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Solubilization of the Epstein-Barr Virus-Determined Nuclear Antigen and Its Characterization as a DNA-Binding Protein

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The Epstein-Barr virus (EBV)-determined nuclear antigen (EBNA) was solubilized from isolated nuclei of two EBV-transformed cell lines, Raji and AW-Ramos, by high-salt treatment. Its DNA-binding properties were studied by DNA-cellulose chromatography and a ^{51}Cr release complement fixation assay. EBNA binds to both double-stranded and single-stranded calf thymus DNA, showing a higher affinity to double-stranded DNA. There was no detectable difference in the DNA binding of EBNA prepared from Raji and AW-Ramos cells.

The Epstein-Barr virus (EBV)-determined nuclear antigen (EBNA) was detected by anticomplement immunofluorescence (ACIF) in all EBV genome-carrying lymphoblastoid cell lines tested, both virus producers and non-producers, and also in fresh biopsies from Burkitt's lymphoma (BL) (25, 33) and nasopharyngeal carcinoma (19). The good correlation between EBNA and EBV DNA (19, 21) makes EBNA a sensitive indicator for the presence of the viral genome.

The nature and function of EBNA are still unknown. Its nuclear localization in transformed cells is reminiscent of the T antigens in adenovirus-, polyoma virus-, and simian virus 40 (SV40)-infected and -transformed cells. Its strong association with the metaphase chromosomes is quite different from the nucleoplasm-localized SV40 T antigen, however.

Conceivably, EBNA is a virus-coded or virus-induced chromosomal protein that participates in the regulation of gene expression in the EBV-transformed cell. Such regulatory proteins are known to have a strong affinity for DNA in other systems. For example, due to its affinity to DNA, the bacteriophage T4-coded DNA-unwinding protein could be isolated by DNA-cellulose chromatography (1, 2). Specific protein-DNA interactions were also utilized for the isolation of the bacteriophage lambda operator with the aid of the lambda repressor protein (29).

Recent evidence shows that the SV40 T antigen, presumably involved in the initiation of viral DNA synthesis and the maintenance of cellular transformation (6, 8–11, 35–37), is a DNA-binding protein (7, 16, 32, 38).

In this paper we report our studies on the interaction of EBNA with DNA.

MATERIALS AND METHODS

Chemicals. Ficoll 400 was obtained from Pharmacia AB (Uppsala, Sweden), cellulose CF11 from Whatman (Springfield Mill, Maidstone, England), calf thymus DNA from Worthington Biochemicals Corp. (Freehold, N.J.), $Na^{125}I$ for protein labeling and $Na_2{}^{51}CrO_4$ from the Radiochemical Centre (Amersham, England), Bio-Beads SM2 from Bio-Rad Laboratories (Richmond, Calif.), lyophilized guinea pig serum and rabbit anti-sheep erythrocyte serum from Flow Laboratories, Inc. (Rockville, Md.).

Cell Lines. Raji, Daudi, Namalwa, Rael, Ramos, AW-Ramos, P3HR-1, B95-8, Molt 4, and BJAB cells were used in this study. Their origin and some relevant properties are listed in Table 1. The cells were grown in RPMI 1640 medium containing 5% fetal calf serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml). They were harvested at a density of 5 $\times 10^5$ to 8 $\times 10^5$/ml.

^{51}Cr release CF assay. A more senstive modification of the standard complement fixation (CF) assay has been used, employing ^{51}Cr-labeled sheep erythrocytes (12, 24). For this purpose phosphate-buffered saline-washed sheep erythrocytes were incubated in $Na_2{}^{51}CrO_4$ (1 mCi/ml) for 45 min at 37°C. After washing with gelatin-veronal buffer (pH 7.2) (0.15 M NaCl-5 mM 5,5-diethylbarbituric acid [sodium salt]-0.5 mM $MgCl_2$-0.15 mM $CaCl_2$), labeled sheep erythrocytes were resuspended in gelatin-veronal buffer at a final concentration of 5% and sensitized with rabbit anti-sheep erythrocyte serum. For the assay, labeled, sensitized sheep erythrocytes were diluted to 0.25%. At this concentration they could be stored at 4°C for 1 week. Total hemolysis of 25 μl of this suspension released 6,000 cpm on an average. The amount of complement was chosen such that one CH_{80} unit was kept free for the specific antigen-antibody reaction (12). Antigens were assayed using BL sera, pooled early infectious mononucleosis sera, and EBV-negative (E.K.) and EBV-positive (E.G.) sera from healthy donors (see Table 2). The titers

TABLE 1. *Properties of cell lines used in this study*

Cell line	EBV genome	Producer status	Antigen-positive cells (%)			Reference
			EA	*VCA*	*EBNA*	
Molt 4	−	(−)	(−)	(−)	(−)	28
BJAB	−	(−)	(−)	(−)	(−)	27
Ramos	−	(−)	(−)	(−)	(−)	20
AW-Ramos	+	−	0.0	0.0	100	20
Raji	+	−	0.05–0.1[a]	0.0	100	30
Daudi	+	+	1.0	0.25	100	17
B95-8	+	+	5.0	2.0	100	27
P3HR-1	+	+	12.0	4.0	100	14

[a] As determined by in situ radioantibody and fluorescence labeling (Moar et al., manuscript in preparation).

are expressed as the dilution of antigen leading to a 50% release of ^{51}Cr.

Some of the BL sera showed a high anticomplementary activity, which could be removed by kaolin adsorption (4).

Preparation of crude cell homogenates. Cells were washed, suspended in isotonic buffer (0.150 M NaCl-20 mM Tris-hydrochloride [pH 7.4]-4 mM $MgCl_2$-1 mM Na_3 EDTA) at a density of 100 × 10^6/ml, and sonically treated for 6 cycles of 15 s in ice. This was followed by centrifugation for 30 min at 6,000 rpm and 4°C in a Sorvall SS-34 rotor.

Removal of detergents from soluble proteins. Nonidet P-40 (NP-40), Triton X-100, and deoxycholate (DOC), initially present at 1%, were removed from protein solutions by adding an equal volume of Bio-Beads SM2 (20 to 40 mesh) (15) and gently shaking the bead suspension for 2 h in the cold. During this time, the detergents are adsorbed to the beads. The concentration of detergents remaining in the solution is so low that no disturbing effect is observed on the CF reaction. The beads were pretreated by the method of Holloway (15) and washed with isotonic buffer.

Isolation of nuclei. Cells were harvested by centrifugation in a Sorvall GS-3 rotor at 2,500 rpm for 15 min at 4°C, suspended, and washed in isotonic buffer. A 5-ml amount of pelleted cells was suspended in 40 ml of hypotonic buffer (50 mM NaCl-20 mM Tris-hydrochloride [pH 7.4] 4 mM $MgCl_2$-1 mM Na_3 EDTA) and swollen in ice for 15 min. Cells were disrupted by 10 strokes in a tight-fitting Dounce homogenizer, and the effect of the disruption was controlled by phase-contrast microscopy. Thereafter nuclei were diluted to a concentration of 100 × 10^6/ml with isotonic buffer and separated from membranes, cytoplasm, and unbroken cells by centrifugation through a cushion of 22.5% Ficoll 400 in isotonic buffer for 15 min at 1,200 rpm and 4°C. The partially purified nuclear pellet was used for further processing.

Isolation of nuclear proteins. EBNA-containing nuclear proteins were isolated by the method of Alberts and co-workers (1, 2). Briefly, nuclei prepared from 5 ml of pelleted cells were treated with 20 ml of lysis buffer (1.7 M NaCl-20 mM Tris-hydrochloride [pH 7.4]-1 mM Na_3 EDTA-1 mM beta-mercaptoethanol) and sonically treated twice for 60 s. DNA was separared from solubilized proteins by the addition of 10 ml of polyethylene glycol (PEG) 6000 (30%), incubation for 30 min in ice, and centrifugation in a Sorvall SS-34 rotor for 15 min at 10,000 rpm and 4°C. The supernatant was dialyzed overnight against "elution buffer" (50 mM NaCl-20 mM Tris-hydrochloride [pH 7.4]-1 mM Na_3 EDTA-8.5% glycerol-1 mM beta-mercaptoethanol). The dialyzed extracts were centrifuged (6,000 rpm for 15 min in a Sorvall RC2-B with an SS34 rotor) to remove a light precipitate.

Chromatography on DNA-cellulose. DNA-cellulose (2) was prepared by mixing Whatman CF 11 cellulose powder with a solution of either single-stranded (ss) or double-stranded (ds) calf thymus DNA (2 mg/ml). ssDNA was prepared by heat denaturing dsDNA for 15 min at 100°C in low-salt buffer and rapid quenching in ice (2). The mixture was then dried at room temperature for several days, followed by freeze-drying.

After washing with elution buffer, a DNA-free cellulose column (2 by 20 cm) was coupled to a DNA-cellulose column (2 by 20 cm) and loaded with nuclear protein extract. Nonbinding proteins were washed out with the same buffer. The DNA-free cellulose column was removed and proteins binding to DNA were eluted with a linear NaCl gradient in the concentrations shown in the figures. Finally the columns were washed with 2 M NaCl. The columns were run at 10 ml/h. Fractions of 1.5 ml were collected and monitored for protein content at 280 nm. As the protein content was usually below the level of detectability, we monitored ^{125}I-labeled proteins in some of the experiments. For this purpose we labeled a small amount of the nuclear proteins with ^{125}I, using the lactoperoxidase method (39). The iodinated proteins were admixed to the unlabeled nuclear proteins prior to chromatography. The fractionated proteins were assayed for EBNA activity by the ^{51}Cr release CF test. To reduce the nonspecific binding of iodinated proteins to the cellulose, proteins were first passed through a column (2 by 20 cm) containing cellulose without DNA. This column was connected to the DNA-cellulose column. After washing and before starting the elution, it was disconnected and discarded.

ACIF absorption tests. ACIF adsorption tests were carried out as described by Reedman and Klein (33). The antigenic extract was incubated with serial dilutions of anti-EBNA-positive BL sera for 2 h at room temperature. As a control, corresponding extracts from the EBNA-negative BJAB cells were

used. The incubated mixtures were tested for regular EBNA staining by ACIF on Raji smears.

RESULTS

Specificity of the CF assay. In the CF reaction BL patient sera were used for the detection of EBNA. As shown in Table 2, these sera contain antibodies against EBNA, viral capsid antigen (VCA), and early antigen (EA). Raji cells do not contain VCA, but may have some EA in a low proportion (<0.1%) of the cells as determined by immunofluorescence and in situ radioantibody labeling (M. Moar et al., manuscript in preparation). It is therefore important to examine the possibility that the CF reaction may detect other antigenic activities in addition to EBNA. Therefore we assayed crude, sonically treated material from several cell lines, representing different antigenic combinations (Table 1). Three types of sera were used: EA+/VCA+/EBNA+, EA+/VCA+/EBNA−, and EA(low)+/VCA+/EBNA− (Table 2).

The results of the antigen titrations are shown in Fig. 1 to 3. In the combination of the Raji extract (Fig. 1) and the EA+/VCA+/EBNA+ serum, the 50% ^{51}Cr release value indicates a CF titer of 1:100 in this particular antigen preparation. Anti-EBNA negative, but anti-VCA and/or EA-positive, sera were not inhibited by the Raji extract. Identical results were obtained with Namalwa, Daudi and Rael (Fig. 1) and AW-Ramos cells (Fig. 2). All lines except Daudi are nonproducers; i.e., they contain EBNA as the sole EBV-related antigen. Daudi is a spontaneous producer of EA and VCA, but at a very low level.

These findings, illustrated in Fig. 1, indicate that EBNA was the only antigen detected by the CF assay in nonproducer or low-level producer cells. To assess whether some of the antigens associated with the viral cycle could be detected in producer cells, we repeated the test with P3HR-1 and B95-8 extracts (Fig. 2). Clearly positive reactions were obtained with the EA(low)+/VCA+/EBNA− serum (titer, 1:16) and the EA+/VCA+/EBNA− serum (titer, 1:25). This shows that the assay can detect EA and VCA in producer cells. EBNA-negative cells, such as Ramos (Fig. 2) and BJAB and Molt 4 (Fig. 3), were entirely negative with all three types of antisera. EBV-negative serum from a healthy donor was negative against all extracts.

These results thus suggest that the CF reaction can detect a variety of EBV-determined antigens, including EBNA, EA, and VCA. The system can be rendered operationally monospecific by the choice of the target cell-antiserum combination.

Solubilization of EBNA. Mechanical disruption and freeze-thawing followed by low- and high-speed centrifugation were reported to give good yields of soluble EBNA (23). This method is not suitable for DNA column-binding studies, however, since the endogenous DNA of the preparation competes with the DNA on the column. To avoid this endogenous DNA, DNA was removed by precipitation with PEG in the presence of 1.7 M NaCl (1, 2). In view of the antigen purification envisaged, it appeared advantageous to start this procedure with partially purified nuclei rather than with whole cells. The nuclei are known to contain most of the antigen.

One side effect of the PEG had to be considered. It interacts with the antiserum and may therefore reduce the CF titers of the initial EBNA preparation. The different sera tested differed in their sensitivity to PEG. The rea-

TABLE 2. *Properties of human sera used in this study*

Serum	KCC[a] no.	Date	Diagnosis	Titer		
				VCA	EA	EBNA
A.O.	1,723	9/23/75	BL	1,280	160	640
A.O.	812	2/28/69	BL	1,280	80	640
R.B.	1,453	9/5/75[b]	BL	640	10	160
A.O.	1,311		BL	1,280	80	640
J.M.	1,003	1969[b]	BL	160	10	160
W.W.	1,021	6/19/70		1,280	320	<2
E.K.		1975	BL	<2	<2	<2
E.G.		4/3/76	Healthy	160	<10	80
§1[c]		1976[b]	IM[d]	80	40	<2
§2		1976[b]	IM	80	10	<2

[a] KCC, Kenya Cancer Center.
[b] Serum pool.
[c] §, Pooled sera from several Swedish patients.
[d] IM, Infectious mononucleosis.

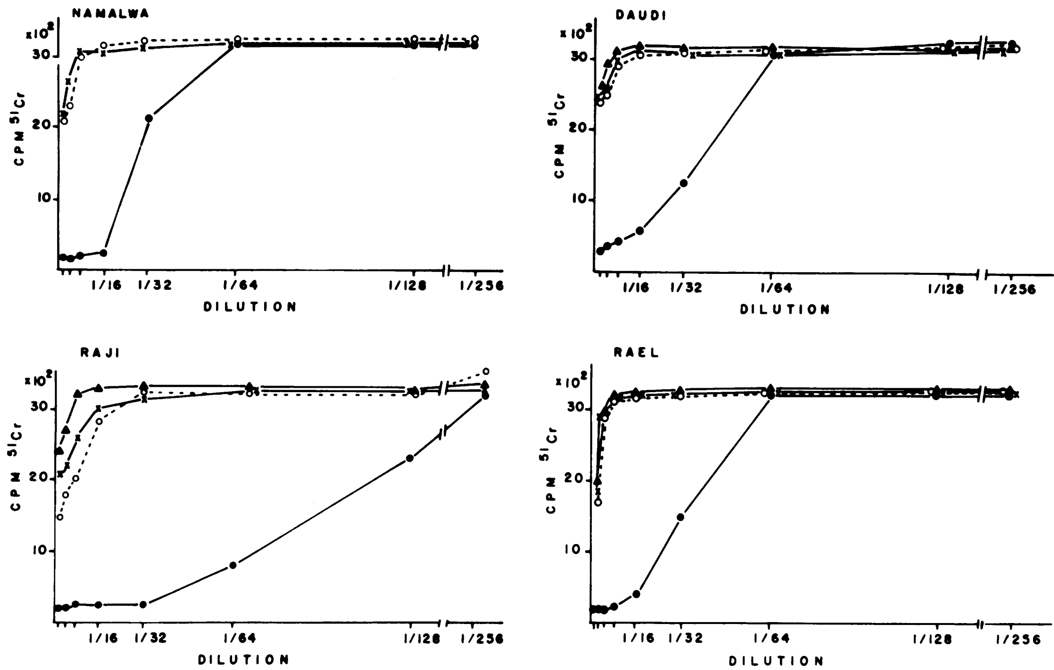

FIG. 1. *Titration of extracts from Namalwa, Daudi, Raji, and Rael cell lines by CF. The curves correspond to the* ^{51}*Cr release at the various antigen dilutions. Symbols:* ▲, *without addition of serum;* ●, *with EA+/VCA+/EBNA+ serum;* ○, *with EA+/VCA+/EBNA− serum;* ×, *with EA(low)+/VCA+/EBNA− serum.*

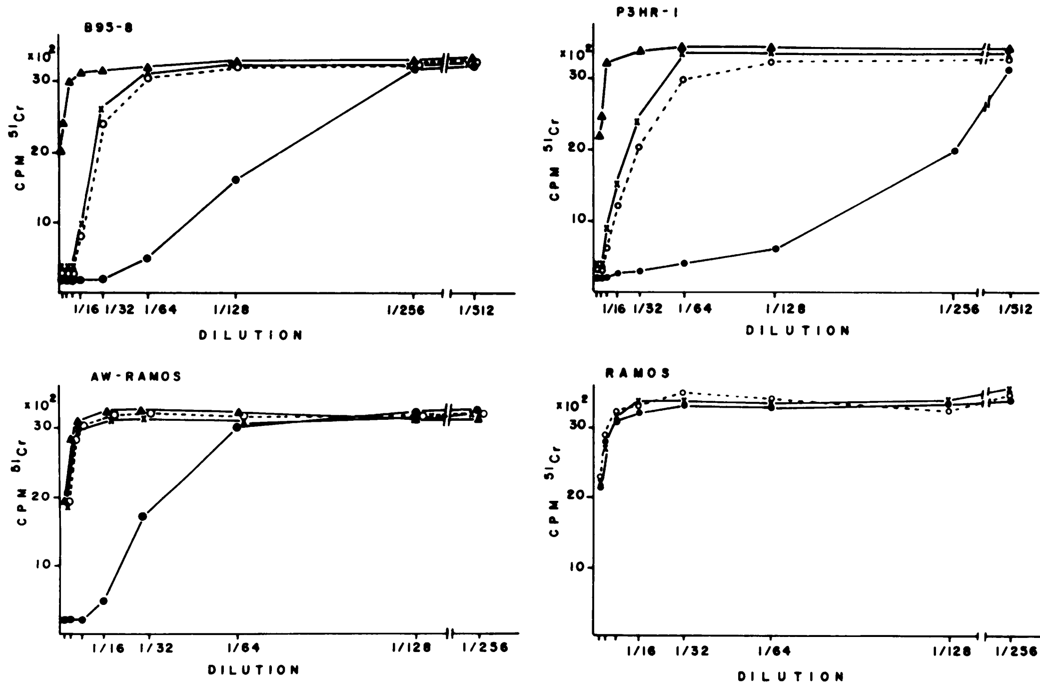

FIG. 2. *Titration of extracts from B95-8, P3HR-1, AW-Ramos, and Ramos cell lines by CF. The curves correspond to the* ^{51}*Cr release at the various antigen dilutions. Symbols are the same as those given in the legend to Fig. 1.*

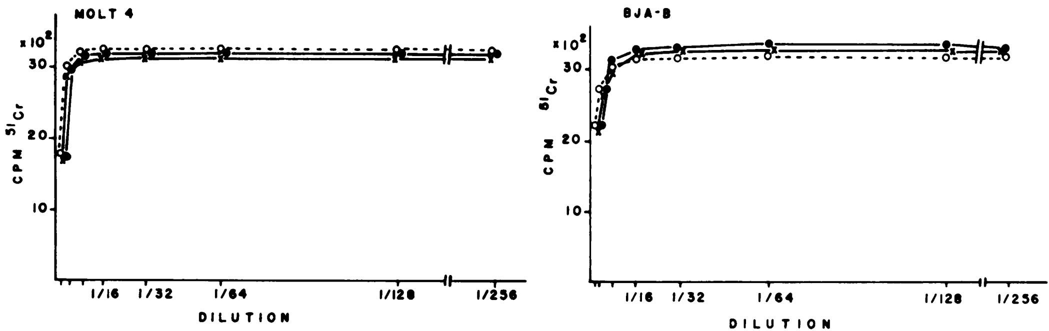

FIG. 3. *Titration of extracts from Molt 4 and BJAB cell lines by CF. The curves correspond to the ⁵¹Cr release at the various antigen dilutions. Symbols are the same as those given in the legend to Fig. 1.*

sons for this are unknown. The PEG effect can be avoided by removing PEG from the antigen preparation by DNA-cellulose chromatography prior to the CF test.

We also tested the ability of urea and detergents to solubilize EBNA (Table 3). Incubation with urea for 30 min at room temperature and subsequent dialysis against isotonic buffer destroyed the antigenic activity; this effect increased with increasing urea concentration. After treatment with 3 M urea, only 12% of the initial activity was left.

The detergents NP-40, Triton X-100, and sodium DOC were added to the nuclear proteins at a final concentration of 1%. After 1 h of incubation in ice, the detergents were removed with Bio-Beads (see above). Triton X-100 and sodium DOC destroyed 75% of the antigenic activity, whereas NP-40 only reduced it slightly.

High-salt treatment, combined with PEG precipitation, solubilized the antigen rapidly and efficiently, while removing most of the DNA. This method was chosen for the experiments described in this paper.

DNA-cellulose chromatography. To assess the DNA-binding properties of EBNA, proteins isolated from nuclei by high-salt treatment were chromatographed on DNA-cellulose columns. EBNA prepared with Raji and AW-Ramos were compared on columns carrying ssDNA and DNA, respectively. Figures 4 and 5 show that EBNA activity prepared from Raji (Fig. 4a) and AW-Ramos (Fig. 5a) nuclear proteins elutes from dsDNA as one homogenous, symmetric peak at 0.38 M NaCl.

The analysis of the same protein material on ssDNA and dsDNA, respectively. Figures 4 and 5 show that EBNA activity prepared from Raji remained unbound and appeared in the low-salt wash fractions. Moreover, the salt gradient eluted two activity peaks, one at 0.27 M and the other at 0.38 M NaCl. The second peak corresponded to the one obtained by elution from dsDNA. No further activity could be eluted with salt concentrations up to 2 M NaCl. The values were identical for EBNA isolated from Raji and AW-Ramos.

TABLE 3. *Sensitivity of EBNA to treatment with urea and detergents*

Treatment	CF titer	Remaining activity (%)
Untreated	1,024	100
Urea, 1.5 M	512	50
Urea, 3.0 M	128	12
Urea, 6.0 M	128	12
NP-40, 1%	920	90
Triton, 1%	256	25
Sodium DOC, 1%	256	25

DNA-cellulose columns are highly efficient in purifying and concentrating proteins, provided they are bound to the column. To exclude the possibility that an EBNA unrelated complement-fixing protein was enriched, we tested nuclear proteins from the EBNA-negative Ramos cell line on dsDNA as a control (Fig. 6). There was no detectable CF activity in the eluate.

These results show that EBNA binds efficiently to dsDNA. The relatively high salt concentrations required for its elution make it likely that we are dealing with a specific DNA-protein interaction. ssDNA binds EBNA much less effectively, however.

The two separate activity peaks eluted from the ssDNA may be due to the presence of a low proportion of dsDNA in this preparation. This idea is supported by the fact that the second peak is eluted at 0.38 M NaCl, i.e., the same concentration required for the elution of EBNA from dsDNA.

ACIF absorption tests. Crude cell or nuclear homogenates of the EBNA-positive Raji or the EBNA-positive BJAB cells were incubated with BL sera in final dilutions of 1:20, 1:40 and

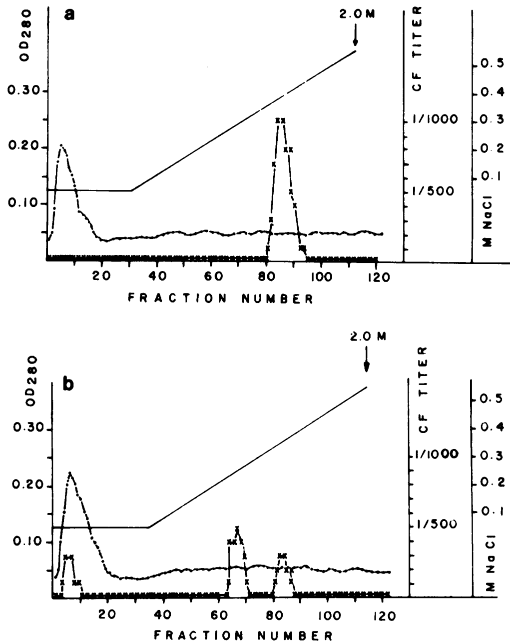

FIG. 4. *Chromatography of nuclear proteins from Raji cells on dsDNA (a) and ssDNA (b). Fractions were tested for their protein content by measuring the optical density at 280 nm (●) and their complement-fixing activity (×). Elution was carried out with a salt gradient (——).*

1:80. Fivefold-concentrated CF antigen from a dsDNA-cellulose column was incubated under the same conditions and the mixtures were tested for ACIF on Raji smears (Table 4). The data show that the purified CF antigen efficiently absorbs the anti-EBNA antibodies of the BL sera.

DISCUSSION

In view of its regular association with the chromosomes of EBV-carrying cells, EBNA may have a regulatory function in viral and/or cellular gene expression. If regulation acts at the DNA level, a close interaction would be expected between EBNA and the cellular DNA. We have approached this question by studying the binding properties of EBNA to DNA, using DNA-cellulose chromatography.

This was only feasible after solubilization of EBNA. High-salt treatment of isolated nuclei gave the best results. Denaturing agents, such as urea and detergents, failed to increase the yield and even destroyed antigenic activity. An analysis of the nuclear protein fraction on DNA-cellulose clearly showed that EBNA binds to DNA. It had a considerably higher affinity for dsDNA than for ssDNA. Its affinity to DNA may be related to the exclusive nuclear localization of EBNA in the ACIF test. It is also compatible with the idea that EBNA may be involved in regulatory processes at the DNA level.

There seems to be some parallelism between the properties of EBNA and the T antigens of the oncogenic adenovirus and SV40 systems. Recently, two proteins were isolated from adenovirus 12-infected cells that could bind efficiently to ssDNA. Genetic evidence suggested that they were early viral products, required for viral DNA replication (34). The possible relationship between these proteins and the T antigen is not yet clear (13, 31).

SV40 T antigen is known to bind to dsDNA from calf thymus and SV40 (7, 16, 32, 38). Electron microscopic evidence showed that it binds

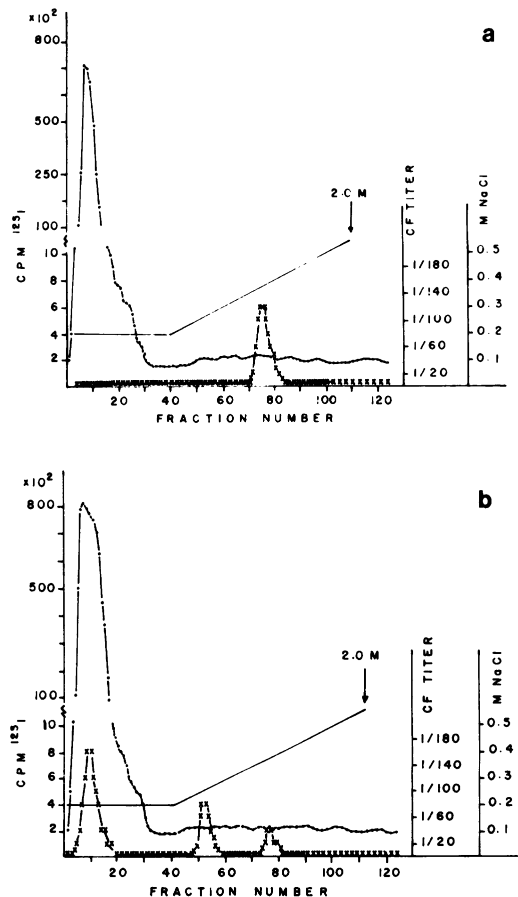

FIG. 5. *Chromatography of nuclear proteins from AW-Ramos cells on dsDNA (a) and ssDNA (b). The eluates were tested by monitoring ^{125}I-labeled proteins (●) and measuring complement-fixing activity (×). Elution was carried out with a salt gradient (——).*

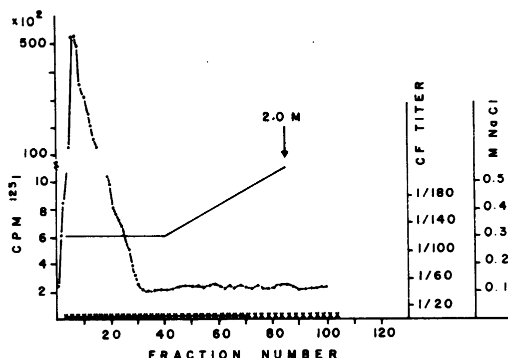

FIG. 6. *Chromatography of nuclear proteins from Ramos cells on dsDNA-cellulose. The fractions were monitored for ^{125}I-labeled proteins (●) and complement-fixing activity* (×). *Elution was carried out with a salt gradient.* (——).

TABLE 4. *Absorption of ACIF*[a]

Absorbing antigen extracts	Absorption of ACIF at BL reference serum dilution of:		
	1:20	1:40	1:80
	+++	++	+
Raji cells, 100 × 10⁶	–	–	–
Raji cells, 50 × 10⁶	Weak +	–	–
Raji cells, 10 × 10⁶	+++	++	Weak +
Raji nuclei, 100 × 10⁶	Weak +	–	–
Raji nuclei, 50 × 10⁶	++	Weak +	–
Raji nuclei, 10 × 10⁶	+++	++	–
BJAB cells, 100 × 10⁶	+++	++	+
BJAB nuclei, 100 × 10⁶	+++	++	Weak +
CF antigen from dsDNA, 5 × concn	–	–	–
CF antigen from dsDNA, 5 × concn and 1:2 dilution	–	Weak +	–

[a] All sera were tested by the ACIF test of Reedman and Klein (33) on acetone-methanol-fixed Raji smears. +++, Brilliant nuclear fluorescence; ++, intense nuclear fluorescence; +, nuclear fluorescence; –, no detectable nuclear fluorescence.

to SV40 DNA, preferentially at the origin of replication (32). Moreover, DNA-binding experiments with partially purified T antigen from SV40, temperature-sensitive mutants in the gene *A* showed that in vitro DNA binding of the T antigen was reduced at the restrictive temperature (38). This suggested that the T antigen may be a gene *A* product, and it may exert its effect by or through binding to viral DNA.

The elution of antigenic activity in a narrow salt concentration range suggests that EBNA is either a single molecule or a relatively homogeneous, tight molecular complex. We have also provided evidence that EBNA derived from different cells has identical binding characteristics. This may imply that EBNA isolated from the in vivo transformed Raji cell line (of BL origin) is not only antigenically, but also even biochemically, related to the EBNA of AW-Ramos derived from the EBNA-negative Ramos line by in vitro conversion with P3HR-1 virus (20).

Finally, the antigen detection system has to be mentioned. We used a modification of the standard CF assay for the detection of EBNA. In the past, the CF reaction was used to demonstrate the "CF" or "S" antigen (3, 40). EBNA was demonstrated by ACIF, however (33). The ACIF absorption experiments show (Table 4) that the EBNA-positive Raji cell homogenates, nuclei, and CF antigen inhibited the immunofluorescent EBNA reaction, whereas EBNA-negative BJAB cells and nuclei did not inhibit. This means, in other words, that the CF antigen is co-existing in the same peak as the antigen that inhibits EBNA fluorescence and has the same binding properties to calf thymus dsDNA-cellulose. It is therefore likely that the CF antigen and EBNA are identical.

In conclusion, our data show that the CF reaction is able to discriminate between the known EBV-determined antigens EA, VCA, and EBNA by using appropriately selected serum-cell combinations. While this work was in progress, a similar conclusion was reached by Lenoir et al. (22), and Baron et al. (5) briefly reported binding to DNA-cellulose of CF antigen from Raji cells.

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