Interaction between Epstein–Barr Virus-determined Nuclear Antigen (EBNA) and the Viral DNA

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SUMMARY

Epstein–Barr virus (EBV) nuclear antigen (EBNA) was purified from the Burkitt lymphoma line Raji and its EBV DNA-binding properties were characterized. EBNA binding protected fragments of about 30 bp of B95-8 cell-derived EBV DNA from an excess of DNase I. Human anti-EBNA antibodies prevented DNA binding. Purified extracts from EBNA-negative cells did not protect EBV DNA against DNase I digestion. Mapping of the EBV DNA fragments protected from endonuclease (EcoRI, HindIII, SalI) digestion revealed many binding sites. Similar results were obtained following mixing of crude cell extracts and HindIII-digested fragments of EBV DNA and subsequent immunoprecipitation of the EBNA–DNA complex. In experiments involving the analysis of EBV DNA, fragments were protected from DNase I digestion by purified EBNA.

The Epstein–Barr virus (EBV) nuclear antigen (EBNA) has been purified and analysed biochemically. EBNA clearly binds to heterologous DNA such as calf thymus DNA, but it is not known whether the antigen binds equally well to all DNA sequences (Luka et al., 1978). Immunofluorescence studies have shown that EBNA is associated with metaphase chromosomes (Ohno et al., 1977). It is possible to convert EBNA-negative nuclei (Ramos) in vitro to EBNA-positive nuclei by addition of the solubilized nuclear antigen from Raji cells (Hirsch et al., 1978). EBNA shows higher binding affinity to double-stranded than to single-stranded DNA (Luka et al., 1977). The biologically active form of EBNA has a molecular weight of 170000 to 200000 (Luka et al., 1978), but a variety of polypeptides between 39K and 81K have been shown to possess EBNA activity as visualized on Western blots (Strnad et al., 1981). According to recent evidence EBNA is encoded by the BamHI fragment K of the EBV DNA (Summers et al., 1982), and Hennessy et al. (1983) have presented data that EBNA has a core of simple amino acid composition.

The biological functions of EBNA may stem from its DNA-binding properties. Conceivably, it may restrict the virus from entering the lytic cycle and/or it may be responsible for the transformation (immortalization) of the target cells. Further biochemical and functional characterization of EBNA is very important for the understanding of the mechanisms of transformation, viral DNA replication and selective gene expression. Towards this end, we have studied the interaction of purified EBNA with EBV DNA in vitro.

EBNA was purified from Raji cells by dsDNA–cellulose chromatography followed by hydroxyapatite chromatography and chromatofocusing according to the method of Luka et al. (1983). To prevent proteolytic breakdown, 2 mM-phenylmethylsulphonyl fluoride (PMSF), 1 mM-tosylphenylalanine chloromethyl ketone and 10 µg/ml pepstatin A were included in all buffers. Analysis of the purified EBNA by SDS–gel electrophoresis showed a major 65K and a minor 70K EBNA component, essentially without further contaminating bands. Chromatofocusing suggested that the EBNA polypeptides were acidic proteins, with a pI of about 5. The higher and the lower molecular weight components were structurally related according to the

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Table 1. **Effect of human anti-EBNA antibody on the binding of the EBNA–DNA complex to the membrane filter***

<table>
<thead>
<tr>
<th>Proteins added to EBV DNA</th>
<th>% DNA retained on filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBNA (approx. 250 ng)</td>
<td>Without DNase I digestion</td>
</tr>
<tr>
<td>Elephant</td>
<td>3.0</td>
</tr>
<tr>
<td>Mock EBNA (approx. 250 ng)</td>
<td>0.3</td>
</tr>
<tr>
<td>EBNA + anti-EBNA-positive serum</td>
<td>0.4</td>
</tr>
<tr>
<td>EBNA + EBV-negative serum</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Experiments were carried out as described in the text. EBV DNA was labelled *in vitro* by nick translation (Rigby *et al.*, 1977) to a sp. act. of 10⁶ c.p.m./µg DNA. 50 ng DNA was used for each test.

Results of peptide mapping and comparative amino acid analyses. DNA-binding proteins were prepared from the EBV genome-negative BJAB cells as described by Luka *et al.* (1980). EBV was purified from B95-8 cell supernatants according to the method of Bornkamm *et al.* (1980). The purified viral DNA was dissolved in 20 mM-Tris–HCl pH 7-5, 0.1 mM-EDTA in 1 µg DNA/10 µl buffer concentration and stored at −20 °C. Lambda DNA was obtained from Boehringer Mannheim.

All binding and protection reactions were at 20 °C. Between 0.05 and 0.1 µg DNA was diluted into 100 µl buffer containing 20 mM-Tris–HCl pH 7-5, 150 mM-KCl, 0.1 mM-EDTA, 1 mM-2-mercaptoethanol (2-ME), 10% glycerol, 200 µg/100 µl bovine serum albumin and 2 µg/100 µl calf thymus DNA. The excess of calf thymus DNA was always present as a competitive DNA to reduce possible non-specific binding of EBNA to EBV DNA. After addition of purified EBNA, the binding reaction was allowed to proceed for 10 min. Digestion was then initiated by addition of MgCl₂ to 10 mM and of 5 µg DNase I, and the mixture was incubated for 6 min at 20 °C. The DNA bound to protein was collected on 13 mm diam. 0.45 µm nitrocellulose filters and washed thoroughly with 20 mM-Tris–HCl pH 7-5, 150 mM-KCl, 0.1 mM-EDTA, 1 mM-2-ME, 150 mM-KCl and 10% glycerol. The filters were transferred to 1.5 ml Eppendorf tubes in 100 µl elution buffer (20 mM-Tris–HCl pH 7-5, 0.1% SDS, 0.1 mM-EDTA) and incubated at 44 °C for 30 min. When the protected fragments were analysed by gel electrophoresis, the elution buffer was 10 mM-Tris–borate pH 8-3, 0.05 mM-MgCl₂, 0.1% SDS, 10% glycerol, 0.025% bromophenol blue, 0.25% xylene cyanol FF.

First we tested the ability of EBNA to protect DNA from nuclease digestion in the presence or absence of specific anti-EBNA serum. Fifty µl human serum (anti-EBNA-positive or -negative serum) was mixed with Protein A–Sepharose diluted 50% in 20 mM-Tris–HCl pH 7-5, 150 mM-KCl, 1 mM-EDTA. After 12 h at 4 °C the Protein A–Sepharose–IgG complexes were centrifuged (500 g). The pellets were washed five times with the same buffer, EBNA (250 ng in the same buffer containing 10% glycerol) was mixed with them and left for 1 h at 4 °C. After centrifugation the supernatant was used for further absorption with fresh Protein A–Sepharose–IgG complexes. Usually this was repeated twice, and the remaining supernatant was used for DNA binding.

The binding of EBV DNA to the membrane filter was dependent on EBNA, since EBNA antibody-containing human serum inhibited the binding reaction (Table 1); EBV-negative serum had no effect. Table 1 also shows that mock EBNA (from BJAB cells) failed to induce a binding reaction.

Mapping of the binding sites of EBNA to EBV DNA showed that EBNA-protected DNA sequences hybridized to practically all restriction fragments, particularly to the larger ones (Fig. 1, lanes 2). No hybridization was seen with mock EBNA-exposed EBV DNA fragments or with EBNA-protected lambda DNA fragments. When the molarity of KCl was increased the hybridization pattern remained the same (data not shown). Therefore, 150 mM-KCl was used throughout.
Fig. 1. Hybridization of protected fragments to EBV DNA after digestion by DNase I. Samples (1 μg) of EBV DNA were cleaved by (a) EcoRI, (b) HindIII or (c) SalI, electrophoresed in 0.4% agarose slab gel and transferred to nitrocellulose sheets (Southern, 1975). The sheets were cut into strips that were hybridized (Smith & Summers, 1980) to protected fragments of EBV DNA, labelled in vitro by nick translation ([32P]dCTP; sp. act. 10⁸ c.p.m./μg) and digested by DNase I in the presence of EBNA. Fifty ng DNA and 500 ng EBNA were used in each test. Lanes 1, total probe; lanes 2, hybridization of protected fragments after DNase I digestion; lanes 3, hybridization of protected fragments after elution from gel. Fragments are marked at the left side of the fluorograms.

The protected fragments separated by acrylamide gel electrophoresis were also used for hybridization to blots containing EcoRI, HindIII or SalI endonuclease-digested fragments of EBV DNA. For this purpose the gel slices containing protected fragments were excised and DNA was eluted as described by Maxam & Gilbert (1977). Fig. 1 (lanes 3) shows the results: the protected pieces hybridized to many different restriction fragments of EBV DNA. Taken together, these findings indicate that EBNA has many binding sites on the viral DNA. To estimate the size of protected fragments, the DNA–protein complexes retained on the filter after nuclease digestion were eluted and analysed on 15% non-denaturating acrylamide gels (Fig. 2) using the method described by Chandler & Gralla (1980). EBNA protected about 30 base pairs of viral DNA against DNase I digestion. Partially digested protected fragments represent a heterogeneous mixture of fragments between 20 and 50 base pairs. Purified EBNA also protected
Fig. 2. Fragments of EBV and λ DNA protected from DNase I digestion by EBNA. Nick-translated DNA (50 ng) was mixed with purified EBNA (250 ng). DNA binding assays and analysis of native DNA by gel electrophoresis were as described in the text. Lane 1, EBV DNA protected by EBNA from DNase I digestion for 3 min; lane 2, EBV DNA protected by EBNA from DNase I digestion for 6 min; lane 3, lambda DNA protected by EBNA from DNase I digestion for 6 min. The positions of HaeII-digested, 3' end-labelled pBR322 DNA fragments (mol. wt. markers) are indicated.

Fig. 3. Immunoprecipitation of DNA fragments bound to EBNA. Raji cell extracts (300 μl) were incubated with HindIII-digested EBV DNA fragments which were 3' end-labelled with 3'-[32P]dATP using the New England Nuclear 3' end-labelling system, and immunoprecipitated with 20 μl human anti-EBNA-positive (lane 2) or -negative (lane 3) serum. Protein A–Sepharose was added to the complex and EBNA-bound DNA was eluted. Hybridization was done in the same way as in Fig. 1 on blots containing HindIII-digested fragments of EBV DNA. Lane 1, total probe. Fragments are marked at the left side of fluorograms.

heterogeneous fragments of lambda DNA, between 5 and 25 base pairs (Fig. 2). The differences in the size of the protected fragments of EBV and lambda DNA suggest a different alignment of the protein on the DNA. Since the same protein was used in both cases, the primary or secondary DNA structure may determine the EBNA alignment. Protection of EBV DNA cannot be attributed to contaminating cellular proteins, since mock EBNA, purified in parallel from EBV genome-negative BJAB cells, did not protect EBV DNA.
Specific DNA binding by EBNA was also examined by immunoprecipitation of EBNA bound to EBV DNA HindIII restriction fragments at 4 °C. Frozen Raji or BJAB cell pellets were suspended 1:4 in buffer (20 mM-Tris–HCl pH 7.5, 150 mM-KCl, 1 mM-EDTA, 1 mM-2-ME, 1 mM-PMSF). The cells were then centrifuged at 38,000 g for 60 min at 4 °C. The supernatant was removed and used for further experiments. To 300 μl cell extract 50 ng 3' end-labelled HindIII fragments of EBV DNA, 50 ng calf thymus DNA and 1 μg bovine serum albumin were added and the reaction was kept at 4 °C for 4 h. Twenty μl of human convalescent anti-EBNA serum (or negative serum) was added for an additional 1 h followed by 50 μl 50% Protein A–Sepharose diluted in the same buffer. After 30 min incubation at 4 °C the immune complexes were pelleted by centrifugation (500 g), the pellets were washed five times in extraction buffer, DNA fragments were dissociated from the immune complex by incubation in 100 μl 20 mM-Tris–HCl pH 7.5, 10 mM-2-ME and 1% SDS for 30 min at 20 °C as described by Scheller et al. (1982), 300 μl of water was added and the mixture was centrifuged. The supernatant was removed, phenol-extracted and used for hybridization. The results were similar to those of the previous experiments, showing many binding sites on EBV DNA (Fig. 3). When the anti-EBNA serum was replaced with the serum of an EBV-negative human donor, there was no hybridization (Fig. 3, lanes 3), nor was there any hybridization when BJAB cell extracts were used instead of the Raji cell extracts (data not shown).

Our experiments provided further results on the DNA-binding properties of EBNA. In our experimental system using highly purified EBNA and EBV DNA a strong protein–DNA interaction was demonstrated, with many binding sites on linear virion DNA. The immunoprecipitation experiment with crude EBNA preparation further confirmed that the binding of EBV DNA to the filters was mediated by EBNA, through many binding sites. It is still possible that EBV DNA has specific binding sites for EBNA, but that this could not be detected under our experimental conditions. Further experiments are required to study the EBNA–EBV DNA interaction using different forms of viral DNA under different conditions.

We can conclude from our data that EBNA is probably tightly bound to EBV DNA in vivo and may protect the large episomal viral genome copies from breakdown.

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REFERENCES


Short communication


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