Studies of Epstein–Barr Virus
(EBV)-associated Nuclear Antigen: Solubilization from Raji Cell Chromatin with 5 M-Urea-2 M-NaCl and Fractionation on Hydroxyapatite

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SUMMARY
EBNA-containing chromatin from Raji cells was solubilized by treatment with high concentrations of urea and salt and fractionated by hydroxyapatite chromatography. Fractions eluting at different phosphate concentrations were analysed for the presence of EBNA by means of an anti-EBNA-specific 125I-labelled IgG absorption assay. The antigen was found in fractions containing non-histone chromatin proteins.

INTRODUCTION
An EBV-associated nuclear antigen (EBNA) has been detected by anticomplementary immunofluorescence in EBV-genome-carrying lymphoid cell lines (Reedman & Klein, 1973). The antigen is present in essentially all living cells of such cell lines irrespective of their virus producing status. It has also been demonstrated in Burkitt’s lymphoma and nasopharyngeal carcinoma tumour biopsies (Reedman et al. 1974). An EBV-associated antigen has also been detected in non-producer cell lines by means of complement fixation tests (Gerber & Deal, 1970; Pope et al. 1969; Reedman et al. 1972; Vonka et al. 1970; Walters & Pope, 1971). The relationship between this antigen and EBNA has been demonstrated by serological and biochemical studies (Klein & Vonka, 1974; Lenoir et al. 1976; Ohno et al. 1977).

In previous papers the development of an 125I-IgG absorption assay capable of detecting EBNA in particulate and soluble fractions prepared from EBV-genome-carrying lymphoid cell lines has been described (Brown et al. 1975; Brown & Klein, 1975). In this paper the assay has been employed to detect EBNA solubilized from Raji cell chromatin by treatment with high concentrations of urea and salt. The chromatin has been fractionated into histone and non-histone protein fractions on hydroxylapatite (HAP; MacGillivray & Rickwood, 1974). The use of a discontinuous elution system employing various concentrations of phosphate in the presence of urea and either NaCl or guanidinium chloride has also permitted the subfractionation of non-histone proteins (Rickwood & MacGillivray, 1975).
METHODS

Cell culture. Raji (Pulvertaft, 1964), U698M (Klein et al. 1974) and Molt 4 (Minowada et al. 1972) cells were grown in RPMI 1640 medium containing 10% foetal bovine serum. The medium also contained penicillin and streptomycin at 100 μg/ml.

Sera and IgGs. IgG was prepared from serum and iodinated with 125I as described previously (Brown et al. 1975). The anti-EBNA specificities of the pair of sera used in this study, N (anti-EBNA-positive) and BA (anti-EBNA-negative), are demonstrated in Fig. 1.

125I-IgG absorption assay for EBNA. This was carried out by a modification of the procedure of Brown et al. (1975). Samples (0.6 μg) of 125I-IgG purified from EBNA-positive (N) or EBNA-negative (BA) sera were absorbed with varying quantities of antigen preparation contained in 200 μl of 0.15 M-NaCl, 10 mM-NaHEPES, 4 mM-MgCl2, 1 mM-EDTA, 0.1 mM-DTT, pH 7.4, for 18 h at 4°C. The absorption mixture was then centrifuged at 300 g for 15 min; 50 μl samples were removed and mixed with 10⁸ Raji nuclei (containing EBNA) in 350 μl of 0.065 M-NaCl, 10 mM-NaHEPES, 2 mM-MgCl2, 0.25 M-sucrose, 10% dialysed foetal bovine serum was added and the mixture incubated for 18 h at 4°C. It was then centrifuged for 15 min at 300 g and the supernatant removed. The nuclei were then re-suspended in 1 ml of 0.15 M-NaCl, 10 mM-tris-Cl, 4 mM-MgCl2, 1 mM-EDTA, 10% dialysed foetal bovine serum, pH 7.4, filtered on a 25 mm glass fibre filter (GF/C, Whatman) and washed with 3 × 5 ml of buffer. The filters were counted in a crystal scintillation gamma counter. Duplicate determinations were carried out. Parallel absorptions of anti-EBNA-positive 125I-IgG and anti-EBNA-negative 125I-IgG were carried out at all dilutions of antigen-containing fractions. Specific binding was considered to be the difference between the binding of the anti-EBNA-positive 125I-IgG and the anti-EBNA-negative 125I-IgG. The anti-EBNA specificity of the assay is demonstrated in Fig. 1 and has been extensively studied previously (Brown et al. 1975).

Preparation, fractionation and analysis of Raji cell chromatin. This was carried out as described by Rickwood & MacGillivray (1975) for tissue culture cells. The HAP 1 (histone) fraction was analysed on SDS-containing polyacrylamide tube gels using a system described by Thomas & Kornberg (1975). Acrylamide (18%), at an acrylamide:bisacrylamide ratio of 30:0.15, was used in the separation gel. The separation gel was 10 cm × 0.6 cm. Electrophoresis was carried out at 4 mA/gel. Gels were stained in a dye solution containing 75 ml methanol, 186 ml water, 30 g trichloroacetic acid, 9 g sulphosalicylic acid and 0.1% Coomassie brilliant blue R for 20 min at 65°C. Destaining was carried out by elution in a solution containing 100 ml ethanol, 260 ml water and 32 ml acetic acid. The HAP 2, 3 and 4 (non-histone) fractions were analysed on SDS/urea-containing polyacrylamide tube gels using the system described by MacGillivray & Rickwood (1974). Acrylamide (15%) and 0.23% bisacrylamide were used in the separation gel. The separation gel was 10 cm × 0.6 cm. Electrophoresis was carried out at 8 mA/gel. Gels were stained and destained as described for the HAP 1 fraction. Gels were scanned at 260 nm with a Joyce-Loebl u.v. scanner. The protein concentration of each fraction was determined using the method of Schaffner & Weissmann (1973) with bovine serum albumin as a standard.

RESULTS

Raji cell chromatin solubilized in 2.0 M-NaCl/5 M-urea containing 1 mm-sodium phosphate and 2 mm-tris-Cl (pH 6.8) was fractionated on a hydroxyapatite column by application of a discontinuous gradient of phosphate in 2 M-NaCl/5 M-urea or 2 M-guanidinium
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Fig. 1

Fig. 1. Specificity of $^{125}$I-IgG absorption assay. The assay was carried out as described in Methods using as absorbing antigen preparation nuclei prepared from EBNA-containing Raji (●) and EBNA-negative U698M (▲) and Molt 4 (■) cell lines. The nuclei were prepared as described previously (Brown et al. 1975). The dashed line indicates the specific binding in the absence of absorbing antigen.

Fig. 2. Densitometric scans of Coomassie brilliant blue 250R stained SDS/polyacrylamide gels of HAP fractions. Electrophoresis was carried out and the gels stained and scanned as described by MacGillivray & Rickwood (1974) and Thomas & Kornberg (1975). The top of the gel is shown to the left; the scan ended at the bromothymol blue marker on the right. Analysis of HAP fractions 1, 2, 3 and 4 was carried out. The assignment of histone bands in the HAP 1 fraction was made by comparison with a standard calf thymus histone preparation. The mol. wt. calibration (mol. wt. x 10$^{-3}$) refers to the HAP 2, 3 and 4 gels only.

chloride/5 M-urea as described by Rickwood & MacGillivray (1975). The following fractions were obtained: (1) HAP 1 – eluted from the column with 2 M-NaCl/5 M-urea/1 mM-phosphate; (2) HAP 2 – eluted from the column with 2 M-NaCl/5 M-urea/50 mM-phosphate; (3) HAP 3 – eluted from the column with 2 M-NaCl/5 M-urea/200 mM-phosphate; (4) HAP 4 – eluted from the column with 2 M-guanidinium chloride/5 M-urea/200 mM-phosphate.
Fig. 3. Absorption of anti-EBNA-specific 125I-IgG by fractions prepared from Raji cell chromatin. The preparation and hydroxyapatite fractionation of chromatin were carried out as described by Rickwood & MacGillivray (1975), using 2 x 10⁹ Raji cells. All HAP fractions were concentrated to 2 ml by dialysis against Carbowax 20M and dialysed against two changes of 500 ml of 0-15 m-NaCl, 10 mm-NaHEPES, 4 mm-MgCl₂, 1 mm-EDTA, 0-1 mm-DTT, pH 7-4, prior to assay. (a) 125I-IgG absorption assays were carried out as described in Methods. The dashed line indicates the level of specific binding in the absence of added absorbing fraction. (b) The distribution of protein in HAP fractions was as follows: HAP 1 (●) 4-37 mg (73-5%); HAP 2 (▲) 1-17 mg (19-7%); HAP 3 (▼) 0-15 mg (2-5%); HAP 4 (■) 0-26 mg (4-3%).

Hydroxyapatite chromatography of salt/urea solubilized chromatin carried out under the conditions employed in these experiments has been demonstrated to separate histone and non-histone proteins effectively (MacGillivray et al. 1972). The gel scan in Fig. 2 shows that the putative histone-containing fraction (HAP 1) contains all species of histone with little evidence of contaminating material. This fraction constitutes 74% of the total protein recovered from the fractionation. The gel scans also show the complexity of the non-histone proteins present in fractions HAP 2, 3 and 4 (MacGillivray & Rickwood, 1974; Rickwood & MacGillivray, 1975). The major peaks running at mol. wt. of approximately 14 and 17 x 10⁶ in HAP fractions 2 and 4 are unlikely to be histones as histones run anomalously under the conditions used and are not found in this mol. wt. range. More than 90% of the DNA is retained on the column under the conditions used (Rickwood & MacGillivray, 1975).

The fractions from the hydroxyapatite column were concentrated by dialysis against Carbowax 20M and then dialysed into 0-15 m-NaCl, 10 mm-NaHEPES, 4 mm-MgCl₂, 1 mm-EDTA, 0-1 mm-dithiothreitol, pH 7-4. The total fraction was retained for assay and all fractions were concentrated to the same volume. These fractions were assayed for the presence of EBNA by means of the 125I-IgG absorption assay (Brown et al. 1975). The results are presented in Fig. 3. These results demonstrate that EBNA can be detected in fractions prepared using a system capable of dissociating chromatin into its protein and nucleic acid components and from which the bulk of nucleic acid has been removed. They also show that the antigenic activity is fractionated by the discontinuous gradient. Very little antigenic activity is present in the histone-containing HAP 1 fraction, but activity is readily detected in the three fractions containing non-histone proteins (HAP 2, 3 and 4). The lack of antigenic activity in the HAP 1 fraction which contains all histone species suggests that the antigenic...
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Fig. 4. Test of nucleolytic activity in HAP fractions. A 25 μl sample of each fraction was incubated with 2 x 10⁷ Raji nuclei for 42 h at 4 °C in the presence of 100 μl of foetal bovine serum and 100 μl of 0.05 M-NaCl, 10 mM-HEPES, 2 mM-MgCl₂, 0.25 M-sucrose, 10% foetal bovine serum, pH 7.4. The incubation mixture was centrifuged at 600 g for 10 min and the supernatant removed. The nuclei were assayed for direct binding of anti-EBNA-specific ¹²⁵I-IgG as described in Methods; 1, 2, 3 and 4 refer to incubations in the presence of HAP fractions 1, 2, 3 and 4. Control without added fraction is labelled 5.

Specific ¹²⁵I-IgG binding, cpm x 10⁻¹

The shape of the absorption curves obtained with the non-histone protein-containing fractions varies. Thus the antigenic activity present in HAP 3 is capable of absorbing a considerable fraction of anti-EBNA-specific ¹²⁵I-IgG even at low concentrations. This differs from the behaviour of the HAP 4 fraction which has only a low absorptive capacity at low concentrations, but which is capable of absorbing 80% of the anti-EBNA-specific ¹²⁵I-IgG at the highest concentration tested. These data suggest a fractionation of antigenic specificities. The protein concentrations of the HAP fractions are given in Fig. 3. It can be seen that the fractions containing the bulk of the antigenic activity (HAP 3 and 4) contain only 7% of the total chromatin protein. These data were confirmed in a series of hydroxyapatite fractionations and in tests with an alternative pair of anti-EBNA-specific ¹²⁵I-IgGs.

The possibility that these observations might be explained simply by the action of endogenous nucleases and/or proteases known to be present in chromatin was considered (Burgoyne et al. 1974; Carter & Chae, 1976; Hewish & Burgoyne, 1973a, b). These enzyme activities might simply solubilize the EBNA present in target nuclei thereby mimicking the absorption of anti-EBNA-specific ¹²⁵I-IgG by the added fractions. EBNA-containing target nuclei were therefore incubated with the maximum concentration of fraction tested in the absorption assay under essentially the same conditions. The fraction was then removed from the target nuclei by centrifugation and the residual binding of anti-EBNA-specific ¹²⁵I-IgG to the treated and control nuclei measured. The results presented in Fig. 4...
demonstrate that the fractions did not contain levels of nucleases or proteases capable of interfering with the assay system.

**DISCUSSION**

The results presented in this paper suggest that EBNA is a non-histone chromatin-associated protein. This is based on the separation of histone and non-histone protein fractions and hydroxyapatite (MacGillivray & Rickwood, 1974; Rickwood & MacGillivray, 1975). Non-histone proteins have been demonstrated to control, at least in part, the transcriptional activity of isolated chromatin (Gilmour, 1974; MacGillivray & Rickwood, 1975). It is possible that EBNA is involved in the control of transcription of EBV sequences in transformed cells. The methods used to fractionate the chromatin and remove from the proteins the bulk of nucleic acid are similar to those used in many studies of chromatin dissociation and reconstitution including those in which reconstitution of transcriptional control in chromatin by non-histone proteins has been demonstrated (Gilmour & MacGillivray, 1976). The data presented here show that EBNA antigenic activity can be recovered after these vigorous treatments. This observation may be useful in the planning of studies of transcriptional control by EBNA.

The differences in the absorption curves obtained for the HAP fractions suggests that fractionation of the antigenic species has been achieved by hydroxyapatite chromatography. The difference in the absorption patterns of fractions HAP 3 and 4 may be related to the observation that traces of DNA are present in the HAP 4 fraction (Rickwood & MacGillivray, 1975).

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**REFERENCES**


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