Epstein-Barr Virus Latent Gene Expression in Uncultured Peripheral Blood Lymphocytes

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In this study of Epstein-Barr virus (EBV) latency, the polymerase chain reaction was used in modified form for amplification and detection of viral mRNA sequences in peripheral blood lymphocytes from healthy seropositive adults. Six known promoters for latent gene expression and eight known gene products were identified in in vitro-immortalized lymphocytes and in the cell lines established spontaneously from seropositive adults. We examined whether mRNA expression in uncultured B cells from four seropositive adults was the same as that which occurred in spontaneously established EBV-positive B-cell lines from the same individuals. A minimum of 17 polymerase chain reaction targets was required to circumscribe the known latent mRNA structures. Expression of the C promoter for the EBNA genes was detected in B-cell RNA from three of the four subjects. Transcripts initiated from the alternative W promoter for EBNA expression were not detected. The spliced transcripts detected in the B cells contained only the C2-to-W1 alternative splice, which was nonproductive for EBNA4 gene expression. None of the other EBNA open reading frames were detected spliced onto the 3' ends of the C promoter-initiated RNAs. Spliced RNA from the TP gene was detected in all four subjects. Expression of the TP gene was restricted to TP1 promoter-initiated RNAs, as no TP2 promoter-initiated transcripts were detected. Expression of RNA from the LMP gene was not detected. The F promoter which is active in the restricted expression latency that occurs in Burkitt's lymphoma cells was not detected being expressed in peripheral blood B cells. This pattern of latent gene expression is unique to uncultured B cells, indicating that there are profound differences between viral latent states in vitro and in situ and suggesting a central role for the TP gene in the latency of EBV.

Epstein-Barr virus (EBV) is a ubiquitous lymphotropic human herpesvirus that is the causative agent of infectious mononucleosis and is associated with African Burkitt's lymphoma (BL) and undifferentiated nasopharyngeal carcinoma (12, 15, 19, 22). The virus infects the host through the oropharynx, where it is capable of infecting epithelial cells and completing all of the steps of virus multiplication (29). From this initial site, the virus also infects and replicates in infiltrating B lymphocytes (19). Upon resolution of the primary infection, the virus persists in the host for life, being periodically detectable in throat washings and recoverable from the peripheral blood of seropositive adults as spontaneous immortalized B lymphocytes harboring latent virus (8, 16, 40). EBV can also infect B cells in vitro and convert them into immortal lymphoblastoid cell lines (LCLs) (11).

Two lines of evidence suggest that the cells in which the virus remains latent are resting B lymphocytes. (i) During prolonged acyclovir treatment, virus shedding in the oropharynx ceases; however, the frequency of establishment of spontaneously immortalized B-cell lines remains unchanged (39). (ii) Analyses of EBV strains recovered from bone marrow transplant recipients strongly suggest that EBV can be eradicated from seropositive recipients during replacement of their hematopoietic cell lineages with bone marrow from seronegative donors (9, 14). A still unanswered question about these latently infected B cells in circulation is how they are able to escape cell death induced by antigen-specific cytotoxic T cells. Such T-cell populations obtained from immunocompetent individuals readily kill autologous EBV-carrying LCLs in vitro (20, 21).

These in vitro-maintained, latently infected cells express six nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA4) and two membrane proteins (LMP and TP) (13). Some of these gene products (EBNA2, EBNA3B, and LMP) have been shown to contain epitopes for cytotoxic T cells, and therefore it seems unlikely that the LCL-like cells could survive in situ (14). EBV-carrying BL cells face the same challenge but are able to survive in the host by invoking a latent state in which viral gene expression is restricted to EBNA1 and host histocompatibility and adhesion molecules are reduced in number on the cell surface (10, 12, 18, 25, 33). The surface characteristics of an immune resistant BL cell are very similar to those of a resting B cell.

It is not clear how the restricted latent viral gene expression is achieved, but studies on LCLs and BLs in vitro have shown that the controls include promoter switching, alternative splicing, and poly(A) site selection (26, 32, 34). Classically, all of the EBNA genes are in a single 100-kbp transcription unit originating from one or the other of two promoters. The TATA-like element of the C promoter (so called because it lies within the BamHI C fragment) is located 26 bases upstream of the most complete cDNA and is directed toward IR1, the major internal repeat, just 304 bp away (3, 4). The TATAAA element of the W promoter lies within the 3.1-kb repeating unit (the BamHI W fragment), 2.3 kb into the first repeat, and is reiterated in every successive repeat throughout the IR1 region (23, 28, 32, 35, 36). There is some evidence that C and W promoters act in a mutually exclusive manner in LCLs and that during the initial stages of infection, use of the W promoter precedes use of the C promoter (37, 38). Five of the EBNA open reading frames (ORFs) are scattered as far downstream as 60

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kb (EBNA1), and inclusion in the final mRNA product appears to depend upon alternative splice and poly(A) site selections. The kinetics of EBNA protein expression suggest that EBNA2 and EBNA4 are expressed earlier than EBNA1 and EBNA3, indicating a potential relationship between promoter usage and gene expression (1, 2).

Further support for this intriguing notion has recently emerged from studies of BL cell lines in which latent EBV gene expression is restricted to just EBNA1. These cells utilize neither C nor W promoters but drive EBNA1 expression from a newly discovered F promoter located downstream of the EBNA2 ORF in the BamHI F fragment (28). The regulation of the use of the three EBNA promoters and the three membrane protein promoters in peripheral blood lymphocytes (PBLs) seems likely to be a key feature of the survival strategy of latent EBV in circulating B lymphocytes (14). We investigated EBV gene expression in purified uncultured PBLs from normal, healthy seropositive adults by using RNA polymerase chain reaction (PCR) to amplify the minute quantities of viral mRNA that might be present in those rare latently infected cells. We detected transcripts from the C promoter and the TP1 promoter and identified a unique state of latent viral gene expression that exists only in the resting B cells in peripheral blood.

MATERIALS AND METHODS

Subjects. Four healthy adults, three males and one female ranging in age from 24 to 37 years, were used in the study because spontaneous LCLs either existed previously or were generated during the study for these individuals. Blood samples of 50 to 100 ml were obtained and processed into lymphocytes and plasma (see below). Plasma obtained at the time of phlebotomy was used to determine EBV antibody status.

Cell cultures. IARC/BL16 was a BL cell line (gift of G. Lenoir, International Agency for Research on Cancer, Lyon, France) contains a type B EBV genome and a fast-migrating EBNA1 and was used to determine whether the plasma contained anti-EBNA2B antibodies. The X50-7, Raji, and B958 cell lines (gift of G. Miller, Yale University, New Haven, Conn.) were used to determine levels of antibodies to latent, early, and viral capsid proteins in plasma. EBV-negative cell lines BJAB and MOLT-4 were used as negative controls in all of the experiments.

The spontaneous LCLs were established from 10^5-cells-per-well seedings of PBLs into 96-well microtiter plates essentially as described previously (40). Cyclosporin A was present in the medium at 100 ng/ml to inhibit T-cell activity, which might otherwise prevent growth of EBV-infected B cells. The plates were refed twice weekly. All cell lines were grown in RPMI 1640 containing 10% fetal bovine serum, 2 mM glutamine, 200 µg of streptomycin per ml, and 60 µg of penicillin per ml.

B-cell fractionation and RNA purification. Immediately upon collection, the blood was layered onto Ficoll-Hypaque (Histopaque 1077; Sigma Diagnostics) and centrifuged at 900 × g for 30 min. The layer of mononuclear cells was harvested, washed once with phosphate-buffered saline (pH 7.2), and suspended in RPMI 1640 medium without serum at 4°C. The cell suspension was then incubated with a 5:1 ratio of anti-CD19 magnetic beads (Dynal) for 30 min at 4°C. CD19-positive B cells were collected with a magnet.

The magnet-attached B cells were immediately placed in a guanidinium isothiocyanate-phenol extraction buffer (RNAzol; Cinna/Biotex). The volume used was 0.2 ml/10^6

cells, and the cell number estimate was based on assumptions that B cells were approximately 10% of the total mononuclear cells and recovery was greater than 80%. Cells were homogenized by pipetting, 0.1 volume of chloroform was added, and the suspension was mixed vigorously. The homogenate was centrifuged at 12,000 × g for 15 min, and the aqueous phase was recovered. RNA was precipitated by addition of an equal volume of ice-cold isopropanol and collected by centrifugation at 1,000 × g for 15 min. The RNA pellet was washed once with ice-cold 75% ethanol and dried under vacuum. The RNA was then suspended in sterile diethylpyrocarbonate-treated RNase-free water and stored at −70°C until use in the PCR.

cDNA syntheses and PCR. cDNA reactions were performed on the RNA extracted from approximately 2 × 10^6 to 5 × 10^6 cells. A 5- to 10-µl RNA sample was used in a reaction mixture consisting of 10 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM MgCl, 0.05% gelatin, 0.05% Tween, 0.25 mM deoxynucleotides, 10 mM dithiothreitol, 10 pmol of the 3′ primer oligonucleotide, 1 U of RNasin RNase inhibitor (Promega), and 1 U of avian myeloblastosis virus reverse transcriptase (Promega). The total reaction volume of 25 µl was incubated at 42°C for 30 min. Aliquots in quantities ranging up to the entire cDNA reaction mixture were subjected to PCR. For PCR, the 5′ and 3′ primers were adjusted to 40 pmol per reaction in buffer conditions identical to those used for the cDNA synthesis reaction. Reaction volumes of 50 µl were overlaid with mineral oil and subjected to 30 to 35 cycles of temperature variation (94°C for 1 min, 55°C for 1 min, and then 72°C for 1 to 2 min). Products were analyzed on 1.8% agarose gels containing 0.5× Tris-borate-EDTA electrophoresis buffer. cDNA control amplifications were performed on a human β-actin target with all RNA preparations to check RNA size, integrity, and performance in a commercially available standard reaction (β Amplimers; Clontech). Mock amplification with B95-8 total RNA was performed to determine the efficiency of amplification. After 35 cycles of amplification, all of the target sequences from 50 cell equivalents of RNA were detected by ^{32}P-labeled oligonucleotide probes of Southern blots.

Design of RNA PCR primers for EBV latent genes. In general, the oligonucleotides were required to be between 18 and 24 bases long and have an annealing temperature within 2° of 55°C in 50 mM KCl. In addition, the oligonucleotides were designed to be free of potential secondary structure, self-complementarity, and complementarity with the other amplification primer oligonucleotides. The OLIGO primer design software (National Biosciences) was used to assist in locating sequences which fulfilled these criteria.

Expression of the six EBNA proteins is controlled by promoter switching, alternative poly(A) site selection, and alternative splicing (32). In Fig. 1, the structures of known cDNAs for EBV latent genes are shown. The ORFs for EBNA1 to EBNA3 are scattered over a 60-kb region downstream of IR1. These ORFs are all linked to W2 through splicing, as far as has been determined, when the W or C promoter drives EBNA gene expression (Fig. 1). It is therefore possible to position antisense oligonucleotide primers near the most 5′ edge of the ORF of the EBNA genes and synthesize short cDNA products that contain the W2 exon. Six such oligonucleotides (K1, E3, E2, L1, Y3/H1, and Y3/p) were synthesized (Fig. 2). Y3/H1 has been designated as the EBNA4 primer and lies across the Y3-to-H1 splice. Messages with this splice are monocistronic, containing only the EBNA4 ORF, but it should be noted that messages containing other EBNA ORFs at their 3′ ends may
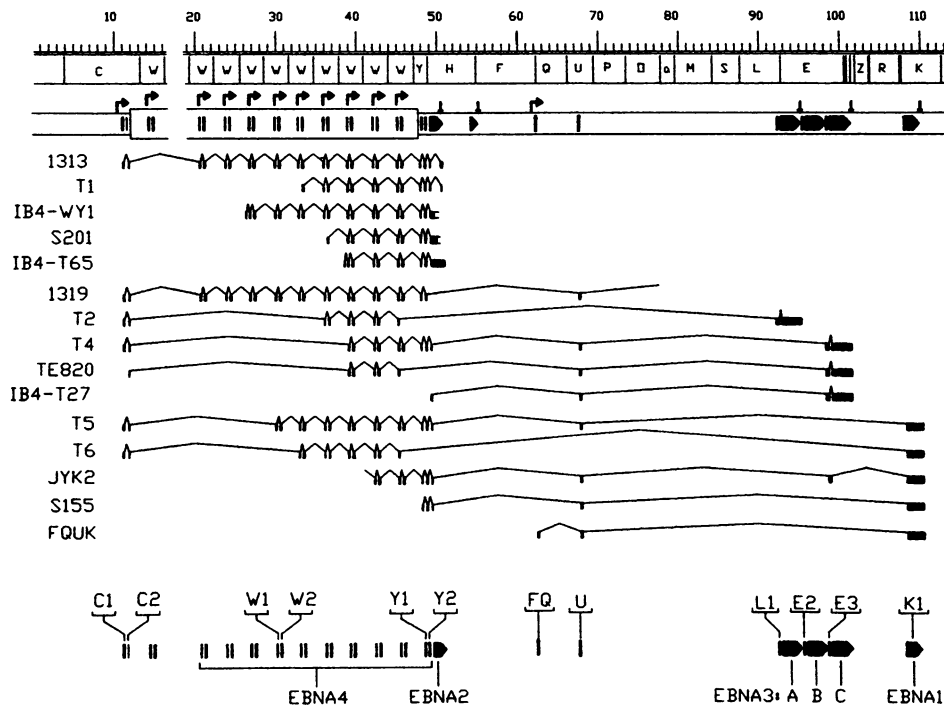
FIG. 1. A map of the published cDNA sequences for EBNA messages. The scale in kilobases and the *Bam*HI restriction fragment map of the left 110 kb of EBV DNA are shown at the top. The bar represents the physical genome, and the exons incorporated into the EBNA mRNAs are shown as horizontal arrows and vertical stripes. Downward vertical arrows represent poly(A) addition signals, and hooked arrows represent latent promoters. The structures of 15 published cDNAs left 5' to right 3' are shown below the physical map. The exon nomenclature used in this report is shown below the cDNAs, and the EBNA protein ORFs are indicated at the bottom.

be polycistronic and also express the EBNA4 protein (31, 35). The E3 primer lies within the short 5' exon which encodes the amino-terminal end of EBNA3C. This exon has been spliced into the leader of at least one EBNA1 message (Fig. 1, JYK2) and therefore may not be solely representative of EBNA3C messages (i.e., T4, TE820, and IB4-T27).

To detect promoter usage and EBNA4 gene expression, we used a set of four sense primers which distinguish between C and W promoter-initiated transcripts and between alternative EBNA4-productive and nonproductive splices (see below). The recent finding of EBNA1 cDNAs (FQ) which apparently are expressed by a unique promoter located downstream of the EBNA2 gene in *Bam*HI-F required the use of a unique 5' primer (FQ) to detect messages of this type, since these mRNAs do not contain W2 exons. The transcription units for the other latent gene products (the membrane proteins) are not as complicated as the EBNA transcription unit. TP1 and TP2 share eight common 3' exons and have unique promoter-associated 5' exons. Therefore, a common T2 antisense primer was used in conjunction with T1 and T1' sense primers to detect TP1 and TP2 expression, respectively. The LMP message was the least complicated, with only one latent structure and one pair of primers (N1 and N3) necessary to detect LMP messages.

Southern blotting. For analysis, the PCR products were size separated on agarose gels in the presence of $^{32}PO_4$-end-labeled size markers consisting of *Msp*I-digested pBR322 (New England Biolabs) and *Hin*fI-, *Rsa*I-, and *Sin*I-digested

pGEM-37 (Promega). The DNA fragments were transferred to nitrocellulose by a modification of the Southern technique involving the use of positive pressure applied above the gel (Stratagene). Transferred DNA was covalently attached to the filter by UV cross-linking. Probes consisted of antisense oligonucleotides $^{32}PO_4$ labeled at the 5' end by T4 polynucleotide kinase. Hybridization was carried out at 48°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–20 mM NaH_2PO_4 (pH 8.4)–0.4% sodium dodecyl sulfate (SDS)–100 μl of denatured, sonicated salmon sperm DNA per ml for 16 h. Filters were washed three times at 42°C in 6× SSC–0.1% SDS, patted damp dry, and wrapped in plastic wrap for exposure to XAR-5 X-ray film (Kodak) for periods of 1 h to 10 days.

Western immunoblotting. Whole-cell extracts were prepared by 30 s of sonication, followed by boiling in sample buffer (0.625 M Tris [pH 6.8], 2% SDS, 0.1% 2-mercaptoethanol, 10% glycerol, 0.02 mM bromphenol blue). Proteins were electrophoretically separated on 1.5-mm-thick, 180-mm-long gels consisting of 10% resolving gel and 5% stacking gel (acrylamide-bisacrylamide ratio, 30:0.8). Generally, 30-μl samples representing 2.3 × 10⁶ cells were electrophoresed at 60 V for 12 h. Proteins were electrophoretically blotted onto nitrocellulose, and the nonspecific antibody-binding sites were blocked with 5% skim milk in Tris-saline (pH 8.0). The filters were then incubated at 4°C for at least 12 h with a dilution of human plasma and then washed with Tris-saline. The blots were probed with I¹²⁵-labeled protein A (NEN), washed again, and exposed to XAR-5 X-ray film for 16 h.
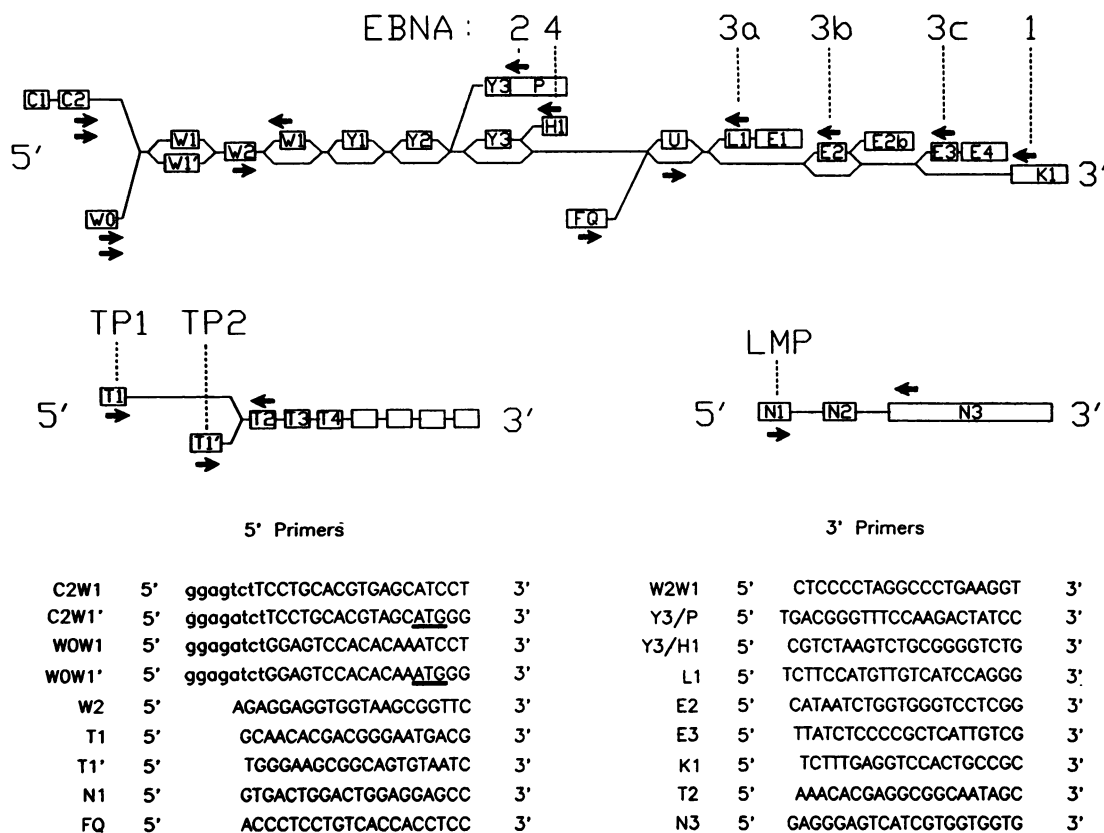
FIG. 2. PCR primers for EBV latent genes. In the schematic drawings, the exons for EBNA, TP, and LMP gene products are represented by open boxes with letter-number designations. The horizontal rightward and leftward arrows represent the locations of 5' and 3' primers, respectively. Each of the EBNA targets is composed of a 3' primer located in the downstream ORF and the 5' W2 primer, which acts as a common anchor for C or W promoter-initiated messages. A separate FQ 5' primer is employed as the common anchor for F promoter-initiated messages. Four 5' primers were used to detect C or W promoter activity and alternative splicing to the W1 exon. The TP and LMP targets are shown below. The sequences of the primers are listed at the bottom.

RESULTS

Anti-EBV antibody status of seropositive subjects. The anti-EBV response has been used to characterize the state of the virus infection in a host (reviewed in reference 19). Early in the infection process, immunoglobulin M antibodies against a variety of viral capsid antigens (VCAs) and early antigens (EAs) are produced. Within 2 to 3 weeks, the anti-VCA and -EA response has switched to the immunoglobulin G class. Although anti-EA antibodies are transient, a low titer of anti-VCA immunoglobulin G persists in most people into later life. During this period, an anti-EBNA response appears, usually directed solely at EBNA1 but sometimes including EBNA2 and other latent proteins.

The subjects used in this study were evaluated for anti-EBV antibody status to determine the titer of antibody against VCAs, EAs, and EBNA antigens. We used Western immunoblotting for this purpose because it is the most sensitive assay for EBV antibodies and provides information on the nature of the proteins involved in provoking the antibody response. All of our donors displayed a typical pattern of reactivity against a panel of antigen sources (Fig. 3). All of the donors had antibodies against EBNA1, and three had antibodies against EBNA2 which were specific for the type A virus strain (type A viruses were present in the spontaneous LCLs established from these individuals). The sera also contained antibodies to viral proteins which

are expressed late in the replication cycle and are components of the virion (unpublished data). Titers of antibodies (VCA, EA, and EBNA, respectively) for the four donors as determined by Western blotting were as follows: donor 1, >3,200, <50, and 1,600; donor 2, >3,200, <50, and 3,200; donor 3, 3,200, <50, and 100; donor 4, 3,200, <50, and 400.

RNA PCR for products initiated from the C and W promoters of the EBNA transcription unit. One of the key features of EBNA messages is their origin or 5' end. The structure there indicates promoter usage, and the first splice onto a W1 exon determines whether the message is bicistronic (expressing EBNA4) or monocistronic (no expression of EBNA4). This latter property is due to alternative splicing of the C2 or W0 exon onto one of two closely spaced W1 acceptor sites. We made four oligonucleotides (C2W1, C2W1', W0W1, and W0W1') which amplify 5' ends in conjunction with a common 3' oligonucleotide (W2W1) and indicate whether a PCR target is derived from a C start or W start message and whether or not it contains a splice creating the AUG initiator codon of EBNA4. The common downstream primer spans the W2W1 splice junction so the amplification target contains the W1W2 splice junction. Specificity for C2 or W0 is provided by the first 15 nucleotides for the 5' primer, while the 3' mismatch created by the alternative splice provides splice junction specificity. Products of these PCR reactions
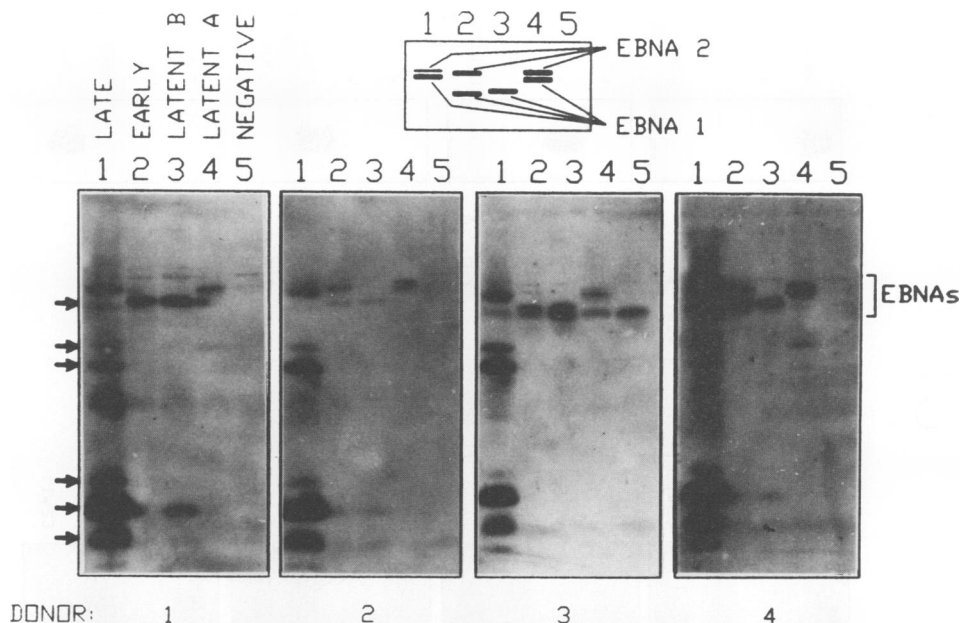
FIG. 3. Western immunoblotting with sera from four healthy seropositive donors. The filters were probed with a 1:50 dilution of serum obtained at the time lymphocytes were collected for RNA processing. In each panel, lane 1 contained B95-8 induced with TPA and butyrate, lane 2 contained Raji induced with TPA and butyrate, lane 3 contained BL16 BL cells harboring latent type B virus, lane 4 contained the X50-7 cord blood line harboring latent type A virus, and lane 5 contained BJAB BL cells with no EBV genome present. The diagram identifies the bands corresponding to EBNA1 and EBNA2, and the arrows on the left indicate VCA bands present in the induced B95-8 lane.

were completely specific when the primers were tested against inappropriate cDNA targets (Fig. 4A).

These four 5' primers were tested on total RNAs extracted from EBV-positive LCLs B95-8 (a C promoter-utilizing line) and X50-7 (a W promoter-utilizing line). The RNA was reverse transcribed by using the 3' common W2W1 splice junction oligonucleotide primer. The cDNA representing 10^4 cells was then aliquoted into PCR reactions with the four 5' primers and cycled through 25 amplifications. The products were electrophoresed on 2% agarose, blotted, and probed with a W1W2 splice junction probe (Fig. 4B). Two observations were made. (i) The cell lines did appear to be exclusive in the use of C (B95-8) or W promoters (X50-7). Even at the PCR level of sensitivity, no leakiness of the nonfunctional promoter was observed. (ii) In both cell lines, the two alternative splices seemed to be made with approximately equal frequencies. This latter observation was difficult to confirm by the rudimentary quantitative PCR analyses we performed, but the conclusion is supported by S1 primer extension analyses on unamplified RNA (23). We made reconstructions by using B95-8 or X50-7 cells diluted into a background of MOLT-4 cells and with 10 additional cycles of amplification were able to detect viral RNA from fewer than 10 cells, a range we suspected would be sufficient to detect EBV messages in PBLs.

For these studies, we used spontaneous LCLs isolated from healthy seropositive adults as controls for PCR amplification. The total RNA preparation for each of four spontaneous LCLs was amplified with the four primers for C and W as described above for B95-8 and X50-7 (Fig. 4C). All of the cell lines were C start users, as anticipated from previous studies, and made both C2W1 and C2W1' splices. In addition, all of the LCLs used the W promoter but appeared to make more W0W1 splices than W0W1' splices. It is not clear why these LCLs display dual promoter usage, but it may

have to do with the spontaneous lytic activation state, which is very high in LCLs of adult PBL origin. All of these spontaneous LCLs produce early antigens and VCAs, as determined by Western immunoblotting with human antiserum and RNA PCR with immediate-early, early, and late gene targets (data not shown). This observation was fortunate, because it allowed us to establish that all four 5' primers work on the in situ latent viruses from these individuals.

PBLs were collected from the four subjects and immediately processed by positive selection into B and non-B cells. The B cells were lysed, and total RNA was prepared. An aliquot representing approximately 5×10^6 cells was used in a cDNA reaction with the W2W1 splice junction oligonucleotide as the primer. This quantity of B cells might be expected to contain between 5 and 200 latent EBV-infected cells (16, 39, 40). Our preliminary estimates using PCR and spontaneous-outgrowth assays suggest that the number is closer to 5 than to 200 (data not shown). We did not obtain ratios of infected to uninfected B cells that ever approached the 1:25,000 range that other investigators have reported for normal, healthy seropositive adults (40).

The cDNA product was amplified through 35 cycles of PCR with each of the four 5' primers and analyzed by Southern hybridization with a W1W2 splice junction oligonucleotide probe (Fig. 4D). The results revealed that the PBLs from three of the four donors had latently infected B cells which contained RNA initiated from the C promoter and processed only the C2W1 splice. RNA from one donor did not produce a detectable amplification product. This experiment was repeated twice with subsequent isolations of RNAs from these donors spanning a 6-month period. One of the positive donors scored negative on one occasion, and the consistently negative donor never gave a positive result for C promoter expression.
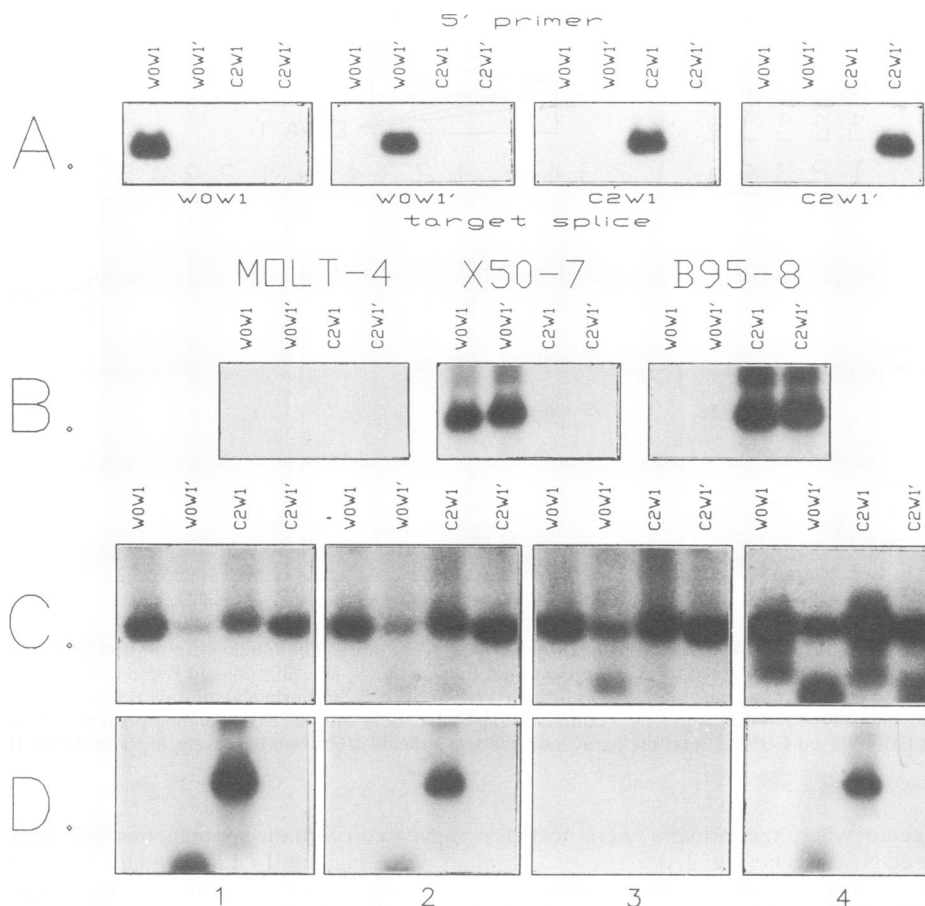
FIG. 4. RNA PCR for the EBNA transcription unit C or W promoter. (A) Specificity of oligonucleotide primers used to detect alternative splices in C or W promoter-initiated RNA. Target cDNAs with the four alternative splices were amplified in four separate reactions with the W2W1 primer as the 3' anchor and one of the four splice-specific 5' primers. Target cDNA was amplified for 30 cycles, blotted, and probed with a ^{32}P-labeled 20-mer spanning the W1W2 splice junction. (B) Total RNAs from MOLT-4, B95-8 (C promoter user), and X50-7 (W promoter user) were amplified with the four alternative 5' primers. (C) RNA PCR with spontaneous LCLs and B cells from four healthy seropositive donors. Total RNA from spontaneous LCL was amplified with the C and W 5' promoter and splice-specific primers. The four primers amplified mRNAs with the requisite structure from each cell line. (D) RNA extracted from 5×10^6 purified peripheral blood B cells was amplified for 35 cycles under the same conditions. Only the C2W1 target was detected by Southern hybridization.

Expression of EBNA1, EBNA2, EBNA3A, EBNA3B, and EBNA3C. The primers used for analysis of C and W promoter usage were designed to demonstrate that the spliced transcripts contained both W1 and W2 exons. In our experiences with PCR, and especially with RNA PCR, we have found that targets of fewer than 500 bases are the most efficient and therefore the easiest to detect from complex mixtures after amplification. When the PCR primers for the downstream ORFs were anchored at or near the 5' end of the transcript and contained a 'W1W2' repeating unit internally, the target size was greater than 1,000 bp and the yield from PCR reactions with LCL RNA targets was poor. In reconstruction experiments diluting LCL RNA into a background of EBV-negative RNA, these targets were found to be unsuitable for potential detection of expression from B-cell RNA preparations in which the number of expressing cells might be below 100 per reaction.

It was therefore necessary to use primers located in downstream leader exons as 5' anchors for the EBNA genes. For the EBNA2 and monocistronic EBNA4 messages, the 5' oligonucleotide anchor was placed within the W2 exon of the repeats. Although multiple initiation sites were present in long W1W2 repeat-containing cDNAs, successive rounds of amplification strongly favored short (<500 per bp) single-repeat or lone W2-containing fragments. Reconstructions were performed in which B95-8 cells were diluted into a background of 10^6 MOLT-4 cells and RNA was extracted and subjected to 35 cycles of RNA PCR amplification (Fig. 5A). These mock experiments showed that when the 5' W2 primer was used in combination with the 3' Y3P and Y3H1 primers, the products of both of these targets were detectable by Southern hybridization down to a dilution of 1 in 5×10^6 cells.

The tracks in Fig. 5B which contained the LCL RNA amplifications for the EBNA2 and EBNA4 targets contained multiple species. In the EBNA2 LCL amplifications, the band at 381 bp represents a cDNA with the structure W2-Y1-Y2-Y3P. The bands at 577 and 773 represent similar products which contained one or two additional W1-W2 repeats, respectively. The band at 513 bp has the structure W2-W2-Y1-Y2-Y3P, and the bands at 709 and 905 bp include additional W1-W2 repeats. These results come from probing
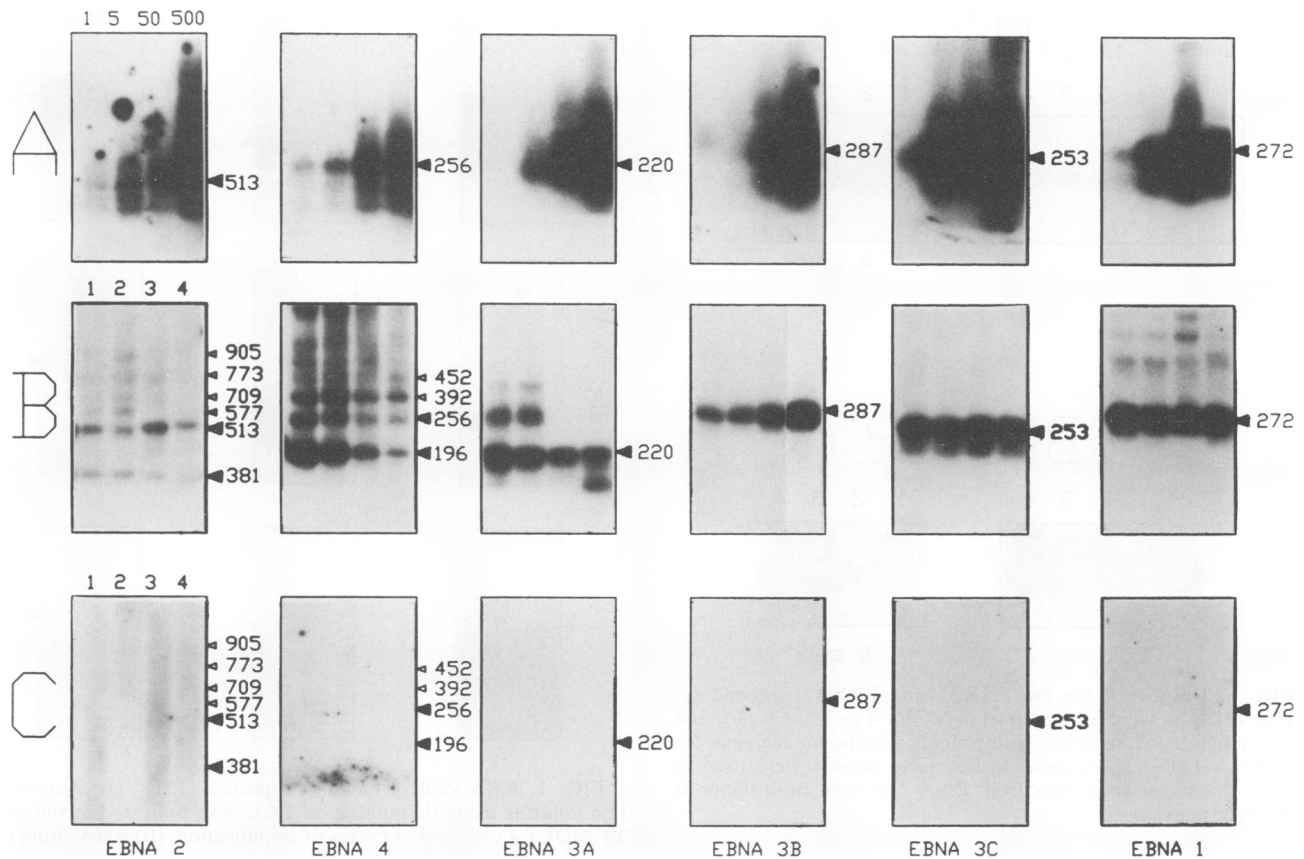
FIG. 5. RNA PCR of EBNA ORFs. (A) Amplification controls consisting of the number of LCL cells indicated mixed with 10^6 MOLT-4 cells, RNA extracted, and amplified for 35 cycles with targets for the EBNA genes. (B) RNAs from spontaneous LCLs amplified with the same primers. Dark triangles indicate the positions and sizes (in base pairs) of the predicted bands. Open triangles locate the positions of minor components which appear in one or more of the cell lines. (C) B-cell RNA from the same donors amplified through 35 cycles with the same target primers. Each blot was probed for an oligonucleotide sequence near the 3' end of the target in the ORF for the EBNA product.

of the Southern blots with probes for different exons and limited preliminary direct sequencing experiments on isolated bands. Use of the same techniques showed that the band at 256 bp in the EBNA4 LCL panel has a structure of W2-Y1-Y2-Y3-H1 and the band at 196 bp has the structure W2-Y1-Y2-H1. The bands at 452 and 392 bp, respectively, represent similar cDNA structures that contain additional W1-W2 repeat exons. None of the products characteristic of EBNA2 or EBNA4 monocistronic cDNA structures were detected in the B-cell RNA preparations (Fig. 5C).

To screen for the presence of cDNA products representing expression of the downstream EBNA gene products (EBNA1, EBNA3A, EBNA3B, and EBNA3C), we used 3' primers located near the 5' end of the EBNA ORF and 5' primers located in W2, the F exon, and the U1 exon. The banding patterns of amplified cDNA products from LCLs using W2 and FQ as the 5' anchors were complex, largely as a result of alternative splicing, and are being investigated separately (data not shown). No comparable bands were detected in amplifications from B-cell RNA using these primers (data not shown). An important point, however, is that the principle bands detected in amplifications of LCL-derived cDNAs of EBNA1, EBNA3A, EBNA3B, and EBNA3C messages all contained the U1 exon. A 5' primer located in U1 used in combination with the L1, E2, E3, or K1 3' primer produced readily interpretable diagnostic band-

ing on Southern blots. The 220-bp (U1-L1), 287-bp (U1-E2), 253-bp (U1-E3), and 272-bp (U1-K1) bands represented splices from U1 to the ORFs for EBNA3A, EBNA3B, EBNA3C, and EBNA1, respectively. The structures of the additional bands that appeared in the L1- and K1-primed amplifications were not determined. Reconstructions using LCLs diluted into a background of MOLT-4 cells produced detectable banding after 35 cycles of amplification down to 5 LCL cells per reaction for L1, E3, and K1 targets and 50 cells per reaction for the E2 target (Fig. 5A). By using hyperamplification with the same primers or nested primer sets, we were able to detect diagnostic bands from single-cell-containing reactions (data not shown). We were unable to detect U exon-EBNA ORF splice-containing transcripts in the RNA from PBLs (Fig. 5C). This strategy was promoter independent in the sense that products of any promoter, known or unknown, would have been detected so long as that promoter was located upstream of the U exon and the U exon was spliced into the final product. One possible reason for the failure to detect transcripts from the EBNA genes is that the DNA encoding these genes is not present in the B cells used to make the RNA. Although we used spontaneous LCLs derived from the peripheral blood of the same donors as the positive controls (Fig. 5), it has been suggested that cells grown in culture may not be representative of EBV genomes in the circulation. We
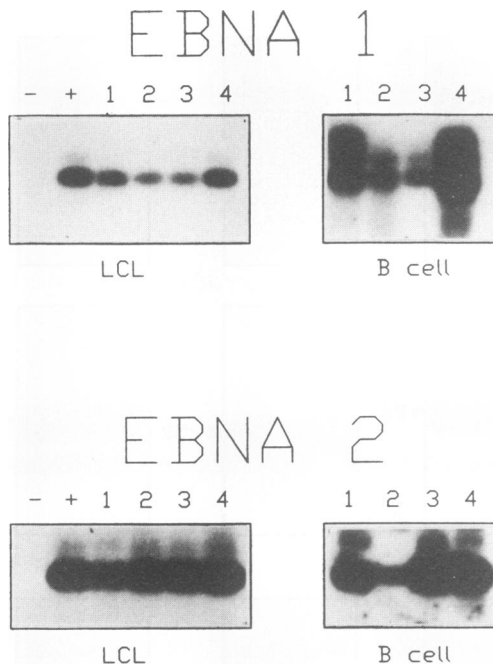
FIG. 6. DNA PCR for the EBNA1 and EBNA2 genomic sequences. DNAs were extracted from MOLT-4 (−), B958 (+), and the four LCLs and amplified with primers specific for the gene for EBNA1 or EBNA2 (left panels). The same primers were used to amplify the target sequences from DNA extracted from donor B cells (right panels).

therefore took purified peripheral blood B cells, extracted DNA rather than RNA, and analyzed the samples for the presence of the unexpressed sequences of the genes for EBNA1 and EBNA2. The EBNA1 target sequence from map positions 107838 to 108048 was 210 bp long and contained the EBNA1 splice acceptor site, the initiator methionine codon, and the coding sequences for the first 33 amino acids. The 210-bp band was detected in all of the LCLs and the B cells, indicating that the 5' side of the EBNA1 gene was intact and present in the PBLs of all of the donors (Fig. 6). The EBNA2 target sequence from map positions 48419 to 48580 was 161 bp long and contained the 3' splice donor of the Y3 exon, the upstream untranslated region, the initiator methionine, and coding sequences for the first 26 amino acids of EBNA2. Similarly, the 161-bp band was detected in all LCLs and B cells (Fig. 6), indicating that the reason for the lack of transcripts from the EBNA2 and EBNA1 regions in B cells was not absence of the DNA encoding these genes.

Expression of mRNA for the viral membrane proteins in PBLs. Membrane proteins TP1 and TP2 are made from two alternative promoters that produce messages which have a unique 5' exon (T1 or T1') spliced onto nine common downstream exons (T2 to T9). A 3' primer located in T2 was used in conjunction with T1 (for TP1 messages) or T1' (for TP2 messages) to amplify RNA from the spontaneous LCLs and uncultured B cells. Amplification of LCL RNA with the T1 primer produced the diagnostic 168-bp band predicted from the published cDNA structure and a series of minor bands (Fig. 7). The TP2 amplification target also yielded a doublet band of 173 bp (the predicted fragment) and 140 bp when LCL RNA was used in the PCR. Reconstructions in which LCLs were titrated into a background of MOLT-4
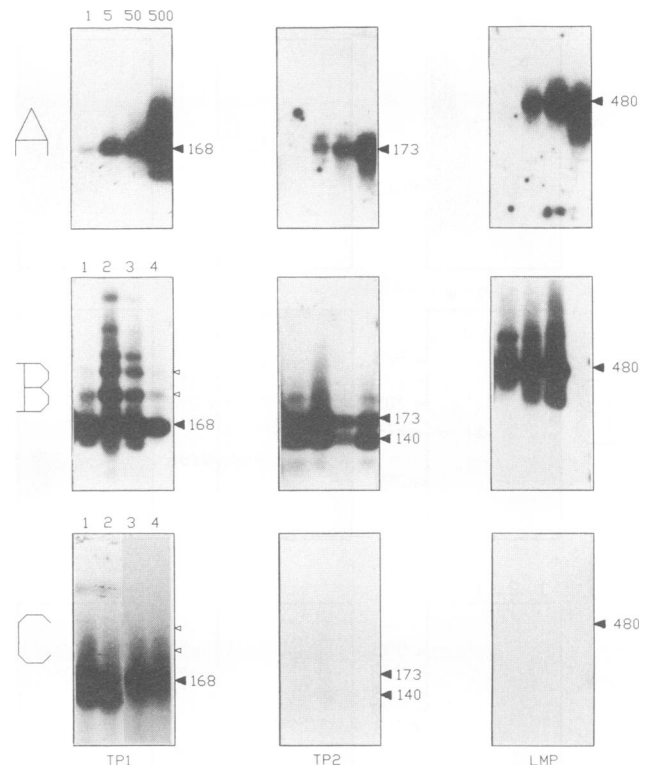

FIG. 7. RNA PCR of membrane protein ORFs. (A) Amplification controls using the number of LCL cells indicated mixed with 10^6 MOLT-4 cells and 35 cycles of amplification. (B) RNAs from the spontaneous LCLs. The 168-, 173-, and 480-bp bands are diagnostic fragments corresponding to TP1, TP2, and LMP mRNA structures. (C) Donor B-cell RNA amplified with the same primer sets. Each blot was probed with a ^{32}P-end-labeled oligonucleotide specific for a sequence near the 5' end of the target molecule.

EBV-negative lymphocytes and then RNA extracted and PCR amplified showed that the TP1 target was detectable at the one-cell-per-reaction level while the TP2 target was detected at the five-cell-per-reaction level. All of the B-cell RNA preparations were positive for the 168-bp diagnostic TP1 fragment, and two also had the lower 140-bp fragment detected in some of the LCLs. The TP2 amplification target was not detected in the same B-cell RNA preparations (Fig. 7).

The LMP gene product is expressed from a promoter operating in latently infected cells or from a separate promoter which is active in productive lytic viral replication (7, 27, 32). We designed the LMP target for PCR to detect the latent type of transcript which includes two splices in a product of 480 bp (Fig. 2). Reconstructions using the LMP primers and LCLs showed that the LMP target fragment of 480 bp was detectable at the five-cell-per-reaction level (Fig. 7A). In the positive-control spontaneous LCLs, one cell line failed to score positive (Fig. 7B). This cell line was also negative for LMP by Western immunoblotting. A coarse examination of the viral DNA structure for mutations was not done; however, both the TP1 and TP2 promoters which flank the LMP gene are active in this cell line. The latter promoter actually overlaps and shares control sequences with the LMP latent promoter. The exact reason why this cell line does not show LMP expression by PCR is not known. All of the peripheral B-cell RNA preparations were

negative for LMP expression by PCR. This gene does not appear to be expressed in latently infected, uncultured B cells.

DISCUSSION

We have attempted to define the role of EBV gene expression in viral latency in healthy adult carriers. Several types of latent virus-cell interactions have already been described (14). These occur in immortalized cell lines maintained in vitro, BL cells, nasopharyngeal carcinoma cells, and other lymphoproliferative syndromes. The LCLs express the full range of six EBNA proteins, LMP, TP1, and TP2. The C promoter is most often used, and the productive EBNA4 splice is made in roughly half of the messages (23). BL cells, and the group I phenotype cell lines established with them, express EBNA1 only and utilize a newly described latent promoter (F promoter) to accomplish this feat of restricted expression (28). Nasopharyngeal carcinomas display similarly limited EBNA expression but also express the LMP gene, and the only cell line established from nasopharyngeal tumors also expresses TP2 RNA (5, 6, 30).

None of the above virus-cell interactions necessarily reflects the normal condition of viral gene expression in latency, since all occur under peculiar circumstances with abnormal cells. Normal latent B cells are accessible through the blood circulation but represent a minute fraction of the total lymphocytes. When cultured, these cells have the morphology, growth characteristics, and viral latent gene expression of in vitro-immortalized cells (16, 39). To circumvent the in vitro changes, we chose a rapid positive selection procedure to purify B cells from blood lymphocyte preparations and lyse them in RNA extraction buffer within 3 h of phlebotomy. We developed a set of PCR primers for the spliced latent genes and their promoters on the basis of structures known from cDNA sequence analyses. This work therefore defined the latent viral gene expression in uncultured B cells only in terms of the latent states observed in classical tumors and in vitro-immortalized cells. Alternative mRNA structures and other genes were not considered. In addition, it should be noted that the PCR assay we used is only an indirect measure of promoter activity. The positive results obtained can be interpreted as the long-lived stable remnants of past expression in cells that no longer actively synthesize RNA for these genes.

Spliced transcripts from two of the latent promoters were detected. The products of the C promoter for the EBNA genes were detected in three of the four B-cell preparations from the subjects for whom spontaneous LCL controls were available. Including uncontrolled B-cell samplings from other individuals, C promoter activity was detectable in about 50% of the cases. Only the C2W1 nonproductive splice for EBNA4 gene expression was detected, indicating that a level of control over EBNA4 gene expression that has been suspected to occur appears to happen in peripheral blood B cells. Despite repeated attempts and hyperamplifications of RNA PCR reactions, it was not possible to detect the 3' structures of EBNA genes in the RNA samples that were positive for C promoter-initiated messages. These failures may mean that these structures do not occur in peripheral blood B cells and that a novel undefined 3' end exists. The only other latent promoter whose transcripts were detected was TP1. TP1 RNA was present in all of the B-cell RNA samples we examined, including uncontrolled samplings from six other individuals.

The state of latent viral gene expression in uncultured B

cells thus appears to be unique. The TP1 gene seems to be always expressed, and the C promoter is often active. These findings are consistent with the hypothesis that a minimally active viral episome evades immune recognition of the cytotoxic T-cell epitopes on its EBNA2, EBNA3, and LMP proteins in a resting B cell. EBNA1 would not be necessary to maintain a viral genome in a nondividing cell. The function of TP1 in this latent scheme remains unclear; however, we have noted that it is a highly labile protein in LCLs (24). TP1 has recently been found in plasma membrane-containing fractions of lymphoblastoid cells and may be associated with protein tyrosine kinases (17, 27). A role for TP1 in reactivation of the latent virus in response to appropriate stimuli could be considered very possible.

One question that arises from these studies concerns the generation and maintenance in healthy adults of the antibody responses against the EBNA proteins and the EBV capsid components (Fig. 3). We had anticipated that at least EBNA1 messages would be found since all our subjects and most, if not all, humans latently infected with EBV produce anti-EBNA1 antibodies. We did not find evidence of EBNA gene expression in latently infected cells. It therefore seems more likely that these responses are not made against latently infected B cells but rather might develop because of periodic reactivation of virus in latently infected cells (presumably not in circulation) and/or from the persistent challenge of new infections from the environment.

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