein-Barr virus-infected B cells persist in the circulation of acyclovir-treated virus carriers. Int. J. Cancer **43:**67–71.