Molecular and Cellular Probes (1996) **10,** 453–461

Analysis of Epstein–Barr virus (EBV) type and variant in spontaneous lymphoblastoid cells and Hu-SCID mouse tumours

Chiara Menin,[1,2] Lucia Ometto,[1] Arianna Veronesi,[1] Silvio Roncella,[2] Barbara Corneo,[1] Marco Montagna,[1] Vincenzo Coppola,[1] Maria Luisa Veronese,[1] Stefano Indraccolo,[1,2] Alberto Amadori,[1] Anita De Rossi,[1] Manlio Ferrarini,[3] Luigi Chieco-Bianchi[1] and Emma D'Andrea[1]*

[1]*Institute of Oncology, Interuniversity Center for Research on Cancer,* [2]*IST Biotechnology Section, University of Padova and* [3]*Istituto Nazionale per la Ricerca sul Cancro, IST, Genova, Italy*

(Received 4 February 1996, Accepted 7 August 1996)

Epstein–Barr virus (EBV) type and strain variations were examined using both lymphoblastoid cell lines (LCLs), spontaneously derived *in vitro* from peripheral blood mononuclear cells (PBMC) of 15 HIV-1-seropositive individuals, and SCID mouse tumours induced by inoculation of PBMC from 11 healthy human donors (Hu-SCID tumours). Polymerase chain reaction (PCR) analysis disclosed that all but one of the 26 EBV+ samples harboured EBV nuclear antigen (EBNA) 2 and 3C type A virus. On the other hand, single strand conformation polymorphism (SSCP) analysis using Epstein–Barr encoded RNA (EBER) specific primers detected an AG876-like (type B) band pattern in 21 of the 26 EBV+ samples. Three Hu-SCID tumours scored as B95.8-like (type A), and two showed neither a type A nor a type B SSCP migration pattern. Sequence analysis of the amplified EBER fragments confirmed the PCR-SSCP findings; moreover, additional mutations were present not only in the two EBV+ samples with anomalous SSCP pattern, but also in two other samples with a standard SSCP profile. Thus, EBER analysis did not correlate with EBNA typing, and appeared to be unsuitable for EBV type assessment. Latent membrane protein (LMP) analysis disclosed, on the whole, seven size variants: as expected, the differences were due to the variable numbers of a 33-bp repeat in the amplified fragment, as assessed by direct sequencing. The broader variability detected by LMP analysis should prove more useful than typing for assessing the presence of single and/or mixed variants resulting from EBV reactivation and/or reinfection.

KEYWORDS: EBV nuclear antigen (EBNA), Epstein–Barr encoded RNA (EBER), latent membrane protein (LMP), PCR-SSCP, EBV typing.

INTRODUCTION

Epstein–Barr virus (EBV), an ubiquitous herpesvirus, is the causative agent of infectious mononucleosis, and is also involved in the pathogenesis of a variety of human tumours in patients with either an apparently

* Author to whom all correspondence should be addressed at: Istituto di Oncologia, Via Gattamelata 64, 35128 Padova, Italy.

normal or compromised immune system.[1] Analysis of the EBV genomes isolated from various regions of the world and from patients with different EBV-associated diseases has, thus far, failed to identify disease-specific viral subtypes.[2] Nonetheless, the finding of divergent sequences for EBV nuclear antigen (EBNA) 2[3] and EBNA 3[4,5] has led to the identification of two EBV types, A and B, also known as EBV 1 and 2, which show differing *in vitro* biological properties.[6] B cells immortalized by type A grow more rapidly, are less concentration-dependent, and reach a higher saturation density, compared with type B transformants. Type A virus shows a worldwide distribution, and is regarded as the overwhelmingly predominant type in Western communities. In contrast, type B virus has been found mainly in Central Africa and New Guinea[3,7] even though a wider distribution has been more recently suggested.[8,9]

DNA sequence analysis of Epstein–Barr encoded RNAs (EBERs) has, less frequently, been used as an additional typing parameter,[10,11] and found to be discordant with EBNA typing data in putative type A and B recombinants.[12] In addition to the broad distinction between EBV types (A and B), different viral isolates exhibit a high degree of heterogeneity, which can be partly assessed by latent membrane protein (LMP) sequence analysis.[13–15] Most healthy EBV carriers harbour a single, latent, viral isolate in their blood.[16–18] In immunocompromised (HIV-infected and transplanted) subjects, the immune system impairment might allow reactivation of, or reinfection with, different EBV variants.[19–22] In particular, changes in the dominant EBV strain have been reported during HIV infection.[23] In addition to EBV typing, more analytical approaches for the identification of EBV variants during single and/or mixed infections are needed. To this end, we explored LMP heterogeneity as well as EBV type in two kinds of EBV infected material; spontaneously *in vitro*-derived lymphoblastoid cell lines (LCLs) from 15 HIV-1-seropositive individuals, and Hu-SCID mouse tumours (Hu-SCID tumours) induced by the inoculation of PBMC from 11 healthy human donors. Our data do not support the reliability of EBER sequences as A and B typing determinants, but do suggest that LMP size variation may be useful in analysing EBV infections.

MATERIALS AND METHODS

Lymphoblastoid cell lines (LCLs)

Spontaneous LCLs were established as previously described[24] from 15 HIV-1-seropositive subjects. Briefly, peripheral blood mononuclear cells (PBMC) were isolated on Ficoll–Hypaque density gradients, and then cultured for 7 days in RPMI 1640 medium supplemented with 10% foetal calf serum (RPMI-FCS; Gibco, Grand Island, NY) at a concentration of 1×10^6 cells ml^{-1} in 24-well plates (Costar, Cambridge, MA) in the presence of 50 ng ml^{-1} Cyclosporin A (CyA; Sandoz, Basel, Switzerland). The cultures were fed weekly with fresh medium, and scored for proliferation by microscopical observation every 4 to 5 days. Small colonies began to appear within 20 to 50 days.

The HIV-1-seropositive subjects, 12 males and 3 females, ranged in age from 23 to 38 years; only one individual (LCL#6) had full-blown AIDS, 4 were asymptomatic, 7 had generalized lymphoadenopathy (LAS), and the remaining 3 had AIDS-related complex (ARC). All 15 were also seropositive for EBV.

Animals and PBMC cell inoculation

SCID mice were purchased from IFFA-Credo (L'Abreisle, France), and bred in our facilities under germ-free conditions. Blood was obtained from 11 EBV seropositive volunteers undergoing lymphapheresis, and PBMC were prepared by Ficoll–Hypaque centrifugation.[25] Groups of three to five SCID mice were injected intraperitoneally with $80–100 \times 10^6$ unfractionated PBMC from a corresponding donor, observed daily for signs of sickness, and eventually sacrificed. At autopsy, tumour masses were collected and divided into aliquots for biological and molecular studies.[26] As all mouse tumours induced by PBMC of a single donor harboured the same EBV variant,[18] a single abundant tumour specimen (representative of one donor) was used throughout this study.

EBV specific oligonucleotide primers

EBV was typed using oligonucleotide primers[11] common for EBNA 2 type and B sequences (Forward (F), 5'AGGCTGCCCACCCTGAGGAT3'; Reverse (R), 5'GCCACCTGGCAGCCCTAAAG3') which amplify a type A fragment of 168 bp (nucleotides 48 170–48 339 of the prototype B95.8 virus isolate)[27] and a type B fragment of 184 bp from the AG876 isolate, respectively.

To confirm EBNA 2 typing, we used a set of primers for EBNA 3C (F, 5'AGAAGGGGAGCGTGTGTTGT3'; R, 5'GGCTCGTTTTTGACGTCGGC3') which amplify a type A fragment of 153 bp (nucleotides 99 939–100 091 of B95.8) and a type B fragment of 246 bp from the AG876 isolate.[5] Primers F,

5′GTGGTCCGCATGTTTTGATC3′, and R, 5′GCAACGGCTGTCCTGTTTG3′ (nucleotides 6780–6969 of B95.8), were used to amplify EBER sequences.[11] A set of primers (F, 5′GGCGCACCTGGAGGTGGTCC3′; R, 5′TTTCCAGCAGAGTCGCTAGG3′) that immediately flank a portion of the LMP sequence of tandem repeats (nucleotides 168 594–168 374 in B95.8) was used to investigate viral variants.[13,14]

PCR and SSCP analysis

DNA from EBV-infected samples was phenol/chloroform extracted as previously reported.[28] PCR was performed using 0·5 μg of DNA, 0·5 μM of each primer, 200 μM of each dNTP, 10 mM Tris-HCl (pH 8·8), 50 mM KCl, 1·5 mM $MgCl_2$, 0·2 mg/ml BSA, and 2·5 U of *Taq* polymerase (Perkin–Elmer Cetus, Norwalk, CT) in a final volume of 100 μl. Thirty cycles of denaturation (94°C for 45 s), annealing (62°C, 45 s for EBNA 2; 55°C, 90 s for EBNA 3C; 60°C, 45 s for LMP), and extension (72°C for 90 s) were carried out in an automated thermal cycler (9600 Perkin–Elmer Cetus). The amplified mixture (20 μl) was electrophoresed either on 1·5% NuSieve (FMC, Rockland, ME), and 1·5% agarose, or a 10% polyacrylamide gel.

For EBER analysis, the reaction was performed in 50 μl for 25 cycles with annealing at 58°C for 30 s. The reaction mixture (10 μl) was then mixed with 1 μCi ^{33}P-dATP and 0·1 U of *Taq* polymerase, and five more amplification cycles were run. The amplified product was diluted 1:3 in 0·1% sodium dodecyl sulphate (SDS)—10 mM EDTA, and further mixed 1:3 with a stop solution in 95% formamide. Samples (10 μl) were heated at 95°C for 5 min, and loaded onto a 0·5 × polyacrylamide MDE gel solution (AT Biochem-Malvern, PA) containing 5% (v/v) glycerol in 0·6 × TBE. Electrophoresis was performed at 20 W for 4–5 h.

Direct DNA sequencing

A phosphorylated PCR product was generated by amplification with one 5′-phosphorylated primer using the same conditions described for PCR analysis. The PCR product was converted to a single-stranded form with 10 U of lambda exonuclease (Pharmacia, Uppsala, Sweden), an enzyme which selectively degrades double-stranded DNA starting from a phosphorylated 5′ terminus. Reaction components were removed from the template prior to and following lambda exonuclease treatment by extraction with an equal volume of phenol/chloroform, and purification by MicroSpin Columns containing Sephacryl S-400 HR resin (Pharmacia).

Single-stranded sequencing was performed according to the dideoxy chain termination method using the fmol DNA Sequencing System (Promega, Madison, WI) and a ^{33}P-5′-end-labelled primer which annealed to the same strand as the phosphorylated PCR primer. Electrophoresis was performed on 8% polyacrylamide gel containing 8 M urea at 65 W.

RESULTS

EBV typing by EBNA specific primers

As the vast majority of EBV typing data are routinely obtained by analysing 4 EBV genes coding for EBNA 2, 3A, 3B, and 3C,[4,5] the type of the 26 EBV+ samples in this study were first assessed by means of PCR analysis with amplimers located 5′ to the EBNA 2 coding region.[11] Figure 1 shows that all 15 LCLs harboured EBNA 2 type A virus (Fig. 1a); only one Hu-SCID tumour (donor G.C.) had EBNA 2 type B (Fig. 1b), in line with a previous report using different sets of primers.[18] EBNA 2 typing data were fully confirmed by PCR analysis for EBNA 3C (data not shown). Therefore, all but one of the 26 EBV+ samples studied harboured type A virus.

EBER specific PCR-SSCP analysis

Ten bp differences within the 1 kb EBER coding region were shown among three representative type A and B viruses;[10] using EBER specific primers, Lin *et al.*[11] more recently genotyped six EBV+ cell lines, and confirmed typing by EBNA 2 analysis. These same workers extended their study to nine additional EBV+ cell lines, and 16 EBV+ Hodgkin's disease biopsies. As reported in their tables, EBER and EBNA typing data were discordant in five of seven cell lines, and in five of 11 tumour specimens harbouring a single EBV type. In all cases, the EBV consensus type was assessed according to the EBNA analysis, and the discordant cases were interpreted as recombinants, i.e. either as a new EBV type or as a type A subtype.[12]

To better ascertain the relevance of EBERs as typing determinants, the 26 EBV+ samples were examined by PCR-SSCP analysis using the same EBER-specific amplimers. In all but two cases (LCL#10 and Hu-SCID tumour of donor T.S.), the PCR-SSCP patterns were superimposable on those of B95.8 or AG876 prototype viruses (Fig. 2); however, contrary to EBNA genotyping, most of the samples [14 LCLs (Fig. 2a) and 7 Hu-SCID tumours (Fig. 2b)] scored as type B

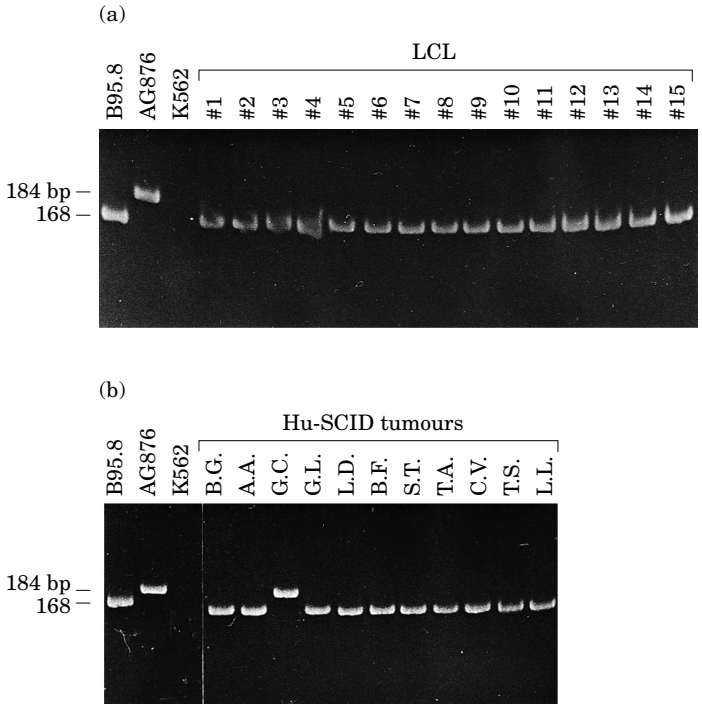

Fig. 1. EBNA 2-specific EBV typing of (a) LCLs spontaneously derived *in vitro* from 15 HIV seropositive individuals and (b) Hu-SCID tumours induced by injection of PBMC from 11 healthy human donors. EBNA 2 PCR products were identified by ethidium bromide staining following acrylamide gel electrophoresis. DNAs from B95.8 and AG876 cells were used as type A and B reference controls, respectively, while K562 DNA served as negative control. Numbers on the left refer to the length (bp) of the amplified, reference products.

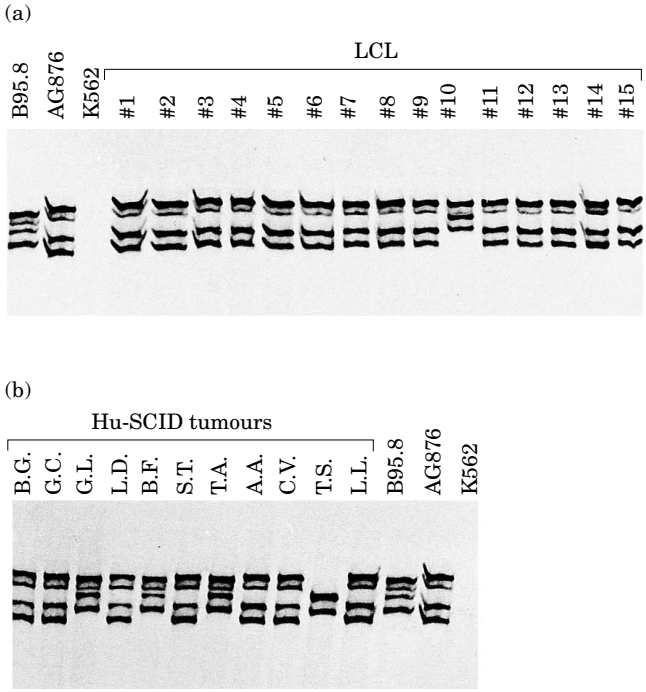

Fig. 2. PCR-SSCP analysis of EBER specific fragments of (a) LCLs spontaneously derived *in vitro* from 15 HIV seropositive individuals and (b) Hu-SCID tumours induced by injection of PBMC from 11 healthy human donors. EBER-specific primers and ^{33}P-dATP were used to obtain radioactive PCR products, which were denatured and separated by polyacrylamide/glycerol gel electrophoresis. The dried gels were then processed for autoradiography. Reference and negative controls as in Fig. 1.

	6799			856	872 871	886			6949
	808	834	854	870	884	911	927	939	944
B95.8	T	c	g g	c c c	G T	A	G	g	G
AG876	A	c	g g	c c c	A G	G	A	g	A
LCLs:									
1	A	c	g g	c c c	A G	G	G	g	A
2	A	c	g g	c c c	A G	G	G	g	A
3	A	c	g g	c c c	A G	G	G	g	A
4	A	c	g g	c c c	A G	G	G	g	A
5	A	c	g g	c c c	A G	G	G	g	A
6	A	c	g g	c c c	A G	G	G	g	A
7	A	c	g g	**t t t**	A G	G	G	g	A
8	A	c	g g	c c c	A G	G	G	g	A
9	A	c	g g	c c c	A G	G	G	g	A
10	A	c	g g	c c c	A G	G	G	**a**	A
11	A	c	g g	c c c	A G	G	G	g	A
12	A	c	g g	c c c	A G	G	G	g	A
13	A	c	g g	c c c	A G	G	G	g	A
14	A	c	g g	c c c	A G	G	G	g	A
15	A	c	g g	c c c	A G	G	G	g	A
Hu-SCID tumours:									
A.A.	A	c	g g	c c c	A G	G	G	g	A
S.T.	A	c	g g	c c c	A G	G	G	g	A
B.G.	A	c	g g	c c c	A G	G	G	g	A
C.V.	A	c	g g	c c c	A G	G	G	g	A
G.C.	A	c	g g	c c c	A G	G	G	g	A
L.D.	A	c	g g	c c c	A G	G	G	g	A
L.L.	A	**t**	g g	c c c	A G	G	G	g	A
T.A.	T	c	g g	c c c	G T	A	G	g	G
T.S.	T	c	**a t**	c c c	G T	A	G	g	G
B.F.	T	c	g g	c c c	G T	A	G	g	G
G.L.	T	c	g g	c c c	G T	A	G	g	G

Fig. 3. Sequence analysis of EBER-specific amplified fragments from the 26 EBV+ samples. The top bar represents the 150-bp EBER fragment from position 6799 to 6949 of the B95.8 genome. Six of the numbers above the bar indicate the position of the bases (capital letters) which differ between B95.8 and AG876 prototype viruses. The remaining seven numbered positions correspond to the bases (small letters) that were found altered (bold) in the samples examined.

(AG876-like), while only 3 Hu-SCID tumours presented a type A (B95.8-like) profile. Therefore, no correlation was found between EBNA and EBER genotyping among the viruses present in our material.

Sequence analysis of EBER amplified fragments

To confirm the PCR-SSCP data, all 26 EBER fragments were sequenced directly on both strands (Fig. 3). While type A guanine at position 6927 was present in all the samples, all the LCLs were characterized by a B-type nucleotide at the remaining 5 positions (capital letters in Fig. 3), which differ between B95.8 and AG876 viruses. A few additional mutations were also detected (small, bold letters in Fig. 3): a G to A at position 6939 in LCL#10, which could account for the corresponding anomalous PCR-SSCP band pattern in Fig. 2; and a CCC to TTT stretch at positions 6870–6872 in LCL#7 with, surprisingly, no apparent change in PCR-SSCP migration pattern (compare with Fig. 2). The Hu-SCID tumours (Fig. 3) with a type A PCR-SSCP pattern (donors T.A., B.F., G.L.) disclosed a type A sequence at all six positions, while a further transition (G to A at position 6854) and transversion (G to T at position 6856) could have caused the anomalous PCR-SSCP pattern in the otherwise type A donor, T.S. Among the seven Hu-SCID tumours harbouring type B virus, an additional C to T mutation at position 6834 (with no alterations in the PCR-SSCP pattern) was detected. On the whole, sequencing data confirmed PCR-SSCP findings at five out of six positions; the two 'silent' exceptions (LCL#7 and Hu-SCID tumour of donor L.L.) should caution against straight-forward generalizations based on SSCP migration patterns.[29]

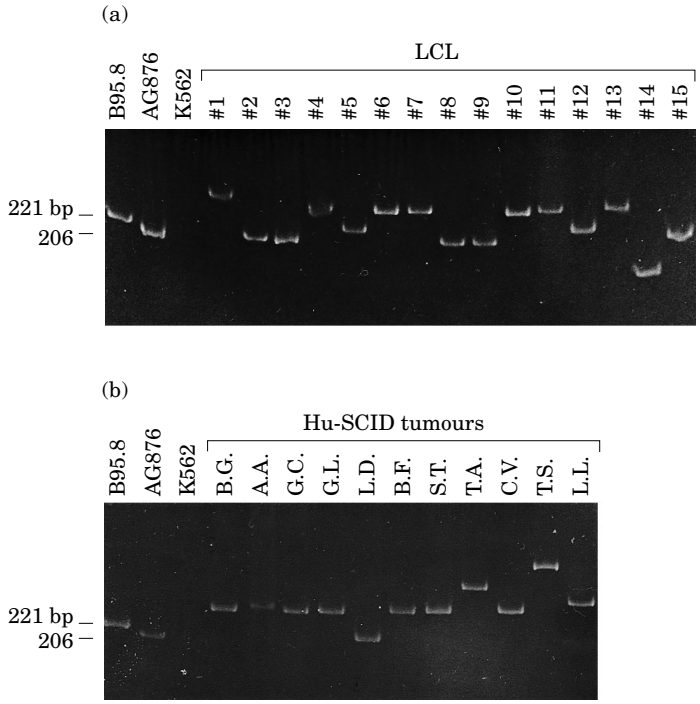

Fig. 4. LMP strain variability in (a) LCLs spontaneously derived *in vitro* from 15 HIV seropositive individuals and (b) Hu-SCID tumours induced by the injection of PBMC from 11 healthy human donors. LMP-specific PCR products were separated by polyacrylamide gel electrophoresis and detected by ethidium bromide staining. Positive and negative controls as in Fig. 1.

LMP sequence variants in LCLs and Hu-PBMC-SCID tumours

Sequence variations at both terminal regions of LMP have been described. An *Xho*I restriction polymorphism is apparently confined to strains from broadly different geographical regions,[15] while nasopharyngeal carcinoma (NPC)-like deletions of amino acids (aa) 343–352 were detected in a third of Hodgkin's disease (HD) specimens, but not in reactive conditions by a Swiss study.[30] In our samples, which indirectly represent natural isolates within northern Italy, the higher degree of LMP heterogeneity should be present in its tandem repeat region.[15] All 26 EBV+ samples were therefore examined for strain variability using LMP-specific primers encompassing the repeat region. Figure 4 shows that ethidium bromide staining distinguished LMP fragments of different size in both LCLs (Fig. 4a) and Hu-SCID tumours (Fig. 4b). Visual inspection disclosed that electrophoretic separation through a polyacrylamide matrix yielded a good fragment resolution (compared with the two prototype viruses), and five bands with different sizes could be readily detected in each set of samples (LCLs in Fig. 4a and Hu-SCID tumours in Fig. 4b).

Sequence analysis of the seven different LMP fragments detected in the 26 EBV+samples

The LMP region of B95.8 (type A) virus is characterized by a 33-bp element which is repeated three times; a fourth 48-bp element derives from the insertion of a 15-bp stretch, while a 30-bp element is localized at the 3' terminus.[13]

Direct sequencing of the LMP variant fragments in the study samples further defined LMP repeat composition (Fig. 5), and seven variants could be identified. The most common variant (239 bp) was carried by 13 EBV+ samples (six LCLs and seven Hu-SCID tumours), while four additional variants (305, 272, 221, 173 bp) were each detected in one case; of the remaining two variants, one (206 bp) was found in five LCLs and one Hu-SCID tumour (donor L.D.), and the other (254 bp) in LCL#1 and Hu-SCID tumour of donor L.L.

DISCUSSIONS AND CONCLUSIONS

EBV type and strain were analysed in 26 EBV+ samples consisting of spontaneously derived LCLs

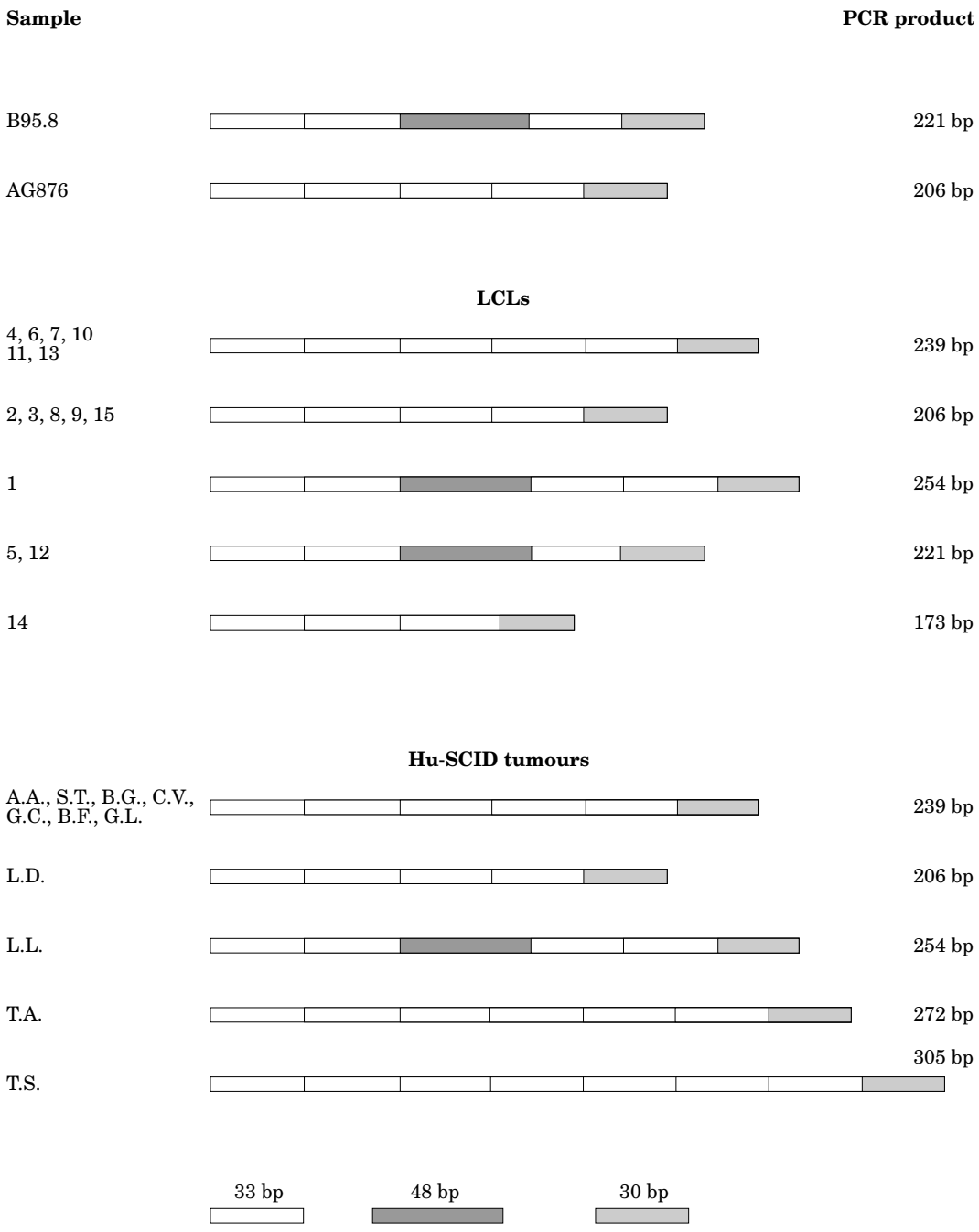

Fig. 5. Structural organization, as established by direct sequencing, of LMP-specific PCR products from the 26 EBV+ samples. On top, the structural organization of LMP repeats in B95.8 and AG876 viral prototypes. On the left, the size in bp of the PCR-amplified LMP product. At the bottom, schematic representation of single 33, 48 and 30-bp repeats.

from 15 HIV-1-seropositive individuals, and tumours induced in SCID-mice by the inoculation of PBMC from 11 normal human subjects. Samples were either oligo- or mono-clonal, with either only episomal (latent) or linear (productive) and episomal EBV genomes (Menin *et al.*[18] and data not shown). PCR analysis with primers specific for EBNA 2 and 3C gave concordant typing results for, and confirmed the presence of, type A virus in all the samples, except one (Hu-SCID tumour of donor B.G.) in which type B virus was found. On the contrary, EBER evaluation by means of PCR-SSCP analysis gave discordant results. Indeed, all LCLs but one (LCL#10), and seven of 11 Hu-SCID tumours (and donors) disclosed an EBER type B profile. Direct sequencing of the amplified EBER fragments confirmed the PCR-SSCP findings, and detected a few additional mutations which did not cause any anomaly in the PCR-SSCP pattern.

Similar variations were previously interpreted as an indication that a third type (or a subtype of type A) of EBV might have arisen by recombination. This was, apparently, not the case in our samples as evidence of mixed infections could not be obtained (data not shown). Indeed, identical EBNA2 (and LMP) fragments were detected in: (1) mouse tumours induced by cells from two separate lymphaphereses from the same donor;[18] (2) five independent LCL#5 clones derived immediately after immortalization; and (3) five different samples of LCL#6,[31] despite its origin from an AIDS patient. To assess the putative existence of additional EBV types or subtypes, it would be necessary to identify functional parameters associated with EBNA type A and EBER type B 'hybrid' sequences directly in patients' samples, in order to avoid any possible selection phenomenon.

However, detection of viral variants by LMP analysis might prove to be more important than EBV typing. Indeed, different LMP constructs, derived from B95.8 virus or a nasopharyngeal carcinoma (NPC) isolate, showed strikingly different transforming capacities following transfection into murine 3T3 cells.[32] LMP is also expressed in HD[33] and NPC[34] tumour tissue, while EBNA 2 is absent.[35,36] Furthermore, variations at the LMP carboxy-terminal domain might disrupt some of the biological functions of this protein.[37,38]

Among the 26 studied EBV+ samples, seven EBV variants could be readily detected, thus confirming LMP sequence heterogeneity. The major variations among the different EBV+ samples were due to different reiterations of the 11-aa repeat, and therefore they could be readily detected by ethidium bromide staining of standard polyacrylamide gels (Fig. 4). LMP analysis, therefore, might represent a further valuable means for studying single or multiple EBV variants resulting from EBV reactivation and/or reinfection that are known to occur in some immunocompromised patients.[8,19,21,23,39–43]

ACKNOWLEDGMENTS

We are grateful to Dr G. W. Bornkamm for providing AG876 cells. This work was supported in part by grants from the Italian Association for Research on Cancer (AIRC); National Research Council (Target Projects ACRO, BTBS, and FATMA); ISS, AIDS Project; MURST 40% and 60%; EU Program: Human Capital and Mobility (ERBCHRXCT940651 to L. C-B. and M.F.). We thank Mrs S. Cargnin, M. Quaggio, and D. Zullato for excellent technical assistance, Mr M. Ghidotti for animal care, P. Gallo for artwork, and Mrs P. Segato for help in preparing the manuscript.

REFERENCES

1. Miller, G. (1990). Epstein–Barr virus: biology, pathogenesis, and medical aspects. In *Virology* (Fields, B. N., Knipe, D. M., Chanock, R. M., Hirsch, M. S., Melnick, J. L., Monath, T. P. & Roizman, B., eds) Pp. 1921–1958. New York: Raven Press.
2. Chen, X., deV Pepper, S. & Arrand, J. R. (1992). Prevalence of the A and B types of Epstein–Barr virus DNA in nasopharyngeal carcinoma biopsies from Southern China. *Journal of General Virology* **73**, 462–6.
3. Zimber, U., Adlinger, H. K., Lenoir, G. M. *et al*. (1986). Geographical prevalence of two types of Epstein–Barr virus. *Virology* **154**, 56–66.
4. Rowe, M., Young, L. S., Cadwallader, K., Petti, L., Kieff, E. & Rickinson, A. B. (1989). Distinction between Epstein–Barr virus type A (EBNA 2A) and type B (EBNA 2B) isolates extends to the EBNA 3 family of nuclear proteins. *Journal of Virology* **63**, 1031–9.
5. Sample, J., Young, L., Martin, B. *et al*. (1990). Epstein–Barr virus type 1 and 2 differ in their EBNA-3A, EBNA-3B, and EBNA-3C genes. *Journal of Virology* **64**, 4084–92.
6. Rickinson, A. B., Young, L. S. & Rowe, M. (1987). Influence of the Epstein–Barr virus nuclear antigen EBNA2 on the growth phenotype of virus transformed B cells. *Journal of Virology* **61**, 1310–17.
7. Young, L. S., Yao, Q. Y., Rooney, C. M. *et al*. (1987). New type B isolates of Epstein–Barr virus from Burkitt's lymphoma and from normal individuals in endemic areas. *Journal of General Virology* **68**, 2853–62.
8. Sixbey, J. W., Shirley, P., Chesney, P. J., Buntin, D. M. & Resnick, L. (1989). Detection of a second widespread strain of Epstein–Barr virus. *Lancet* **ii**, 761–5.
9. Apolloni, A. & Sculley, T. B. (1994). Detection of A-type and B-type Epstein–Barr virus in throat washings and lymphocytes. *Virology* **202**, 978–81.
10. Arrand, J. R., Young, L. S. & Tugwood, J. D. (1989). Two families of sequences in the small RNA-encoding region of Epstein–Barr virus (EBV) correlate with EBV types A and B. *Journal of Virology* **63**, 983–6.
11. Lin. J.-C., De, B. K. & Lin, S.-C. (1993). Rapid and sensitive genotyping of Epstein–Barr virus using single-strand conformation polymorphism analysis of polymerase chain reaction products. *Journal of Virological Methods* **43**, 233–46.
12. Lin, J.-C., Lin, S.-C., De, B. K., Chan, W.-P. & Evatt, B. L. (1993). Precision of genotyping of Epstein–Barr virus by polymerase chain reaction using three gene loci (EBNA 2, EBNA 3C, and EBER): predominance of type A virus associated with Hodgkin's disease. *Blood* **81**, 3372–81.
13. Fennewald, S., vanSanten, V. & Kieff, E. (1984). Nucleotide sequence of an mRNA transcribed in latent growth-transforming virus infection indicates that it may encode a membrane protein. *Journal of Virology* **51**, 411–9.
14. Hennessy, K., Fenwald, S., Hummell, M., Cole, T. & Kieff, E. (1984). A membrane protein encoded by Epstein–Barr virus in latent growth-transforming infection. *Proceedings of the National Academy of Science, USA* **81**, 7207–11.
15. Miller, W. E., Hood Edwards, R., Walling, D. M. & Raab–Traub, N. (1994). Sequence variation in the

Epstein–Barr virus latent membrane protein 1. *Journal of General Virology* **75**, 2729–40.
16. Yao, Q. Y., Rowe, M., Martin, B., Young, L. S. & Rickinson, A. B. (1991). The Epstein–Barr virus carrier state: dominance of a single growth-transforming isolate in the blood and in the oropharynx of healthy virus carriers. *Journal of General Virology* **72**, 1579–90.
17. Kunimoto, M., Tamura, S., Tabata, T. & Yoshie, O. (1992). One-step typing of Epstein–Barr virus by polymerase chain reaction: predominance of type 1 virus in Japan. *Journal of General Virology* **73**, 455–61.
18. Menin, C., Ometto, L., Veronesi, A. *et al.* (1995). Dominance of a single Epstein–Barr virus strain in SCID mouse tumors induced by injection of peripheral blood mononuclear cells from healthy human donors. *Virus Research* **36**, 215–31.
19. Sculley, T. B., Apolloni, A., Hurren, L., Moss, D. J. & Cooper, D. A. (1990). Coinfection with A- and B-type Epstein–Barr virus in human immunodeficiency virus-positive subjects. *Journal of Infectious Diseases* **162**, 643–8.
20. Kyaw, M. T., Hurren, L., Evans, L. *et al.* (1992). Expression of B-type Epstein-Barr virus in HIV-infected patients and cardiac transplant receipients. *AIDS Research and Human Retroviruses* **8**, 1869–74.
21. Gratama, J. W., Lennette, E. T., Lonnqvist, B. *et al.* (1992). Detection of multiple Epstein–Barr viral strains in allogeneic bone marrow transplant receipients. *Journal of Medical Virology* **37**, 39–47.
22. Gratama, J. W., Oosterveer, M. A. P., Weimar, W. *et al.* (1994). Detection of multiple "ebnotypes" in individual Epstein–Barr virus carriers following lymphocyte transformation by virus derived from peripheral blood and oropharynx. *Journal of General Virology* **75**, 85–94.
23. Buisson, M., Morand, P., Genoulaz, O., Bourgeat, M. J., Micoud, M. & Seigneurin, J. M. (1994). Changes in the dominant Epstein–Barr virus type during human immunodeficiency virus infection. *Journal of General Virology* **75**, 431–7.
24. Roncella, S., Caretto, P., Forni, G. *et al.* (1990). Assessment of the potential malignancy of Epstein–Barr virus (EBV)-infected B cells in AIDS-related disorders. *Cancer Detection and Prevention* **14**, 325–9.
25. Veronese, M. L., Veronesi, A., D'Andrea, E. *et al.* (1992). Lymphoproliferative disease in human peripheral blood mononuclear cell-injected SCID mice. I. T. lymphocyte requirement for B cell tumor generation. *Journal of Experimental Medicine* **176**, 1763–7.
26. Veronesi, A., Coppola, V., Veronese, M. L. *et al.* (1994). Lymphoproliferative disease in human peripheral blood mononuclear cell-injected SCID mice. II. Role of host and donor factors in tumor generation. *International Journal of Cancer* **59**, 676–83.
27. Baer, R., Bankier, A. T., Biggin, M. D. *et al.* (1984). DNA sequence and expression of the B95.8 Epstein–Barr virus genome. *Nature* **310**, 207–11.
28. Amadori, A., Veronese, M. L., Mazza, M. R. *et al.* (1992). Lymphoma induction by human B cells in SCID mice. *Leukemia* **6**, 23s–25s.
29. Hayashi, K. Yandell, D. W. (1993). How sensitive is PCR-SSCP? *Human Mutation* **2**, 338–46.
30. Knecht, H., Bachmann, E., Brousset, P. *et al.* (1993). Deletions within the LMP1 oncogene of Epstein–Barr virus are clustered in Hodgkin's disease and identical to those observed in nasopharyngeal carcinoma. *Blood* **82**, 2937–42.
31. Roncella, S., Francia di Cele, P., Cutrona, G. *et al.* (1993). Cytogenetic rearrangement of c-myc oncogene occurs prior to infection with Epstein–Barr virus in the monoclonal malignant B cells from an AIDS patient. *Leukemia and Lymphoma* **9**, 157–64.
32. Chen, M.-L., Tsai, C.-N., Liang, C.-L. *et al.* (1992). Cloning and characterization of the latent membrane protein (LMP) of a specific Epstein–Barr virus variant derived from the nasopharyngeal carcinoma in the Taiwanese population. *Oncogene* **7**, 2131–40.
33. Murray, P. G., Young, L. S., Rowe, M. & Croker, J. (1992). Immunohistochemical demonstration of the Epstein–Barr virus-encoded latent membrane protein in paraffin sections of Hodgkin's disease. *Journal of Pathology* **166**, 1–5.
34. Brooks, L., Yao, Q. Y., Rickinson, A. B. & Young, L. S. (1992). Epstein–Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts. *Journal of Virology* **66**, 2689–97.
35. Brousset, P., Chittal, S., Roya, L. & Delsol, G. (1991). Absence of Epstein–Barr virus nuclear antigen 2 in tumor cells of Hodgkin's disease. *Lancet* **337**, 1107.
36. Deacon, E. M., Pallesen, G., Niedobitek, G. *et al.* (1993). Epstein–Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells. *Journal of Experimental Medicine* **177**, 339–49.
37. Moorthy, R. K. & Thorley-Lawson, D. A. (1993). Biochemical, genetic, and functional sites on the Epstein–Barr virus-encoded oncogenic latent membrane protein LMP-1. *Journal of Virology* **67**, 2637–45.
38. Izumi, K. M., Kaye, K. M. & Kieff, E. (1994). Epstein–Barr virus recombinant molecular genetic analysis of the LMP1 amino-terminal cytoplasmic domain reveals a probable structural role, with no component essential for primary B-lymphocyte growth transformation. *Journal of Virology* **68**, 4369–76.
39. Katz, B. Z., Niederman, J. C., Olson, B. A. & Miller, G. (1988). Fragment length polymorphisms among independent isolates of Epstein–Barr virus from immunocompromised and normal hosts. *Journal of Infectious Diseases* **157**, 299–308.
40. Preiksaitis, J. K., Diaz-Mitoma, F., Mirzayans, F., Roberts, S. & Tyrrel, D. L. J. (1992). Quantitative oropharyngeal Epstein–Barr virus shedding in renal and cardiac transplant receipients: relationship to immunosuppressive therapy, serologic responses, and the risk of posttransplant lymphoproliferative disorder. *Journal of Infectious Diseases* **166**, 986–94.
41. Borisch, B., Finke, J., Henning, I. *et al.* (1992). Distribution and localization of Epstein–Barr virus subtypes A and B in AIDS-related lymphomas and lymphatic tissue of HIV-positive patients. *Journal of Pathology* **168**, 229–36.
42. Shibata, D., Weiss, L. M., Nathwani, B. N., Brynes, R. K. & Levine, A. M. (1991). Epstein–Barr virus in benign lymph node biopsies from individuals infected with human immunodefiency virus is associated with concurrent or subsequent development of Non-Hodgkin's Lymphoma. *Blood* **77**, 1527–33.
43. Shibata, D., Weiss, L. M., Hernandez, A. M., Nathwani, B. N., Bernstein, L. & Levine, A. M. (1993). Epstein–Barr virus-associated non-Hodgkin's lymphoma in patients infected with the human immunodeficiency virus. *Blood* **81**, 2102–9.