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Two Families of Sequences in the Small RNA-Encoding Region of Epstein-Barr Virus (EBV) Correlate with EBV Types A and B

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DNA sequence analysis was carried out on the 1-kilobase *Sac*I-*Eco*RI region of the *Eco*RI J fragment of four strains of Epstein-Barr virus (EBV) (MABA, P3HR-1, FF41, and NPC-5), and the sequences were compared with the prototype sequence from strain B95-8. Ten single-base changes which grouped the strains into two families (1 and 2) were found. Restriction endonuclease polymorphisms predicted from the sequences were used to classify the EBV DNA from a further 26 EBV-positive cell lines into these two families. The EBNA-2 types (A or B) of the strains were found to correlate with the J region type; EBNA-2 type A DNA regularly contained J region sequence type 1, while EBNA-2 type B DNA generally carried J region sequence type 2. These data are consistent with the notion of there being two distinct families of EBV with discrete, conserved differences in DNA sequence.

Epstein-Barr virus (EBV) is a member of the herpesvirus family of viruses. It is one of the most common infectious agents of humans and has been found in all populations worldwide. It is estimated that 80 to 90% of the total human population is infected with EBV (17). Within such a huge natural pool of virus there exists the potential for extensive evolutionary variability, but the extent of any such variation is, however, not clear. Analysis of the restriction enzyme cleavage patterns of several isolates (5, 7, 11, 14, 35, 36, 38) revealed restriction enzyme fragment polymorphisms and differences in the extent of some repeated sequences and identified a few deletions. Recently, some of the deletions in Raji DNA were precisely mapped by DNA sequence analysis (15). The same study compared the *Eco*RI D fragment of the Raji strain with that of the prototype B95-8 strain. In addition, analysis of the DNA around the EBNA-2-encoding region in several strains defined two types (A and B) of EBV (1, 10, 41) which encode antigenically distinct forms of EBNA-2. Nevertheless, the general conclusions are that all strains that have been studied are closely related (5) and that there is no evidence for subtypes specific for the different EBV-associated diseases (7).

We decided to address the question of potential evolutionary variation at the nucleotide level by comparing the sequence of a defined region of EBV DNA in several strains. The rightmost *Sac*I-*Eco*RI subfragment of EBV *Eco*RI-J is a little over 1 kilobase (kb) in length and contains the transcriptional units for two EBV-specified small RNAs (J-RNAs or EBERs), each about 175 bases in length (2, 25). These RNAs, which in some cells are the most abundant EBV-encoded transcripts (2, 34), are transcribed by RNA polymerase III (20, 33), and it appears that no other RNA species are transcribed from this region of the genome (4, 19). This segment of EBV DNA is suitable for a comparative sequence study because (i) it is relatively short; (ii) it contains two transcribed regions which specify (presumably) functional RNA molecules and which therefore most likely have conservational pressures on them; (iii) it contains the sequences of the intragenic controlling signals for RNA polymerase III which are known to vary between different polymerase III-transcribed genes (8), but it is not known whether there is variation within a given gene in different individuals of the same species; and (iv) it contains transcriptionally silent regions between and flanking the genes which may be relatively free of conservational pressure and show a greater degree of divergence.

We present the results of the sequence analysis of this region of the genome in four isolates from different geographical and pathological backgrounds and compare the sequence with the known sequence (2, 4, 33) of the prototype B95-8 strain. These sequences predicted restriction endonuclease polymorphisms which were used to type a further 26 strains. A broad correlation was found between the sequence in the small-RNA region and EBNA-2 type A or B.

Cloned *Sal*I A fragments (which contain the *Eco*RI J fragment) from EBV strains MABA and P3HR-1 were kindly provided by G. Bornkamm. The strain FF41 J fragment cloned into pACYC184 was a gift from G. Miller. The *Eco*RI J fragments from these three strains plus that from the B95-8 strain (3) were subcloned into pAT153. Cellular DNA obtained from a nasopharyngeal carcinoma (NPC) biopsy (NPC-5; 39) was cleaved with *Eco*RI and, following agarose gel electrophoresis, the 2.8- to 4-kb fraction was cloned into lambda gt10 (Vector Cloning Systems). Phage containing the *Eco*RI J fragment were selected, and the J fragment was subcloned into pAT153.

The cloned *Eco*RI J DNA fragments from the five EBV strains were initially screened for variation by restriction enzyme cleavage and gel electrophoresis. When several multicut endonucleases were used, only a single polymorphism was observed: the MABA J fragment contained one extra *Sau*3AI site (data not shown).

For sequence analysis the 1-kb *Sac*I-*Eco*RI fragment was isolated and cloned into M13 mp18 and mp19 either intact or after further cleavage with *Sau*3AI. Sequences were obtained by the chain termination method of Sanger et al. (37) (Fig. 1). The sequences from strains FF41 and NPC-5 were

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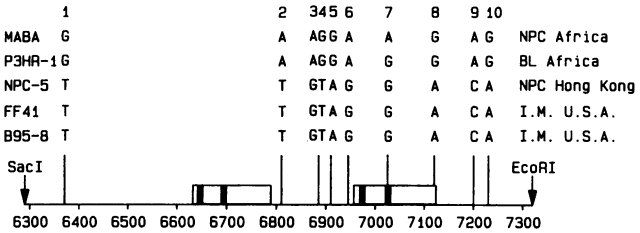

FIG. 1. Summary of the DNA sequences in the EBER region between the *Sac*I site within *Eco*RI-J and the *Eco*RI site at the right end of the J fragment of five strains of EBV. Nucleotides within the EBV DNA sequence are numbered as described by Baer et al. (4) and are shown at the bottom. The positions of the coding sequences for the two small RNAs (2, 20) are shown as open boxes; the intragenic transcriptional controlling sequences are shown as filled boxes. The sites of sequence variation are shown by the vertical lines and are numbered 1 to 10. The nucleotide found at each variant position in the five EBV strains listed on the left is shown above the corresponding line. The geographical origin and disease association of each strain are shown on the right. BL, Burkitt's lymphoma; I.M., infectious mononucleosis. Site 1, Position 6370; site 2, position 6808; site 3, position 6884; site 4, position 6886; site 5, position 6911; site 6, position 6944; site 7, position 7023; site 8, position 7123; site 9, position 7198; site 10, position 7231.

identical to that of B95-8. However, P3HR-1 and MABA belonged to a second "family" of viruses, showing, respectively, 9 and 10 single-base changes as compared with the first family. The changes were clearly nonrandom since, of the 10 base changes in MABA, compared with B95-8, 9 were also found in P3HR-1.

As anticipated, variations tended to cluster in the 160-base-pair "spacer" region between the termination site of EBER-1 and the initiation point of EBER-2, whereas the genes themselves were more conserved. The sequence of EBER-1 was completely maintained in all strains, while EBER-2 showed a change in the last base before the termination T cluster in both MABA and P3HR-1. In MABA there was a second change which created the observed *Sau*3AI polymorphism and which fell within the second intragenic control region (box B) for polymerase III transcription. This change, which appears to be rare (see Table 1), did not give the box B sequence any closer resemblance to the consensus sequence (8). It did, however, indicate that between individuals of a given species variations can occur in the intragenic promoter regions of the same gene.

The function of the EBER RNAs is unknown, although, owing to their abundance in latently infected B cells and in many NPC biopsies, they are often assumed to be important in transformation (2, 39). At first sight it appears unlikely that the observed sequence variation has much functional significance, since (i) most changes occur outside the coding regions, and polymerase III transcription signals are intragenic; (ii) the box B polymorphism is very rare, and there does not appear to be any correlating biological feature in the two cell lines harboring this change; and (iii) the single-base change at the 3′ end of EBER-2 in type 2 sequences falls within a region of strong homology between EBER-1 and EBER-2 (2), and both variants of this sequence can bind to the La protein (13).

Some of the cell lines used in this study have been maintained in tissue cultures for many years, raising the possibility of in vitro-induced changes. This seems unlikely, since the NPC-5 sequence, which has never been passaged in tissue cultures and was maintained in *Escherichia coli* for only a few weeks prior to sequencing, is identical to the

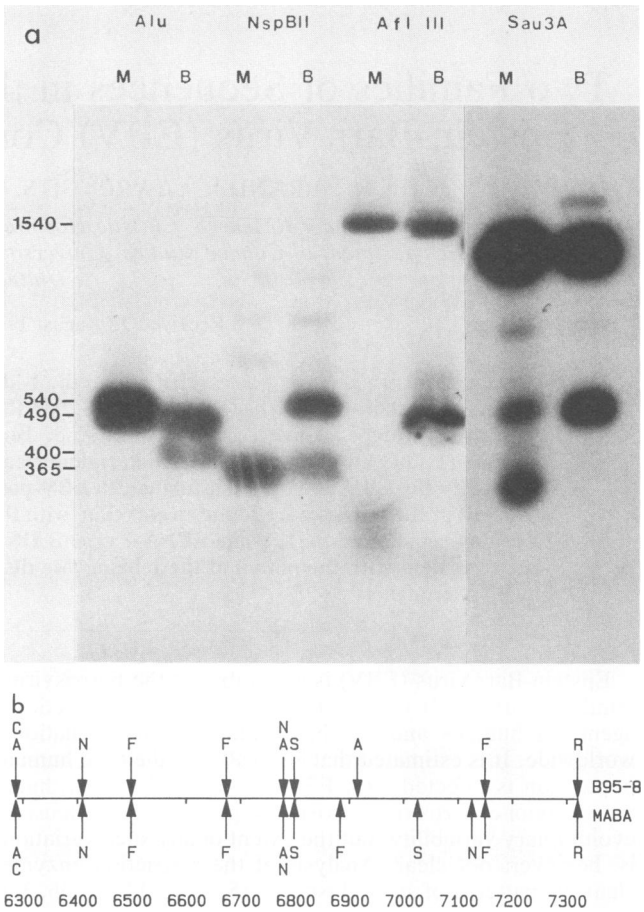

FIG. 2. Restriction enzyme fragment patterns produced by cleavage at polymorphic sites. DNA from plasmid pAT153 containing the *Eco*RI J fragment from B95-8 EBV (B) or MABA EBV (M) was cleaved with *Eco*RI plus *Alu*I, *Nsp*BII, *Afl*III, or *Sau*3AI as indicated. The fragments were fractionated by electrophoresis on a 1.5% agarose gel, transferred to nitrocellulose membranes, and hybridized with the ^{32}P-labeled *Sac*I-*Eco*RI 1-kb subfragment of *Eco*RI-J (Fig. 1) by previously described methods (3). The numbers on the left indicate fragment lengths in base pairs. Restriction endonuclease map of the EBER region of B95-8 EBV and MABA EBV between the *Sac*I (C) and *Eco*RI (R) sites showing the polymorphic sites for *Alu*I (A), *Nsp*BII (N), *Afl*III (F), and *Sau*3AI (S). Note that some fragments in panel a extend to the left of the *Sac*I site.

B95-8 sequence, which has been maintained in tissue cultures since the early 1970s (27) and cloned in *E. coli* since 1979 (3).

The occurrence of two sequence families prompted us to look for changes in restriction endonuclease recognition sequences which could be used as a simple screen for larger numbers of samples. Four such sequences were found at variant site 4 (*Afl*III), site 5 (*Alu*I), site 7 (*Sau*3AI), and site 8 (*Nsp*BII) (Fig. 1). The fragment patterns produced by these enzymes are shown in Fig. 2a, and a map is shown in Fig. 2b.

Cellular DNA was prepared from 26 EBV-positive cell lines, digested with *Eco*RI plus each of the four enzymes individually, and fractionated on 1.5% agarose gels alongside similarly digested markers of cloned *Eco*RI J fragments from B95-8 and MABA. Following transfer to nitrocellulose or Hybond (Amersham Corp.) membranes, the filters were probed with the ^{32}P-labeled 1-kb *Sac*I-*Eco*RI subfragment of

TABLE 1. Summary of restriction endonuclease polymorphisms in EBV DNA from various sources

DNA	Derivation	Sequence type[a] following cleavage with:				Consensus type	EBNA-2 type
		*Sau*3AI	*Alu*I	*Nsp*BII	*Afl*III		
Wangu	N[b]	1	1	2	1	1	A
Alvoch	N	1	2	2	2	2	B
Igunga	N	1	1	1	1	1	A
Ojango	N	1	2	2	2	2	B
Nekesa	N	1	1	1	1	1	A
Ogada	N	1	2	2	2	2	A
Nzivo	N	1	2	2	2	2	B
Odhiambo	N	1	1	1	1	1	A
Babu	N	1	2	2	2	2	B
Makena	N	1	2	2	2	2	A
Muthoki	N	1	1	1	1	1	A
Wan BL	BL[b]	1	1	1	1	1	B
BL18	BL	2	2	2	2	2	A
BL16	BL	1	2	2	2	2	B
WW1 BL	BL	1	2	2	2	2	A
WW2 BL	BL	1	2	2	2	2	B
Mwika BL	BL	1	1	1	1	1	A
Chep BL	BL	1	1	1	1	1	B
BL74	BL	1	1	2	1	1	A
BL72	BL	1	1	2	1	1	A
Eli BL	BL	1	2	2	2	2	B
BL29	BL	1	2	2	2	2	B
BL36	BL	1	1	1	1	1	A
Liv BL	BL	1	1	2	1	1	A
Daudi	BL[c]	1	1	1	1	1	—[d]
Raji	BL[e]	1	1	1	1	1	A[f]
P3HR-1	BL[g]	1	2	2	2	2	B[h]
B95-8	IM[i]	1	1	1	1	1	A[j]
FF41	IM[k]	1	1	1	1	1	ND[l]
MABA	NPC[m]	2	2	2	2	2	A[j]
NPC-5	NPC[n]	1	1	1	1	1	ND

[a] Type 1, B95-8 class; type 2, MABA class.
[b] DNA was obtained from spontaneous lymphoblastoid cell lines obtained from normal individuals (N) or Burkitt's lymphoma biopsies (BL) (24, 31, 32).
[c] From reference 23.
[d] —, Daudi DNA is deleted in the EBNA-2-encoding region (21).
[e] From reference 29.
[f] From reference 10.
[g] From reference 18.
[h] P3HR-1 DNA is deleted in the EBNA-2-encoding region (6, 16, 22, 30). However, Jijoye, the parental strain of P3HR-1, is a B-type virus (1).
[i] DNA was obtained from an infectious mononucleosis (IM) patient-derived strain of EBV (27).
[j] From reference 41.
[k] From reference 12.
[l] ND, Not determined.
[m] From reference 9.
[n] *Eco*RI J fragment cloned from an NPC biopsy (this work).

*Eco*RI-J. From the fragment pattern the EBV DNA could be assigned to the B95-8 (type 1) or MABA (type 2) family (Table 1).

In arriving at the "consensus type" (Table 1), little weight was given to the rare *Sau*3AI polymorphism since, with this enzyme, the type 2 sequence was found in only two strains (MABA and BL18). In addition, although the other three enzymes generally indicated the same family, in four strains (Wangu, BL72, BL74, and Liv BL) *Nsp*BII did not follow the same pattern as *Alu*I and *Afl*III.

It is well established that a variation in the EBNA-2 gene defines two families of EBV. It was of interest to determine whether the two sequence families within *Eco*RI-J correlated with EBNA-2 type. The cell lines were typed as expressing EBNA-2 type A or B as previously described (40) (Table 1).

P3HR-1 (17) is derived from the Burkitt lymphoma cell line Jijoye (28) and is the prototype B-type virus (1). Thus, although P3HR-1 has a deletion of the EBNA-2 region, it is considered to be a B-type EBV.

It is evident that in general, J sequence family 1 parallels EBNA-2 type A and vice versa, although there are some exceptions. MABA, Makena, Ogada, BL18, and WW1 BL have a type 2 J region and a type A EBNA-2 gene, whereas Chep BL and Wan BL have a type 1 J region and a type B EBNA-2 gene. These "mixed" viruses could have arisen by recombination between a type A virus and a type B virus somewhere in the approximately 40 kb which separate the J region from the EBNA-2 gene. In this context it is worth noting that dual EBV infections do occur naturally (26).

The mixed virus DNA from Wan BL has previously been shown to be heterogeneous (40), and a spontaneous lymphoblastoid cell line from the same patient contained a type A EBNA-2 region (L. S. Young, unpublished observations). The two strains which carry the rare *Sau*3AI polymorphism are both mixed viruses. However, owing to the small numbers it is not clear whether this observation has any significance.

Previous studies on the A and B classification of EBV DNA have utilized cell lines of Burkitt lymphoma origin and, thus far, this has been the only disease associated with type B EBV. It is therefore notable that MABA is of NPC origin and, although it is EBNA-2 type A, it is type 2 for the J region. However, like most other type B viruses, it is of African origin.

A comparison of the sequences of the BALF2 and BALF3 open reading frames from Raji and B95-8 DNAs revealed high homology, suggesting that these two strains "are very close in evolutionary terms" (15). This conclusion is in agreement with the results of the present work.

This study has demonstrated specific microheterogeneity in the sequence of a region of EBV DNA which seems to be conserved as two families and maintained essentially in parallel with the A and B types of EBV previously defined. Interestingly, recent data (M. Rowe, L. S. Young, K. Cadwallader, L. Petti, E. Kieff, and A. B. Rickinson, J. Virol., in press) indicate analogous familial antigenic variation in the EBNA-3 (*Bam*HI-E) region of EBV. The phenotypes formerly ascribed to EBNA-2 type A or B should therefore now be correlated with the whole viruses, since clearly there are multiple changes linked to these distinct alleles of EBNA-2. It will be of interest to see to what extent this two-family relationship is evident in the rest of the EBV genome.

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