Epstein–Barr Virus Nuclear Antigen (EBNA): Size Polymorphism of EBNA 1

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SUMMARY

The mol. wt. of the polymorphic Epstein–Barr virus (EBV) nuclear antigen (EBNA) molecule (EBNA 1) encoded by the BamHI K fragment of the EBV DNA has been determined in 14 EBV-carrying lymphoblastoid and Burkitt's lymphoma cell lines. There is no obvious correlation between the size of this polypeptide and any properties of the cells from which it is derived, other than those related to the strain of transforming virus. We confirm that the polymorphic region of this molecule is the glycine–alanine copolymer encoded by the third internal repeat of the EBV genome (IR3) and we consider the significance of this domain.

The Epstein–Barr virus (EBV) nuclear antigen (EBNA) was first demonstrated in EBV-transformed cells by a complement-enhanced immunofluorescence assay, ACIF (Reedman & Klein, 1973). Recently, it has been shown that EBNA, as demonstrated by ACIF using polyspecific human antisera, is not a single entity but rather a complex of at least two and possibly more antigenic components. The major component has been identified as a polypeptide encoded by the large HindIII subfragment of the BamHI K fragment of the EBV genome (Hennessy & Kieff, 1983; Fischer et al., 1984; our unpublished data). This molecule apparently exists in two forms, both with the same mol. wt.: Class I EBNA, which is readily extracted from the nucleus, and Class II which is tightly bound to chromatin (Spelsberg et al., 1982; Sculley et al., 1983). Further, the polypeptide is subject to a size polymorphism (Strnad et al., 1981) which correlates with the length of the third internal repeat region of the genome, IR3 (Hennessy et al., 1983; Fischer et al., 1984), and binds to EBV DNA in an apparently specific manner (Gergely et al., 1984). In addition to the polymorphic EBNA, Hennessy & Kieff (1983) have also described a non-polymorphic 82K mol. wt. polypeptide (designated EBNA 2) which is serologically distinct from the BamHI K-encoded molecule (designated EBNA 1).

To establish whether there is a prevalent form of the polymorphic EBNA 1, SDS extracts from 10 EBV-carrying cell lines were separated by polyacrylamide gel electrophoresis and immunoblotted using EBNA-positive human serum. The results from studies on a variety of cells, shown in Fig. 1 and Table 1, suggest that 79K is the most common mol. wt. form of EBNA 1. This size polypeptide is found in B95-8, MST, Namalwa and Wil2 as well as in other B95-8 transformed lines, IBW9, MSP, WT46 and Maja (data not shown). This antigen has been identified as a 78K species in Daudi, a 75K species in P3HR1, AW-Ramos and Putko, and a 69K species in Raji cells. In BL8, it is found as a 94K species, the largest EBNA 1 so far reported. A second fine band of about 81K was visible in some lanes. It is not, however, consistently detected and similar-sized bands appear in extracts from EBV-negative cells, such as Ramos (data not shown). The significance of these bands is uncertain, but they are unlikely to be EBV-associated antigens.

The variation in molecular weight of EBNA 1 does not appear to alter its nuclear distribution and there is obvious correlation between its size and the properties of various cells, except those related to the strain of transforming virus (see Table 1).

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Fig. 1. Immunoblots of proteins from 10 EBV-carrying cell lines. In each case a pellet of 10^6 to 2 \times 10^6 cells was washed twice in phosphate-buffered saline (PBS) and solubilized in SDS-PAGE sample buffer (Laemmli, 1970). These samples were then electrophoresed on a 10% polyacrylamide gel in the presence of 0.1% SDS, using the discontinuous buffer system of Laemmli (1970). The ratio of acrylamide to bisacrylamide used was 40:1. Proteins were transferred to nitrocellulose as described by Burnette (1981) and probed with EBNA-positive human serum (diluted 1/100) followed by anti-human IgG-peroxidase conjugate used in conjunction with a substrate mix containing 0.4 mg/ml 3,3'-diaminobenzidine tetrahydrochloride and 0.0125% hydrogen peroxide. (a, d) B95-8; (b) BL8; (c, l) MST; (e) Raji; (f) P3HR1; (g) AW-Ramos; (h) Putko; (i) Daudi; (j) Namalwa; (k) Wil2.

The observation that BL8 (Harris et al., 1984) has a very large EBNA 1 (94K) and that this is a comparatively new cell line (< 2 years) suggested to us that smaller antigens found in older cell lines could be an artefact produced by culture in vitro, extensive passage resulting in the gradual deletion of DNA encoding EBNA 1. To test this hypothesis, we immunoblotted proteins from four cell lines recently established from African Burkitt's lymphomas (C. Rooney, personal communication). The results (Fig. 2 and Table 1) show, however, that even newly established cell lines (< 50 passages) show polymorphism (Chep, 73K; Muk, 73K; Eli, 75K; Obagi, 79K) and have neither particularly large nor small EBNA 1 polypeptides. This suggests that there is a natural polymorphism in the 'wild' population of viruses.

The variable region of EBNA 1 is encoded by the IR3 repeat, and in the marmoset lymphoblastoid line B95-8, it consists entirely of glycine and alanine residues (Hennessy & Kieff, 1983; Baer et al., 1984; Allday & MacGillivray, 1985). The absence of arginine and lysine from this sequence of over 200 residues makes the protein resistant to a number of proteases, including trypsin. Trypsinized nuclear extracts (see Allday & MacGillivray, 1985) from cell lines Raji, MST and BL8 were immunoblotted using EBNA-positive human antiserum. Fig. 3 shows that the resulting major antigenic product is polymorphic. This confirms that the IR3-encoded region (together with eight additional residues isolated with it in the proteolytic cleavage) is responsible for the size polymorphism and that it may account for up to 46% of the total EBNA 1 molecule. In each case the difference between the mol. wt. of the native EBNA 1 and that of the IR3 peptide is approximately 50K. This would be expected if only the IR3-encoded region of the molecule is polymorphic.

Any increase in the length of the IR3 repeat DNA sequence (as seems to have occurred in BL8; Harris et al., 1984, and confirmed by us) could have arisen by unequal crossover (Smith,
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Source of virus/cells</th>
<th>Cell type</th>
<th>Producer (N) non-producer</th>
<th>Copies of genome</th>
<th>Age of line</th>
<th>Mol. wt. of EBNA 1</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B95-8</td>
<td>Infectious mononucleosis isolate</td>
<td>Marmoset B-lymphoid</td>
<td>P</td>
<td>63</td>
<td>&gt;10 years</td>
<td>79K</td>
<td>Miller et al. (1972)</td>
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<td>Raji</td>
<td>Burkitt's lymphoma</td>
<td>Human B-lymphoid</td>
<td>NP</td>
<td>+</td>
<td>50</td>
<td>&gt;10 years</td>
<td>69K</td>
</tr>
<tr>
<td>Daudi</td>
<td>Burkitt's lymphoma</td>
<td>Human B-lymphoid</td>
<td>P (weak)</td>
<td>+</td>
<td>120-150</td>
<td>&gt;10 years</td>
<td>78K</td>
</tr>
<tr>
<td>Namalwa</td>
<td>Burkitt's lymphoma</td>
<td>Human B-lymphoid</td>
<td>NP</td>
<td>2-3</td>
<td>&gt;10 years</td>
<td>79K</td>
<td>See Arrand et al. (1983)</td>
</tr>
<tr>
<td>P3HR1</td>
<td>Burkitt's lymphoma</td>
<td>Human B-lymphoid</td>
<td>P*</td>
<td>+</td>
<td>300-1000</td>
<td>&gt;10 years</td>
<td>75K</td>
</tr>
<tr>
<td>Putko</td>
<td>P3HR1-MLK562 fusion</td>
<td>Hybrid</td>
<td>NP</td>
<td>34</td>
<td>&gt;10 years</td>
<td>75K</td>
<td>See Arrand et al. (1983)</td>
</tr>
<tr>
<td>AW-Ramos</td>
<td>P3HR1-infected Ramos</td>
<td>Human B-lymphoid</td>
<td>NP</td>
<td>1</td>
<td>&gt;10 years</td>
<td>75K</td>
<td>See Arrand et al. (1983)</td>
</tr>
<tr>
<td>BL8</td>
<td>Burkitt's lymphoma</td>
<td>Human B-lymphoid</td>
<td>P (weak)</td>
<td>225</td>
<td>approx. 2 years</td>
<td>94K</td>
<td>Harris et al. (1984)</td>
</tr>
<tr>
<td>Wil2</td>
<td>EBV-positive splenic lymphocytes</td>
<td>Human B-lymphoid</td>
<td>?</td>
<td>?</td>
<td>17</td>
<td>&gt;10 years</td>
<td>79K</td>
</tr>
<tr>
<td>MST</td>
<td>B95-8-infected peripheral lymphocytes</td>
<td>Human B-lymphoid</td>
<td>?</td>
<td>?</td>
<td>approx. 10 years</td>
<td>79K</td>
<td>de Kretser et al. (1983)</td>
</tr>
<tr>
<td>Muk</td>
<td>Burkitt's lymphoma</td>
<td>Human B-lymphoid</td>
<td>P</td>
<td>?</td>
<td>&gt;10 years</td>
<td>73K</td>
<td>C. Rooney, pers. comm.</td>
</tr>
</tbody>
</table>

* The virus produced is mainly non-transforming.
Fig. 2. Immunoblot (from SDS–10% polyacrylamide gel, as described in Fig. 1) of proteins from four Burkitt’s lymphoma-derived cell lines. The blot was probed with EBNA-positive human serum. (a) Partially purified EBNA 1 (see Fig. 3) from MST; (b) Obagi; (c) Muk; (d) Chep; (e) Eli.

Fig. 3. Immunoblot (from SDS–10% polyacrylamide gel) of trypsin-digested, partially purified EBNA 1 from three EBV-carrying cell lines. EBNA 1 was extracted from nuclei in essentially the same manner used to prepare Class I EBNA by Sculley et al. (1983). The clarified nuclear extract was then rapidly heated to 70 °C and shaken at this temperature for 10 min before cooling on ice. After centrifugation at 10000g for 20 min, the supernatant containing EBNA 1 was mixed with at least 2 vol. ethanol and allowed to precipitate overnight at −20 °C. This precipitate was pelleted at 10000g for 30 min and resuspended in a volume of extraction buffer equivalent to one-tenth of that of the original extract. The concentrated extract was stored in aliquots at −70 °C until use. One-hundred µl aliquots were incubated for 1 h at 37 °C with 10 µl 0.25 µg (w/v) trypsin. The reaction was terminated by mixing with 100 µl SDS–PAGE sample buffer and boiling for 3 min. Forty µl samples were immediately electrophoresed and immunoblotted as described in Fig. 1. (a) Raji; (b) MST; (c) BL8.

1976). This in turn could cause frameshifts which would introduce arginine residues (Heller et al., 1982) and hence potential cleavage sites for trypsin. Our results suggest that this has not taken place in BL8. It is not possible, however, to say whether the alternative frameshift (which could introduce glutamine and glutamate) or any other minor amino acid changes have occurred.

Recent reports (Yates et al., 1984a, b) show that EBNA 1 has an important function in the efficient maintenance of extrachromosomal EBV episomes in infected (and transfected) cells; the IR3 repetitive region can be deleted without apparent loss of this function. This suggests that the triplet repeats found in the DNA are dispensable for the trans-acting function that EBNA 1 provides for the putative origin of EBV plasmid replication. These data pose something of a paradox: the glycine–alanine domain appears to be dispensable for a major function of EBNA 1 and can be deleted (Yates et al., 1984b; see also Fischer et al., 1984) yet it is retained by all strains of EBV examined and can represent up to almost 50% of the native molecule. This could mean that the IR3-encoded region has no role other than separating functional (e.g. DNA-binding)
protein domains. It is equally likely, however, that this region of EBNA 1 has an as yet unidentified role which is vital to the virus although it is not concerned with plasmid maintenance and is independent of the size polymorphism. A suggestion regarding function comes from a consideration of the potential structure(s) of this unusual domain.

Although glycine-rich peptides are generally non-helical and disordered (Zubay, 1983), Rhodes et al. (1984) have proposed that the EBNA 1 glycine–alanine polymer may form a relatively ordered and rigid structure. They also present evidence that intermolecular interactions, which produce dimers or even multimers, may occur. This would be consistent with the observations that EBNA(1) forms aggregates in vivo (Lenoir et al., 1976; Luka et al., 1978) and lends support to the suggestion of Hennessy & Kieff (1983) that the glycine-rich region of EBNA 1 may be involved in such assemblies. Interaction between individual molecules appears to be a feature of the 'cooperative binding' exhibited by proteins, such as histone H1, which bind to DNA in large clusters (see Alberts et al., 1983). This may also be the case with EBNA 1 binding. Further indirect support for this notion comes from evidence that glycine-rich domains in other polypeptides, e.g. the cytokeratins (Fuchs & Hanukoglu, 1983), may be involved in the assembly of polymeric structures.

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REFERENCES


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