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Epstein-Barr Virus Latency in Blood Mononuclear Cells: Analysis of Viral Gene Transcription during Primary Infection and in the Carrier State

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Epstein-Barr virus (EBV) can display different forms of latent infection in B-cell lines in vitro; however, the types of infection normally established by the virus in vivo remain largely unexplored. Here we have approached this question by analyzing the types of viral RNAs present in mononuclear cells freshly isolated from the blood of 14 infectious mononucleosis patients undergoing primary EBV infection and 6 long-term virus carriers. Reverse transcription-PCR amplifications were carried out with a panel of oligonucleotide primers and probes which specifically detect (i) the EBER1 RNA common to all forms of latency, (ii) transcripts either from the Cp and Wp promoters generating all six nuclear antigen (EBNA1, -2, -3A, -3B, -3C, -LP) mRNAs or from the Fp promoter generating a uniquely spliced EBNA1 mRNA, (iii) the latent membrane protein (LMP1 and 2A) mRNAs, and (iv) the BZLF1 mRNA, an immediate-early marker of lytic cycle. Viral transcription in infectious mononucleosis mononuclear cells (and in the B-cell-enriched fraction) regularly included the full spectrum of latent RNAs seen during EBV-induced B-cell growth transformation in vitro, i.e., EBER1, Cp/Wp-initiated EBNA mRNAs, and LMP1/LMP2 mRNAs, in the absence of lytic BZLF1 transcripts. In addition, transcripts with the splice pattern of Fp-initiated EBNA1 mRNA, hitherto seen only in vivo in certain EBV-positive tumors, were frequently detected. In long-term virus carriers, the mononuclear cells were again positive for latent (EBER1) and negative for lytic (BZLF1) markers; Cp/Wp-initiated RNAs were not detected in these samples, but in several individuals it was possible to amplify both Fp-initiated EBNA1 mRNA and LMP2A mRNA signals. We suggest (i) that primary infection is associated with a transient virus-driven expansion of the infected B-cell pool through a program of virus gene expression like that seen in in vitro-transformed cells and (ii) that long-term virus carriage is associated with a switch from Cp/Wp to Fp usage and thus to a more restricted form of latent protein expression that may render the infected cells less susceptible to recognition by the virus-specific cytotoxic T-cell response.

Epstein-Barr virus (EBV), a gammaherpesvirus widespread in human populations, naturally infects two major target cell types in vivo, pharyngeal epithelium and mature B lymphocytes (25). Pharyngeal epithelial cells appear to be naturally permissive, with virus replication in differentiating squamous cells leading to the release of infectious virions into buccal fluid (10, 42). In contrast, B lymphocytes harbor the virus as a nonproductive (latent) infection (28), and it is this interaction which appears central to the phenomenon of viral persistence (9, 52). Studies on infectious mononucleosis (IM) patients show that a large pool of latently infected B cells is generated in vivo during the course of primary infection (16, 20, 47). Although this pool is substantially reduced in size once the host cell-mediated immune response develops (32, 34), small numbers of infected B cells persist in the blood and lymphoid tissues of virus carriers, detectable by virtue of their ability to give rise to EBV-transformed lymphoblastoid cell lines (LCLs) by spontaneous outgrowth in vitro (28, 53). An important objective, therefore, is to identify the types of EBV–B-cell interaction which will allow on the one hand efficient colonization of the lymphoid system during primary infection and on the other hand long-term persistence of infected cells in the face of sustained host immune responses.

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The present approach to this question draws upon information already at hand from the various in vitro models of EBV latency. The best characterized of these models comes from the experimental infection of normal resting B lymphocytes and their consequent growth transformation to LCLs (19). Such transformed cells carry episomal virus genomes and constitutively express nine EBV latent proteins, namely, the nuclear antigens EBNA1, -2, -3A, -3B, -3C, and -LP and the latent membrane proteins LMP1, -2A, and -2B. Viral transcription in this situation is quite complex; long primary transcripts driven first from the viral *Bam*HI W promoter (Wp) and then later in the transformation process from the adjacent *Bam*HI C promoter (Cp) are differentially spliced to produce the individual EBNA mRNAs (49, 50), whereas the three LMP transcripts are expressed from different promoters in the *Bam*HI N region of the genome (14, 21, 40). In addition, there is abundant expression of the small noncoding EBER1 and EBER2 RNAs (13, 23) and of a family of transcripts in the *Bam*HI A region of the genome (4, 15) whose coding capacity is still in question. We now refer to this characteristic LCL pattern of gene expression as latency III.

More recently, work with EBV-positive tumors has revealed alternative forms of viral latency which, although sharing EBER and *Bam*HI A RNA transcription with latency III (4, 8, 12, 37), express different patterns of latent mRNAs and hence of latent proteins. Thus, in latency I, as seen in Burkitt's lymphoma (BL) biopsy cells and early-passage BL lines, there is selective expression of a single viral protein, EBNA1, via a

unique *Bam*HI Q/U/K-spliced EBNA1 mRNA transcribed from the *Bam*HI F promoter (Fp); in this situation the Wp, Cp, and LMP promoters are silent and hence the other latent proteins are not expressed (37, 38, 41). Latency II was first recognized in the context of an epithelial tumor, nasopharyngeal carcinoma (7, 55), but can also be induced in B cells under certain circumstances in vitro (36). Here again, Fp is active and Cp and Wp are silent (3, 44), resulting in expression of EBNA1 in the absence of the other nuclear antigens. However, in this form of infection there is detectable transcription from the LMP promoters (3, 5, 43), resulting in expression of the LMPs at anything from trace amounts (in which case distinctions between latency I and II become somewhat arbitrary) up to LCL-like levels.

Given the spliced nature of the viral mRNAs expressed in latent infections, it is possible to design oligonucleotide primers and probes which can specifically detect individual transcripts (or families of transcripts from a common promoter) by reverse transcription and PCR (RT-PCR) amplification. We have already used such an approach to identify the patterns of virus promoter usage, and hence the resident form(s) of virus latency, in EBV-infected cells either in experimentally manipulated in vitro systems (17) or in snap-frozen tumor biopsy material (3, 6). In the present work, we have developed an extended panel of such oligonucleotide primers and probes to analyze by RT-PCR the types of EBV RNAs detectable in mononuclear cells directly isolated from the blood of acute-IM patients and of healthy virus carriers.

MATERIALS AND METHODS

Cell lines. The following cell lines were used as reference populations: Ramos is an EBV-negative sporadic BL line; Akata and WW2-BL are EBV-positive BL lines showing a stable latency I phenotype (36, 37); ODHI-BL and MAK-BL are EBV-positive BL lines studied in relatively early passage, when most cells exhibited latency I, but in which a small subpopulation had switched to latency III (11); AG876 is a late-passage EBV-positive BL line which has fully progressed to latency III (22); IB4 and B95.8 are EBV-transformed LCLs exhibiting latency III and known either to be tightly latent (IB4) or to contain approximately 5% cells in lytic cycle (B95.8) (22). All cell lines were maintained in exponential growth in RPMI 1640 supplemented with 1 mM glutamine and 10% fetal calf serum prior to snap-freezing of the cells (or of appropriate mixtures of cells from different lines) in liquid nitrogen and storage at −80°C.

Donors. Heparinized blood samples (60 ml) were taken from 14 patients in the acute phase of IM within 1 to 3 weeks of the appearance of symptoms at a time of elevated blood leukocyte counts and the presence of atypical lymphoblasts in the leukocyte population; diagnosis of IM was confirmed by detection of immunoglobulin M antibodies to the EBV capsid antigen. In all cases, unfractionated mononuclear (UM) cells were prepared from blood by isopycnic centrifugation, using lymphocyte separation medium (Flow Laboratories), and washed twice in RPMI 1640, and then a cell pellet was immediately snap-frozen in liquid nitrogen prior to storage at −80°C. In certain cases with high cell yields (IM 32 and 33), part of the UM cell population was further separated into T-cell-depleted and T-cell-enriched fractions by E rosetting and rescue of rosetted cells by NH_4Cl lysis (26); cells of each fraction were likewise immediately snap-frozen and stored. In certain other cases with high cell yields (IM 12, 16, 17, and 27), part of the UM cell population was seeded at 10^7 cells per ml in complete medium (see above) and cultured for 2 days at 37°C before harvesting and snap-freezing. Some patients (IM 12, 19, and 20) also provided a second blood sample 3 months after the resolution of symptoms. Blood samples (120 ml) were also taken on three occasions from each of six healthy EBV-seropositive donors (i.e., long-term virus carriers) and two EBV-seronegative control donors.

RNA extraction and RT-PCR amplification. RNA was isolated from the above snap-frozen cell pellets (usually within 1 to 4 days of storage) by extraction in 8 ml of guanidinium isothiocyanate-phenol (RNAzol; Cinn/Biotex). Cells were homogenized by pipetting, 0.8 ml of chloroform was added, and the suspension was mixed vigorously. The homogenate was centrifuged at 9,000 rpm for 30 min, and RNA was precipitated from the resultant aqueous phase by the addition of an equal volume of ice-cold isopropanol for 30 min on ice. The precipitate was then pelleted by centrifugation at 9,000 rpm for 15 min, washed in ice cold 70% ethanol, then resuspended in 100 μl of sterile diethylpyrocarbonate-treated RNase-free water, and stored at −80°C.

RT-PCR amplifications were carried out on stored RNA samples representing the yield from between 10^6 and 10^7 cells, depending on the cell populations being analyzed. RNA samples were initially heated for 2 min at 90°C and then rapidly cooled on ice. RT-PCR was carried out in a one-tube reaction using components and conditions described in detail elsewhere (3). The products from 40 cycles of amplification were analyzed by electrophoresis through 3% agarose gels, then Southern transferred onto Hybond N+ nylon membranes (Amersham), and detected by hybridization to an end-labeled oligonucleotide probe specific for the amplified sequence. In one set of experiments, a second round of 40 cycles of amplification was carried out with nested primers and, as a substrate, one-fifth of the product of the first round of amplification. The primer-probe combinations used in this work are identified in Table 1, which shows their individual transcript specificities, exon derivation (where appropriate), coordinates on the B95.8 EBV genome, and nucleotide sequences (1).

RESULTS

Sensitivity of oligonucleotide primer-probe combinations used in RT-PCR analysis. The specificity of the various oligonucleotide primer-probe combinations listed in Table 1 for individual EBV transcripts either has already been documented (17, 38) or was confirmed in a series of RT-PCR assays conducted as a preliminary to the present work on RNA from reference cell lines. We then sought to determine the relative sensitivities of these reagents by screening on cell mixtures containing a standard number of EBV-negative Ramos cells and serial 10-fold dilutions (10^{-2} to 10^{-6}) of the following EBV-positive reference lines: the tightly latent Akata-BL and WW2-BL lines representing latency I and the tightly latent IB4 LCL and the virus-productive B95.8 LCL representing latency III. The RNA extracted from aliquots (2×10^6 cells each) of these mixed populations was subjected to RT-PCR analysis using the different primer-probe combinations. Typical results from experiments with the Akata-Ramos, IB4-Ramos, and B95.8-Ramos mixtures are illustrated in Fig. 1 and 2; unless otherwise stated, titrations involving WW2-BL gave endpoints identical to those shown for Akata-BL.

We first targeted for amplification the small noncoding EBER1 RNA, since this is the most abundant EBV latent transcript (23) and is also common to all known forms of latency (8, 37). As shown in Fig. 1, EBER1 could readily be detected in mixtures containing 10^{-6} dilutions of EBV-positive cells in a Ramos cell background, i.e., at a loading of two

TABLE 1. Oligonucleotide primers and probes used in RT-PCR analysis

Transcript	Primer or probe designation[a]	Genome co-ordinates[b]	Oligonucleotide sequence
	5′ primer	6776–6795	5′-AAAACATGCGGACCACCAGC
EBER1 RNA	3′ primer	6648–6629	5′-AGGACCTACGCTGCCCTAGA
	Probe	6718–6737	5′-ACGGTGTCTGTGGTTGTCTT
Cp- and Wp-initiated mRNAs			
Cp initiated	5′ primer (C_1C_2 exon splice)	11470–11479/11626–11635	5′-CATCTAAACCGACTGAAGAA
Wp initiated	5′ primer (W_0W_1 exon splice)	14399–14410/14554–14558	5′-GTCCACACAAATCCTAG
Cp and Wp initiated	3′ primer (W_2 exon)	14832–14813	5′-CCCTGAAGGTGAACCGCTTA
Cp and Wp initiated	Probe (W_2 exon)	14740–14721	5′-TGGGCGACCGGTGCCTTCTT
EBNA1 mRNAs			
Cp and Wp initiated	5′ primer (Y_3 exon)	48397–48416	5′-TGGCGTGTGACGTGGTGTAA
Fp initiated	5′ primer (Q exon)	62440–62457	5′-GTGCGCTACCGGATGGCG
Cp, Wp, and Fp initiated	5′ primer (U exon)	67483–67502	5′-TTAGGAAGCGTTTCTTGAGC
Cp, Wp, and Fp initiated	3′ primer (K exon)	107986–107967	5′-CATTTCCAGGTCCTGTACCT
Cp, Wp, and Fp initiated	Probe (U exon)	67544–67563	5′-AGAGAGTAGTCTCAGGGCAT
	5′ primer (W_2 exon)	14802–14822	5′-AGAGGAGGTGGTAAGCGGTTC
EBNA2 mRNA	3′ primer (Y_3/P)	48583–48562	5′-TGACGGGTTTCCAAGACTATCC
	Probe (Y_2 exon)	47885–47904	5′-GAGAGTGGCTGCTACGCATT
	5′ primer (exon 1)	169262–169243	5′-CTTCAGAAGAGACCTTCTCT
LMP1 mRNA	3′ primer (exon 2)	169081–169100	5′-ACAATGCCTGTCCGTGCAAA
	Probe (exon 1/2 splice)	169216–169206/169128–169119	5′-CTACTGATGATCACCCTCCT
	5′ primer (exon 1)	166874–166893	5′-ATGACTCATCTCAACACATA
LMP2A mRNA	3′ primer (exon 3)	380–361	5′-CATGTTAGGCAAATTGCAAA
	Probe (exon 2)	62–81	5′-ATCCAGTATGCCTGCCTGTA
	5′ primer (exon 1)	102719–102700	5′-TTCCACAGCCTGCACCAGTG
BZLF1 mRNA	3′ primer (exon 2/3 splice)	102330–102341/102426–102433	5′-GGCAGCAGCCACCTCACGGT
	Probe (exon 2)	102450–102469	5′-CTTAAACTTGGCCCGGCATT

[a] Some of the primer-probe combinations used here (for Cp- and Wp-initiated mRNAs, for EBNA1 mRNAs, and for LMP2A mRNA) have been validated in previous work (17); note that the combination used here for LMP1 mRNA proved at least 10-fold more sensitive than that used in earlier work (17).
[b] Genome coordinates are given with reference to the B95-8 genomic sequence (1).

infected cells per sample from which RNA was prepared. We then turned to sets of primers which specifically amplified transcripts either with the $C_1/C_2/W_1/W_2$ splice structure typical of Cp-initiated EBNA mRNAs or with the $W_0/W_1/W_2$ splice structure typical of Wp-initiated EBNA RNAs. Using the C_1/C_2 5′ and W_2 3′ primer combination specific for Cp-initiated transcripts, it was possible to detect the presence of such RNAs at 10^{-5} dilutions of the Cp-using B95.8 line; similar results could also be obtained with other Cp-using LCLs (data not shown). The specificity of these Cp primers is here illustrated by the absence of signals from Akata-BL, in which Cp is silent (22), and from the IB4 LCL, in which Cp is deleted (50). Conversely the W_0/W_1 5′ and W2′ 3′ primer combination clearly detected Wp-initiated EBNA transcripts at 10^{-3} dilutions of the IB4 line (and of a second Wp-using line, AG876; data not shown); the ability of these primers also to amplify RNA from B95.8 must reflect either some low-level Wp usage in that line or possibly the presence of some erroneously spliced Cp-initiated RNAs. Figure 1 also shows the results obtained with primer-probe combinations which were designed to distinguish between the two alternative forms of EBNA1 mRNA. Thus, EBNA1 transcripts with the *Bam*HI Y3/U/K splice structure, expressed from Cp/Wp and diagnostic of latency III, could easily be detected down to 10^{-3} dilutions either of the IB4 or of the B95.8 cell line but were never seen in Akata-BL. Conversely, transcripts with the *Bam*HI Q/U/K splice structure, expressed from Fp and a feature of latency I/II, were detectable even at 10^{-4} dilutions of Akata-BL (albeit as a weak signal) but not at all in the tightly latent IB4 line. It is important to note that Fp is activated in LCL cells when they switch from latency III into lytic cycle (22), and this explains the low levels of Q/U/K-spliced transcripts that are detectable in the virus-producing B95.8 cell line. Amplification with the primer combination specific for EBNA2 mRNA, another individual member of the Cp/Wp-initiated transcript family, gave a pattern of results and a sensitivity of detection similar to that observed with the primer combination specific for the Y3/U/K-spliced EBNA1 mRNA (data not shown).

The corresponding data for RT-PCR detection of LMP transcripts in the same cell mixtures are shown in Fig. 2. Expression of the LMPs is a consistent feature of LCLs, and indeed both LMP1 and LMP2A mRNAs were detectable at 10^{-5} dilutions both of IB4 and B95.8 cells. In parallel assays, there was no detectable LMP transcription in WW2-BL cells (data not shown) while Akata-BL cells were negative for LMP1 transcripts but, as illustrated, did yield a weak positive signal for LMP2A mRNA although only in the undiluted cell sample. Such findings are in line with earlier studies in which we have on occasions been able to detect low-level LMP2A transcription in serially passaged BL cell lines in the absence of any detectable Cp/Wp activation or cellular drift toward an LCL-like phenotype (reference 22 and unpublished observations). Note that an alternatively spliced LMP2 transcript encoding the LMP2B form of the protein is also regularly expressed in LCLs but at lower abundance than the LMP2A mRNA (38, 40); RT-PCR analysis for this transcript (3) was not included in

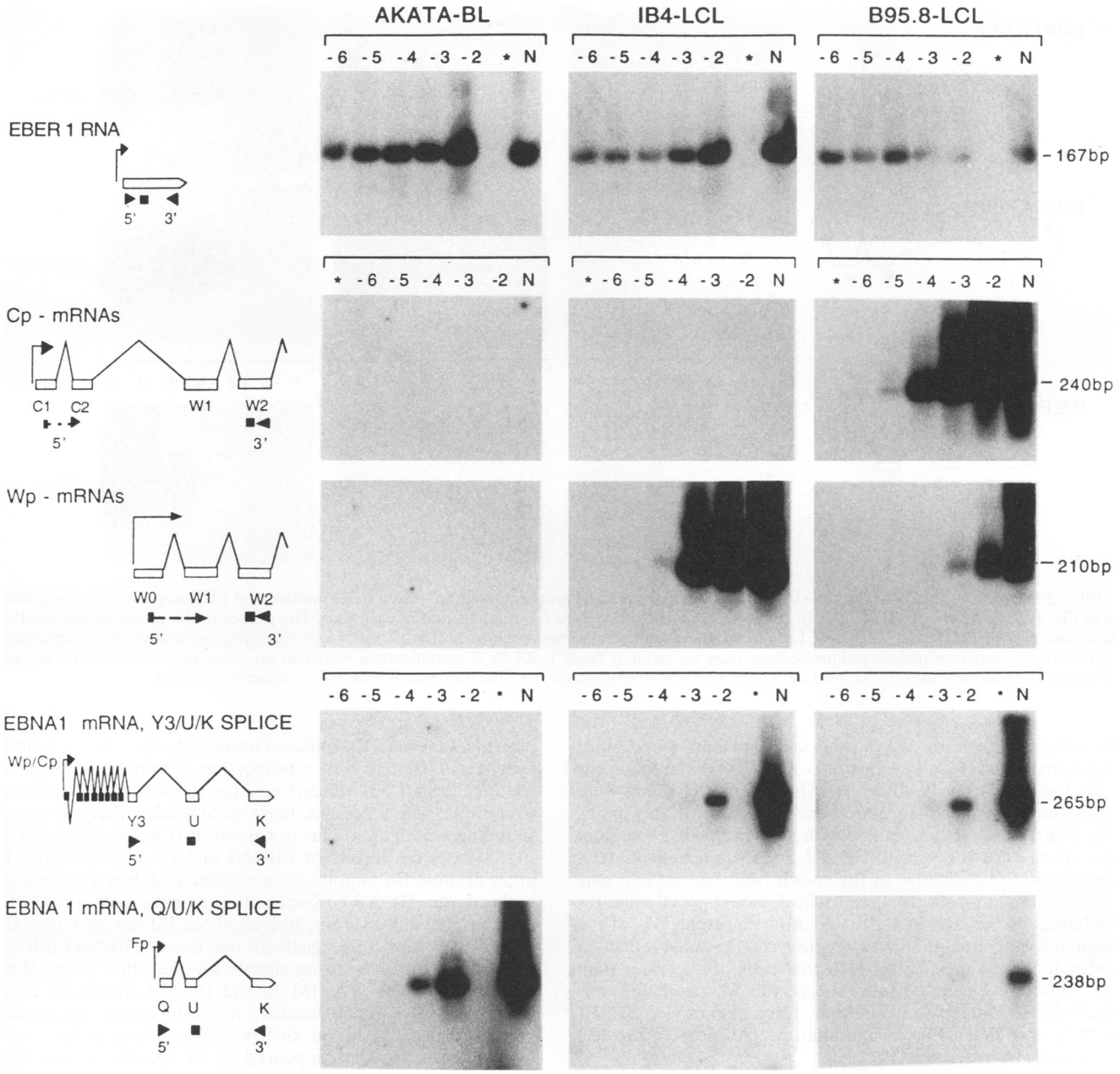

FIG. 1. Detection of EBV latent transcripts by RT-PCR analysis in RNA preparations from mixtures of EBV-positive and EBV-negative B-cell lines. Results are shown for the Akata-BL line (latency I), the IB4-LCL (latency III), and the B95.8 LCL (predominantly latency III, with 5% cells in lytic cycle), each tested as an undiluted population (N) or after dilution at 10^{-2} down to 10^{-6} in the EBV genome-negative Ramos line; RNA from a total of 2×10^6 cells was used as the template for amplification in each case (* denotes an empty track). The primer-probe combinations used for detection of EBER1 RNA, Cp-initiated mRNAs, Wp-initiated mRNAs, and the Y3/U/K-spliced and Q/U/K-spliced forms of EBNA1 mRNA are shown at the left below diagrams of the relevant transcripts; full details of primer and probe sequences are given in Table 1. Note that both Cp-initiated and Wp-initiated transcripts can display an alternative splice acceptor site (W_1') five nucleotides downstream of the W_1 splice acceptor (35). The Cp primer-probe combination used in this study will amplify both C_2/W_1-spliced and C_2/W_1'-spliced RNAs, yielding products of 240 and 235 bp, respectively, which cannot be resolved on these gels; the Wp primer-probe combination used will not detect RNAs with the W_0/W_1' splice. RT-PCR amplifications were carried out as described in Materials and Methods, the products were hybridized overnight with ^{32}P-labeled probe, and the autoradiographs were exposed either overnight (for EBER1 RNA, Cp-initiated mRNA, and Wp-initiated mRNA detection) or for 3 days (for EBNA1 mRNA detection). In each case, the RT-PCR products detected were of the correct predicted size (indicated at the right) based on already identified viral mRNA splice structures.

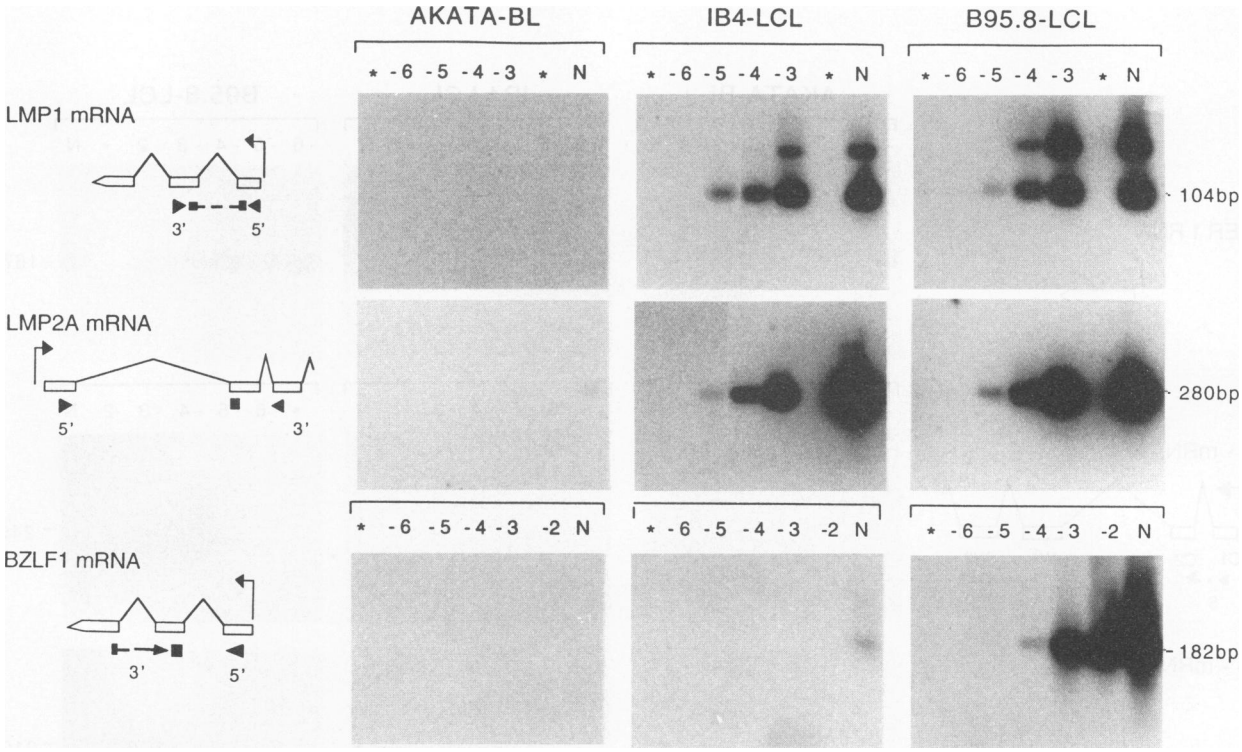

FIG. 2. Detection of EBV transcripts by RT-PCR analysis in RNA preparations from mixed EBV-positive and EBV-negative cell populations as in Fig. 1; RNA from a total of 2×10^6 cells was used as the template for amplification in each case. The primer-probe combinations used for detection of the latent cycle LMP1 and LMP2A mRNAs, and of the immediate-early BZLF1 mRNA of lytic cycle, are shown diagrammatically at the left; full details of primer and probe sequences are given in Table 1. RT-PCR amplifications were carried out as described in Materials and Methods, the products were hybridized overnight with ^{32}P-labeled probe, and the autoradiographs were exposed overnight.

the standard range of assays used in the present work, since preliminary assays on cell mixtures and on IM mononuclear cells indicated that LMP2B signals, where detected, were always much weaker than those observed for LMP2A. Finally, Fig. 2 also shows data obtained with a primer-probe combination specific for the spliced BZLF1 mRNA, an immediately-early transcript indicative of the switch from latency into lytic cycle (2). Such transcripts were easily detected in cell mixtures containing B95.8 cells at a 10^{-4} dilution; since only 5% of the original B95.8 population was in lytic cycle, this must reflect an ability to detect productively infected cells at a greater than 10^{-5} dilution. By these same criteria, the Akata-BL line was tightly latent whereas, interestingly, trace amounts of BZLF1 mRNA were detectable in undiluted preparations of the IB4 LCL (see also reference 39).

Transcriptional analysis of IM mononuclear cells. In the next series of experiments, blood mononuclear cells were prepared from 14 patients with serologically confirmed IM and processed to provide RNA for RT-PCR analysis of EBV transcripts. Each RNA preparation was tested on at least two occasions with each of the primer-probe combinations specified above, and consistent results were obtained. Figures 3 and 4 show the series of autoradiographs obtained when RNA samples from 11 of the 14 patients were assayed in parallel; each track contains the product from RT-PCR amplification of RNA from 5×10^6 IM mononuclear cells, or from 10^6 cells in the case of positive (B95.8) and negative (Ramos) control tracks.

From Fig. 3, it can be seen that EBER1 RNAs were easily detected in the mononuclear cells from all 11 of these IM patients. Likewise, Cp-initiated transcripts were also present in every case, whereas only a proportion of samples (IM 12, 16, 19, 27, 32, and 33) yielded positive signals for Wp-initiated transcripts; where present, these were weaker than the corresponding Cp signals. The remaining two autoradiographs in Fig. 3 show the results of EBNA1 mRNA amplifications. In many of these IM samples, we were able to detect the presence both of *Bam*HI Y3/U/K-spliced transcripts and of *Bam*HI Q/U/K-spliced transcripts. Indeed, those IM samples (e.g., IM 32 and 33) with strong signals for one form of EBNA1 mRNA tended also to give strong signals for the other form. With other IM samples (e.g., IM 16 and 19), the signals for these transcripts were much weaker, requiring longer autoradiographic exposures to be clearly visible. Nevertheless, such amplification products appeared to be specific in that they hybridized with the relevant internal sequence-specific oligonucleotide probe, were of the correct predicted size (i.e., 265 bp for Y3/U/K-spliced RNA and 238 bp for Q/U/K-spliced RNAs), and were observed in two independent RT-PCR assays on the same RNA preparations; no such products were ever detected in EBV-negative control cell tracks on the same autoradiographs. Given the frequent coincidence of both types of EBNA1 mRNA structure in IM samples, we considered the possibility that the Q/U/K amplification signals might reflect the presence of unusually spliced Cp/Wp-initiated transcripts containing *Bam*HI Q-derived sequences. This did not seem to be the case, however, since amplifications using 5′ primers from the W_2, Y_2 and Y_3 exons of Cp/Wp-initiated mRNAs (Table 1) in combination with the K 3′ primer never yielded a product which could be detected with the Q 5′ primer as a

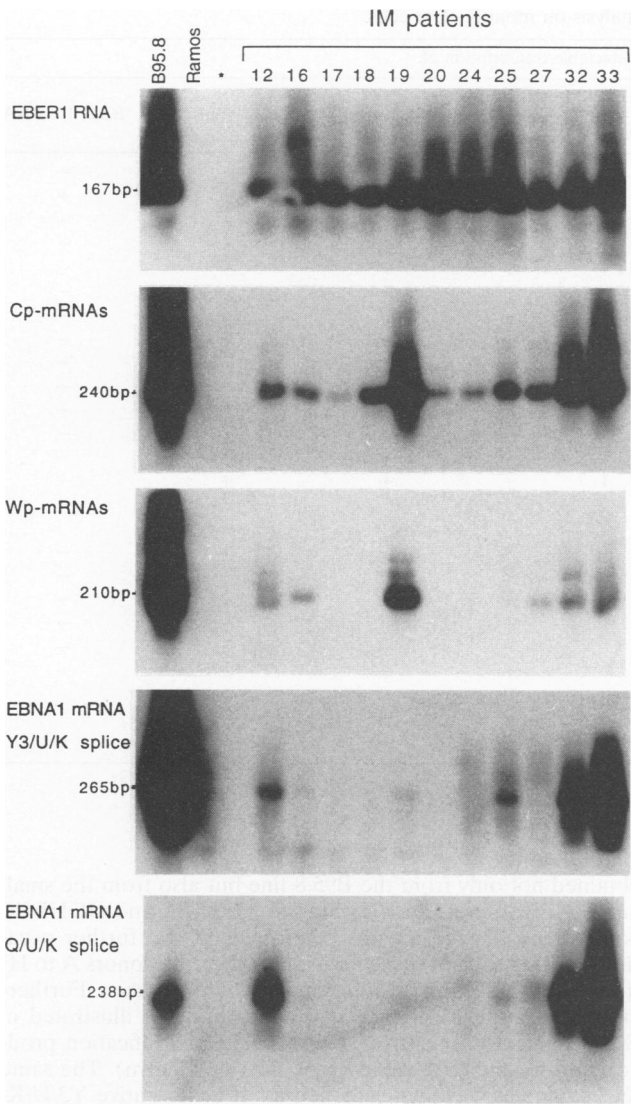

FIG. 3. RT-PCR screening for EBER1 RNA, Cp- and Wp-initiated mRNAs, and the Y3/U/K-spliced and Q/U/K-spliced forms of EBNA1 mRNA in RNA preparations from freshly isolated IM mononuclear cells; RNA from 5×10^6 mononuclear cells was used as the template for amplification in each case. Control cells were the EBV-positive B95.8 LCL (predominantly latency III, with 5% cells in lytic cycle) and the EBV-negative Ramos line, amplifying RNA from 10^6 cells in each case. RT-PCR amplification was carried out with the same primer-probe combinations as used for Fig. 1, the products were hybridized overnight with ^{32}P-labeled probe, and the autoradiographs were exposed either overnight or for 3 days as in Fig. 1.

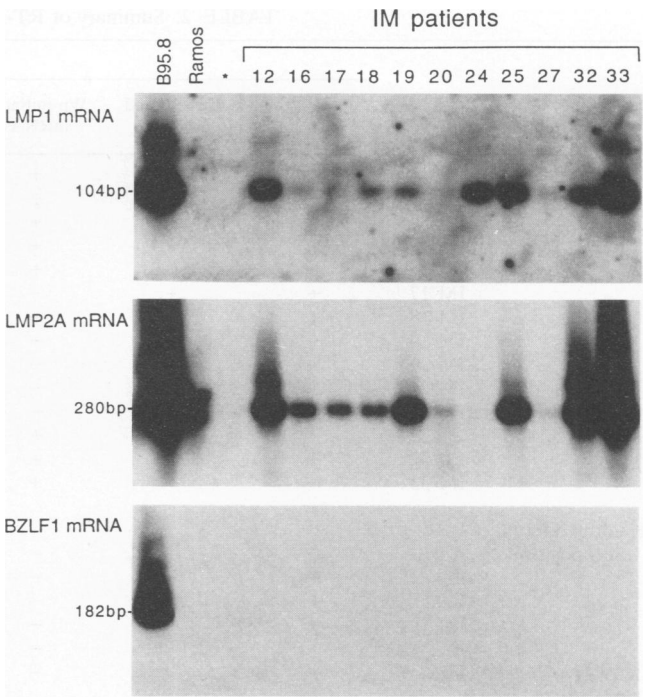

FIG. 4. RT-PCR screening for LMP1, LMP2A, and BZLF1 mRNAs in RNA preparations from the same IM mononuclear cells and control cells as used for Fig. 3. RT-PCR amplification was carried out with the same primer-probe combinations as used for Fig. 2, the products were hybridized overnight with ^{32}P-labeled probe, and the autoradiographs were exposed overnight as in Fig. 2.

probe (data not shown). A smaller set of IM samples was also screened specifically for the presence of Cp/Wp-initiated EBNA2 mRNAs; clear evidence of such transcripts was again seen in samples such as IM 32 and IM 33 (see below), which gave the strongest signals for Cp usage.

Figure 4 shows data obtained from the same set of 11 IM samples following RT-PCR amplification with EBV primer-probe combinations specific for the LMP transcripts. Expression of appropriately spliced LMP1 RNA was detectable in all 11 IM samples, although in certain cases clear evidence of the relevant amplification product required longer autoradiographic exposure of the gel. Likewise, LMP2A transcripts were relatively easily detected in most samples, and even IM tracks 24 and 27 in Fig. 4 were detectably positive with longer exposure. Here again, a repeat experiment using LMP1-specific and LMP2A-specific primer-probe combinations confirmed the initial results and reproduced the same pattern of quantitative differences between individual IM samples. Finally, screening of these same RNA preparations with a primer-probe combination specific for the immediate-early BZLF1 mRNA failed to detect any evidence of lytic gene transcription (Fig. 4). Only in one case (IM2; not shown) did we ever detect a BZLF1 signal in freshly isolated mononuclear cells. Because at least some infected cells in IM blood are known to enter lytic cycle on explantation in vitro (30), in four cases (IM 12, 16, 17, and 27) we also prepared RNA from an aliquot of mononuclear cells after 48 h in culture; positive signals for BZLF1 transcription were obtained in two cultured populations (IM 12 and 16), whereas freshly isolated cells were consistently negative in the same assay (data not shown). The overall data from RT-PCR analysis of freshly isolated mononuclear cells from all 14 IM patients studied in this work are presented in summary form in Table 2.

In two cases (IM 32 and 33), the yield of UM cells was sufficient also to prepare RNA from T-cell-depleted and T-cell-enriched fractions of the mononuclear population. When RNA from these fractions was subjected to similar RT-PCR analysis, it was clear that EBV transcripts were in both cases much more easily detected in the T-cell-depleted than in the T-cell-enriched population. Figure 5 illustrates the point with reference to results for IM 33, in which case RNA from the equivalent of 5×10^6 cells from the UM, T-cell-

TABLE 2. Summary of RT-PCR analysis on mononuclear cells

Donor group	Donor	Detectable transcription of:							
		EBER1 RNA	Cp-initiated mRNAs	Wp-initiated mRNAs	EBNA1 mRNAs		LMP1 mRNA	LMP2A mRNA	BZLF1 mRNA
					Y3/U/K	Q/U/K			
IM patients	IM 1	+	+	+	−	+	+	+	−
	IM 2	+	+	+	+	+	+	+	+
	IM 3	+	+	+	−	−	+	+	−
	IM 12	+	+	+	+	+	+	+	−
	IM 16	+	+	+	+	+	+	+	−
	IM 17	+	+	−	−	−	+	+	−
	IM 18	+	+	−	−	−	+	+	−
	IM 19	+	+	+	+	+	+	+	−
	IM 20	+	+	−	−	−	+	+	−
	IM 24	+	+	−	−	−	+	+	−
	IM 25	+	+	−	+	+	+	+	−
	IM 27	+	+	+	−	+	+	+	−
	IM 32	+	+	+	+	+	+	+	−
	IM 33	+	+	+	+	+	+	+	−
Healthy donors									
Seropositive	A	+	−	−	−	+	−	+	−
	B	+	−	−	−	+	−	+	−
	C	+	−	−	−	+	−	+	−
	D	+	−	−	−	+[a]	−	−	−
	E	+	−	−	−	−	−	−	−
	F	+	−	−	−	+[a]	−	+	−
Seronegative	G	−	−	−	−	−	−	−	−
	H	−	−	−	−	−	−	−	−

[a] Detected after second round of amplification.

depleted, and T-cell-enriched cell fractions was amplified and probed for Cp-initiated transcripts and for the EBNA2 and LMP1 mRNAs.

Transcriptional analysis of mononuclear cells from long-term virus carriers. In the final set of experiments, RNA preparations were made from the freshly isolated blood mononuclear cells of the following healthy individuals: three of the original IM patients rebled several months after the resolution of their symptoms (post-IM donors 12, 19, and 20), six EBV-seropositive individuals with no prior history of IM (virus carriers A to F), and two EBV-seronegative control individuals (G and H). Aliquots of RNA representing the yield from 10^7 UM cells were subjected to RT-PCR amplification analysis using the various primer-probe combinations as described above. As controls, this set of amplifications used aliquots of RNA (the yield from 10^6 cells) from the EBV-negative Ramos and the EBV-positive virus-producing B95.8 cell lines and from two EBV-positive tightly latent BL lines, MAK-BL and ODHI-BL, which in very early passage had displayed a uniform latency I transcription pattern but which by the time of study had begun to show evidence of low-level Cp/Wp and LMP promoter usage, as small numbers of cells with a latency III phenotype appeared in the culture (11).

Figure 6 presents the results of such an experiment analyzing samples from healthy individuals. There was clear evidence for the presence of EBER1 transcripts in the mononuclear cells of all six seropositive donors A to F and of at least two of the post-IM donors, 12 and 19, whereas RNA from seronegative donor G never gave an EBER1-specific signal. When the same RNA preparations were screened for Cp-initiated EBNA transcripts, however, no specific amplification products were detected from any of these donors despite long gel exposures; note that as positive controls in the same assay, signals were obtained not only from the B95.8 line but also from the small number of Cp-using cells within the MAK-BL and ODHI-BL populations. The result was confirmed on two further occasions, testing each of those individuals (healthy donors A to H) from whom additional blood samples could be taken. Furthermore, all of the mononuclear cell preparations illustrated in Fig. 6 were also negative for Wp-specific amplification products and for the EBNA2 mRNA (data not shown). The same set of samples was then screened for the alternative Y3/U/K-spliced and Q/U/K-spliced forms of EBNA1 mRNA. Figure 6 shows the results obtained when the products of amplification with the Y3 5′-K 3′ primer combination, and of a parallel amplification with the Q 5′-K 3′ primer combination, were hybridized with the same U-exon-derived oligonucleotide probe and exposed on the same autoradiograph. Whereas a Y3/U/K-spliced mRNA-specific product was never detected in any of the test samples, three individuals (seropositive donors A to C) gave weak but definite positive signals, indicating the presence of a Q/U/K-spliced EBNA1 transcript.

This same panel of healthy donor and post-IM samples was also screened for the presence of LMP transcripts. As illustrated by the results in Fig. 6, LMP2A mRNA-specific signals could be detected in five of these individuals (seropositive donors A, B, and F and post-IM donors 12 and 19). The strength of the signals observed for the positive control cell populations in this particular autoradiograph reflects the longer exposure time that was necessary to visualize the appropriate product in some of the test samples; under the same conditions, no such products were ever obtained from the EBV-negative control cells (data not shown) or from the seronegative donor samples (Fig. 6). Repeat assays on further samples from the same healthy donors confirmed these positive results and on one occasion also detected an LMP2A

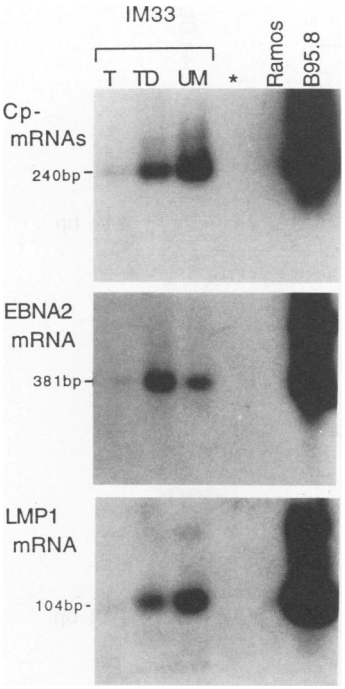

FIG. 5. RT-PCR screening for Cp-initiated mRNAs and for the EBNA2 and LMP1 mRNAs in RNA preparations from UM cells of IM patient 33 and from T-cell-enriched (T) and T-cell-depleted (TD) fractions derived from this same cell population. RNA from a total of 5×10^6 cells was used as the template for amplification in each case. Control cells were the B95.8 LCL and Ramos line as in Fig. 3 and 4, amplifying RNA from 10^6 cells in each case. RT-PCR amplification was carried out with the relevant primer-probe combinations from Table 1, the products were hybridized overnight with ^{32}P-labeled probe, and the autoradiographs were exposed either overnight (for Cp-initiated mRNA and LMP1 mRNA detection) or for 3 days (for EBNA2 mRNA detection).

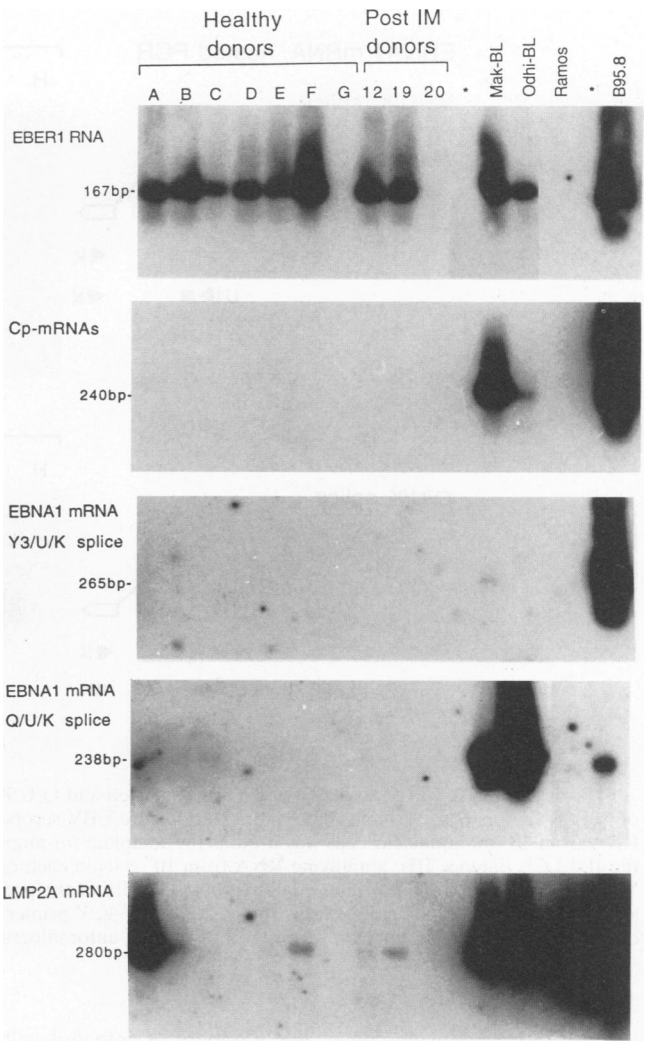

FIG. 6. RT-PCR screening for EBER1 RNA, Cp-initiated mRNAs, the Y3/U/K-spliced and Q/U/K-spliced EBNA1 mRNAs, and LMP2A mRNA in RNA preparations from the mononuclear cells of healthy donors A to G and post-IM donors 12, 19, and 20. Note that donors A to F were EBV seropositive whereas donor G was an EBV-seronegative control individual; RNA from 10^7 mononuclear cells was used as the template for amplification in each case. Control cells were the EBV-positive B95.8 LCL (predominantly latency III, with 5% cells in lytic cycle), the EBV-negative Ramos line, and the EBV-positive Mak-BL and Odhi-BL lines (predominantly latency I, but with a small proportion of cells having switched to latency III); RNA from 10^6 control cells was used in each case. RT-PCR amplification was carried out with the relevant primer-probe combinations from Table 1, the products were hybridized overnight with ^{32}P-labeled probe, and the autoradiographs were exposed for up to 3 days.

mRNA-specific signal from seropositive donor C. Interestingly, however, we did not detect evidence of LMP1 mRNA-specific amplification products when the same panel of RNA preparations as specified above was screened in parallel assays using the LMP1 primers, or even when subsequent preparations from the same donors were subjected to two successive rounds of amplification using these primers (data not shown).

As part of the repeat experiments carried out on further fresh blood samples from healthy donors A to H, we included a series of additional amplifications designed to recheck the question of EBNA1 mRNA splice usage in healthy donor cells. Thus, aliquots of the first-round amplification products obtained with the Y3 5′ and K 3′ primers and with the Q 5′ and K 3′ primers were both subjected to a second round of amplification using the nested U1 5′-K 3′ primer combination; the products of this second round were then hybridized in parallel by using the common U-exon probe. The results of such an experiment involving RNA preparations from five seropositive donors (B to F) and from two seronegative donors (G and H) are illustrated in Fig. 7. Specific signals were obtained from four of the seropositive donors but only when the initial round of amplification had used a primer combination (Q 5′-K 3′) specific for Fp-initiated EBNA1 transcripts. The overall data from RT-PCR analysis of healthy donor mononuclear cells are summarized in Table 2 alongside the corresponding results from IM patients.

DISCUSSION

The EBV-cell interactions underlying primary infection and persistence remain largely unexplored, not least because virus-infected cells constitute a very small fraction of the total B-cell pool. Only in the most acute phase of IM has it been possible to visualize infected cells in the blood by immunofluorescence staining for the EBNA complex; even then these cells numbered only 1 in 10^2 to 10^3 circulating B cells (16, 20, 47) in all but exceptional cases (33). Later in the disease and subse-

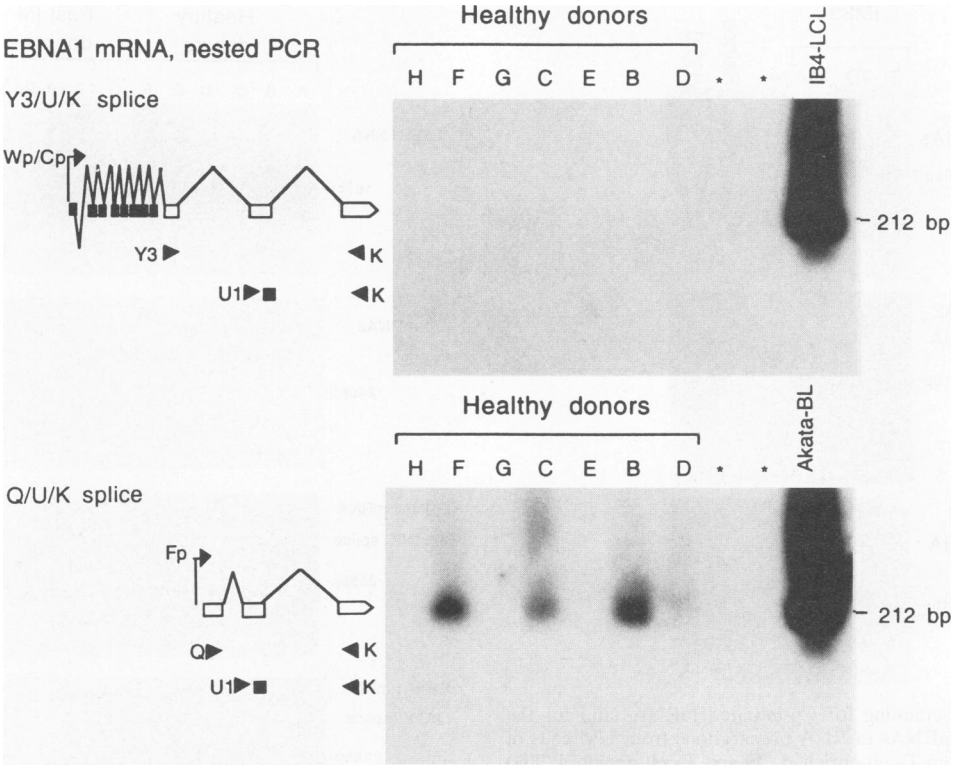

FIG. 7. Nested RT-PCR screening for Y3/U/K-spliced and Q/U/K-spliced EBNA1 mRNAs in RNA preparations from the mononuclear cells of healthy donors B to H. Note that donors B to F were EBV seropositive whereas donors G and H were EBV-seronegative control individuals; RNA from 10^7 mononuclear cells was used as the template for amplification in each case. Control cells were the Akata-BL line (latency I) and the IB4 LCL (latency III), amplifying RNA from 10^6 cells in each case. A first round of RT-PCR amplifications was carried out with either the Y3 5′-K3′ or the Q 5′-K 3′ primer combination. One-fifth of the products from these first-round amplifications was then used as the template in a second round of amplifications using the nested U1 5′-K 3′ primer combination. The products of this second round were hybridized in parallel overnight, using the ^{32}P-labeled U-exon probe, and the autoradiographs were exposed overnight.

quently during convalescence, the numbers of infected cells decline, eventually reaching the much lower steady-state levels characteristic of the life-long virus carrier state (34). These steady-state levels are difficult to quantitate directly but can be inferred from the propensity of seropositive donor lymphocytes to give rise to EBV-transformed LCLs by spontaneous outgrowth in vitro (28). Such outgrowth assays indicate that numbers of cells ranging from 2×10^4 to 2×10^6 circulating B cells per culture well, depending upon the individual donor, are required to establish spontaneous LCLs (51, 53). However, it must be remembered that the process of spontaneous outgrowth in vitro is itself inefficient, given its dependence upon virus replication, release, and secondary infection of coresident B lymphocytes (31) and given the inherently low cloning ability of such virus-infected indicator cells (46). Hence, the inferred range of 1 virus-infected cell per 2×10^4 to 2×10^6 B cells in the circulatory systems of healthy individuals is likely to be an underestimate of the actual frequency range. It was with these considerations in mind that we set out to use RT-PCR amplification as a means of screening freshly isolated mononuclear cell preparations for EBV transcripts.

The panel of oligonucleotide primer-probe combinations used for this purpose was designed (i) to detect transcription of EBER1 RNA and of BZLF1 mRNA as markers of latent and lytic infections, respectively, (ii) to identify whether latent EBNA mRNAs were being driven from Cp/Wp or from Fp, and (iii) to determine whether the individual LMP transcripts were being expressed. The initial titrations (Fig. 1 and 2) show that even with relatively short autoradiographic exposures, it is possible to detect specific viral transcripts in RNA prepared from cell mixtures containing very small numbers of EBV-positive cells. Thus, the RT-PCR amplification protocols could easily detect EBER1 transcripts from a 2-cell input, Cp-initiated EBNA transcripts and LMP1, LMP2A, and BZLF1 mRNAs from a 20-cell input, and the Fp-initiated EBNA1 mRNA from a 200-cell input. Assuming that viral RNAs are not markedly less abundant in latently infected cells in vivo than in the in vitro models, we considered these results sufficiently encouraging to begin the analysis of RNA from freshly isolated mononuclear cell populations.

The results from IM patients (Fig. 3 and 4) are important in demonstrating that the spectrum of viral RNAs expressed during EBV-induced B-cell transformation in vitro (19, 49, 50) is indeed also expressed in circulating cells in vivo during the course of primary infection. Thus, RNAs with the splice structure typical of Cp-initiated transcripts were detectable in the mononuclear cells of every IM patient studied, whereas Wp-initiated transcripts were detectable in most but not all cases, a fact we attribute to the apparently lower sensitivity of the Wp primers. In many cases, it was also possible to amplify individual EBNA mRNAs (e.g., the Y3/U/K-spliced EBNA1 and W_2/Y3P-spliced EBNA2 transcripts) that are known to be expressed from Cp/Wp. All IM samples were also positive for

transcripts with the typical LMP1 and LMP2A mRNA splice structures, another feature of in vitro-transformed LCL cells. We interpret these results, along with the data from cell fractionation experiments (Fig. 5), as indicating that during primary infection, EBV gains access to B cells within the circulating pool and initiates in at least some, and perhaps all, of these infected cells a program of latent gene transcription in vivo like that seen when resting B cells are infected and growth transformed to LCLs in vitro. The ability to adopt this form of infection, and thereby to drive the proliferation of virus-infected cells, would offer considerable advantages to the virus at this time, since it would allow efficient colonization of the B-lymphoid system before the primary cytotoxic T-cell response, which targets growth-transformed LCL-like cells (45, 48), has had time to develop.

A very interesting additional feature of the results with IM mononuclear cells is the frequency with which transcripts could be detected with the Q/U/K splice structure typical of Fp-initiated EBNA1 mRNAs (Fig. 3). Though these were often seen most clearly in IM samples in which Cp/Wp transcripts were also relatively abundant, we could find no evidence that they represented unusually spliced forms of Cp/Wp-initiated mRNAs; the frequent coincidence of easily detectable levels of the putative Fp-initiated and Cp/Wp-initiated transcripts in IM blood may reflect a common dependence upon overall virus load in the B-cell pool. We explored the further possibility that these Q/U/K-spliced were arising from some lytically infected cells in the blood, since in vitro the entry of LCL cells into lytic cycle is accompanied by a marked activation of Fp and at least some of these Fp-driven lytic transcripts display the Q/U/K splice structure (22). However, screening of the IM mononuclear cells for evidence of BZLF1 mRNA, a much more sensitive indicator of lytic cycle entry than Q/U/K-spliced transcripts (Fig. 2), gave negative results in all but one case (Fig. 4; Table 2). We infer, therefore, that during the course of IM, some latently infected B cells in which EBNA1 is being expressed via transcription from Fp are generated. This is indeed the first demonstration that a Q/U/K-spliced latent transcript, hitherto observed only in certain EBV-positive malignancies, is expressed in normal virus-infected cells in vivo. What the present work cannot determine, however, is whether this putative Fp-initiated transcription is occurring in infected cells in which Cp/Wp is also active or in a separate population. The evidence from in vitro systems would suggest that Fp and Cp/Wp function as mutually exclusive promoters (22, 38, 41), but one cannot assume that such rules will necessarily be operative in the in vivo situation.

Finally, encouraged by the findings presented above and by a recent study detecting EBV transcripts in the lymphocytes of four seropositive donors (29), we extended the present study to asymptomatic virus carriers. The results in Figure 6 show that EBER1 transcripts are regularly detectable by RT-PCR amplification in RNA preparations from 10^7 seropositive donor lymphocytes, and indeed more recent limiting dilution experiments suggest that EBER1 signals can be obtained from at least 100-fold-lower input numbers of cells (unpublished data). Unlike the situation in IM patients, however, here we found no evidence of Cp/Wp-initiated transcripts or of any individual EBNA mRNAs (such as the Y3/U/K-spliced EBNA1 mRNA) that are expressed from these promoters. The negative results on amplification for Cp-initiated RNAs are particularly significant since the Cp primers are known from the earlier titration experiments (Fig. 1) to be particularly sensitive at detecting LCL-like cells in mixed populations. There is a discrepancy here between our data and those of an earlier study by Qu and Rowe (29) in which three of four seropositive donors gave positive signals for RNA with a $C_2/W_1/W_2$ splice. We would have anticipated detecting such a species by using our Cp primers if the RNA in question were initiated from Cp and contained the usual $C_1/C_2/W_1/W_2$ exon structure; thus, the identity of that RNA remains uncertain, particularly since in the same report there was no evidence of any transcripts with the expected downstream splicing into EBNA open reading frames (29). On the other hand, we were able to detect low levels of transcripts with the Q/U/K-splice structure typical of an Fp-driven EBNA1 mRNA in the circulating mononuclear cells of five of six healthy seropositive donors. Levels were such that detection required either prolonged exposure of the autoradiograph following a standard amplification with the Q 5′ and K 3′ primers or a second round of amplification with nested primers to improve sensitivity (Fig. 6 and 7). Interestingly, many of these same RNA preparations, though apparently LMP1 mRNA negative, contained low but detectable levels of LMP2A transcripts, a result which is in accord with the earlier observations of Qu and Rowe (29). It is again worth noting that there are situations in vitro in which both the Q/U/K-spliced EBNA1 mRNA and the LMP2A mRNA can be expressed as lytic rather than latent cycle transcripts (22, 36). However, in the present context, these RNAs must be derived from latently infected cells within the circulating pool, since the same RNA preparations were negative for the BZLF1 lytic cycle transcript.

Clearly much more work is required if we are to understand the various forms of latency which EBV can establish in vivo and how these relate to the forms of latency currently recognized in in vitro models. The present analysis is limited to EBV-carrying cells in the blood and does not address the more complex situation which could exist in lymphoid tissues; even in the circulation, however, the virus-infected cell pool may well be heterogeneous in terms of cellular phenotype and of viral gene expression. Caution must be exercised, therefore, in extrapolating from data on whole populations to the situation at the single-cell level. Our provisional conclusion is that the form(s) of latency predominant in the circulating B-cell pool of IM patients is distinct from the form(s) predominant in healthy virus carriers. Thus, whereas primary infection appears to generate a substantial number of cells expressing the Cp/Wp-driven EBNA transcripts and LMP mRNAs characteristic of virus-driven B-cell proliferation, long-term persistence seems more commonly associated with Fp-driven EBNA1 mRNA and LMP2A mRNA expression. In this latter context, it is interesting that neither of the proteins encoded by these transcripts has growth-transforming activity per se; rather, EBNA1 plays an essential role in virus genome maintenance (54), whereas LMP2A appears to be capable of interfering with membrane signaling events involved in virus reactivation (24). Both functions would be compatible with the concept of viral latency in a long-lived resting B-cell population in vivo (9, 52). Furthermore, latent infections involving Fp rather than Cp/Wp usage would offer the virus an additional advantage in immunological terms. Thus, the dominant target epitopes for virus-specific cytotoxic T-cell memory responses are frequently derived from the EBNA proteins other than EBNA1 (18, 27), and latently infected cells lacking expression of these target antigens would be much more likely to survive in the face of immune T-cell surveillance.

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ADDENDUM IN PROOF

Normal circulating B cells expressing EBNA1 transcripts in the absence of EBNA2 and LMP1 transcripts have also been detected by workers in another laboratory (F. Chen, J. Z. Zou, L. diRenzo, G. Winberg, L. F. Hu, E. Klein, G. Klein, and I. Ernberg. Submitted for publication).

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