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Evidence for Two Classes of Chromatin-Associated Epstein-Barr Virus-Determined Nuclear Antigen

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A new class of Epstein-Barr virus nuclear antigen (EBNA) was identified by the complement fixation assay. This new species of EBNA is more tightly bound to chromatin and was termed class II EBNA, as opposed to the more weakly associated species, class I EBNA. Preparations of this new antigen(s) specifically reduced absorption with the titer of anti-EBNA antibodies as determined by the anticomplement immunofluorescence assay. Therefore, the complement fixation antigens (class II EBNA) appear to be related to the classical EBNA (class I EBNA). The class I EBNA was found to focus at the same pH (4.6) as the soluble antigen found in the cytosol. The class II EBNA differed from the class I EBNA with regard to its overall charge, molecular size, antigenicity, and affinity for chromatin. The class II EBNA appeared to be a basic protein, based on its apparent pI of 9.2 and its binding to cation-exchange resins. It differed from histones with regard to its molecular size (molecular weight between 60,000 and 70,000) and its elution from hydroxylapatite chromatography. Steps were taken to prevent proteolysis and artifacts in the immunological assays and in the overall charge estimation of the new antigen by nonspecific basic histone protein-acidic protein interactions. Both class I and class II EBNA were identified by radioimmunoelectrophoresis on two-dimensional polyacrylamide gels with pI values of 5.0 and 8.5, respectively, and a molecular weight range of 60,000 to 70,000 for both. A lower-molecular-weight antigen identified by molecular sieve chromatography appeared to be due to interference by histones in the immunoassays since it was not observed by the two-dimensional gel electrophoresis. Further characterization of this class II EBNA is in progress.

Of all the Epstein-Barr virus (EBV)-associated antigens in nonproducing cells carrying the genome for the virus, only the EBV-induced nuclear antigen (EBNA) and possibly the lymphocyte-detected membrane antigen are expressed in transformed cells (23). EBNA appears to be of prime importance in viral transformation because of the coincidence of its appearance and that of transformation (23), its association with metaphase chromosomes (2, 32) and chromatin (36, 37), and its early appearance after EBV infection but before DNA replication (22).

Rapid progress has been made over the past 5 years in the chemical characterization of EBNA (see references 26 and 29 for reviews). Many laboratories have partially purified and characterized the classical EBNA which appears to be identical to the soluble (S) antigen detected by complement fixation (CF) (3, 14, 20). However, discrepancies in the molecular sizes of the classical EBNAs exist. Molecular weights of 47,000 to 50,000 to heterogeneous groups of 50,000, 70,000, and 100,000 species have been reported. Matsuo et al. (19, 21) and Luka et al. (16, 17) have reported purification of the classical EBNA to homogeneity. However, there are discrepancies in these isolated species. The latter reported a monomer molecular weight of 48,000 and a native polymeric form of 170,000 to 200,000. Only the polymeric form was antigenic. Matsuo et al. (19, 21) reported forms of 100,000, 70,000, and 50,000 for the classical EBNA. These investigators believe that these subunits form the multimeric high-molecular-weight polymers observed in the crude cytosols.

There is increasing evidence that there is more than one species of EBNA. zur Hausen and Fresen (40) reported evidence for two distinct forms of EBNA, based on immunofluorescent staining in EBV-infected cells. As mentioned above, Matsuo et al. reported three species of the classical EBNA. Brown et al. (5) also identi-

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fied more than one species of EBNA by using strong denaturants to remove most of the chromatin proteins. Recently, Strnad et al. (38) found multiple EBNA species in transformed cells carrying the EBV genome. The existence of two classes of chromatin-associated EBNAs, one more tightly bound to the DNA than the classical EBNA, was reported by Pikler et al. (28, 29). The loosely bound EBNA was termed class I EBNA, and the tightly bound antigen was termed class II EBNA. This paper presents further evidence that the class I and class II EBNAs are distinct in regard to both immunological and physicochemical properties.

MATERIALS AND METHODS

Cells. Several EBV genome-positive lines were used in these studies: the African Burkitt lymphoma Raji line (10), the human NC_{37} cell line (9), and the EBV-converted Ramos line. A negative line, Ramos, was also used on occasion as a control. All cell lines were grown in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum and gentamicin (50 μg/ml) at 37°C in the presence of 5% CO_2 (27). Cultures were harvested every 3 to 4 days and reseeded at a concentration of 5×10^5 cells per ml. Cells not used immediately after harvesting were stored in 10 mM Tris–0.1 mM EDTA–5% glycerol (pH 7.2) at −20°C for no longer than 2 weeks before use.

ACIF. Anticomplement immunofluorescence (ACIF) was carried out as a modification of the method described by Reedman and Klein (30), using a three-step procedure of Huang et al. (11). Standard anti-EBNA-positive sera were negative for antinuclear antibodies as determined by immunofluorescence on EBV genome-negative cell lines.

^{51}Cr release-CF assay. The quantitative ^{51}Cr-CF assay was carried out by following the microtechnique of Sever (31), using ^{51}Cr-labeled sensitized sheep erythrocytes as described by Cikes (8). The test was performed in the presence of 2 U of complement, with total hemolysis releasing approximately 10,000 cpm. Each dilution of antigen was tested for anticomplementary activity. All assays included an antibody-negative antiserum as a control, and all sera used were negative for antinuclear antibodies as determined by immunofluorescence against EBV genome-negative cell lines.

Single radial immunodiffusion assay. To semi-quantitatively assay for immunological activity of class I and class II EBNA antigens, a modification of the single radial immunodiffusion (SRD) test of Mancini et al. (18) was used. The sample solvent contained 0.05 M barbital buffer (pH 8.6) with 0.1% (wt/wt) NaN_3. For samples containing the class II EBNA, the sample solvent, called SRD buffer, also contained 2.25 M urea and 0.5 M NaCl to maintain the solubility of the protein and to reduce the nonspecific ionic interactions between basic and acidic proteins without interference with the antibody-antigen reactions. This modification prevented such interactions in all but the very concentrated histone solutions.

Double radial immunodiffusion assay. For comparison of the class I and II antigens, a double radial immunodiffusion assay was used. In this case, the antiserum was not incorporated into the gel; rather, 20 μl of the antiserum was placed in a 5-mm hole in the center of the gel. The gels contained sodium chloride-urea as described above for the SRD assay. The precipitin lines in these gels were observable directly.

Isolation of the soluble EBNA. The S EBNA is defined in this paper as that antigen detected in cytosol extracts of EBV-infected cells. All isolation steps were performed at 4°C unless otherwise specified. Harvested cells were washed twice by low-speed centrifugation in Veronal-buffered diluent (pH 7.3 to 7.4) and suspended to a final concentration of approximately 10^8 cells per ml in the diluent containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF). After three cycles of rapid freezing (in an acetone-dry ice bath) and thawing (to 37°C), the cells were homogenized in a Dounce all-glass homogenizer (tight-fitting pestle) with 15 strokes and further disrupted by sonication in an ice bath with a Bronson sonifier (cell disrupter 200) at approximately 1.5 A for three bursts of 2 s each, with intervening 30-s cooling periods. The suspension was centrifuged at 12,000 × g for 15 min in the HB-4 rotor of a Sorvall RC2-B centrifuge. The supernatant was further centrifuged at 100,000 × g for 1 h in an SW50.1 rotor. The clear supernatant contained the soluble EBNA, which for purposes of clarity is defined as the S antigen to distinguish it from the chromatin-associated EBNAs. The supernatant was analyzed for protein concentration before being lyophilized and stored at −70°C. Before use, crude S antigen was suspended in the desired volume of Veronal-buffered diluent–PMSF solution, and insoluble material was removed by sedimentation at 10,000 × g for 5 min.

Chromatin isolation. Nuclei were first isolated by the technique of Brown et al. (5), with minor modifications. The chromatin from these purified nuclei was prepared by the procedure of Spelsberg et al. (32, 33, 35), with minor modifications.

The quantitative analyses of the DNA, histone, and acidic proteins in chromatin were performed as described by Spelsberg et al. (31–33, 35) and Ansevin et al. (1). Protein was determined by the method of Lowry et al. (15) and by the filter assay method of Bramhall et al. (4), substituting Coomassie blue as the protein stain. The latter method was used when urea, guanidine hydrochloride (GuHCl), or sodium dodecyl sulfate (SDS) was present. DNA was measured by the diphenylamine method of Burton (7).

Isolation of class I and class II EBNA. Class I EBNA was removed by incubating the chromatin for 90 min at 4°C with 20 volumes of a solution containing 1.0 M NaCl with 10 mM Tris, 1 mM EDTA, and 1 mM PMSF (pH 7.4). The solution was centrifuged at 100,000 × g overnight, and the resulting supernatant, containing crude class I EBNA, was dialyzed against water and lyophilized. Class II EBNA was isolated by two methods. Initially, the class II EBNA was dissociated from chromatin by using 4 or 6 M GuHCl (pH 6.0) followed by centrifugation at $10^5 \times g$ for 36 h to pellet the DNA. This long procedure for isolating class II EBNA was replaced by a more rapid method involving hydroxylapatite (HA). In the second method, class II EBNA was prepared from chromatin previously extracted with 1.0 M NaCl to remove class I EBNA. This extracted chromatin was suspended in a solution containing 0.002 M Tris (pH 7.5) by homogenization with

a Teflon pestle-glass homogenizer, using a pestle rotation of about 200 rpm. The chromatin was bound to HA, and the proteins were removed with modifications of the method described by Spelsberg et al. (34, 36, 37). Briefly, Bio-Gel HTP (Bio-Rad Laboratories, Richmond, Calif.) was prepared by allowing 25 g of the resin to hydrate in 500 ml of 0.10 M sodium phosphate (pH 6.0) overnight at 4°C. The solution was then filtered on a scintered glass funnel, and the HA was suspended in 125 ml of the same phosphate buffer containing 1 mM PMSF. The HA preparation (600 mg of HA per mg of DNA) was then mixed with the chromatin described above (devoid of class I EBNA). The HA and the chromatin were allowed to incubate in a beaker for 1 h with periodic mixing. The solution was then filtered, and the chromatin-HA resin was rinsed with 2 volumes of the same phosphate-PMSF buffer and suspended in a beaker, using 1 to 2 volumes of a solution containing 4 M GuHCl and 0.1 M sodium phosphate (pH 6.0). The suspension was allowed to incubate for 0.5 h with periodic mixing. The mixture was filtered, and the resin was rinsed with 1 to 2 volumes of the 4 M GuHCl buffer (pH 6.0). The combined filtrates containing the crude class II EBNA were then dialyzed for 36 h against distilled water (pH 5.5 to 6.0), lyophilized, and stored at 4°C until needed. In the initial isolations of class II EBNA with this method, serial extractions with solutions containing increasing unit molarities of GuHCl were performed to establish the elution zone of the EBNA.

Isoelectric focusing. The antigens were solubilized in a solution containing 6 M urea and 0.002 M Tris-hydrochloride (pH 7.0) and subjected to isoelectric focusing for hydrophobic proteins as described by Pikler et al. (29) and Spelsberg et al. (34).

Molecular sieve chromatography in CL-Sepharose 6B. Molecular sieve chromatography of the class II EBNA was performed in CL-Sepharose 6B resin packed in a column (2.5 by 95 cm) as described elsewhere (34, 36), using as a mobile phase a solution containing 6.0 M GuHCl, 0.01 M phosphate buffer (pH 6.0), 0.001 M EDTA, and 0.01 M ethanethiol.

Anion-exchange chromatography of class II EBNA with DEAE-cellulose. The lyophilized class II EBNA isolated from the chromatin-HA procedure was suspended in 6.0 M GuHCl–0.01 M phosphate buffer (pH 6.0) at one-fifth the prelyophilized volume and dialyzed against a solution containing 3.0 M urea and 0.05 M Tris-hydrochloride (pH 7.4). Insoluble protein found not to contain EBNA activity was removed by centrifugation at 10,000 × g for 10 min. The preparation was mixed with prewashed DEAE-cellulose (DE-52, Whatman Biochemicals, Ltd., Maidstone, Ky.). The resin was placed in a column and washed with 4 column volumes of sample buffer. Then the resin was eluted stepwise with 4 volumes each of buffers containing increasing concentrations of NaCl in 3.0 M urea–0.05 M Tris-hydrochloride (pH 7.4). Each fraction was dialyzed against deionized, distilled water (pH 5.5), lyophilized, and stored. The dry proteins were then suspended in 1/10 the volume (of the prelyophilized solution) of a solution containing 6.0 M GuHCl and 0.01 M phosphate (pH 6.0), dialyzed against the SRD buffer (2.25 M urea, 0.5 M NaCl, 0.1% [wt/wt] NaN_3, 0.05 M barbital buffer [pH 8.6]) and analyzed for protein by the Coomassie blue method and for antigens by the SRD method.

Radioimmunoelectrophoresis. The electrophoretic transfer of proteins from the polyacrylamide gels to nitrocellular paper (BA 85; Schleicher & Schuell Co., Keene, N.H.) and detection of EBNA with antibody and radioiodinated protein A (New England Nuclear Corp., Boston, Mass.) were done by the method of Burnette (6).

Polyacrylamide gel electrophoresis. The composition and electrophoresis of 18% SDS-polyacrylamide slab gels were by the method of Laemmli (13), with modifications as described by Jackson (12). Two-dimensional gel electrophoresis was performed as described by O'Farrell (24). Samples of NC_{37} cells were collected by centrifugation and dispersed in 2% SDS–10 mM $NaPO_4$ (pH 6.8)–10 mM 2-mercaptoethanol by sonication. The sample was heated to 90°C for 2 min and cooled, and solid urea was added to 8 M. Nonidet P-40 was added to 0.5%, and the solution was centrifuged at 100,000 × g for 24 h. The top lipid layer was discarded, and samples of the remaining supernatant were subjected to two-dimensional gel electrophoresis.

RESULTS

EBNA was initially detected in isolated nuclei and chromatin from both the NC_{37} and Raji cell lines, using the ACIF assay and the ^{51}Cr-CF assay (28, 29). Studies with the ^{51}Cr-CF assay demonstrated that 70% of the total EBNA was dissociated from chromatin by 0.5 M NaCl. All EBNA activity appeared to be removed when measured by the standard ACIF method. The residual 30% of the EBNA (detected by the ^{51}Cr-CF assay) remained bound to the DNA even after extraction with 2.0 M NaCl (pH 6.0). When the chromatin was extracted with 2.0 M NaCl and 5.0 M urea (pH 6.0) or 4.0 M GuHCl (pH 6.0), this residual (30%) EBNA was removed and could be detected in the extracts. This tightly bound EBNA was termed class II EBNA, and the more commonly studied, loosely bound EBNA was termed class I EBNA.

To assure that the antigen detected by the ^{51}Cr-CF assay was related to EBNA, an anti-EBNA-positive serum was absorbed with equal concentrations of a CF antigen-positive or CF antigen-negative extract and then titrated for residual antibodies by both ACIF and CF. An extract prepared from NC_{37} cells which contained class II EBNA was used as the positive extract in this absorption, and a similar extract from Raji cells which lacked CF activity was used as the antigen-negative preparation (Table 1). The anti-EBNA titer of the unabsorbed serum was 240, and the CF titer was 80. Both titers were unaffected by absorption with the CF antigen-negative preparation. However, titers in both assays were reduced to less than 30, the lowest dilution tested, after absorption with the CF antigen-positive NC_{37} extract. These results indicated that the EBNA II antigen detected by CF was related to EBNA de-

TABLE 1. Absorption of anti-EBNA antibodies with CF antigen-positive extracts prepared from NC_{37} cells

Serum absorbed with:[a]	Antibody titer	
	EBNA	CF
Unabsorbed	240	80
CF(+) extract[b]	<30	<30
CF(−) extract[c]	240	80

[a] Anti-EBNA antibody-positive human serum (1:30 dilution) was absorbed one time with CF(+) or CF(−) extract and then titrated for anti-EBNA antibodies by ACIF and for CF antibodies by the ^{51}Cr-CF test. Absorption took place for 1 h at 37°C and overnight at 4°C.

[b] CF(+) extract was prepared from NC_{37} cells and contained class II EBNA.

[c] CF(−) extract was prepared from Raji cells but did not contain CF antigen in the CF assay.

tected by ACIF, confirming results by others (26).

Since many antisera recognize only the tertiary structure of protein antigens, and since denaturing conditions were required to dissociate the majority of proteins from the chromatin, there was concern that the dissociated antigens would not be detected in subsequent studies due to the loss of their antigenic determinants. Therefore, studies on the effects of protein-denaturing agents on the antigenicity of the S antigen (soluble EBNA) and class I and class II EBNA were performed. The antigenic activities of all three antigens as measured with the ^{51}Cr-CF assay were not destroyed by a variety of denaturing conditions (Table 2). Heat, extreme pH, and high concentrations of GuHCl and urea did not destroy the antigenicity after these chemicals were totally removed from the protein. Although it has been reported that urea destroys the antigenic activity of EBNA (25), we found that the apparent denaturing effects of urea on the antigenic activity in our sample was due to the insufficient removal of urea from the antigen mixtures by dialysis before the immunoassay. These results permitted the further fractionation of these antigens by using these chaotropic agents, without the worry of destroying the antigenicity. All solutions contained either the protease inhibitor, PMSF, in the dilute buffers or chaotropic agents, both of which retard proteolysis. Also, when storage over extended periods was desired, the extracts were lyophilized to dry powders. These precautions preserved the antigenic activity throughout the handling and analyses and reduced variations in the analytical methods.

Early methods to isolate class II EBNA required long periods of centrifugation in 4 to 6 M GuHCl. An alternate, more rapid method involved binding the chromatin to HA and extracting the resin with increasing concentrations of GuHCl. The phosphate concentration was selected empirically to minimize protein binding to the HA after its dissociation from the DNA, but it was less than the concentration needed to dissociate the DNA from the resin. Figure 1 shows the elution pattern of the protein as well as class I and class II EBNAs from chromatin. In Fig. 1A, the elution of class I EBNA by the increasing concentrations of NaCl is shown. Free chromatin was first subjected to a series of dilute salt extractions, followed by low-speed centrifugations. The chromatin, extracted with 1.5 M NaCl, was then bound to HA, and the remaining protein was dissociated by increasing concentrations of GuHCl. Figure 1B shows the elution of the class II EBNA from the chromatin which is bound to the resin. This EBNA eluted from the resin between 2 and 4 M GuHCl. Since the dissociation of class II EBNA from unbound

TABLE 2. Effects of protein denaturation on the antigenicity of S antigen and class I EBNA

Treatment[a]	Remaining antigenicity (% of control)[b]		
	S antigen	Class I EBNA	Class II EBNA[c]
Control	100	100	100
Heating to 90°C for 1 h	50	50	
0.3 N KOH, 37°C for 1 h, neutralize and cool to 4°C	100	100	
0.3 N HCl, 23°C for 10 min, neutralize and cool to 4°C	50	50	
6.0 M GuHCl, 4°C for 1 h, dialyze vs buffer for 24 h	100	100	100
6.0 M GuHCl, 4°C for 1 h, dialyze vs buffer for 6 h	0	0	
6.0 M urea, 4°C for 1 h, dialyze vs buffer for 24 h	100	100	100
6.0 M urea, 4°C for 1 h, dialyze vs buffer for 6 h	0	0	

[a] The isolation of S, class I, and class II antigens, as well as the ^{51}Cr-CF assays, were carried out as described in the text. Three independent experiments were carried out, with the same results. All preparations were assayed by ^{51}Cr-CF within several hours after the treatments were completed.

[b] Values are based on the highest dilution of antigen giving >75% complement fixation.

[c] S antigen and class I EBNA were obtained as described in the text. The class II EBNA was obtained by extraction of chromatin with 6.0 M GuHCl (pH 6.0), centrifugation to sediment the DNA, and processing of the supernatant. Briefly, the supernatants were dialyzed against distilled water for 16 h with several changes and then lyophilized. When needed, the particular EBNA was suspended in the Tris-EDTA-PMSF buffer for the control or for the particular solvent indicated.

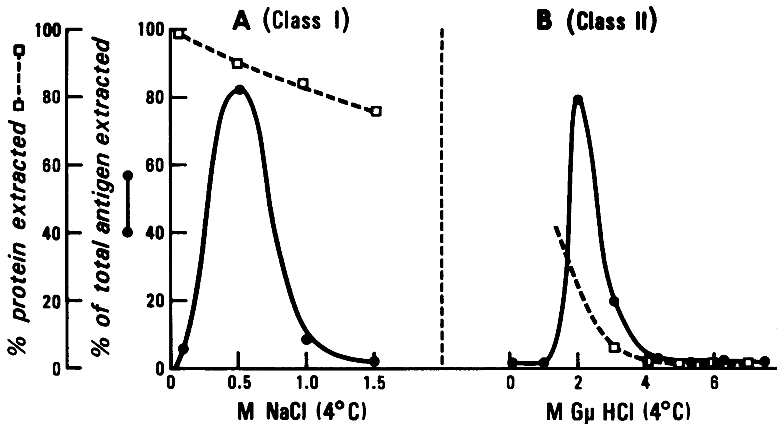

FIG. 1. Dissociation of class I and class II EBNAs from chromatin by using HA. The dissociation of both class I and class II EBNAs from chromatin with HA occurred in two steps. (A) The extraction of class I EBNA from 1 mg (DNA) of unbound chromatin at various salt concentrations was followed by centrifugation to sediment the residual chromatin. (B) The residual chromatin was suspended in dilute Tris buffer (pH 7.5) and bound to 0.5 g of prewashed HA (HTP). The class II EBNA was then eluted from the chromatin-HA resin by using increasing concentrations of GuHCl (pH 6.0). (----) Percentage of total chromatin protein extracted. This was determined by the Coomassie method as described in the text. (——) Percentage of total antigen extracted as determined by the ^{51}Cr-CF assay described in the text. The total recovery of EBNA and protein was approximately 50%.

chromatin also occurred in GuHCl between 2 and 4 M by the centrifugation method, the chromatin-HA chromatography thereby represents more of a DNA affinity chromatography than does HA chromatography. The DNA contamination in the eluted protein fractions was less than 1% (wt/wt). Furthermore, treatment of the eluted fractions with DNase or RNase failed to reduce the antigenic activity, whereas treatment with the proteases abolished the antigenicity. All subsequent studies utilized this HA method to obtain the class II EBNA due to its ease and rapidity.

To help identify whether class II EBNA isolated by this method differed immunologically from class I EBNA, double radial immunodiffusion assays were run, using high-titered anti-EBNA-positive sera. Figure 2 shows the results with one anti-EBNA-positive serum. The precipitin lines between class I and class II EBNA displayed a spur or discontinuity, suggesting that the two antigens were in part antigenically different. This pattern was noted with four different anti-EBNA-positive sera. No precipitin lines were observed with these sera against EBV genome-negative cell lines or with EBNA-negative sera. These gels contained 0.5 M NaCl and 2.5 M urea, which helped to prevent artificial precipitin lines due to basic protein-acidic protein interactions or to premature hydrophobic protein precipitations (see above). Only very high concentrations of histones still caused artificial precipitations under these conditions. The concentrations of proteins used in these double radial immunodiffusion assays were far less than that required for the nonspecific precipitin lines to occur. Thus, the precipitin lines probably reflect true antibody-antigen interactions.

The class II EBNA was then characterized

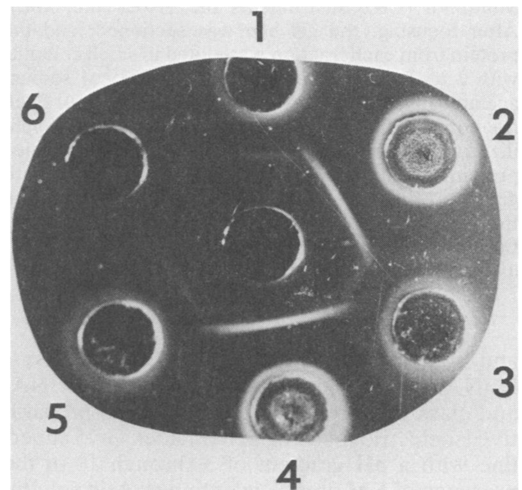

FIG. 2. Double immunodiffusion gel of class I and class II EBNA from NC_{37} cell chromatin. Center well, Anti-EBNA positive antiserum; well 1, class I EBNA (640 μg of protein); well 2, class II EBNA (700 μg of protein); well 3, class I EBNA (640 μg of protein); well 4, class II EBNA (700 μg of protein); well 5, class I EBNA (640 μg of protein); well 6, phosphate-buffered saline.

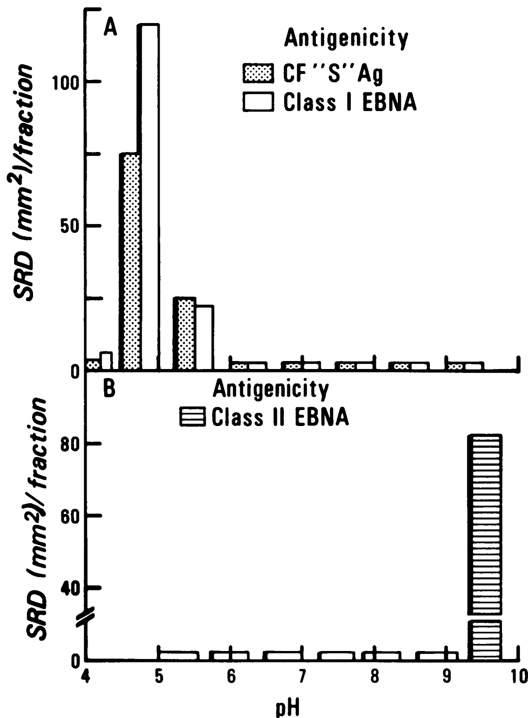

FIG. 3. Preparative isoelectric focusing of the S antigen, class I EBNA, and class II EBNA from NC_{37} cell chromatin. The S antigen, class I EBNA, and class II EBNA obtained from the 3 M GuHCl extract from chromatin-HA chromatography were subjected to preparative flatbed isoelectric focusing with an LKB 2117 Multiphor (LKB Instruments Inc., Rockville, Md.). After focusing, the gel bed was sectioned, and the protein from each fraction was eluted in small columns with 2 to 3 volumes of 6 M GuHCl–0.05 M sodium acetate–0.01 M EDTA–0.5 mM PMSF (pH 6.0). Each fraction was dialyzed against distilled water and lyophilized. The protein recovery was 55% of the applied protein. The bars represent the antigenicity. The antigens were detected by the SRD method as described in the text. The EBNA recovery was estimated at 60% by titration of the chromatography eluants in the SRD assay.

and compared with the S antigen and class I EBNA. The three antigens (S, class I EBNA, and class II EBNA) were subjected to preparative isoelectric focusing in Sephadex G-75 superfine with a pH gradient of 3 through 10 in the presence of 6 M urea to maintain protein solubility. The S antigen and the class I EBNA focused at exactly the same pH (4.5 to 5.0) (Fig. 3A). Further analysis of these two antigens by isoelectric focusing in more sensitive pH gradients with a pH range of 3 to 5 showed that the two antigens still focused together at pH 4.6 (data not shown). Interestingly, the class II EBNA preparation focused in the pH range of 9.2 (Fig. 3B). Since this is the upper limit of the available focusing ampholines, an accurate pI could not be ascertained with this system. The SRD assay used in these analyses included urea-sodium chloride to maintain solubility and to reduce artificial precipitin lines due to basic protein-acidic protein interactions or to premature precipitation of insoluble hydrophobic proteins.

The class II EBNA preparation from the chromatin-HA chromatography still contained histones. Because a high concentration of histones could still bind to the antigen and cause it to focus at a higher pH value, even in the presence of 6 M urea, alternate methods were utilized to assess the charge of the class II EBNA. The class II EBNA preparation was applied to DEAE-cellulose chromatography in the presence of urea. Most of the protein and the antigen eluted in the void volume of the resin (Fig. 4). The class II EBNA preparations were then applied to cation-exchange resins, also in the presence of GuHCl. The bulk of the protein and the antigenic activity bound to the cation exchanger Bio-Rex-70 at pH 6.8 in 5% (wt/wt)

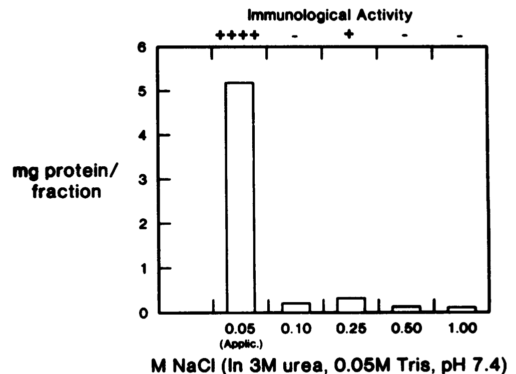

FIG. 4. Anion-exchange chromatography of class II EBNA from Raji cell chromatin, using DEAE-cellulose at 4°C. The class II EBNA fraction (7 mg of protein) extracted from the chromatin-HA resins by 3 M GuHCl (pH 6.0) was applied to DEAE-cellulose chromatography by using a protein-DEAE mixture of 1:20 (wt/wt). The eluting fractions were dialyzed against water and lyophilized. The total recovery was 5.92 mg of protein, or 84% of the applied protein. When ready for analysis, the fractions were suspended in a 6 M GuHCl solution (pH 6.0), dialyzed against 2.25 M urea–0.5 M NaCl, buffered at pH 8.6, and analyzed by the SRD assay as described in the text. The antigenic activity is shown in the upper abscissa. "++++" represents a greater antigen concentration than "+", and "−" represents no detectable antigen activity. The bars represent milligrams of protein per fraction, as determined by the Coomassie assay method. The recovery of the EBNA was estimated at 60% by titration of the chromatography eluants in the SRD assay.

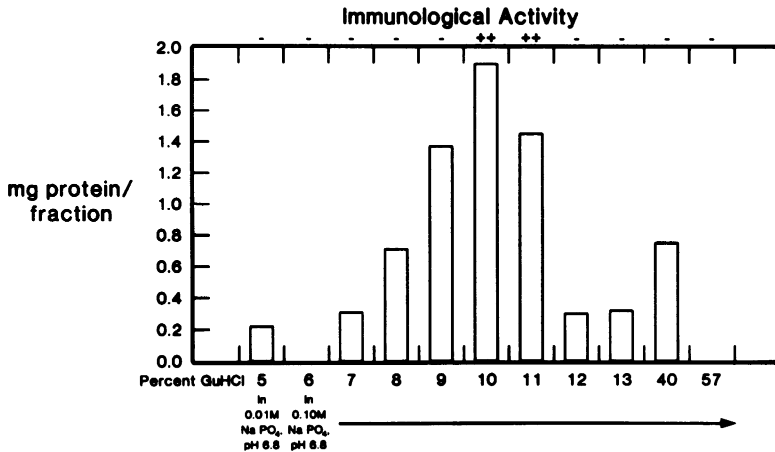

FIG. 5. Cation-exchange chromatography of class II EBNA from Raji cell chromatin, using Bio-Rex-70. The class II EBNA preparation, isolated by the chromatin-HA method and containing 20.7 mg of protein, was applied to prewashed Bio-Rex-70 resin at 22°C with a protein–Bio-Rex-70 mixture of 1:20 (wt/wt). The resin was extracted after 45 min at 22°C with increasing concentrations of GuHCl (pH 6.8), as described in the text. The eluted fractions were processed, dialyzed, lyophilized, and quantitated for protein by the Coomassie assay and for antigenicity by the SRD method. The recovery of protein was 7.33 mg, or 35% of the applied protein. (++) Positive precipitate rings (i.e., presence of antigen) in the SRD method. (−) No antigenic activity. The recovery of EBNA was estimated at 50% by titration of the chromatography eluants in the SRD assay.

GuHCl and eluted in a distinct region between 10 and 12% (wt/wt) GuHCl (Fig. 5). Similarly, the class II EBNA bound tightly to carboxymethyl cellulose at 22°C, using as a solvent 3.0 M urea in 0.1 M NaCl at pH 4.2. These results confirmed that the class II EBNA was a basic protein.

Two methods were used to separate class II EBNA from histones to assure that the class II EBNA was not an artifact of the binding of histones to EBNA. First, the class II EBNA preparations were fractionated by molecular sieve chromatography in 6.0 M GuHCl, and the antigenic activity generally eluted as two fractions in molecular weight ranges (for globulin proteins) of 25,000 to 35,000 and 65,000 to 75,000, respectively (Fig. 6). The antigenic activity in the 65,000- to 75,000-molecular-weight range eluted as a class of nonhistone chromatin proteins. The activity in the 24,000 to 35,000 range, however, eluted in the range of the histones. This antigenic activity could be caused by histones which at high concentrations were found to cause artificial precipitin lines in the SRD assay, even in the presence of sodium chloride-urea. Therefore, other methods were applied which not only separated the EBNA activity from histones but also detected the antigens by alternate means. In one instance, the class II EBNA was separated from histones by a modified HA chromatography method. In this method, class I EBNA-deficient whole chromatin was bound to the resin in 2.0 M NaCl–5.0 M urea in 0.001 M phosphate buffer. The proteins were eluted with increasing concentrations of phosphate. Figure 7 shows the elution patterns of protein and EBNA activity from the HA, using the ^{51}Cr-CF assay to detect the latter. Beside the figure are patterns of SDS-polyacrylamide gels of the pooled fractions from the column. Clearly, the polyacrylamide gel electrophoretic patterns of the fractions containing the class II EBNA verified that the histones did not bind to this resin under these conditions and eluted first from the column. Class II EBNA activity eluted at 0.2 M phosphate. The material eluting with 500 mM sodium phosphate was RNA and DNA. Neither the histones nor the RNA or DNA displayed antigenic activity in the ^{51}Cr-CF assay. Thus, the class II EBNA was separated from the histones and DNA.

As a separate approach to demonstrating the presence of the basic antigen and to obtain a better idea as to the heterogeneity of the EBNA species, whole cell extracts were applied to two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). The first dimension employed isolectric focusing, and the second dimension involved molecular sieve electrophoresis on 10% SDS-polyacrylamide slab gels. This was followed by electrophoretic transfer of the proteins from the polyacrylamide gel to nitrocellulose paper and incubation with serum and ^{125}I-labeled protein A. The positive serum reacted with acidic as well as basic antigens, both in the molecular weight range of 65,000 to 75,000 (Fig.

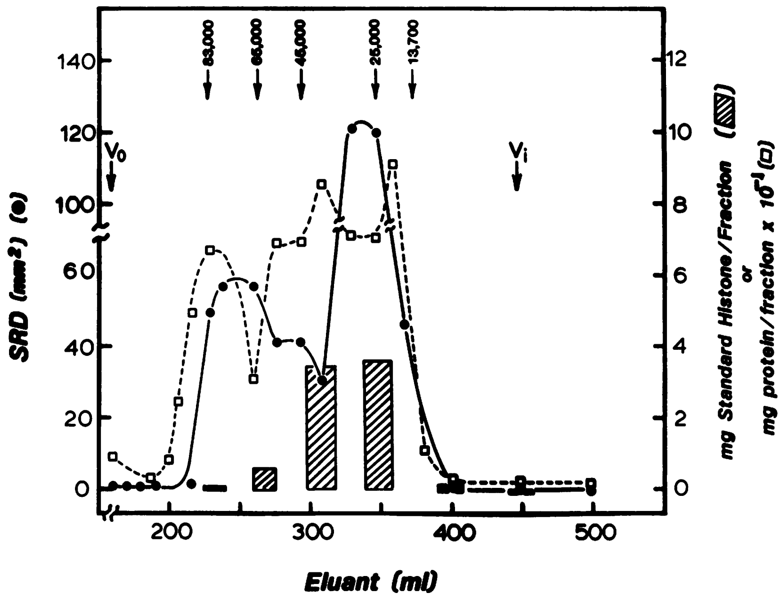

FIG. 6. Molecular-sieve chromatography of class II EBNA from NC_{37} cell chromatin on CL-Sepharose-6B in 6 M GuHCl. The class II EBNA obtained from the chromatin-HA chromatography was dialyzed, lyophilized, and suspended at a concentration of 4 mg of protein per ml in a small volume of buffered 6 M GuHCl (pH 6.0) as described in the text. The preparation, containing 12 mg of protein, was applied to a column of CL-Sepharose-6B (2.5 by 95 mm), and the sample buffer was used as the eluting solvent. At the completion of the run, every 10 fractions were pooled, dialyzed, lyophilized, and analyzed for protein (---) by the Coomassie blue assay and for antigens (——) by the SRD assay as described in the text. The recovery of protein was 6.9 mg, or 57.5% of the applied protein, and the recovery of EBNA was estimated at 60% by titration of the chromatography eluants in the SRD assay. The bars represent standard histones eluting from this resin. The elutions of standard globular proteins from the column are shown as arrows above the class II EBNA profile.

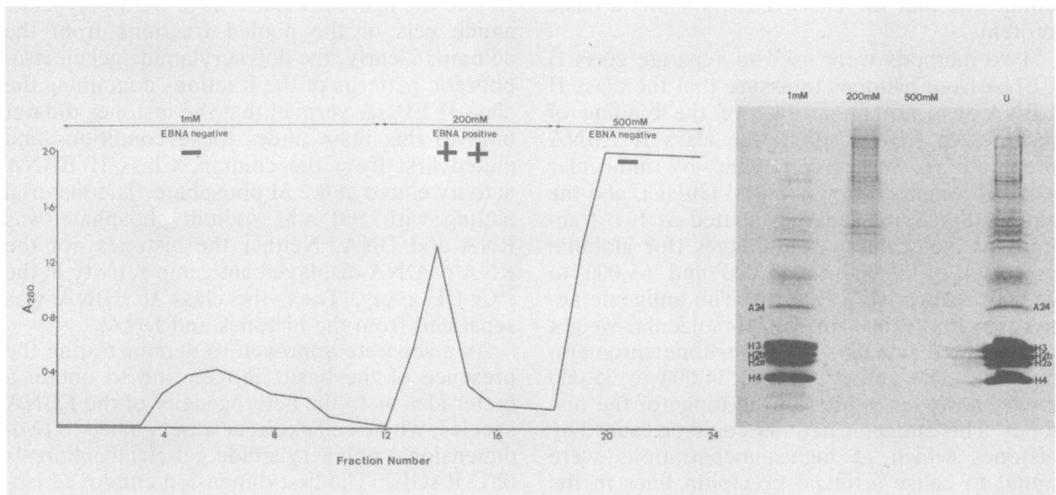

FIG. 7. Fractionation of class II EBNA from Raji cell chromatin on HA chromatography. Salt-washed Raji chromatin was suspended in a solution containing 2.0 M NaCl, 5.0 M urea, and 1 mM phosphate (pH 6.8). This solution was applied to a column of HA equilibrated in 2.0 M NaCl–5.0 M urea–1 mM phosphate (pH 6.8), and protein was eluted with a stepwise gradient of sodium phosphate. Fractions (4 ml) were collected, and protein was measured by its absorbance at 280 nm (A_{280}) (——). (++) Presence of antigen; (−) no antigen. The overall protein recovery was estimated to be about 80% of that applied to the resin based on the absorbance. EBNA was located by assaying the pooled fractions, using the ^{51}Cr-CF method. Recovery of EBNA was determined to be 65%. Samples from each gradient step were subjected to electrophoresis on an 18% polyacrylamide gel containing SDS as described in the text.

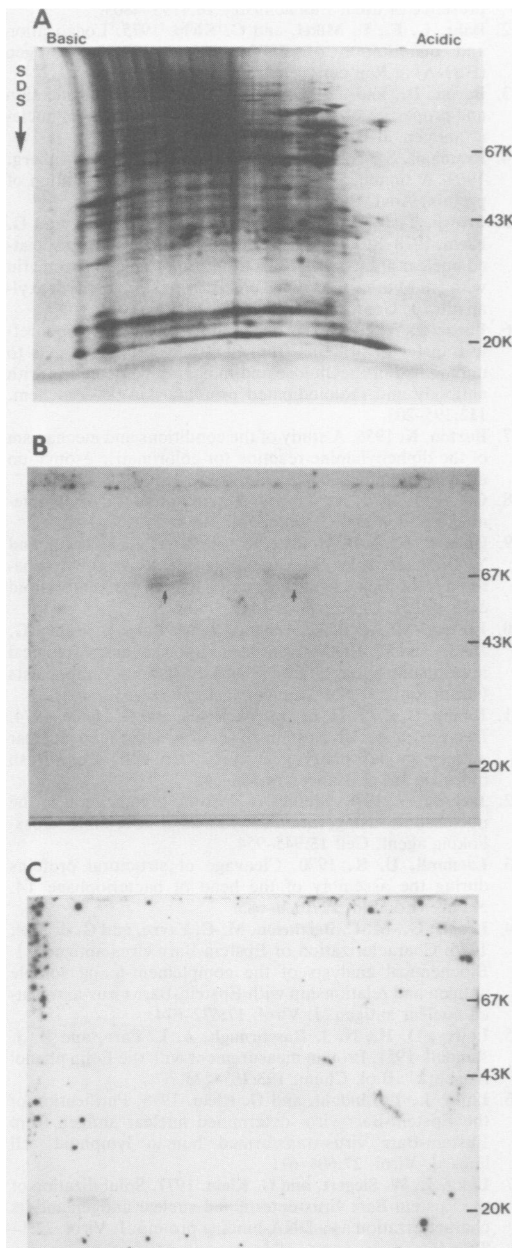

FIG. 8. Radioimmunoelectrophoresis of NC_{37} cell extracts. Samples of NC_{37} cell extracts were subjected to 2-D PAGE as described in the text. Proteins were electrophoretically transferred to nitrocellulose paper, and the papers were incubated with antiserum and ^{125}I-labeled protein A as described in the text. (A) Coomassie blue stained gel; (B) anti-EBNA-positive serum; (C) anti-EBNA-negative serum. The numbers to the side of each figure represent the molecular weights of ^{125}I-labeled standard proteins which were electrophoresed in the second dimension gel.

8B). These results support the existence of the higher-molecular-weight peak of activity from the CL-Sepharose 6B chromatography (Fig. 6) but not the lower-molecular-weight peaks of activity. Thus, the antigenicity in that lower-molecular-weight range in the molecular sieve chromatography (Fig. 6) was probably due to the eluting histones, which are sufficiently concentrated that they interfere with the SRD assay even in the presence of the urea-sodium chloride, as described earlier. The negative serum (Fig. 8C) showed no such reaction with these proteins. Also, extracts from a variety of negative cell lines did not show any specific antigens when tested with anti-EBNA-positive sera (data not shown). These results support the EBNA-specific antigenicity of the antigens (Fig. 8B).

DISCUSSION

These results confirm our earlier reports (36, 37) of the existence of a new class of EBNA bound sufficiently tightly to the DNA to avoid detection in studies performed in other laboratories. The loosely bound or class I EBNA was dissociated from chromatin by 0.4 M NaCl, whereas the class II EBNA was not dissociated by 2.0 M NaCl. The class II EBNA could only be dissociated by 2.0 M NaCl–5.0 M urea or by 4.0 M GuHCl. In short, the class II EBNA was extracted from chromatin only after 80% of the total chromatin proteins were dissociated. The class II EBNA was shown to be related to the class I or classical EBNA when the former was recognized by antibodies which recognize the class I EBNA in the ACIF test.

The class I EBNA was found to be a nonhistone chromatin protein focusing at pH 4.6; it appeared to be identical in this regard to the S antigen, as reported by other laboratories (14, 15). The class II EBNA, in contrast, appeared to be a more basic protein, but also appeared to be of the nonhistone chromatin class (based on its size, its pI, and its behavior on adsorption chromatography). Its basic charge was supported by its binding only to cation exchange resins and its apparently high pI in Sephadex or polyacrylamide gels. The possible artifact due to binding of the antigen by histones was alleviated by the separation of class II EBNA from the histones by molecular-sieve and HA chromatographies and by 2-D PAGE of whole cell extracts. The possible artifactual precipitin lines in the immunological assays, due to basic histone protein-acidic protein interactions or to premature precipitations of hydrophobic proteins, were alleviated in part by including sodium chloride-urea in the SRD assays, by using the ^{51}Cr-CF and radioimmune electrophoretic transfer assays, and by removing the histones before assaying.

In the 2-D PAGE, both a basic antigen which probably represented class II EBNA and an acidic antigen which probably represented class I EBNA were detected. The identification of two species with the basic EBNA by the 2-D PAGE suggests microheterogeneity of the class II EBNA. A molecular weight for the class II EBNA was estimated to be approximately 70,000. The small antigenic species observed by molecular-sieve chromatography were not observed on the 2-D PAGE system, using the same positive antiserum. Thus, it is likely that this apparent antigen is an artifact, based on an artificially induced precipitin line in the SRD assay. This is probably caused by the very high concentration of histones shown in our studies to cause such lines, even in the presence of the sodium chloride-urea.

Most other studies on EBNA have used extraction methods which only dissociate the class I EBNA. Only the studies by Brown et al. (5) included sufficient methodology to extract class II EBNA. The pI of each of the EBNA species studied by Strnad et al. (38) is not known, so comparison with the class II EBNA reported here is not possible. The molecular size of the class II EBNA is in the range of the species of EBNA reported for the class I EBNA by Strnad et al. (38) and certain species by Matsuo et al. (19, 21). The class II EBNA shows both antigenic and charge differences as well as a higher binding affinity to the chromosomal material compared with the class I EBNA. That the class II antigen is not an artifact of class I EBNA bound to DNA is based on the following facts. (i) DNA would make the antigen much more acidic, not more basic. (ii) DNA alone shows no antigenicity with these EBNA-positive antisera. (iii) DNase treatment of the class II EBNA failed to alter its antigenicity. (iv) The class II preparations contained only minute amounts (1% [wt/wt]) of DNA relative to total protein. Definitive conclusions of any molecular relationship between class I and class II EBNAs must await purification and peptide or sequence analysis of these proteins or common immunological recognition sites. Since the class II EBNA is tightly associated with chromatin, it is tempting to speculate on a role in regulating gene expression or in viral DNA replication and transformation, as is indicated for the simian virus 40 large T antigen (39).

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