Blocking of Acid-fixed Nuclear Binding of Epstein-Barr Virus Nuclear Antigen (EBNA) by Different DNA Species

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SUMMARY

Treatment of Epstein-Barr virus-determined nuclear antigen (EBNA) with DNA resulted in blocking of its ability to convert acid-fixed EBNA-negative cell nuclei to an EBNA-positive form. Epstein-Barr virus (EBV) DNA, herpes simplex virus type 2 (HSV-2) DNA and DNA isolated from three lymphoblastoid cell lines differed in their potency to block this reaction. EBV DNA was found to be about three times more effective than cellular DNAs in abolishing the ability of DNA-cellulose-purified EBNA to convert acid-fixed nuclei to the EBNA-positive form; the effect of HSV-2 DNA was of intermediate character. No difference was found between the blocking potency of DNAs isolated from EBV-genome-negative Ramos cells and EBV-genome-positive Raji and P3HR-1 cells.

Recently, Epstein-Barr virus-determined nuclear antigen (EBNA) has been characterized as a DNA-binding protein and chromatography on DNA-cellulose has been used as an efficient step in its purification (Baron & Strominger, 1978; Luka et al., 1977, 1978). Nothing is known about the efficiency of EBNA binding to various DNA sequences. To investigate this, we compared the capacity of different DNAs to bind DNA-cellulose-purified EBNA. The rationale of our experimental design was as follows. If there is a certain degree of specificity in the EBNA-DNA reaction, some DNA species will be more capable of binding EBNA than others. Therefore, after incubation of EBNA with different DNAs, the amount of unbound EBNA would decrease proportionally to the frequency of such sequences in the particular DNAs. The amount of the unbound antigen could then be determined by its reaction with acid-fixed nuclei (Ohno et al., 1977; Hirsch et al., 1978). It will be shown in this report that DNAs of different origin differ in their capacity to block the in vitro conversion of acid-fixed nuclei to the EBNA-positive form.

Epstein-Barr virus (EBV) DNA was extracted from Raji cells superinfected with P3HR-1 EBV as described by Yajima & Nonoyama (1976) and purified by three cycles of CsCl gradient centrifugation. Intracellular herpes simplex virus type-2 (HSV-2) DNA was isolated from human embryo lung cells infected with HSV-2, strain 196, at 0.01 p.f.u./cell, 48 h post-infection, according to Hirsch et al. (1976) and also purified by three cycles of CsCl gradient centrifugation. DNAs of three lymphoblastoid cell lines, the EBV genome negative Ramos cells and two EBV genome positive Raji and P3HR-1 cells (Hirsch et al., 1978), were isolated from cells resuspended in 0.15 M-NaCl, 0.01 M-tris-HCl, pH 8.0, 0.001 M-EDTA. After treatment with 1% sodium dodecyl sulphate and 1 mg of Pronase (nuclease free, Calbiochem) per 1 ml for 5 h at 37 °C, the cell DNA was extracted with phenol and precipitated with ethanol. DNA dissolved in 0.1 × SSC (SSC is 0.15 M-NaCl and 0.015 M-trisodium citrate) was digested with 50 μg of heat-treated RNase (RASE, Worthington) ml at 37 °C for 30 min. After further treatment with phenol and ethanol precipitation it was dissolved in 0.1 × SSC.

EBNA was extracted from Raji cells and purified on DNA-cellulose as described by Hirsch et al. (1978). The partially purified EBNA was treated with various amounts of viral or cellular DNA at 4 °C for 75 min. The reaction mixtures consisted of a constant amount
Fig. 1. Intensity of immunofluorescence of the acid-fixed Ramos cell nuclei after treatment with 20 μg of DNA-cellulose-purified EBNA treated with various amounts of DNA in the total volume of 100 μl. Smear preparations were tested for EBNA by ACIF. (a) Untreated DNA. (b) DNA fragmented by sonication (in an MSE 100 W sonic vibrator at 0 to 4 °C and amplitude 12 μm for 2 min). The average sedimentation coefficient of DNA molecules was 6-8 S as determined by analytical ultracentrifugation. O — O, EBV DNA; △ — △, HSV DNA; ● — ●, Ramos cell DNA; □ — □, Raji-cell DNA; ■ — ■, P3HR-1-cell DNA. The degrees of the ACIF reaction (— to +++) refer to the intensity of staining: brilliant (+++), strong (++), intermediate (+), faint (+) and none (—). Differences between parallel repeated readings by two observers did not exceed one +.

(20 μg) of EBNA-containing proteins and several different amounts of DNA (0·2 to 5·0 μg) in a total vol. of 100 μl of the adsorption buffer (0·05 M-NaCl, 10 mM-sodium phosphate, pH 6·0, 1 mM-β-mercaptoethanol and 5%, v/v, glycerol).

The amount of EBNA that failed to bind to DNA in solution was tested by the acid-fixed nuclear binding (AFNB) technique described by Ohno et al. (1977) and modified by Hirsch et al. (1978). Briefly, Ramos cell nuclei were fixed with methanol-acetic acid, treated with 0·6 M-NaCl and washed with distilled water. Forty-five μl of the reaction mixture containing EBNA and DNA (see above) were added per fixed smear preparation. After incubation at 20 °C for 1 h in a humid box, the smears were washed and samples tested for EBNA by anti-complement immunofluorescence (ACIF) as described by Reedman & Klein (1973). All samples were tested in duplicate. The preparations were coded and examined independently by two persons. No significant differences between the two observations were found. In repeated readings there was a certain variation in classification of the intensity of fluorescence; however, a good agreement within any one experiment was achieved in discriminating between positive and negative preparations.

Results of a representative experiment are indicated in Fig. 1(a). Whereas almost all Ramos nuclei were successfully converted to the EBNA-positive form by the addition of untreated EBNA, no positive reaction was observed when EBNA had been treated with 3 μg of EBV DNA. Treatment with the same or even higher amounts of either Ramos or Raji or P3HR-1 DNA resulted in only a slight decrease in the immunofluorescence. It can also be seen that the effect of the other virus DNA tested, that of HSV-2, was of intermediate character. The results in Fig. 2 illustrate the decrease in Ramos cell nuclei immunofluorescence after treatment of EBNA with increasing amounts of EBV and Raji DNAs. Similar results were obtained in five repeated experiments. In these tests, the amount of EBV DNA necessary to abolish the reaction varied with the different EBNA preparation from 1·3 to 3 μg; the amount of cellular DNA necessary to reach the endpoint of negative immunofluorescence was always three times higher. The relative differences in the blocking potency between EBV DNA and HSV-2 DNA were also nearly identical in all experiments.
Fig. 2. Immunofluorescence of the acid-fixed Ramos-cell nuclei after treatment with 20 μg of DNA-cellulose purified EBNA treated with 0.4 μg (a) and (f), 0.8 μg (b) and (g), 1.6 μg (c) and (h), 2.4 μg (d) and (i), 3.2 μg (e) and (j) of Raji cell and EBV DNA, respectively. The same preparations as documented in Fig. 1(a) are shown. Magnification × 250.

Since the differences in mol. wt. between the EBV and cellular DNAs might be of some importance in the binding of EBNA, DNAs fragmented by sonication to a uniform size of 300 to 400 nucleotide-long chains were used for blocking the AFNB reaction (Fig. 1 b). This experiment was run in parallel with that shown in Fig. 1 (a). It can be seen that the blocking potencies of EBV DNA and HSV-2 DNA were strongly decreased by the fragmentation, whereas no blocking activity was observed after treatment of EBNA with Raji DNA in the interval tested.

These data indicate that EBV DNA exhibited a higher activity in blocking EBNA than the other DNA species tested. The following factors, either separately or in combination, might be involved. (1) Some special features of EBV DNA, e.g. high G+C content and the presence of nicks in the DNA backbone; (2) different complexity and presence of specific DNA binding sequences for EBNA in some DNA molecules. Differences in the blocking of the AFNB reaction between two herpesvirus (namely HSV-2 and EBV) DNAs possessing approximately the same mol. wt. and similar structural features make the first explanation rather improbable. In addition, in one experiment, nicks were artificially introduced to Ramos P3HR-1 cell DNAs by DNase I under conditions resembling those used in the nick translation reaction (Rigby et al. 1977). No visible increase in blocking potency of DNA was observed after treatment of 30 μg of Ramos or P3HR-1 DNA with 25 ng of DNase in 1 ml of 50 mM-potassium phosphate, pH 7.4 and 5 mM-MgCl₂ for 30 min at ambient temperature.

At this stage, however, there is at least one reason which makes it difficult to link the observed differences entirely to the content of specific DNA-binding sequences (in a particular DNA sample). In our experiments EBNA preparations were used which were only partially purified. The impurities present could react in an unpredictable way with different DNA species and interfere with the binding of EBNA.

Recently we have shown that EBNA is eluted from in vitro converted Ramos cell nuclei
at a 0.1 M lower concentration of NaCl than from P3HR-1 nuclei, which might be due to the presence of EBV DNA in the latter cells (Hirsch et al. 1978). In the present series of experiments we failed to demonstrate a difference among Ramos, Raji and P3HR-1 DNAs (Fig. 1a). However, the two observations need not necessarily be contradictory. In the present experiments, residual free EBNA was determined, while in the previous tests residual bound EBNA was monitored. It would apparently be more difficult for the free antigen, present in amounts insufficient to give an appreciable overall reaction, to find rare specific sites, if they actually exist, than it would be for small amounts of antigen which are attached to such sites to leave them.

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