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Isolation of Intertypic Recombinants of Epstein-Barr Virus from T-Cell-Immunocompromised Individuals

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All wild-type isolates of Epstein-Barr virus (EBV) analyzed to date for allelic polymorphisms of the nuclear antigen EBNA2 gene (in the *Bam*HI YH region of the genome) and of the EBNA3A, -3B, -3C genes (tandemly arranged in the *Bam*HI E region) have proved either uniformly type 1 or uniformly type 2 at all four loci. The absence of detectable intertypic recombination in the wild probably reflects the rarity with which individual carriers, and certainly individual target cells, become coinfected with both virus types. Studying a group of human immunodeficiency virus-positive T-cell-immunocompromised patients known to be at enhanced risk of multiple EBV infections, we have isolated intertypic EBV recombinants from 2 of 40 patients analyzed. These recombinants, whose in vitro transforming capacity appeared at least equal to that of type 1 strains, carried a type 1 EBNA2 allele and type 2 EBNA3A, -3B, and -3C alleles. This was clearly demonstrable at the DNA level by PCR amplification using type-specific primer-probe combinations and was confirmed at the protein level (for EBNA2 and EBNA3C) by immunoblotting with type-specific antibodies. In one patient, the recombinant appeared to be the predominant strain, being the virus most commonly rescued by in vitro transformation both from the blood and from the throat washings on two separate occasions 20 months apart. A regular type 1 virus strain was also present in this individual, but this was not related to the recombinant since the two viruses carried type 1 EBNA2 genes with different patterns of variance from the B95.8 prototype sequence. In the other patient, recombinants were isolated on one occasion from the blood and on a separate occasion, 21 months later, from the throat; these recombinants were almost certainly related, being identical at several genomic polymorphisms and differing only in one facet of the "EBNAprint," the size of the EBNA1 protein. Three different type 1 viruses were also isolated from this patient, two of which carried EBNA2 genes with the same pattern of sequence variation from B95.8 as the recombinant; however, since this is a fairly common pattern of variance, the relationship of these viruses to the recombinant remains an open question. We infer that intertypic recombinants of EBV are not uncommon in HIV-positive T-cell-immunocompromised patients, that they arise in such individuals as a consequence of their increased frequency of mixed-type infections, and that they will prove capable of efficient transmission in the human population.

Epstein-Barr virus (EBV), a gammaherpesvirus widespread in human populations, is carried by the vast majority of individuals as a persistent asymptomatic infection characterized by virus shedding from lytically infected cells in the oropharynx and by the presence of latently infected cells within the circulating B-lymphocyte pool (reviewed in reference 30). Much of the interest in this virus stems from its association with malignant disease, and it was work on one such malignancy, the endemic (African) form of Burkitt's lymphoma, which first identified two distinct types of EBV isolate, originally called A and B and now called types 1 and 2 (2, 10). These types are essentially homologous across large stretches of the genome but can be distinguished by polymorphisms of the EBNA2 gene, situated in the *Bam*HI YH region of the genome (2, 10), and of the EBNA3A, -3B, and -3C genes, tandemly arranged over 40 kb away in the *Bam*HI E region (33, 36, 38). Interestingly, the nuclear antigens encoded by these polymorphic genes are all associated with latent, as opposed to lytic, infection and constitute four of the eight latent cycle proteins that are constitutively expressed in EBV growth-transformed B cells in vitro (30). By contrast, obvious type-specific polymorphisms have not been identified in the other latent proteins, namely, EBNA1 and EBNA-LP and the latent membrane proteins LMP1 and -2 (8, 14, 27, 35, 48), or in any of the lytic cycle products thus far analyzed (25, 28, 42).

There are subtle biological differences between the two EBV types in vitro in that after experimental infection of indicator B cells, type 1 virus transformants tend to grow out to lymphoblastoid cell lines (LCLs) more quickly than their type 2 counterparts, to be less dependent on cell aggregation for optimal growth thereafter, and to show slightly lower levels of spontaneous entry in lytic cycle (29a, 31). Recombining exogenous sequences back into the EBV laboratory strain P3HR1 (a rare EBNA2 deletion mutant of the type 2 isolate, Jijoye) formally showed that EBNA2 was essential for transforming ability (9, 19) and also made it clear that the above-described type-specific differences in LCL growth were principally determined by the EBNA2 type rather than by EBNA3A, -3B, or -3C (9). Second-site homologous recombination back into the P3HR1 genome showed that, besides EBNA2, EBNA3A and -3C were also essential for transformation whereas EBNA3B was not (43). The mechanisms whereby EBNA2 contributes to cell growth transformation are beginning to be understood (18, 20), whereas the precise roles of the EBNA3 proteins remain to be determined (26, 32).

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There also appear to be differences between EBV types 1 and 2 in terms of their distributions among world populations. The bulk of evidence on this point has come from the typing of EBV strains rescued by in vitro transformation from the blood and/or throat washings of healthy carriers. This evidence suggests that type 1 strains are predominant in much of the Western world and in Southeast Asia, with relatively few (<5%) of the individuals tested giving rise to type 2 isolates; by contrast, the two virus types appear to be more equally represented in populations in equatorial Africa and New Guinea (1, 16, 49, 51, 52). Significantly, throughout this work, there were no instances of any healthy carrier yielding both type 1 and type 2 viral isolates, not even from communities in which both virus types were relatively common. However, the generally faster growth of type 1-transformed cells in vitro (31) introduces a potential bias into such studies, and it is possible that some type 1-infected individuals also harbor an occult type 2 infection which is not detected by such in vitro isolation protocols. In this context, the analysis of oropharyngeal viral shedding by direct amplification of EBV DNA in throat washings has indicated, in some (39) but not all (23, 49) studies, that a small proportion of healthy carriers do sustain a mixed-type infection. Taken overall, the evidence suggests that coinfection with EBV strains of different types can occur but is relatively rare in healthy individuals. Likewise the coresidence of different viruses of the same type must also be rare, at least from the analysis of Caucasian populations. Thus, when multiple independent isolates have been made from individual donors, any one person appears to carry a single characteristic EBV strain (usually type 1) which is dominant both in the blood and in the throat and which persists over time (16, 49).

The comparative rarity of mixed-type infection may help to explain a second feature noted throughout this work, namely, that all virus isolates from any of the human populations studied were either uniformly type 1 at all four polymorphic loci (EBNA2, -3A, -3B, and -3C) or uniformly type 2 (16, 18, 21, 49). Naturally occurring recombinants between the two virus types have therefore never been observed in the wild, whereas laboratory experiments conducted over a decade ago showed that superinfecting the Raji or BJAB/B95.8 cell line (both carrying type 1 virus strains) with the type 2 virus strain P3HR1 could generate Raji/P3HR1 or B95.8/P3HR1 recombinant genomes with transforming function (41). The generation of intertypic recombinants in vivo remains a formal possibility, therefore, if an individual did become infected with different EBV types; this is particularly so because type 1 and type 2 viruses appear to show the same tropism both for B lymphocytes and for oropharyngeal epithelial cells in vivo (31, 45) and might therefore establish coinfections at the cellular level. We were interested in exploring this possibility in a group of human immunodeficiency virus (HIV)-positive Caucasian patients since type 2 virus infection appears to be more prevalent in such individuals (6, 24). Furthermore, analysis of viral DNA in the oral hairy leukoplakia lesions (epithelial foci of uncontrolled EBV replication) to which these patients are unusually prone has indicated the relatively frequent coresidence of type 1 and type 2 viral sequences (45, 46). From the cohort of 40 T-cell-immunocompromised HIV-positive patients described in the accompanying report (50), here we present data from two particular individuals providing the first evidence for the existence of natural type 1/type 2 EBV recombinants.

MATERIALS AND METHODS

Patients. The two patients, designated EBH5 and EBH34 in the accompanying report (50), were both HIV-positive male homosexuals of Caucasian origin. EBH5 first presented with AIDS symptoms in 1988 and was unique among the 40 patients described in that report (50) in having a history of prior blood transfusion (for anemia) during 1989 to 1991. Blood and throat washing samples were taken from EBH5 in July 1993 and again in April 1995. Patient EBH34 did not have AIDS symptoms but has been HIV positive for several years and when studied had a low $CD4^+$ T-cell count ($<400/mm^3$); this patient was sampled in November 1993 and again in June 1995.

Virus isolation. Methods for virus isolation in LCLs derived from blood and from throat washings, and for the secondary transfer of virus isolates from established LCLs into indicator B cells from seronegative donors, are fully described in the accompanying report (50).

Analysis of viral polymorphism. PCR assays for virus typing at the EBNA2, EBNA3A, EBNA3B, and EBNA3C loci were carried out as described by Sample et al. (36); assays for strain identification at the 30-bp deletion and 33-bp repeat loci in the LMP1 gene were performed as described in the accompanying report (50) and by Miller et al. (27), respectively. Distinctions between individual EBV strains on the basis of their different "EBNAprints" (i.e., the different size patterns of their EBNA1, EBNA2, and EBNA3C proteins as visualized by immunoblotting of sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]-separated LCL extracts) were initially determined by using the human sera Hu-MS (reactive to EBNA1 and type 1 EBNA3C) and Hu-NZ (reactive to EBNA1 and type 2 EBNA3C) and the monoclonal antibodies (MAbs) IH4 (anti-EBNA1), PE2 (anti-EBNA2), and E3CA10 (anti-type 1 EBNA3C) as described in the accompanying report (50). In certain cases, the type of resident EBNA2 protein was confirmed by using the recently developed MAbs R3 and E391, specific for type 1 EBNA2 and for type 2 EBNA2, respectively (22).

EBNA2 gene sequencing. For certain isolates, the entire EBNA2 coding region was amplified by using the flanking oligonucleotide primers E2 seq 1 and E2 seq 2 (see the legend to Fig. 3). The product was run on a 1% low-melting-point agarose gel and stained with ethidium bromide, and the band was cut out and purified by using the Wizard PCR Preps DNA purification system (Promega) as instructed by the manufacturer. Thereafter, a series of sequencing reactions was carried out with an Ampli Cycle sequencing kit (Perkin-Elmer) and a number of ^{32}P-end-labeled primers (see the legend to Fig. 3). These reactions covered most of the unique regions of the EBNA2 gene sequence outside the polyproline-coding repeat domain (10).

RESULTS

Virus isolates from patient EBH34. On a first occasion of sampling, in July 1993, patient EBH34 yielded eight cell lines arising by spontaneous transformation of blood lymphocyte cultures (EBH34 1 to 8) and five cell lines established from infectious virus in throat washings by in vitro transformation of cord blood indicator B cells (EBH34 1* to 5*). These LCLs, plus appropriate type 1 EBV-positive, type 2 EBV-positive, and EBV-negative control lines, were screened for the identity of their resident EBV genomes by PCR amplification across a range of polymorphic markers. Figure 1A shows the results of initial screening at the EBNA2 locus, from which it is clear that all of the EBH34-derived isolates carried type 1 EBNA2 genes. As described in the accompanying report (50), reconstitution experiments had shown that this particular PCR assay is capable of specifically detecting type 2 EBNA2 sequences in the presence of at least a 1,000-fold excess of the type 1 allele, yet repeated testing gave no evidence of type 2 signals in EBH34 LCLs even after prolonged exposure of the autoradiographs.

We were therefore surprised to find, on extending the PCR analysis to the polymorphic EBNA3A, -3B, and -3C loci, that type 2 signals were not only detectable here but predominant. As illustrated in Fig. 1B, the majority of LCLs both from the blood and from the throat washings of patient EBH34 gave exclusively type 2 signals in the EBNA3A, -3B, and -3C gene amplifications, a result confirmed in several assays. By contrast, lines 2 and 5 from the blood and lines 1* and 3* from the throat also produced type 1 signals at all three loci; in lines 2 and 5, these signals were much weaker than the accompanying type 2 signals; in lines 1* and 3*, they were dominant in early-passage cultures and evidence of coresident type 2 sequences at this time often required long autoradiographic exposures (data not shown). We inferred that the majority of EBH34-derived lines carried a single, albeit unusual, virus strain, whereas the remaining lines from this patient were

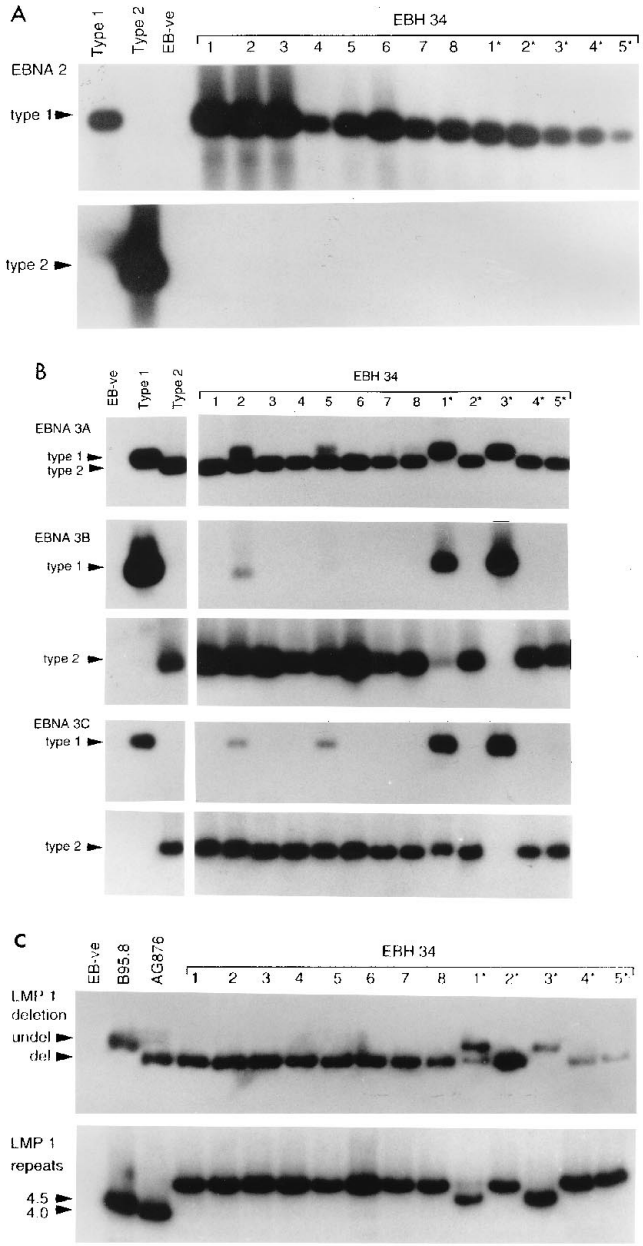

FIG. 1. PCR analysis of EBV strains carried by blood-derived LCLs 1 to 8 and throat washing-derived LCLs 1* to 5* from patient EBH34. For virus typing, EBNA2 gene amplification (A) was carried out with a common 5′ primer and type-specific 3′ primers, whereas EBNA3A, -3B, and -3C gene amplifications (B) were in each case carried out with common 5′ and 3′ primers (36). The products of EBNA2, EBNA3A, and EBNA3C gene amplifications were in each case probed separately with type-specific probes; the product of the EBNA3B gene amplification was probed with a single common probe, the gene type being identified by the size of the product (36). For strain detection, LMP1 amplifications (C) were carried out as described in the accompanying report (50). All assays included reference DNA samples from the type 1 B95.8 cell line, the type 2 AG876 cell line, and the EBV-negative (EB-ve in all figures showing blots) BJAB cell line. Note that several EBH34-derived isolates display a type 1 EBNA2, type 2 EBNA3A, -3B, -3C genotype.

mixtures of some cells carrying that strain and other cells carrying a different strain. The results of PCR analysis across LMP1 polymorphic loci (27) were consistent with this view. Thus, as illustrated in Fig. 1C, the majority EBH34 isolate was characterized by a 30-bp deletion at the LMP1 deletion locus (like the AG876 reference strain) and by an estimated five copies of the 33-bp LMP1 repeat. By contrast, in the two EBH34-derived lines most obviously different from the rest by EBNA3 gene typing (lines 1* and 3*), the dominant viral strain (the B95.8 reference strain) did not have the 30-bp LMP1 deletion and carried an estimated four to five copies of the 33-bp LMP1 repeat.

The most interesting aspect of these PCR data was the identification of a viral isolate which appeared to be type 1 at the EBNA2 locus and type 2 at the EBNA3 loci. This was further investigated at the protein level by immunoblotting of PAGE-separated LCL extracts with human sera having well-characterized EBNA reactivities and with EBNA-specific MAbs. With this approach, virus isolates can be typed on the basis of their EBNA2 and EBNA3C antigenicity (33, 38) and then identified as individual strains on the basis of their EBNA1, EBNA2, and EBNA3C size profiles, i.e., their EBNAprints (16, 49, 50). The immunoblots obtained from a representative panel of EBH34-derived LCLs are shown in Fig. 2. Lines 1, 3, 4, 4*, and 5*, which by PCR had given type 1 EBNA2/type 2 EBNA3A, -3B, -3C as the only detectable viral genotype, all displayed the same unique EBNAprint. This included an EBNA2 protein, as usual running as a characteristic doublet, which appeared to be type 1 by virtue of its position in the 80- to 90-kDa region of the gel (Fig. 2, MAb PE2 blot), as opposed to the 70- to 75-kDa region typically occupied by type 2 EBNA2 proteins (2, 10, 33, 38); its type 1 identity was subsequently confirmed by immunoblotting with an EBNA2 type-specific human serum (data not shown) and with EBNA2 type-specific MAbs (see below). The same lines nevertheless expressed an EBNA3C protein which was clearly type 2 by virtue of its reactivity with a type 2-specific human serum (Hu-NZ blot) and not with a type 1 EBNA3C-specific

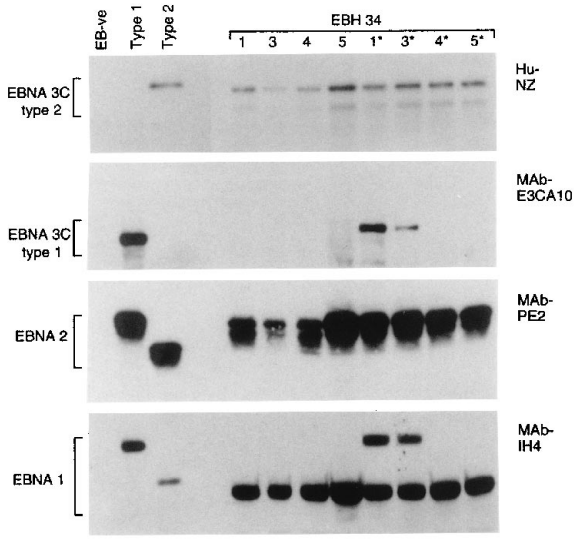

FIG. 2. EBNAprint analysis of EBV strains carried by blood-derived LCLs 1, 3, 4, and 5 and throat washing-derived LCLs 1*, 3*, 4*, and 5* from patient EBH34. Protein extracts were separated by SDS-PAGE, and immunoblots were probed with the type 2 EBNA3C-specific human serum Hu-NZ, with the type 1 EBNA3C-specific MAb E3CA10, with the EBNA2-specific MAb PE2, and with the EBNA1-specific MAb IH4. All immunoblots included reference protein samples from the type 1 LCL C2+OBA, from the type 2 LCL C2+BL16, and from the EBV-negative BJAB cell line (49). Note that several EBH34-derived isolates display an EBNA2 protein which by size is typically type 1 (appearing as a characteristic doublet on shorter autoradiographic exposures) and an EBNA3C protein which antigenically is type 2 (appearing as a major band and a minor breakdown product).

B95.8 coordinates	48628	48905	48990,1	48998	49057	49091	49095	49113	49136|7	49170	49444	49449	49466	49754	49767	49913	49930	49935
EBNA 2 codon	42	134	163	165	185	196	198	204	211|2	223	314	316	321	417	422	470	476	478
B95.8	GAC Asp	CTT Leu	AGG Arg	GTC Val	CAA Gln	ATG Met	CCA Pro	ACC Thr	---	CTG Leu	TTA Leu	CAT His	CCA Pro	ACG Thr	GTT Val	TCA Ser	GAG Glu	CCC Pro
EBH 34.1 / EBH 34.3 rec	GGC Gly	CTG Leu	GTG Val	GTA Val	CAA Gln	ATG Met	TCA Ser	ACC Thr	---	CTG Leu	TCA Ser	CAT His	CCC Pro	ACG Thr	GTT Val	TCA Ser	GGG Gly	TCC Ser
EBH 34.3* type1	GAC Asp	CTT Leu	GTG Val	GTA Val	CGA Arg	ATT Ile	CCA Pro	TCC Ser	CTC Leu	TTG Leu	TTA Leu	AAT Asn	CCA Pro	ACA Thr	GTT Val	TCT Ser	GGG Gly	CCC Pro
EBH 5.1 / EBH 5.5* rec	GAC Asp	CTT Leu	GTG Val	GTA Val	CGA Arg	ATT Ile	CCA Pro	TCC Ser	CTC Leu	TTG Leu	TTA Leu	AAT Asn	CCA Pro	ACA Thr	GTT Val	TCT Ser	GGG Gly	CCC Pro
EBH 5.3 / EBH 5.1* type1	GAC Asp	CTT Leu	GTG Val	GTA Val	CGA Arg	ATT Ile	CCA Pro	TCC Ser	CTC Leu	TTG Leu	TTA Leu	AAT Asn	CCA Pro	ACA Thr	GTT Val	TCT Ser	GGG Gly	CCC Pro
EBH 5.4* type1	GAC Asp	CTT Leu	GTG Val	GTA Val	CGA Arg	ATT Ile	CCA Pro	TCC Ser	CTC Leu	TTG Leu	TTA Leu	AAT Asn	CCA Pro	ACA Thr	ATT Ile	TCT Ser	GGG Gly	CCC Pro
coordinate boundaries	48504 48677	48831		49009	49039					49177	49221		49474	49706				49956
codon boundaries	1 58	109		168	178					225	239		324	401				484

FIG. 3. For each cell line shown, including B95.8 as a reference, the entire EBNA2 gene and flanking regions were initially amplified by using the 5′ primer E2 seq 1 (5′-GCCAACAACCTTCTAAGCAC; B95.8 genome coordinates 48451 to 48470) and the 3′ primer E2 seq 2 (5′-TAACATTTATTTGGGATACATTG; B95.8 genome coordinates 50013 to 49991). PCR sequencing of regions of the amplified fragment was carried out by using as the primers E2 seq 1, E2 seq 2 or one of the following primers within the EBNA2 reading frame: E2 seq 3 (5′-CCCTCTTACTCATCAAAGCA; B95.8 coordinates 49283 to 49302), E2 seq 4 (5′-GTAATG GCATAGGTGGAATG; B95.8 coordinates 49374 to 49355), E2 seq 5 (5′-ACCAGAGCCAAACACCTCCA; B95.8 coordinates 49619 to 49638), or E2 seq 6 (5′-CCCTTGTCCCTGATGAAGAC; B95.8 coordinates 49691 to 49672). Sequenced regions are shaded, and the sequence boundaries identified by the B95.8 coordinates (5) and EBNA2 codon numbers are given at the bottom. Nucleotide and amino acid changes relative to the B95.8 sequence are shown in darker shading, and their positions are identified by the B95.8 coordinates and EBNA2 codon numbers given at the top of the Table.

MAb (MAb-E3CA10 blot) or with a type 1-specific human serum, Hu-MS (data not shown). Hereafter we refer to the resident virus in these lines as the EBH34 recombinant.

In the same analysis, EBH34-derived lines 1* and 3*, which by PCR had scored as type 1 at the EBNA2 locus but as both type 1 and type 2 at the EBNA3 loci, showed a more complex EBNAprint, exhibiting all of the reactivities associated with the recombinant strain plus an additional EBNA1 band of higher molecular weight (Fig. 2, MAb-IH4 blot) and a type 1-specific EBNA3C band (MAb-E3CA10 blot). Such a pattern again suggests the coexistence of two cell populations in these LCLs, one carrying the recombinant virus and the other carrying a typical type 1 virus. We were unable to isolate this latter strain by outgrowth because continued passage of lines 1* and 3* was associated with increasing dominance of cells carrying the recombinant virus, nor could we rescue this type 1 virus by secondary transfer into indicator B cells because these particular throat washing-derived lines were of cord blood origin and completely nonproductive (data not shown).

A very similar pattern of results was obtained when patient EBH34 was sampled on a second occasion, in April 1995. These second samples yielded a further 4 blood-derived LCLs and 14 throat washing-derived LCLs. Once again many of these lines carried the same recombinant virus as the only detectable viral strain, while a minority of LCLs from the blood and from the throat harbored both the recombinant and the same coresident type 1 strain as detected earlier.

EBNA2 gene sequencing of the EBH34 recombinant and type 1 viruses. We wished to determine whether the type 1 virus present in some EBH34-derived LCLs might have been one of the parental strains involved in the generation of the EBH34 recombinant. Selected regions of the EBNA2 gene were therefore amplified and sequenced from two LCLs (EBH34.1 and -34.3 respectively), both of which contained only recombinant virus, and from early-passage cultures of the 34.3* LCL, at which time the type 1 virus was the dominant species. The sequence data (Fig. 3) show nucleotide and predicted amino acid changes relative to the type 1 EBNA2 sequence of the B95.8 reference strain (5). Both recombinant virus-carrying LCLs gave the same pattern of 10 nucleotide changes vis-à-vis B95.8 in the regions of EBNA2 analyzed, leading to seven predicted amino acid substitutions. By contrast, the type 1 virus-carrying LCL showed 11 nucleotide changes vis-à-vis B95.8, only 3 of which were shared with the recombinant, plus the insertion of an additional nucleotide triplet between EBNA2 codons 214 and 215; these changes led to six amino acid substitutions, only two of which were shared with the recombinant, plus the insertion of a leucine 215 residue. Note that both of these EBNA2 sequences were minor variants of the type 1 (B95.8) allele and quite distinct from the type 2 (AG876) allele, which shows substantial divergence from B95.8 throughout the EBNA2 open reading frame (10).

Virus isolates from patient EBH5. Patient EBH5 was originally sampled in November 1993, at which time three blood-derived LCLs (EBH5.1 to -3) and three throat washing-derived LCLs (EBH5.1* to -3*) were established. Sampling on a second occasion, in June 1995, yielded a further 10 lines from the blood (EBH5.4 to -10) and three lines from the throat (EBH5.4* to -6*). Figure 4 shows the results obtained when these lines were screened by PCR amplification across the

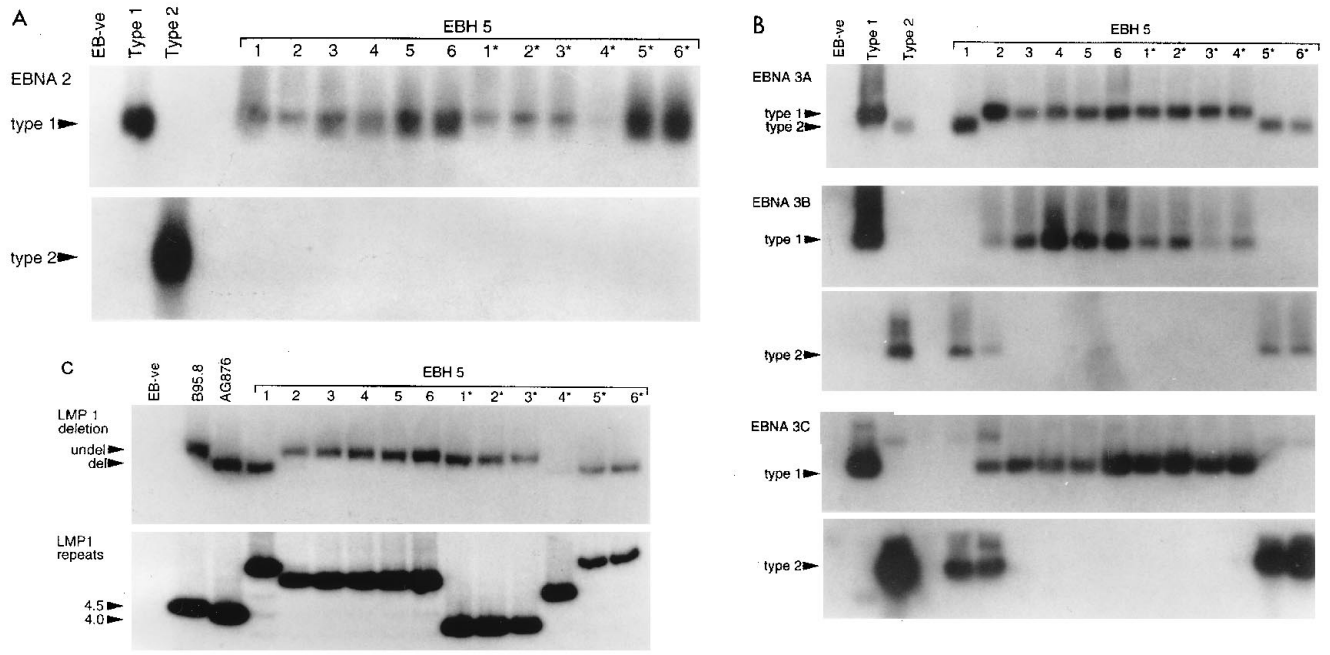

FIG. 4. PCR analysis of EBV strains carried by blood-derived LCLs 1 to 6 and throat washing-derived LCLs 1* to 6* from patient EBH5. Virus typing assays were carried out at the EBNA2 gene locus (A) and at the EBNA3A, -3B, and -3C gene loci (B); strain detection assays were carried out at the LMP1 gene loci (C) as for Fig. 1. Very occasionally in the EBNA3C typing assay, the type 1 probe shows weak hybridization to the type 2 PCR product if the latter is present in abundance (panel B, type 2 control track, EBNA3C type 1 panel); this is easily recognizable as a cross-reaction because of the characteristic size difference between the type 1 and type 2 PCR products. The blots show that some EBH5 isolates (e.g., 1, 5*, and 6*) display a type 1 EBNA2, type 2 EBNA3A, -3B, -3C genotype.

standard range of EBV genome polymorphisms; because lines 4 to 10 were all identical, this group is represented in Fig. 4 by lines 4 to 6. As with patient EBH34, all isolates from patient EBH5 were type 1 at the EBNA2 locus, with no evidence of coresident type 2 sequences even after long autoradiographic exposures (Fig. 3A and data not shown). When the analysis was extended to the EBNA3A, -3B, and -3C loci, most of the isolates again only gave type 1 signals, as would be expected from classical type 1 virus strains. However, blood-derived line 1 and throat washing-derived lines 5* and 6* were exclusively type 2 at all three loci, while blood-derived line 2 gave both type 1 and type 2 signals (Fig. 4B). Differences were also apparent between individual isolates at the polymorphic LMP1 gene loci. Thus, those LCLs which had been consistently type 1 at both EBNA2 and EBNA3 genes were clearly divisible into three subgroups (lines 3 to 6, 1* to 3*, and 4*, respectively) based on the presence or absence of the 30-bp deletion and on the number of 33-bp repeats. Furthermore, those LCLs showing a type 1 EBNA2/type 2 EBNA3 genotype (lines 1, 5*, and 6*) also showed a unique LMP1 genotype characterized by the presence of 30-bp deletion and by an estimated seven copies of the 33-bp repeat (Fig. 4C); note that line 2, which appeared to be a mixture by EBNA3 genotyping, did give very faint LMP1 amplification signals corresponding to those shown by lines 1, 5*, and 6* as well as much stronger signals corresponding to those shown by lines 3 to 6.

The subsequent EBNAprint analysis shown in Fig. 5 confirmed that EBH5 lines 3 to 5, lines 1* to 3*, and line 4* did indeed carry distinct type 1 virus strains. This is most apparent from the different sizes of their EBNA1 proteins (MAb-IH4 blot) and type 1 EBNA3C proteins (MAb-E3CA10 blot) and, for line 4*, from the slightly lower molecular weight of its type 1 EBNA2 protein (MAb-PE2 blot). More importantly, however, the gels clearly show that EBH5 lines 1, 5*, and 6* each carry a recombinant virus expressing a typical type 1 EBNA2 protein but an EBNA3C which must be type 2 because it is recognized by the human serum Hu-NZ and not by MAb E3CA10 (or by the type 1-specific human serum Hu-MS [data

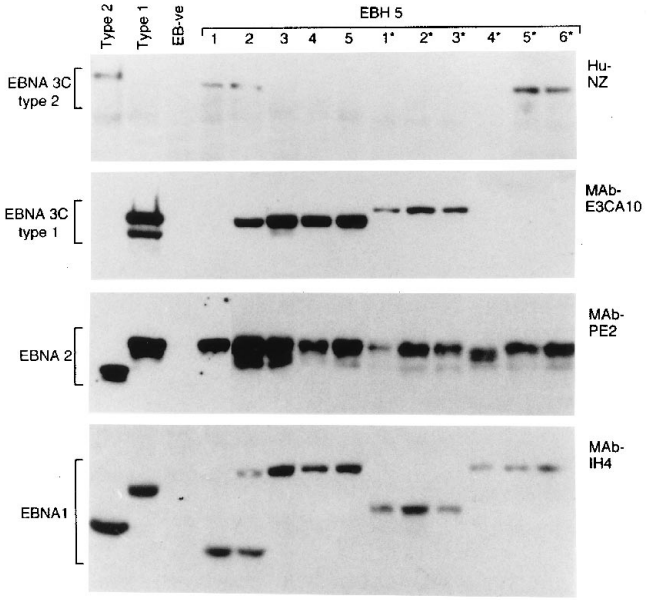

FIG. 5. EBNAprint analysis of EBV strains carried by blood-derived LCLs 1 to 5 and throat washing-derived LCLs 1* to 6* from patient EBH5, using the antibodies used for Fig. 2. Note that some EBH5 isolates (e.g., 1, 5*, and 6*) express an EBNA2 protein (appearing as a characteristic doublet) which by size is type 1 and an EBNA3C protein which antigenically is type 2.

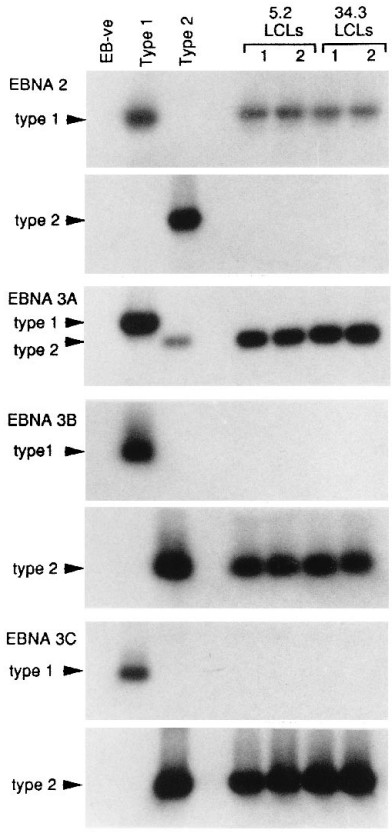

FIG. 6. PCR analysis of the EBV strains carried by in vitro-transformed LCLs established from cocultures between indicator B cells and X-irradiated cells of the EBH5.2 cell line (5.2 LCLs 1 and 2) or of the EBH34.3 cell line (34.3 LCLs 1 and 2). Virus typing assays were carried out at the EBNA2, EBNA3A, EBNA3B, and EBNA3C loci as for Fig. 1. Note that all four in vitro transformants display a type 1 EBNA2, type 2 EBNA3A, -3B, -3C genotype.

not shown]); these same reactivities are also present within the mixed EBNAprint shown by line 2. The type 1 EBNA2 and type 2 EBNA3C proteins appear to be constant in size between the different recombinant virus isolates. However, differences in EBNA1 protein size clearly distinguish the recombinant carried by lines 1 and 2 from that carried by the later-established lines 5* and 6* (MAb IH4 blot).

EBNA2 gene sequencing of EBH5 recombinant and type 1 viruses. The EBNA2 genes carried by different EBH5-derived recombinant and type 1 virus strains were compared by sequence analysis across the same EBNA2 regions as analyzed for the EBH34 isolates. The relevant data for representatives of each of the EBH5 strains are presented in Fig. 3. We found that recombinant viruses 5.1 and 5.5* had the same EBNA2 sequence, and this matched exactly that shown by two of the type 1 isolates, 5.3 and 5.2*. All showed 11 nucleotide changes vis-à-vis B95.8 plus a triplet nucleotide insertion, leading to six amino acid substitutions plus an additional leucine 215 residue. The third type 1 isolate, 5.4*, showed a very similar pattern of changes, with just one more nonconservative mutation compared with the B95.8 sequence. It is worth noting that the EBNA2 sequence shown by four of the five EBH5-derived virus strains is exactly that shown by the type 1 EBH34-derived isolate 34.3* (Fig. 3). In fact, this pattern of variance from the B95.8 sequence has been identified in a number of type 1 EBV strains in the literature (3, 37, 46), constituting a type 1 subgroup for which the W91 isolate is a prototype (46).

Secondary passage of EBH34 and EBH5 recombinants. A final series of experiments sought to determine whether the recombinant viruses noted above could be secondarily passaged by in vitro transformation of indicator B cells. Cocultures were therefore set up between (i) X-irradiated cells of the recombinant virus-carrying lines EBH34.3, EBH34.11, and EBH5.2 and (ii) target lymphocytes freshly isolated from the blood of adult EBV-seronegative donors. These particular lines were selected because they contained detectable numbers of cells spontaneously entering lytic cycle; in addition, we reasoned that use of the mixed line EBH5.2 (Fig. 4 and 5) should allow separation of its coresident type 1 and recombinant viruses.

All three sets of cocultures did indeed yield in vitro-transformed LCLs carrying the relevant recombinant virus. This was clear from PCR amplification data of the kind illustrated in Fig. 6. Here LCLs separately established by virus transfer either from the EBH5.2 line or from the EBH34.3 line can be seen only to contain virus with a type 1 EBNA2/type 2 EBNA3A, -3B, -3C genotype. Analysis at the protein level confirmed these observations, each of the secondarily transferred LCLs reproducing precisely the EBNAprint of the primary recombinant virus strain (data not shown). The recombinant nature of the virus passaged from EBH34.3, EBH34.11, and EBH5.2 is further confirmed by the immunoblots shown in Fig. 7. Here we used the recently developed EBNA2 type-specific MAbs R3 (type 1) and 3E91 (type 2) and the existing EBNA3C type-specific reagents MAb E3CA10 (type 1) and Hu-NZ (type 2) to show that these secondarily passaged viruses do indeed encode a type 1 EBNA2 protein and a type 2 EBNA3C protein.

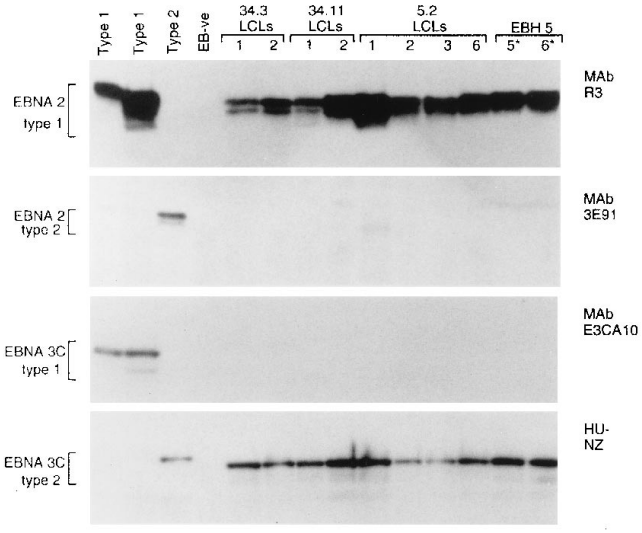

FIG. 7. EBNA typing analysis of EBV strains carried by in vitro-transformed LCLs established from cocultures between indicator B cells and X-irradiated cells of the EBH34.3 cell line (34.3 LCLs 1 and 2), the EBH34.11 cell line (34.11 LCLs 1 and 2), or the EBH5.2 cell line (5.2 LCLs 1, 2, 3, and 6). Also included in the analysis were the EBH5 throat washing-derived LCLs 5* and 6* (see Fig. 5), the standard panel of type 1, type 2, and EBV-negative control lines (see Fig. 2), and (in the extreme left-hand track) an additional type 1-positive control line carrying the B95.8 virus strain. The immunoblots were probed with the type 1 EBNA2-specific MAb R3, the type 2 EBNA2-specific MAb 3E91, the type 1 EBNA3C-specific MAb E3CA10, and the type 2 EBNA3C-specific human serum Hu-NZ. All of the EBH34- and EBH5-derived virus transformants express a type 1 EBNA2 protein and a type 2 EBNA3C protein.

DISCUSSION

Several studies of EBV DNA sequences either cloned directly or amplified by PCR from oral hairy leukoplakia biopsies have shown that nonhomologous recombination events are relatively common during EBV replication in vivo (27, 40, 44, 46, 47). These have been detected both at the EBNA2 (40, 44, 46, 47) and at the LMP1 repeat (27, 44) loci, occurring between genomes either of the same strain (27, 47) or of different coresident strains (44). Recombinations at the EBNA2 locus typically produce deletions and/or insertions in the EBNA2 gene (40, 46, 47); of particular interest in the present context is the report of a rearrangement, presumably arising by intertypic recombination, in which sequences from the *Bam*HI S region had been inserted between fragments of a type 1 EBNA2 gene on one side and of a type 2 EBNA2 gene on the other (44). Genomes containing such aberrant structures appear to be capable of replication in the oropharynx in vivo but, in the absence of an intact EBNA2 gene, would not be expected to retain B-cell growth-transforming capacity (9, 19); their ability to be transmitted and to establish persistent infections of the B-cell reservoir in a new host therefore remains to be determined. The present results, and simultaneous observations in another laboratory (7), have come from in vitro virus isolation studies rather than from the direct analysis of viral DNA in ex vivo samples; they provide the first evidence for the existence in the wild of intertypic EBV recombinants which retain both transforming and replicative functions.

The two patients described in this report each yielded recombinants with a type 1 EBNA2, type 2 EBNA3A, -3B, -3C genotype (Fig. 1, 2, 4, and 5). The ease with which these viruses were isolated and subsequently passaged by secondary transfer into indicator B cells in vitro (Fig. 6 and 7) suggests that naturally occurring recombinants with this structure are at least the equal of standard type 1 virus strains in B-cell-transforming ability. Furthermore, the LCLs with recombinant virus grew as quickly as type 1-carrying lines, consistent with the observation that differences in the outgrowth rates of type 1 and type 2 transformants (31) are genetically determined at the EBNA2 locus (9). It is worth recalling that in the pioneering study of Skare et al. (41), still the best-documented description of in vitro recombination between coresident EBV genomes, the recombinants produced were also type 1 at the EBNA2 locus and type 2 at the EBNA3 loci. In that case, however, the type 2 parental strain was the EBNA2 deletion mutant P3HR1 (29) and one of the type 1 parental strains used was Raji, a viral genome with a deletion in the *Bam*HI E region (34) now known to have resulted in loss of the EBNA3C gene. Not surprisingly, therefore, the recombinants selected for in vitro transforming ability contained the *Bam*HI YH (EBNA2) region from Raji and the *Bam*HI E (EBNA3C) region from P3HR1. Unlike in that earlier study, here we were unable to map the precise positions of recombination because the corresponding parental virus strains could not be isolated from either patient.

In the case of patient EBH34, the type 1/type 2 recombinant virus appeared to be the dominant viral species in vivo. Thus, the majority of LCLs isolated both from the throat washings and from the blood of this individual carried the recombinant virus as the only detectable viral strain (Fig. 1 and 2). This was the case both at the initial sampling in 1993 and on a second occasion 20 months later. A coresident type 1 strain was detected in some of the throat washing- and blood-derived LCLs from this patient on both occasions, but this virus was clearly not the source of the type 1 EBNA2 gene found in the recombinant. Sequencing of the EBNA2 genes in the EBH34 recombinant and EBH34 type 1 isolates revealed two different patterns of variance from the B95.8 prototype (Fig. 3). Respectively, these patterns broadly accord to the MT1444 and W91 EBNA2 prototype sequences described by Walling et al. (46) and to the type 1 EBNA2 variants classified as group 2 and group 3b by Schuster et al. (37). The dominance of the recombinant virus in patient EBH34, and the inability to detect either parental strain, raises the possibility that this patient acquired the recombinant by horizontal transmission from another carrier. Certainly the ability of the EBH34 recombinant to replicate at high levels in the oropharynx, coupled with its potent B-cell-transforming capacity, suggests that such a virus would be capable of efficient transmission and persistence in the human population.

In the case of EBH5, a type 1/type 2 recombinant was detectable in the blood on the first occasion of testing alongside a type 1 virus strain and in the throat on a second occasion of testing 21 months later alongside a different type 1 strain (Fig. 4 and 5). The blood-derived recombinant and that isolated subsequently from the throat are very likely related, not just because of their type 1 EBNA2/type 2 EBNA3A, -3B, -3C genotypes (Fig. 4A and B) and identical EBNA2 gene sequences (Fig. 3) but also because of their characteristic LMP1 gene markers, with the combination of a 30-bp deletion and seven copies of the 33-bp repeat (Fig. 3C). However, their EBNAprints, while identical at EBNA2 and EBNA3C, showed clear differences in EBNA1 protein size (Fig. 5). Such variation at a single locus of the EBNAprint, particularly at EBNA1 or EBNA3C, has been observed in other situations (16, 49) and on its own cannot be taken as an indicator of independent viral strains. On the contrary, it is thought to arise through intrastrain recombination occurring across internal repeat sequences present in these EBNA genes (13). While the blood- and throat washing-derived EBH5 recombinants therefore very likely have a common origin, the history of the original recombination event is less clear. Any of the coresident type 1 viruses isolated from this patient could possibly have been the source of the type 1 EBNA2 gene in the EBH5 recombinant. In particular, the strains represented by LCL 5.3 and by LCL 5.2* had EBNA2 proteins of the same size as that encoded by the recombinant (Fig. 5) and showed sequence identity over the regions of EBNA2 analyzed (Fig. 3). However, since this nucleotide sequence appears to be common to a number of different type 1 virus strains (37), including the EBH34-derived type 1 virus described earlier, then the source of the EBNA2 gene in the EBH5 recombinant cannot be unequivocally identified. Moreover, with this patient as with patient EBH34, no type 2 virus was isolated as a possible source of the EBNA3A, -3B, -3C gene region, raising the possibility that the recombinant strain had been acquired from another donor. In this context, it is worth noting that EBH5 appears to carry a particularly wide range of resident virus strains. This may reflect not just the effects of HIV-mediated T-cell suppression but also the fact that, uniquely among the 40 HIV-positive patients described in the accompanying report (50), EBH5 had a history of prior blood transfusion, another potential source of acquired EBV strains (15).

It would seem highly significant that these novel EBV recombinants have been isolated from immunocompromised patients, in fact from two unrelated individuals out of a random selection of only 40 HIV-positive patients analyzed (50). Such findings contrast markedly with the cumulative evidence from, we estimate, more than 250 independent EBV strains isolated from various human populations and derived either from healthy donors or from patients with EBV-associated tumors such as Burkitt's lymphoma and nasopharyngeal carcinoma

(15, 21, 29a, 49, 51). In every case these isolates were either uniformly type 1 or uniformly type 2 at the EBNA2, -3A, -3B, and -3C loci. Intertypic recombination requires the replication of two distinct virus types in the same target cell. We infer that our isolation of intertypic recombinants specifically from HIV-immunocompromised patients reflects (i) these patients' greater susceptibility to multiple EBV infection, particularly with type 1 and type 2 viral strains (24, 45, 46, 50) and (ii) the generally elevated levels of EBV replication occurring at permissive sites in these individuals (12, 17). Both factors would increase the chances of a permissive target cell, of either epithelial (17) or B-lymphoid (4) origin, being coinfected with both virus types. Interestingly, a simultaneous study from another laboratory has identified a type 1/type 2 recombinant among a range of EBV isolates rescued from apparently healthy donors in Papua New Guinea (7), a region where type 1 and type 2 viral strains appear to share roughly equal prevalence in the population (3, 51). This finding suggests that mixed-type infections, and the subsequent generation of intertypic recombinants, are not entirely restricted to severely immunocompromised individuals but can sometimes occur, albeit probably less frequently, in the general population. It will be interesting now to look more closely at those African populations where both EBV types are known to be prevalent (51) for similar evidence of intertypic recombinants.

Finally, the identification of these novel EBV strains raises questions about their possible disease association, particularly as such viruses are more likely to be found in the very cohorts of T-cell-immunocompromised patients who are at increased risk of EBV-driven immunoblastic lymphoma (6) and possibly also of EBV-positive Hodgkin's disease (11). In this context, by using PCR analysis alone it will be difficult to identify a recombinant virus when it is present against a background of coresident type 1 and type 2 viral strains. We therefore feel that, wherever possible, investigations into the identity of resident EBV strains in biopsy material should be augmented by virus isolations in vitro.

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