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Identification and Characterization of a Major Early Cytomegalovirus DNA-Binding Protein

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We characterized a DNA-binding protein with an approximate molecular weight of 129,000 (DB129) which is present in the nuclei of cytomegalovirus- (strain Colburn) infected cells, but not in virus particles. Results of two types of experiments demonstrated that DB129 is a member of the early class of herpesviral proteins. First, time course pulse-labeling experiments showed that its synthesis begins after that of the immediate-early protein IE94, but prior to the appearance of late viral proteins, and was reduced at late times. Second, in the presence of inhibitors of viral DNA replication, DB129 continued to be made and accumulated to elevated levels. A second set of experiments showed that DB129 bound to single-stranded DNA in vitro and was eluted by a NaCl gradient in two peaks, one at about 0.2 M and the second at about 0.6 M. A similar pattern of release was observed when infected-cell nuclei were serially extracted with increasing NaCl concentrations. In addition, treatment of nuclei with DNase I selectively released DB129, along with a small but significant fraction of another DNA-binding protein, DB51. These results suggest that DB129 is associated with DNA in vivo and that it interacts directly with single-stranded DNA. It was also shown that cells infected with human cytomegalovirus (strain Towne) contain a slightly larger counterpart to DB129, which was designated DB140. Similarities between these proteins and the major DNA-binding protein of herpes simplex virus are discussed.

Virus-specific proteins synthesized in cells after infection by members of the herpesvirus group have been broadly categorized in three temporal classes: immediate-early (alpha), delayed-early or simply early (beta), and late (gamma) (17). Synthesis for each of these classes is coordinately regulated. The immediate-early proteins are those synthesized immediately after infection, the synthesis of which is not dependent on prior viral protein synthesis. The early proteins are synthesized subsequent to and are dependent on prior synthesis of immediate-early protein(s). Their appearance is observed just before the onset of viral DNA replication. The late proteins appear last and, in general, function to package the viral DNA and give rise to the mature enveloped virus particle.

The large genome of the cytomegaloviruses (CMVs) has a theoretical coding capacity 50% greater than that estimated for herpes simplex virus (HSV; e.g., over 100 proteins), yet studies of CMV-infected cells have identified fewer than 40 infected cell-specific proteins (6, 9, 25, 42–44). Many of these are structural constituents of the virion, but the functions of the rest remain largely unknown. To understand the interaction between CMV and its host during the course of infection, further knowledge of viral gene products and their functions is needed. The early proteins are of particular interest because of their involvement in regulating viral gene expression and DNA replication (4, 15, 18, 35). In addition, many antiherpesvirus chemotherapeutic agents act by interfering with the function of members of this group of proteins (5, 7, 16, 19, 41). The identification and further study of CMV early gene products has therefore become an area of increasing interest (2, 9, 10, 13, 18, 23, 24, 35, 42, 43).

Previous reports showed that strain Colburn CMV-infected cells contain a 129,000-molecular-weight, phosphonoformic acid- (PFA) enhanced protein (13) and that cells infected with human strains of CMV (HCMV) contain a 140,000-molecular-weight possible counterpart (11). Evidence presented here shows that this strain Colburn CMV protein, designated DB129, is an early protein, binds single-stranded DNA (ssDNA) in vitro, and is associated with DNA in infected cells. It is also shown that the HCMV 140,000-molecular-weight protein, designated DB140, is indeed the counterpart of DB129.

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MATERIALS AND METHODS

Cells and virus. Human foreskin fibroblasts were prepared, cultured, and infected as described previously (9, 14). Strains Colburn and Towne CMV used in these studies have also been described previously (9, 11, 20).

Radiolabeling of infected cells and use of metabolic inhibitors. Mock- or CMV-infected human foreskin fibroblast monolayer cultures grown in 60-mm-diameter dishes were biosynthetically labeled by adding [^{35}S]methionine (SJ.204; Amersham, Corp., Arlington Heights, Ill.), prepared as a 10-fold-concentrated stock in serum-free medium, to a final concentration of 10 μCi/ml 24 h prior to harvesting. Pulse-labeling was done by removing the medium at the time of labeling and replacing it with methionine-free, serum-free medium containing 20 μCi of [^{35}S]methionine per ml. Labeling was terminated by removing the isotope-containing medium and washing the cells in ice-cold phosphate-buffered saline. Biosynthetic labeling with [^{32}P]P_i (PBS.11A; Amersham) was done by adding the isotope directly to the culture medium to a final concentration of 200 μCi/ml. The labeling intervals varied with the experiment and are described in detail in the text.

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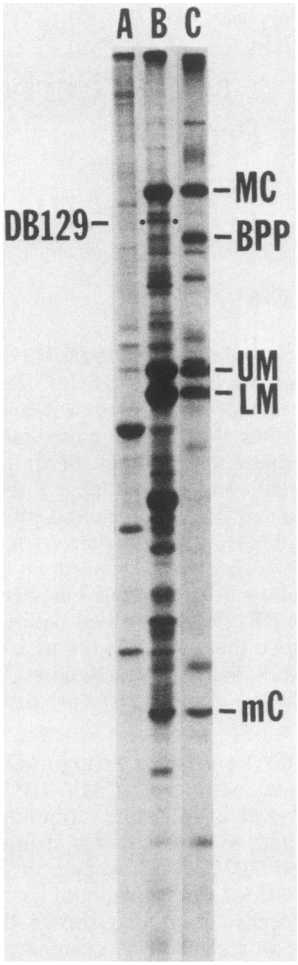

FIG. 1. Colburn DB129 is not a virion protein. Mock-infected or CMV- (Colburn) infected cells were radiolabeled at 72 h postinfection with [^{35}S]methionine, and nuclear fractions were prepared in situ as described in the text. Virions were labeled with [^{35}S] methionine and purified as previously described (21). Positions of the following major virion proteins are indicated for reference: MC, major capsid; BPP, basic phosphoprotein; UM, upper matrix; LM, lower matrix; mC, minor capsid. Lane A, nuclear fraction, mock-infected cells; lane B, nuclear fraction, Colburn-infected cells; lane C, purified Colburn virions.

PFA (Richmond Organics, Ashland, Va.), prepared fresh as a 10-fold-concentrated stock in serum-free medium, or hydroxyurea (H-8627; Sigma Chemical Co., St. Louis, Mo.) was added at the time of infection to the final concentration given in the figure legends. Labeling medium also contained the appropriate concentration of inhibitor.

Fractionation of infected cells. Cell monolayers grown in 60-mm-diameter culture dishes were separated into detergent-soluble and -insoluble components without removing the cells from the substrate, a method referred to as in situ fractionation (12). The medium was removed, and the cell monolayer was washed twice with 0.5 ml of cold phosphate-buffered saline. The cells were then lysed by adding 0.5 ml of buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4], 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, Nonidet P-40) per dish at 4°C, and the dish was kept on ice for 20 min with occasional rocking. The lysate, referred to as the soluble cytoplasmic fraction, was collected and saved. The detergent-insoluble material which remained on the substrate included adherent cytoskeletal elements and nuclei and is referred to as the nuclear fraction. Following a wash with 0.5 ml of buffer B (10 mM Tris [pH 7.4], 1 mM dithiothreitol, 0.1 mM EDTA), the nuclear fraction was extracted with 0.5 ml of 1.0 M NaCl in buffer B on ice for 15 min with occasional rocking. This fraction is referred to as the nuclear salt extract. In some cases a serial salt extraction was done on the nuclear fraction, using concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 M NaCl. Each of the sequential extractions was done as described above for 1.0 M NaCl. The material remaining on the dish after salt extraction was solubilized for subsequent analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), by adding 0.5 ml of SDS-PAGE sample buffer (see below) and is referred to as the nuclear SDS fraction.

DNA affinity chromatography. ssDNA affinity chromatography was done essentially as described by Purifoy and Powell (38). Salt extracts, prepared as described above from nuclei of infected or mock-infected human foreskin fibroblast cells, were dialyzed and concentrated in a collodion bag apparatus (UH100/25; Schleicher & Schuell, Inc., Keene, N.H.) against DNA-binding buffer (20 mM Tris [pH 7.5], 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol); clarified by centrifugation at 10,000 × *g* for 10 min; and chromatographed at 4°C with ssDNA-agarose (5906SA; Bethesda Research Laboratories, Gaithersburg, Md.) columns with an approximate bed volume of 1 ml equilibrated in DNA-binding buffer. Columns were eluted with NaCl in DNA-binding buffer. Salt concentrations in eluant fractions were determined by refractometry. Details of the elution protocol are given in the respective figure legends.

DNase I treatment of nuclei. DNase I (6330 DPFF; Cooper Biomedical, Inc., Malvern, Pa.) digestion was done essentially as described by Knipe and Spang (27) with the following modifications. Nuclei were prepared by the in situ method described above and washed once with 0.5 ml of nuclease digestion buffer (10 mM Tris [pH 7.5], 10 mM NaCl, 1.5 mM $MgCl_2$). Digestion was done in situ by adding 0.5 ml of digestion buffer containing 5.0 μg of DNase I per ml, allowed to proceed at room temperature for 30 min, and stopped by adding EDTA to a final concentration of 5 mM. The supernatant fraction and the material remaining on the substrate were then separately collected and processed for subsequent analysis by gel electrophoresis.

PAGE. SDS-PAGE was carried out as described by Laemmli (28) in gels with an acrylamide monomer concentration of 10%, unless otherwise indicated. Samples were prepared for electrophoresis by adding an equal volume of a solution containing 4% SDS (28365; Pierce Chemical Co., Rockford, Ill.), 20% (vol/vol) 2-mercaptoethanol, 10% (vol/vol) glycerol, 0.02% bromophenol blue, 0.1 M Tris (pH 7.0) (2× SDS-PAGE sample buffer) and by heating at 100°C for 3 min. [^{35}S]methionine-labeled proteins were detected in gels by fluorography (3, 30) with prefogged Kodak X-Omat AR film or by autoradiography with Kodak DEF film. Calculations of the relative amounts of radiolabeled proteins were based on measurements made with an E-C 910 densitometer equipped with a 540-nm interference filter (E-C Apparatus Corp., St. Petersburg, Fla.). The absorbance of a band was calculated as 0.5 (peak height times width at the base line).

RESULTS

Initial observation and characterization. An in situ cell fractionation method was used to study the intracellular

distribution of infected cell-specific proteins in strain Colburn CMV-infected cell cultures. The results obtained by this method of cell fractionation, with respect to previously identified Colburn proteins, are essentially the same as those obtained with protocols based on differential centrifugation (9; data not shown). In the course of these studies we observed a high-molecular-weight protein associated with the nuclear fraction of CMV-infected cells. Calculations based on the previously estimated molecular weights of the major capsid protein (145,000) and the basic phosphoprotein (119,000) (11), indicate that this protein has a molecular weight of 129,000 (Fig. 1, lane B). Because of its DNA-binding properties, described below, this protein has been designated DB129. Although DB129 has not been detected in either mature virions (Fig. 1, lane C) or in any of the intracellular capsid forms (9; data not shown), two lines of evidence indicate that it is probably virus specified. First, no similar protein was seen in mock-infected cells (Fig. 1, lane A). Second, infection of cells with other CMV strains, including both simian and human, gave rise to the appearance of nuclear proteins with similar properties but, in the case of HCMV, with different molecular weights (see Fig. 7).

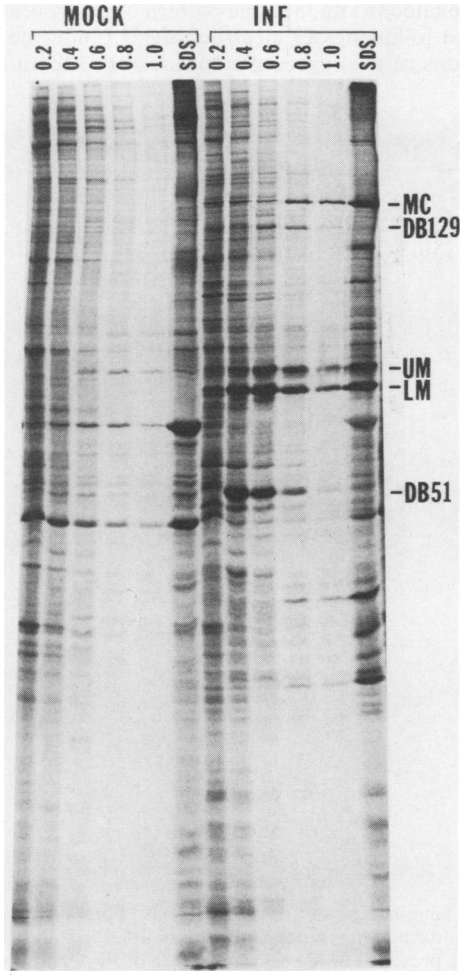

FIG. 2. Serial salt extraction of nuclei from mock-infected (MOCK) and Colburn-infected (INF) cells. Dishes (60-mm-diameter) of cells were mock infected or infected with strain Colburn CMV, labeled with [^{35}S]methionine from 36 to 60 h postinfection, and used to prepare nuclei in situ as described in the text. The nuclei were then serially extracted with 0.2, 0.4, 0.6, 0.8, and 1.0 M NaCl as indicated in the text, and the resulting extracts were analyzed by SDS-PAGE. Shown is a fluorogram of the resulting gel. Positions of major virus-specific proteins (as defined in the legend to Fig. 1) are indicated for reference.

TABLE 1. Effect of PFA and hydroxyurea on virus production and DB129 synthesis

Treatment	DB129 Synthesis		Infectious virus produced	
	Relative amount[a]	Fold increase[b]	Titer (IU/ml)[c]	Reduction factor
PFA (concn [μg/ml])				
0.6	1.0	1×10^7		
100	5.2	9.1	5×10^5	20
200	14.0	24.6	1×10^4	1,000
300	16.7	29.3	1×10^4	1,000
400	10.0	17.6	5×10^2	20,000
500	7.8	13.6	5×10^2	20,000
Hydroxyurea (concn [mM])				
None	2.8	1.0	1×10^8	
0.5	8.0	2.9	1×10^7	10
1.0	19.8	7.1	1×10^7	10
10	20.2	7.2	1×10^4	10,000
25	14.2	5.1	5×10^3	20,000
50	2.5	1.0	5×10^2	200,000

[a] Peak area measurements were made from densitometric scans of fluorograms containing [^{35}S]methionine-radiolabeled Colburn-infected cell nuclear fractions

[b] Calculated by dividing the relative amount of DB129 present in the drug-treated cultures by the relative amount for the nontreated culture.

[c] Infectious units (IU) were determined by cytopathic effect endpoint titration (9).

To determine the relative stability of the association of DB129 with the nuclear fraction, a serial salt extraction of infected cell nuclei was done in situ as described above (Fig. 2). Under these conditions DB129 was seen to behave differently than the closely migrating proteins also present in mock-infected cells. DB129 was released from the nuclei over a broad range of salt concentrations: approximately 30% was released at 0.2 M NaCl, 15% at 0.4 M NaCl, and 50% at 0.6 to 0.8 M NaCl, as estimated by densitometry. Most proteins either eluted at a lower salt concentration or were less salt extractable (e.g., major capsid protein) under these conditions. Consistent with previous results (12), DB51, which also binds to ssDNA in vitro (13), was eluted maximally at 0.4 M NaCl. Unlike DB51, which precipitates at low salt concentrations (13), DB129 remained in solution following removal of salt from the extract by dialysis.

To determine whether DB129, like DB51, is phosphorylated (12, 13), infected and mock-infected cultures were incubated in medium containing [^{32}P]P_i from 24 to 72 h postinfection, harvested, and fractionated as described above; and the resulting fractions were analyzed by SDS-PAGE to identify phosphoproteins. The matrix proteins and DB51, previously shown to be phosphorylated (12, 13, 49), were highly labeled, but no band of ^{32}P radioactivity comigrated with [^{35}S]methionine-labeled DB129 (data not shown). Thus, DB129 is apparently not phosphorylated.

Synthesis of DB129 is insensitive to PFA. Studies of several of the herpesviruses have demonstrated a requirement for viral DNA replication to obtain normal expression of late gene products (4, 13, 19, 36, 43). Therefore, we next examined the sensitivity of DB129 synthesis to inhibitors of DNA replication, relative to that of other Colburn proteins. PFA was used for these studies because it is an effective inhibitor

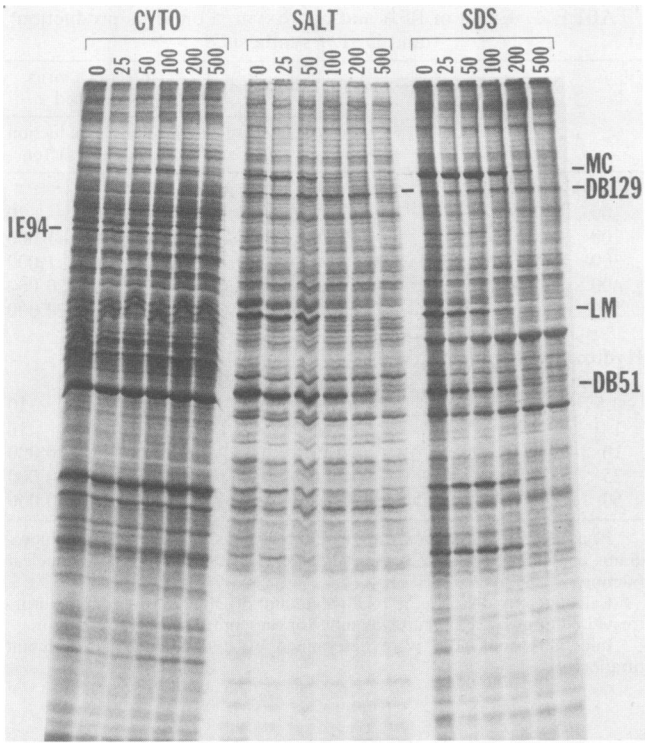

FIG. 3. PFA sensitivity of CMV strain Colburn. PFA was added at the time of infection to final concentrations of 25, 50, 100, 200, and 500 μg/ml. A control culture was maintained without PFA. Cultures were labeled with [^{35}S]methionine from 24 to 60 h postinfection in the continued presence of the respective concentrations of PFA. The cultures were then fractionated in situ as described in the text. Shown is a fluorogram of the SDS-PAGE analysis. Soluble cytoplasmic (CYTO), nuclear salt extract (SALT), and nuclear SDS fractions (SDS) were grouped together, as indicated. The amount of PFA (in micrograms per milliliter) present in the respective cultures is indicated at the top of each lane. Protein designations are as described in the legend to Fig. 1.

of both CMV DNA replication and the production of late viral antigens (16, 46, 47).

The effects of a range of PFA concentrations from 25 to 500 μg/ml were determined. PFA was added to the culture medium at the time of infection and maintained throughout the course of the experiment. Cells were radiolabeled from 24 to 60 h postinfection and then fractionated in situ. Proteins synthesized during the labeling period were then examined following SDS-PAGE (Fig. 3). The addition of PFA to uninfected cells at these concentrations had no apparent effect on host protein synthesis (data not shown). Concentrations of up to 50 μg of PFA per ml had little effect on the incorporation of [^{35}S]methionine into most of the late viral proteins, such as the capsid proteins (e.g., major capsid protein), which were found predominantly in the nuclear SDS fraction. The lower matrix protein was an exception, showing about 50% greater sensitivity to the effects of PFA. Higher concentrations of PFA progressively reduced late viral protein synthesis until, at 500 μg/ml, incorporation of methionine into late proteins was not detected. In contrast, incorporation into DB129 was not reduced but rather increased at PFA concentrations up to 200 μg/ml. Densitometric measurements showed that at 200 μg/ml, for these labeling conditions, incorporation of [^{35}S]methionine into the major capsid protein was reduced by nearly 10-fold, while incorporation into DB129 was increased approximately 4-fold above that into the nontreated culture. Even at 500 μg/ml DB129 synthesis was detectable. Other inhibitors such as aphidicolin (data not shown) and hydroxyurea (compared with PFA in Table 1) gave similar results. Maximal amounts of DB129 were observed at levels of hydroxyurea and PFA which reduced the amount of infectious virus produced by a factor of 10^3 to 10^4.

Time course of DB129 synthesis. The elevated levels of DB129 resulting from PFA treatment were evidence that this protein is a member of the early temporal group of virus proteins. To further investigate this possibility, time course experiments were done to study the rates of viral protein synthesis at various times after infection, both in the presence and absence of PFA. Because 200 μg/ml yielded maximum DB129 synthesis in the experiment described above, this concentration was used for the drug-treated cultures. Infected and mock-infected cells, maintained either in the presence or absence of PFA, were pulse-labeled with [^{35}S]methionine for 1 h at the indicated times after infection and fractionated in situ, and the pattern of incorporation was determined following gel electrophoresis. Only the nuclear salt fractions of the respective cultures are shown (Fig. 4).

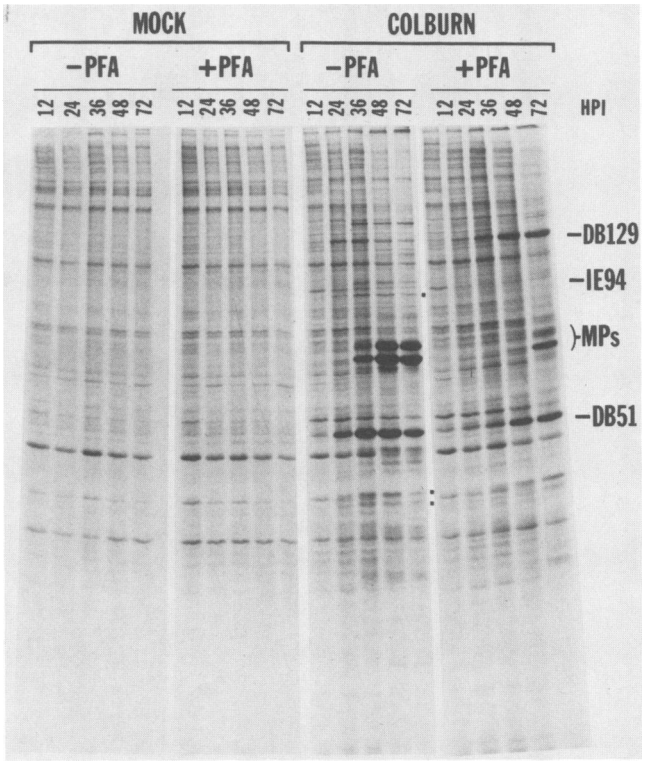

FIG. 4. Time course of DB129 synthesis. Four sets (five dishes each) of cultures were mock infected or infected and incubated either in the presence or absence of 200 μg of PFA per ml added at the time of infection. At 12, 24, 36, 48, and 72 h postinfection (HPI), one dish from each set was pulse-labeled for 1 h, as described in the text, and then fractionated in situ. Cell fractions were analyzed by SDS-PAGE. Presented here is a fluorogram of the gel containing the nuclear salt extract fractions from each time point. Culture conditions and radiolabeling time points are given at the top of the figure. Positions of several proteins, including the upper and lower matrix proteins (MPs), are indicated for reference. Dots indicate the positions of the 86,000-, 42,000-, and 40,000-molecular-weight proteins referred to in the text.

However, all fractions were analyzed to establish that differences in the patterns of incorporation did not result from an abnormal distribution of proteins within the cell caused by PFA treatment. No such changes were evident, nor were any differences observed in the mock-infected controls, with or without PFA.

At least three temporal classes of viral proteins were evident in the infected cells. The predominant immediate-early protein (IE94) was the only viral protein detected at 12 h postinfection, either in the presence or absence of PFA (23, 24). By 24 h postinfection the synthesis of a second group of Colburn proteins, including DB129 and DB51, was evident in both the untreated and the PFA-treated cultures. Other proteins which appeared to be members of this group had estimated molecular weights of 86,000, 42,000, and 40,000 and are indicated by dots in Fig. 4. The synthesis of late proteins, as exemplified by the 66,000-molecular-weight lower matrix protein, was readily detected by 36 h postinfection in nontreated cultures. Comparable amounts of this protein were not present in PFA-treated cultures, however, until 60 to 72 h postinfection. The relative rate of methionine incorporation into DB129 at each time point in this experiment is compared with those of DB51 and lower matrix protein in Fig. 5. Maximal rates of DB129 synthesis in nontreated cultures occurred at 24 to 36 h postinfection and declined thereafter (Fig. 5A). By comparison, maximal rates of DB51 and lower matrix protein synthesis did not occur until 36 to 48 and 48 to 72 h postinfection, respectively. In PFA-treated cultures (Fig. 5B) there were two major differences. First, the onset of late protein synthesis (e.g., lower matrix protein) was delayed by approximately 1 day. Second, the rate of DB129 synthesis was not reduced after 36 h but continued to increase to 48 h postinfection and remained at this level even at 72 h postinfection. The failure to reduce synthesis of DB129 at late times in the presence of 200 μg of PFA per ml, coupled with its elevated rate of synthesis, led to the high levels of this protein observed under these conditions (Table 1). A direct comparison of the rates of

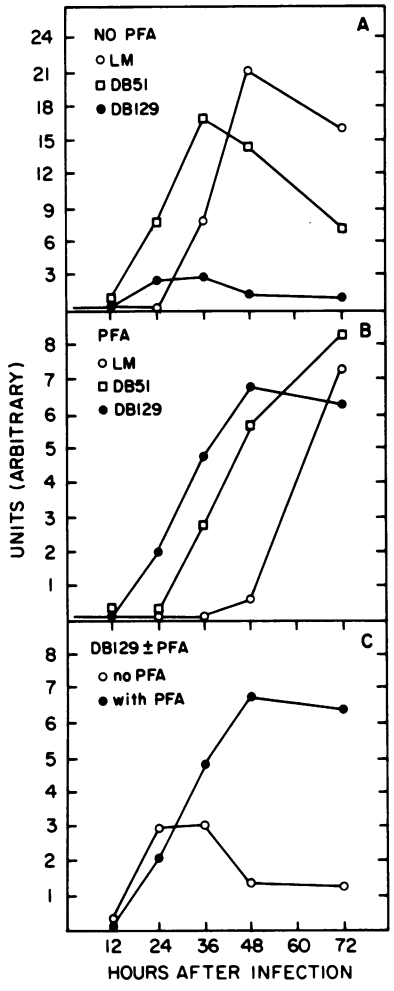

FIG. 5. Comparison of relative rates of DB129, DB51, and lower matrix protein synthesis. Fluorographic exposures of the salt extract fractions from the time course experiment shown in Fig. 4 were scanned, and relative absorbances of representative proteins were calculated as described in the text. (A) Time course comparison of the relative rates of synthesis of the 66,000-molecular-weight lower matrix protein (LM), DB51, and DB129 in the absence of PFA. (B) Comparison of the relative rates of synthesis of the same three proteins in the presence of 200 μg of PFA per ml. (C) Direct comparison of relative rates of DB129 synthesis in the absence or presence of 200 μg of PFA per ml.

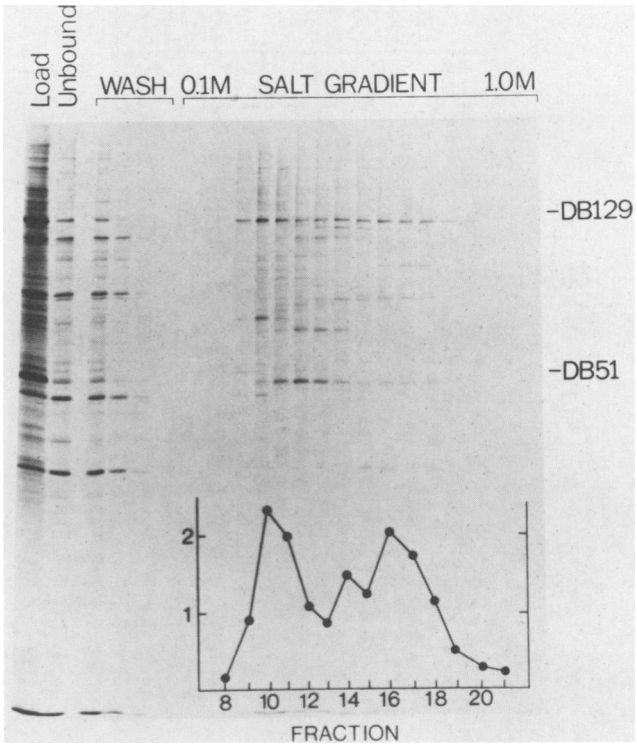

FIG. 6. ssDNA-agarose affinity chromatography of DB129-containing extracts. Cells were infected with Colburn CMV in the presence of 200 μg of PFA per ml and labeled with [^{35}S]methionine from 48 to 72 h postinfection, and extracts were prepared for column chromatography as described in the text. Under these conditions DB129 and DB51 were the major labeled infected cell-specific proteins present in the salt extract fraction. The sample was then loaded onto a 1-ml bed volume ssDNA-agarose column, and the column was washed with three bed volumes of DNA-binding buffer and eluted with a 10-ml linear gradient of NaCl (0.1 to 1.0 M in DNA-binding buffer). Eluant fractions were examined by SDS-PAGE, and relative amounts of DB129 in each fraction were estimated by densitometry. The inset shows the relative absorbance of the DB129 band versus fraction number plotted coincident with the appropriate lane. Shown here is a fluorogram of the resulting gel.

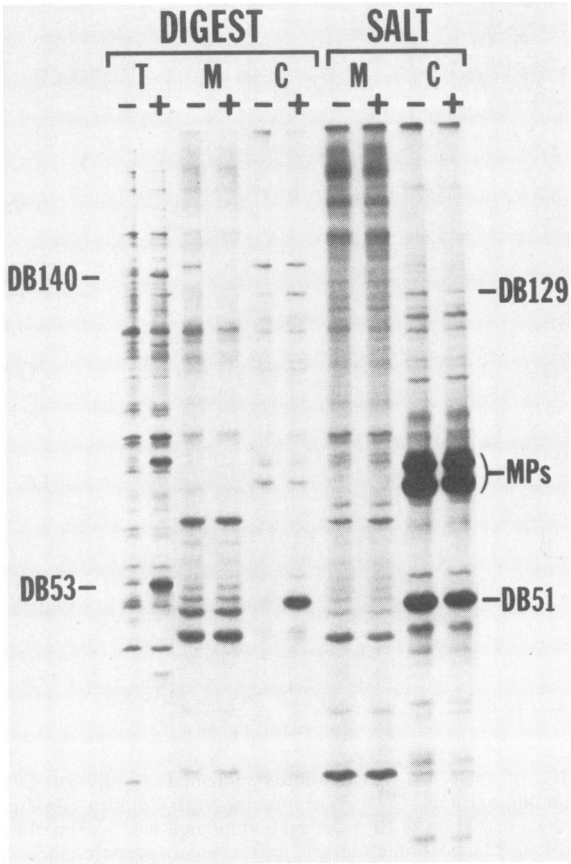

FIG. 7. Treatment of nuclei from Colburn-infected and Towne-infected cells with DNase I. Mock-infected and infected cells were pulse-labeled for 2 h with [^{35}S]methionine at 72 h postinfection. Nuclei were prepared in situ and incubated with DNase I as described in the text. Following digestion, the supernatant was collected. The residual nuclear material was washed with buffer B and then extracted with 1.0 M NaCl in buffer B. Resulting nuclease and salt extracts were then analyzed by SDS-PAGE. Shown here is a fluorogram of the resulting gel. Major capsid protein is present in the mock (−) digest lane (just above the DB140 position) as a result of spillover from an adjacent lane containing Colburn SDS fraction. Positions of several proteins indicated in preceeding figures are shown for reference. Abbreviations: M, mock-infected cells; C, Colburn-infected cells; T, Towne-infected cells; MPs, upper and lower matrix proteins. Buffer control and DNase I incubations are indicated by − and +, respectively.

DB129 synthesis in the presence or absence of PFA is shown in Fig. 5C.

DB129 binds DNA in vitro. Because CMV early proteins may have a role in DNA replication or transcriptional regulation and DB129 was found to be localized in nuclei of infected cells, we next examined the possibility that DB129 associates with DNA. Our first approach was to determine whether DB129 interacts with DNA in vitro. Salt extracts were prepared by in situ fractionation of cells infected with the Colburn strain in the presence of 200 μg of PFA per ml. Following dialysis they were subjected to affinity chromatography with ssDNA-agarose. The column was eluted with a linear gradient of NaCl, the fractions were analyzed by SDS-PAGE, and the relative amounts of DB129 in each fraction were estimated by densitometry (Fig. 6). DB129 bound to ssDNA and eluted primarily in two major peaks, one at approximately 0.2 M NaCl and the second centered at about 0.6 M NaCl. In addition, a minor peak was consistently observed to elute at about 0.5 M NaCl. Similar results were obtained for DB129 in extracts prepared from nontreated cells. No protein of similar size and elution properties was observed in extracts prepared from mock-infected cells. Several other proteins were found to be retained on the ssDNA column and released at characteristic salt concentrations, including DB51 which has previously been shown to bind DNA (12, 13).

DB129 is released from nuclei of infected cells by treatment with DNase I. Because the results described above showed that DB129 can bind DNA in vitro, we next examined the possibility that it interacts with DNA in vivo. Fenwick et al. (8) have demonstrated that DNase I treatment releases ICP8, the major DNA-binding protein of HSV type I, from nuclei of infected cells. If DB129, likewise, is associated with chromatin, nuclease treatment might similarly promote its release. To test this possibility, in situ-prepared infected cell nuclei were incubated with DNase I, and the digest fraction, as well as a 1.0 M salt extract of the material remaining on the dish, was analyzed by electrophoresis (Fig. 7). Greater than 50% of DB129 was released by nuclease treatment under these conditions, as estimated by densitometry. No protein of similar size was released by DNase I treatment of

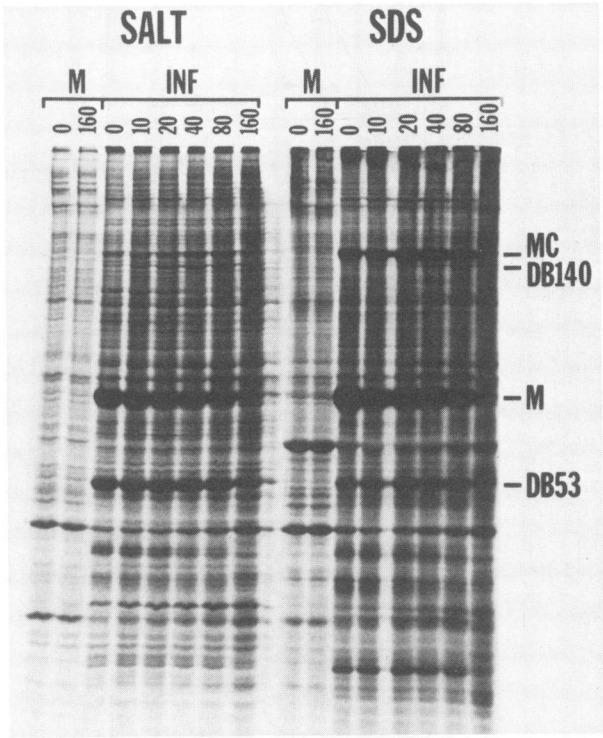

FIG. 8. Effect of PFA on HCMV protein synthesis. PFA was added at the time of infection to final concentrations of 0, 10, 20, 40, 80, and 160 μg/ml. Mock-infected cultures were maintained in 0 or 160 μg of PFA per ml. Cultures were pulse-labeled for 4 h at 5 days postinfection in the presence of the respective concentration of PFA, fractionated in situ, and then analyzed by SDS-PAGE. Shown here is a fluorogram of the gel containing the nuclear salt extracts (SALT) and SDS fractions (SDS). PFA concentrations (in micrograms per milliliter) are indicated at the top of the figure, and positions of several proteins are indicated for reference. HCMV-infected cells contain only one abundant matrix protein (M) (11, 49, 50). Abbreviations: M, mock-infected cells; INF, infected cells; MC, major capsid protein.

mock-infected cells. In addition, a significant fraction of DB51 was released, as were small amounts of other infected cell-specific proteins, including polypeptides with approximate molecular weights of 94,000, 42,000, 40,000, and 35,000. Colburn-specified proteins not released by DNase I treatment under these conditions included the major capsid protein and the matrix proteins, late proteins which are present in the virion. In a parallel experiment, in situ-prepared HCMV- (strain Towne) infected cell nuclei were treated with DNase I. A 140,000-molecular-weight protein was released, which we designated DB140. A small fraction of DB53 was also specifically released by DNase I treatment. DB140 corresponded to the 140,000-molecular-weight protein previously noted as a possible counterpart to DB129 (11).

HCMV-infected cells contain a counterpart to DB129. DNase I release of DB140 from HCMV-infected cell nuclei provided support for the possibility that it is the HCMV counterpart to DB129. To further test this possibility, we examined the effect of PFA on DB140 synthesis. In an initial experiment with 200 μg of PFA per ml, DB140 was synthetized, but it was not enhanced relative to untreated infected cells (data not shown). However, we observed that HCMV exhibits greater sensitivity to PFA than does the Colburn strain; therefore, we tested a range of PFA concentrations from 10 to 160 μg/ml (Fig. 8). Increasing concentrations of PFA led to a progressive decrease in incorporation into late proteins, as exemplified by the matrix protein, with little expression observed at 160 μg of PFA per ml. However, even the lowest concentration of PFA tested (10 μg/ml), was sufficient to promote an approximately fourfold stimulation of incorporation into DB140. Maximal stimulation of DB140 synthesis was observed at 80 μg/ml. At 160 μg of PFA per ml, incorporation of methionine into DB140 was comparable to that seen in nontreated cells.

To determine whether DB140 exhibited other properties similar to those of Colburn DB129, we subjected it to many of the characterization experiments described above. DB140 was found to be localized in the nuclear fraction of infected cells and to exhibit similar salt extraction properties (Fig. 8; data not shown). Figure 9 shows the results obtained when salt extracts were prepared from nuclei of HCMV-infected cells in situ, applied to an ssDNA affinity column, and eluted with a discontinuous gradient of NaCl. For comparison, a similarly prepared and chromatographed extract of Colburn-infected cells is also presented. Greater than 50% of DB140 bound to ssDNA and eluted over the same range of salt concentrations that displaced Colburn DB129 (0.25 to 1.0 M). Figure 9 also documents the fact that DB53, like its Colburn counterpart DB51, is released from ssDNA at a salt concentration of about 0.3 to 0.5 M.

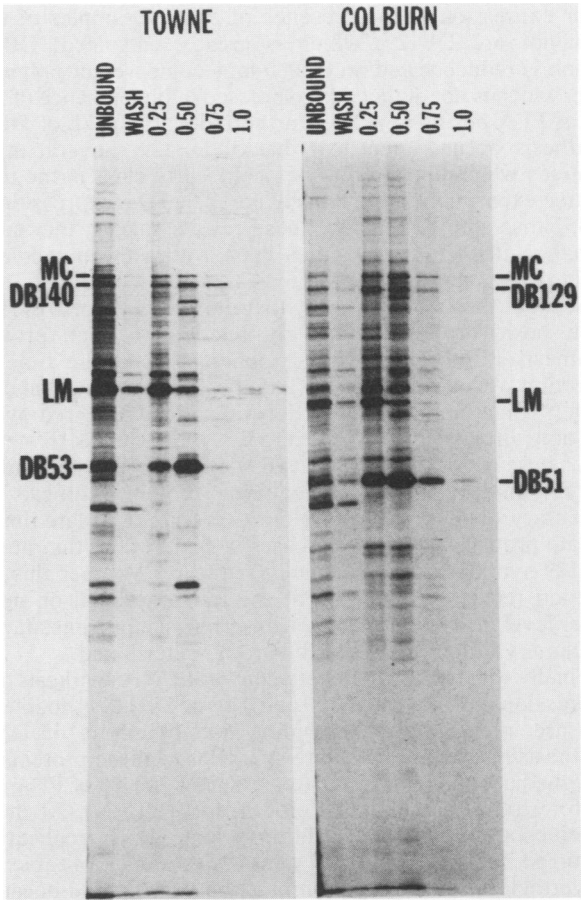

FIG. 9. ssDNA affinity chromatography of the HCMV 140,000-molecular-weight protein. The 1.0 M salt extract fraction prepared from an HCMV-infected culture treated with 80 μg of PFA per ml (the same culture shown in Fig. 8) was diluted to a final concentration of 0.1 M NaCl and applied to a ssDNA-agarose column as described in the text. The column was washed with three bed volumes of DNA-binding buffer and then eluted with 3-ml steps of 0.25, 0.5, 0.75, and 1.0 M NaCl in DNA-binding buffer. For comparison, an extract of nuclei from [^{35}S]methionine-labeled, Colburn-infected cells was prepared and chromatographed identically. Eluant fractions were examined by SDS-PAGE. Shown here is a fluorogram of the resulting gel. Abbreviations: MC, major capsid protein; LM, lower matrix protein.

DISCUSSION

The studies described here were done to characterize a phosphonoformate-enhanceable protein with an approximate molecular weight of 129,000 (DB129) that is present in the nuclear fraction of strain Colburn CMV-infected cells and to compare its synthesis with that of other infected cell-specific proteins. The results presented here demonstrate that this protein is an early, or beta-class, protein, associates with DNA, has an HCMV counterpart, is neither phosphorylated nor present in virions, and shares many properties with the major DNA-binding protein of HSV.

Expression of DB129. Two lines of evidence indicated that DB129 is an early protein. First, time course pulse-labeling experiments showed that synthesis of DB129 reached near maximal rates prior to the onset of late protein synthesis but after the peak of immediate-early protein (IE94) synthesis. Second, inhibition of viral DNA synthesis did not block synthesis of DB129. Previous studies have shown a correlation between expression of a subset of herpesvirus proteins in the absence of DNA replication and their early temporal expression (4, 36, 46). Concentrations of PFA which eliminated late viral protein synthesis (500 μg/ml), even at very late times after infection, did not prevent synthesis of DB129. Similar results were obtained with other inhibitors of DNA synthesis, such as hydroxyurea and aphidicolin. Thus, the failure to decrease DB129 synthesis at late times in PFA-treated cells can probably be ascribed, directly or indirectly, to the block in DNA synthesis rather than some nonspecific effect of PFA.

Several results obtained from these experiments merit further comment. First, proteins which appear to be early by virtue of their time course of synthesis vary in the extent of

their expression in the presence of PFA. Examples of this behavior are DB129, which is greatly enhanced; DB51, which is reduced; and an 86,000-molecular-weight polypeptide, which is not detectably expressed in the presence of 200 μg of PFA per ml (Fig. 4). Furthermore, the peak of DB51 synthesis occurs later than that of DB129. Several other proteins which appeared to be of the early class in the time course experiment more closely paralleled the expression of DB51 than that of DB129. These results suggest that additional regulatory complexities exist within the immediate-early, early, and late-temporal classes of CMV.

Second, these studies imply that the synthesis of DB129 is both positively and negatively regulated. When protein synthesis is inhibited with cycloheximide at the time of infection with strain Colburn and the cells are pulse-labeled with [^{35}S]methionine immediately after the drug is removed, the only viral protein observed to be synthesized is IE94 (10, 24). These results indicate that IE94 synthesis, or some other as yet unidentified event requiring protein synthesis, is necessary for DB129 expression. Conversely, at late times, as late protein synthesis increases to high levels, the rate of DB129 synthesis is dramatically reduced. Whether this reduction reflects modulation at the transcriptional or some other level or competition for transcriptional or translational machinery at late times remains to be determined.

Finally, the relationship between viral DNA synthesis and expression of Colburn DB129 and Towne DB140 is not clear. We are intrigued by the finding that inhibitors of DNA synthesis maximally stimulate synthesis of these proteins at intermediate concentrations (e.g., 80 and 200 μg of PFA per ml for strains Towne and Colburn, respectively). At these intermediate PFA concentrations, some DNA replication occurred because infectious virus was produced (Table 1). Nevertheless, this replication must be very limited because no viral DNA synthesis was detected at 100 μg of PFA per ml or above in a preliminary experiment in which HCMV-infected cells were treated with a series of PFA concentrations and labeled with [^{32}P]P_i from 24 to 72 h postinfection; and their DNAs were extracted, cleaved with *Hin*dIII restriction endonuclease, and analyzed by agarose gel electrophoresis and autoradiography (data not shown). Similar results have been reported with phosphonoacetate (19, 22, 29). We speculate that the overproduction of DB129 (or DB140) at intermediate PFA concentrations reflects a balance between increased gene copy number and failure to reduce DB129 synthesis at late times. If this is the case, then higher concentrations of PFA which more completely inhibit viral DNA synthesis would yield less overproduction of these CMV proteins, which is in agreement with our observations shown in Fig. 3 and 8. Alternatively, their overproduction may be due solely to the accumulation of mRNA resulting from the failure to regulate DB129 transcription. The reduced magnitude of this effect at higher concentrations of PFA may simply reflect deleterious effects on transcriptional or other events that are required for early protein expression.

Association of DB129 with DNA. Two lines of evidence suggest that DB129 associates with DNA. First, DB129 that was present in extracts of nuclei from Colburn-infected cells bound to ssDNA in vitro and was released over a broad range of salt concentrations as two major peaks, one at about 0.15 M NaCl and a relatively salt-stable peak at about 0.6 M NaCl. Although this study does not resolve the question of whether DB129 interacts with ssDNA directly or indirectly, the former seems likely because (i) few other proteins remained associated with ssDNA at NaCl concentrations greater than 0.5 M, and none of these exactly coeluted with DB129; (ii) the salt stability of the interaction was comparable to that of better characterized ssDNA-binding proteins such as the major DNA-binding protein of HSV (37, 38); and (iii) partially purified DB129 retained the capacity for salt-stable interaction with ssDNA (data not shown). We suspect that associations with other proteins or nucleic acids present in the extract account for the lower affinity peaks as well as the generally broad elution pattern. Consistent with this possibility is the fact that the minor peak of DB129 at 0.5 M NaCl coeluted with two other proteins with approximate molecular weights of 120,000 and 80,000 (Fig. 6). Alternatively, or in addition, DB129 may be capable of a range of interactions with ssDNA, as would be expected, for example, if it bound cooperatively.

A second line of evidence which suggests that DB129 also associates with DNA in vivo is its release from nuclei by DNase I treatment. Greater than 50% of DB129 was released. Only a few other polypeptides were specifically released by this treatment, including DB51 and minor proteins with molecular weights of approximately 94,000, 42,000, 40,000,and 35,000. The 42,000- and 40,000-molecular-weight species comigrated with early proteins of the same size observed in the time course experiment (Fig. 4). The magnitude and selectivity of DB129 release strongly suggests that there is an association with DNA in vivo. Furthermore, in serial salt extraction experiments, DB129 was released from nuclei of infected cells over a broad range of salt concentrations, in a pattern much like that observed for its chromatographic elution from ssDNA (compare Fig. 2 and 4). This suggests that the same types of interactions occur in vivo and in vitro.

Finally, the data presented here do not address whether DB129 preferentially associates with viral or host DNA. We note, however, that when infected cells were maintained in the presence of PFA, a greater percentage of DB129 was removed from nuclei by low salt concentrations than from nuclei of untreated cells (unpublished data). This may indicate that DB129 forms the relatively salt-stable complex primarily with viral DNA; i.e., when viral DNA synthesis was selectively inhibited, formation of the salt stable complex was inhibited. Alternatively, this may simply reflect saturation of the salt-stable compartment by the excess DB129 made in the presence of PFA. Further purification of DB129 will allow more detailed studies of the nature and sequence specificity, if any, of its interaction with DNA and make possible studies of its capacity to interact specifically with other proteins as well.

HCMV expresses an apparent counterpart protein. Previous studies have described HCMV counterparts to strain Colburn CMV proteins (11), which in some cases were found to be immunologically cross-reactive (12, 48). The results presented here demonstrate that a protein with properties similar to those of DB129 is expressed in HCMV-infected cells. Although these results do not prove that Colburn DB129 and HCMV DB140 are structurally or functionally related, we believe that this is the case because they share the following properties: relatively high molecular weights, nuclear localization, salt extractability from infected cell nuclei, solubility in 0.05 M NaCl, ssDNA binding and elution, early time course of synthesis, release from infected cell nuclei by DNase I, and overproduction in the presence of PFA. Furthermore, in both Colburn- and Towne-infected cells, only one protein accumulated to abnormally high levels in the presence of PFA, DB129, and DB140, respectively; and these were immunologically cross-reactive

(D. G. Anders and W. Gibson, manuscript in preparation). Similar proteins are expressed by all simian CMV and HCMV strains examined.

In conclusion, we note that the biological and biochemical properties reported here for Colburn DB129 and Towne DB140 are very similar to those described for the major DNA-binding proteins of HSV types 1 and 2 (ICP8 and ICSP11,12, respectively) (1, 4, 26, 34, 38). These proteins bind more strongly to ssDNA than to double-stranded DNA in vitro (1, 38, 40), are associated with chromatin in vivo (27, 31, 32, 33, 39), are required for viral DNA replication (4, 29), interact specifically with other proteins (45), and may be involved in the regulation of viral gene expression (15). All of the properties listed above that are common between DB129 and DB140 are also shared by the HSV major DNA-binding protein. Perhaps most strikingly, these CMV proteins behave very similarly to HSV type 2 ICSP11,12 throughout the purification procedure described by Powell et al. (37) (unpublished data). We suggest that Colburn DB129 and Towne DB140 are CMV counterparts to this HSV protein. Further studies of these proteins and their interaction with other molecules should lead to a better understanding of their function, aid in the identification of other CMV early viral proteins, and provide a model to study the regulation of CMV early gene expression.

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