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DNA-Binding Proteins Induced by Herpes Simplex Virus Type 2 in HEp-2 Cells

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Affinity chromatography on single-stranded and double-stranded DNA-cellulose indicates that 12 proteins previously identified from herpes simplex virus type 2-infected cells, ranging in molecular weight from 28×10^3 to 186×10^3, bind to DNA-cellulose. The DNA-binding proteins found in infected cells differed in relative binding strengths for denatured DNA-cellulose. The virus specificity of these DNA-binding proteins was further studied by comparison with DNA-binding proteins isolated from mock-infected cells, and by immunoprecipitation of infected-cell DNA-binding proteins with antisera specific for viral antigens. The promise this technique holds for the purification and study of polypeptides involved in virus DNA replication, recombination, or repair is discussed.

Herpes simplex virus (HSV) is a large DNA virus with the genetic capacity to code for perhaps 100 proteins. Indeed, some 50 infected-cell-specific polypeptides have been characterized by high-resolution polyacrylamide gel electrophoresis (10, 18). To prove that these polypeptides are viral gene products, it is necessary to synthesize them in vitro from mRNA that is demonstrated to be of viral origin. In the absence of such data, three lines of evidence suggest that these polypeptides are virus specified (10, 18). First, they appear after infection and are synthesized in increasing amounts at times when general host protein synthesis is declining. Second, almost all of those available in soluble form can be immunoprecipitated with HSV general antiserum, which reacts solely with virus antigens. Third, the spectrum of polypeptides induced in the infected cell is dependent on the type of virus used.

Although these polypeptides have been studied in some detail, the function of most of them is unknown. Some 16 (19) to 24 (24) have been identified as structural components of virions, and one has been identified as HSV-1-induced thymidine kinase (11). By analogy with the T-even bacteriophage whose genome, slightly larger than that of HSV, codes for numerous proteins required for DNA synthesis (15), one might expect that a number of HSV gene products would be involved with HSV-DNA replication. This expectation is supported by genetic evidence from several laboratories, which indicates that a high proportion of the HSV genes so far identified is required, directly or indirectly, for DNA replication (23, 25).

DNA-cellulose chromatography, a technique introduced by Alberts et al. (2), allows the selection of proteins that interact with DNA and that may function in DNA replication, repair, or recombination without prior knowledge of their actual function. Such proteins, including DNA and RNA polymerases, ligases, nucleases, and others, such as T-even bacteriophage gene 32 product, have been isolated in a native state by this procedure for further purification and functional characterization (1, 2). DNA affinity chromatography has been used extensively to study such proteins present in prokaryotic (2) and in normal and transformed eukaryotic cells (22), as well as similar proteins induced by bacteriophage (2, 4, 13) and animal viruses (5, 9, 26).

These considerations led us to examine the DNA-binding properties of proteins in HSV type 2 (HSV-2) -infected cells. We have isolated 17 infected-cell-specific polypeptides that bind reversibly to denatured or native DNA-cellulose from HSV-2-infected HEp-2 cells. During the course of this study, Bayliss et al. (3) reported the isolation of 16 DNA-binding proteins in HSV-1-infected BHK cells. Although similar numbers of DNA-binding proteins were isolated in both studies, there is a considerable difference in the spectrum of polypeptides observed. This report deals with the isolation of DNA-binding proteins of HSV-2-infected cells, the reproducibility of their chromatography on DNA-cellulose, and their virus specificity.

MATERIALS AND METHODS

Cells, media, and virus. HEp-2 cells grown in Eagle medium containing 10% fetal calf serum and 0.075% sodium bicarbonate were used for all experi-

ments and for virus propagation. Vero (green monkey kidney) cells were grown in a similar manner and used for virus assay. The virus used in this study was the 186 strain of HSV-2. Virus stocks were grown in HEp-2 cells infected at a low input multiplicity as described previously by Watson et al. (27) to avoid the appearance of defective virus.

Infection and radiolabeling procedure. Procedures for infection and radiolabeling were described recently (18). Briefly, 8×10^6 HEp-2 cells (grown in 100-mm-diameter plastic petri dishes) were infected with 1.6×10^8 PFU of virus, and the virus was allowed to adsorb for 1 h at 37°C. At the end of the adsorption period, virus or mock inocula were decanted, and the monolayers were washed with fresh warm medium. Medium containing 2.5 to 3.0 μCi of ^{14}C-labeled amino acid mixture per ml (specific activity, 57 mCi/m-atom of carbon), or for one experiment, 10 μCi of ^{3}H-labeled amino acid mixture (specific activity, 0.18 to 54.0 Ci/mmol) was added. Isotopes were obtained from Amersham-Searle, Inc., Arlington Heights, Chicago, Ill. After a further 11-h incubation, the cells were harvested by scraping into the medium and washed three times with ice-cold phosphate-buffered saline before being frozen at −70°C as a cell pellet.

Preparation of cell extracts. A greater proportion of radiolabeled protein is in an insoluble form in HSV-2-infected cells than in mock-infected cultures, as determined by sedimentation at $100,000 \times g$ (K. L. Powell, unpublished observations). There may be many reasons for this observation, but one is likely to be that some viral proteins are bound to DNA, as is the case with T-even bacteriophage DNA-binding proteins (1, 2). An extraction procedure using high-molarity salt was developed to solubilize HSV proteins bound to DNA and to remove DNA that could compete with subsequent binding to cellulose-bound DNA.

Before DNA-cellulose chromatography, the cells were thawed and suspended in buffer containing 20 mM Tris-hydrochloride, pH 7.5, 2 mM β-mercaptoethanol, and 500 μg of bovine serum albumin per ml at a cell concentration of 10^7/ml. The cells were then subjected to ultrasonic disruption for 2 min at 9 kc/s. An equal volume of high-salt buffer was added to give final concentrations of 1.7 M NaCl and 5 mM EDTA, and the mixture was allowed to stand for 40 min at 0°C. DNA and protein precipitates were removed by centrifugation for 20 min at $30,000 \times g$, 4°C. This high-salt extract was dialyzed overnight at 4°C against three changes of column buffer (20 mM Tris-hydrochloride, pH 7.5; 50 mM NaCl; 1 mM EDTA; 2 mM β-mercaptoethanol; 10% glycerol). The resulting light precipitate, containing proteins insoluble in low-molarity salt, was removed by centrifugation for 60 min at $100,000 \times g$, 4°C. Preliminary experiments demonstrated this procedure to be more efficient, both in the retention of proteins and in the removal of DNA, than procedures that have been described previously (1–3) using DNase treatments or polyethylene glycol precipitation. As indicated in the text, for certain experiments KCl was substituted for NaCl in extraction and column buffers. The average percentage of total acid-insoluble radiolabeled proteins that is in the final extract using KCl, as well as the percentage of sample bound to denatured DNA-cellulose, was similar to that obtained with NaCl.

DNA-cellulose chromatography. Denatured and native DNA-cellulose was prepared as described by Alberts and Herrick (1) using 9 mg of salmon sperm DNA (Calbiochem, San Diego, Calif.) per g of purified cellulose. Columns (0.6 by 3 cm) were poured and washed with column buffer before loading with samples. After an extensive wash, DNA-binding proteins were eluted with the same buffer containing increasing concentrations of NaCl: routinely, 20 ml of 0.1, 0.3, 0.5, and 1.0 M. For linear gradient elution, proteins were eluted from the column by means of a linear 70-ml gradient of 0.1 to 1.0 M NaCl in column buffer. One-milliliter fractions were collected, and the acid-insoluble radioactivity of 50-μl samples was determined by spotting on 2.5-cm squares of glass-fiber filter paper (Whatman GF/C, Clifton, N.J.). Dried filters were batch-washed through three 5% trichloroacetic acid and two 95% ethanol washes, dried, and counted in scintillation fluid consisting of toluene containing 16 g of 2,5-diphenyloxazole per liter and 1.6 g of 1,4-bis-[2-(4-methyl-5-phenyl-1,3 oxazolyl)]benzene per liter.

Immunoprecipitation. General antiserum to HSV-2 was prepared as described in detail by Watson et al. (27). This hyperimmune rabbit serum was prepared by inoculation with HSV-2-infected rabbit cells that had been grown only in medium containing rabbit serum and is specific for HSV antigens. Immunoprecipitation of radiolabeled proteins from extracts after high-salt treatment and dialysis (sample) and of proteins eluted from DNA-cellulose was performed as described by Honess and Watson (11). All reagents were sedimented at $75,000 \times g$ for 1 h before use. Serum excess conditions were determined by prior titration of the reagents. Samples were mixed with an excess of antisera or preimmune serum from the same rabbit and incubated at 4°C for 48 h. The precipitate was sedimented at 2,000 rpm for 15 min, and washed six times with ice-cold phosphate-buffered saline. The washed pellet was resuspended in 1% SDS, 0.5 M urea, and 1% mercaptoethanol. Tris-hydrochloride (pH 6.7) was added to 0.05 M, and glycerol to 8%; bromophenol blue and phenol red were tracking dyes. Samples were disrupted in a boiling water bath for 2 min for analysis by polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis and quantification. The procedures for high-resolution polyacrylamide slab gel electrophoresis have been detailed previously (18). The results were analyzed by autoradiography of a dried gel slab using Kodak NS2 T film. Polypeptide quantification was done after densitometer scanning of the X-ray films as detailed previously (18).

DNase assay. HSV-2-induced alkaline DNase was assayed by a modification of the procedure of Morrison and Keir (16) that measured the release of acid-soluble DNA fragments from native DNA during incubation at 37°C. The standard assay (0.2 ml) contained 50 mM Tris-hydrochloride, pH 9.0; 2 mM Mg^{2+}; 10 mM 2-mercaptoethanol; 50 μl of enzyme extract; and 10 μg of [^{3}H]thymidine-labeled native HEp-2 cell DNA (10,000 cpm/μg). Reaction mixtures

were preincubated at 37°C for 10 min before addition of enzyme, and the reaction was stopped after an additional 10 min with 1.5 ml of cold 5% trichloroacetic acid. Carrier bovine serum albumin (250 μg/tube) was added, and tubes were kept at 0°C for 30 min. The trichloroacetic acid-precipitable material was sedimented at 2,000 × g for 10 min. The radioactivity solubilized was determined from the supernatant and pellet for each sample. A 0.2-ml sample of the supernatant was used for determination of acid-soluble radioactivity in scintillation fluid containing 10% Bio-Solv (Beckman Instruments, Fullerton, Calif.). The pellet was resuspended in 1 ml of NCS (Amersham/Searle), and radioactivity was determined in scintillation fluid.

RESULTS

Extraction of cells for DNA-cellulose chromatography. To isolate infected cell-specific DNA-binding proteins, HSV-2-infected HEp-2 cells were labeled with ^{14}C-amino acids from 1 to 12 h after infection. This labeling period was previously shown to allow the preferential incorporation of radioactivity into those proteins (designated infected cell-specific polypeptides [ICSP]) that appear and are synthesized in increasing amounts after infection (18).

Preliminary experiments indicated that the widely used extraction procedure for DNA-cellulose chromatography of Alberts and Herrick (1) resulted in very low yields of ^{14}C-amino acid-labeled proteins from HSV-infected cells, with less than 5% of the total acid-insoluble radioactivity remaining in the soluble extract. The extraction procedure we devised, as detailed in the Materials and Methods section, resulted in a substantial increase in the proportion of radiolabeled proteins which was extracted (average of 24%) from infected cells (Table 1). Since any DNA remaining in the extract may compete with cellulose-bound DNA for DNA-binding proteins, the removal of DNA is important in the extraction. Our high-salt extraction procedure was slightly more efficient than that of Alberts and Herrick (1) in removal of DNA from the soluble phase, as determined by extracting [^{3}H]thymidine-labeled (1 to 12 h post-infection) cells by both procedures. Both extraction procedures resulted in less than 1% of the DNA remaining in the soluble phase (0.7% for high salt, 0.8% with high-salt/polyethylene glycol precipitation).

DNA-cellulose chromatography. When proteins extracted by high salt from HSV-2-infected cells were applied to native or denatured

TABLE 1. *Recovery of radioactivity in DNA-cellulose chromatography*

Prepn	Total cpm ×10^{-5}	% Total cpm[a] recovered	% cpm[b] recovered	Avg %[c] total cpm recovered	Avg %[b,d] cpm recovered
Sample					
Whole cells	2084.0	100		100	
High-salt extract	1095.0	46.6		44.3	
Dialysate	338.0	16.2		24.4	
Denatured DNA column					
Column load	135.1	16.21	100		100
0.05 M NaCl	90.0	10.82	80.98		81.28
0.1 M NaCl peak	1.8	0.21	1.65		2.64
0.3 M NaCl peak	8.5	1.02	7.59		9.51
0.5 M NaCl peak	2.1	0.25	1.86		2.41
1.0 M NaCl peak	1.7	0.21	1.52		1.33
Total bound	21.0	2.52	19.02		18.70
Native DNA column					
Column load	135.1	16.21	100		100
0.05 M NaCl	99.6	11.19	88.12		89.69
0.1 M NaCl peak	1.6	0.20	1.44		1.54
0.3 M NaCl peak	6.9	0.82	6.07		5.37
0.5 M NaCl peak	1.3	0.15	1.12		0.89
1.0 M NaCl peak	0.2	0.02	0.14		0.09
Total bound	13.4	1.61	11.88		10.32

[a] Of the dialysate, 2/5 was loaded onto each column in this experiment. The percentage of total recovered has been corrected by this value for column recoveries.

[b] Overall recovery of radioactivity loaded on the column in this experiment was 82.5% for the denatured and 84.0% for the native column. In other experiments, this varied from 75.2 to 104.0%.

[c] Average values for seven sample preparations.

[d] Average percentage of loaded sample recovered in various column eluates from six separate experiments for denatured DNA columns and for two separate experiments for native DNA columns.

DNA-cellulose columns at low-salt concentration (0.05 M NaCl), 10 to 20% of the input radioactivity was retained (Table 1). This radiolabel remained bound to the DNA-cellulose even if 40 bed volumes of low-salt buffer were passed through the column. As can be seen from Fig. 1, which shows the results of typical experiments, increasing the molarity of NaCl in the elution buffer resulted in efficient elution of the remaining radioactivity from the column. Most (60%) of the bound radioactivity eluted by this stepwise elution procedure was removed by the 0.3 M NaCl eluate from both native and denatured DNA-cellulose columns. Less than 1% of the radiolabel applied to the column remained bound after 1.0 M elution and could not be removed by elution with 2.0 M NaCl. A major difference between native and denatured DNA was the elution of bound label with 1.0 M NaCl eluate from denatured, but not from native, DNA. In control experiments, essentially no radioactivity attached to cellulose columns prepared without DNA (Fig. 1D), demonstrating that the proteins retained by the DNA-cellulose columns do so by virtue of interaction with DNA, rather than simply filtering out in passage through the column. The distribution of ^{14}C-labeled infected-cell proteins in DNA-cellulose chromatography is shown in Table 1. This distribution was relatively consistent in different experiments (Table 1, last column).

To determine whether any of the peaks eluted at various molarities of NaCl were enriched for HSV-infected-cell proteins, the following experiment was done. Extracts were prepared from mock-infected cultures labeled with ^{3}H-amino acids and from HSV-2-infected cultures labeled with ^{14}C-amino acids from 1 to 12 h after infection. A mixture of these extracts was then passed over a denatured DNA-cellulose column and the results are shown in Fig. 1C. A major difference in the elution profile of mock-infected proteins was the proportionately higher amount of radioactivity which eluted with 0.1 M NaCl. Similar results have been obtained with ^{14}C-amino acid-labeled mock-infected cell extracts on both denatured and native DNA-cellulose. From two additional experiments, an average of 90% of the bound radioactivity eluted with buffer containing 0.1 M (49%)

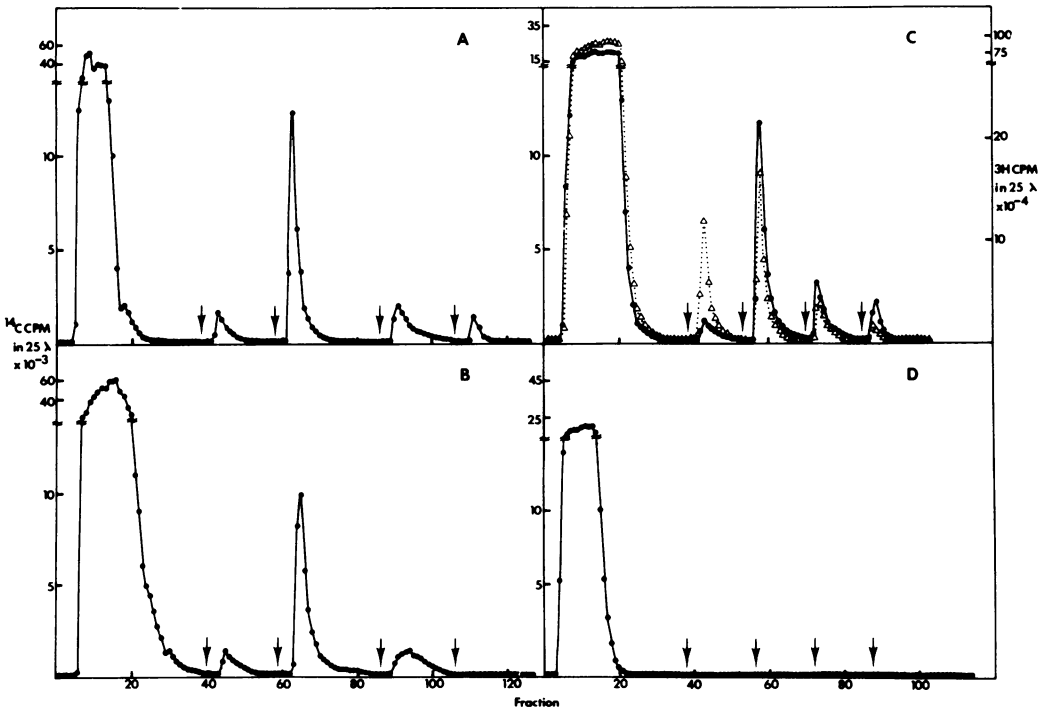

FIG. 1. *DNA-cellulose chromatography of proteins extracted from HSV-2-infected and mock-infected HEp-2 cells. The arrows indicate, from left to right, the position where 0.1, 0.3, 0.5, and 1.0 M NaCl-containing buffer was loaded. (A) HSV-2-infected cell extracts on denatured DNA-cellulose. (B) Infected-cell extracts on native DNA-cellulose. (C) A mixture of ^{3}H-amino acid-labeled mock-infected cell extract plus ^{14}C-amino acid-labeled infected-cell extracts chromatographed on denatured DNA-cellulose. (D) Infected-cell extracts on cellulose containing no DNA.*

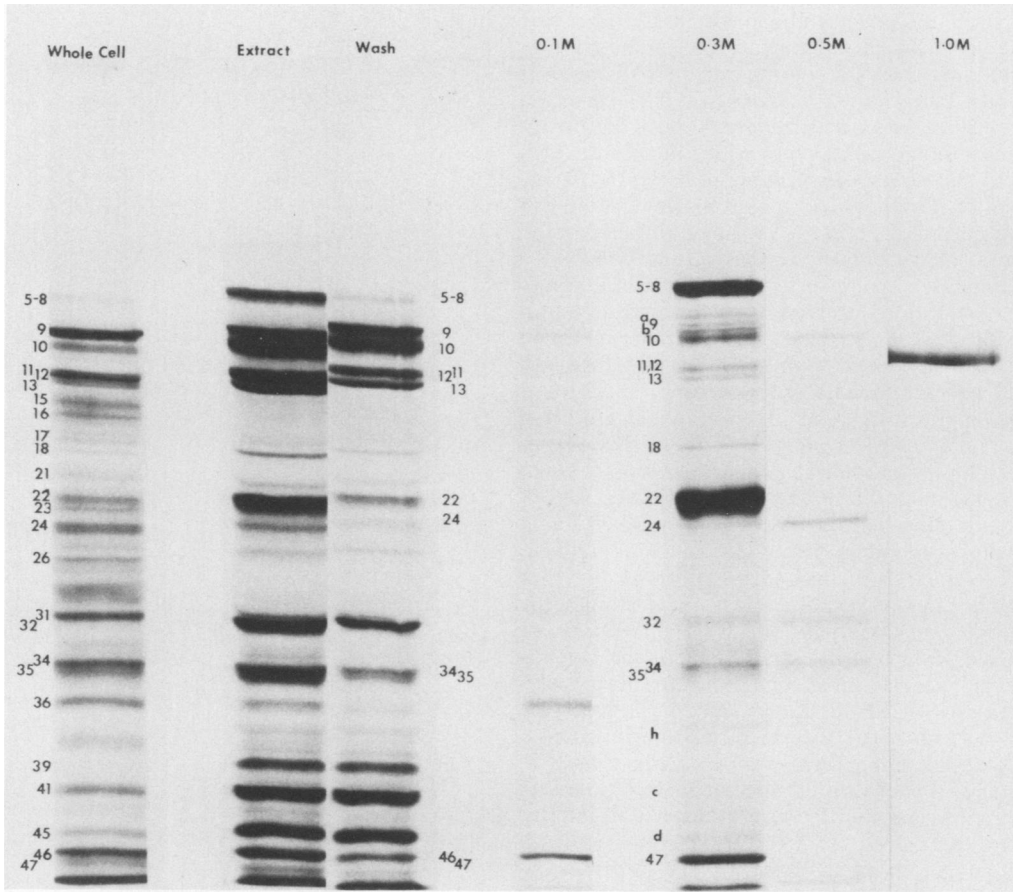

FIG. 2. *Autoradiograph of DNA-binding proteins from infected cell extracts eluted from a denatured DNA-cellulose column with KCl and subjected to electrophoresis in a 8% polyacrylamide slab gel. Polypeptides previously identified in HSV-2-infected cells (18) are designated by ICSP number beside the whole-cell sample and for reference beside that polypeptide present in the 0.05 M wash which includes the void volume and beside that polypeptide in the 0.3 M eluate.*

or 0.3 M (41%) NaCl. Eluates of 0.3, 0.5, and 1.0 M NaCl all exhibited an enrichment for infected cell proteins; however, the maximal enrichment (1.0 M eluate) was only twofold. Peak fractions were pooled, dialyzed overnight against distilled water, and lyophilized before they were run on cylindrical 8% polyacrylamide gels as described previously (19). From concentrates eluting at 0.3 and 0.5 M, major peaks of ^{14}C radioactivity were observed which had insignificant co-migrating tritium label in molecular-weight ranges of about 185×10^3, 145×10^3, 95×10^3, and 55×10^3 (data not shown). This double-labeling technique has been a classical procedure for the identification of DNA-binding proteins from virus-infected cells containing host proteins (1, 2, 26); however, differential radioactive counting procedures using sliced gels do not have the resolution necessary for analyzing HSV-infected cell-specific polypeptides (10). Autoradiographic analysis, required for such resolution, makes obligatory the use of slab gels wherein samples can be co-electrophoresed under identical electrophoretic conditions.

Polyacrylamide gel electrophoresis of DNA-binding proteins. Each of the eluates of infected-cell material bound to the denatured DNA-cellulose was concentrated by lyophilization and subjected to electrophoresis on 8% polyacrylamide slab gels containing SDS. Figure 2 shows the results of one such experiment in which stepwise elution with 0.1, 0.3, 0.5, and 1.0 M KCl was used. This figure shows the polypeptides present in infected cells, in the dialyzed salt extract (column load), the 0.05 M KCl wash, and in the different KCl eluates. (Throughout this report the term 0.05 M wash

is used to indicate the peak fractions of the material which did not adhere to the column including the void volume and 0.05 M wash.) It can be seen from this figure that the extraction procedure efficiently recovers most of the infected-cell-specific polypeptides in soluble form with the notable exceptions of ICSP 15, 16, 23, and 24. Further, the major species binding to the column are observed to be diminished in the material not binding to the column (wash).

Seventeen polypeptides were found to bind to DNA-cellulose, and these are designated tentatively with the previous ICSP number based on co-electrophoretic mobility with the infected-cell extract in adjacent positions for slab gel electrophoresis. ICSP 5–8 are phosphorylated polypeptides (K. L. Powell, unpublished observations) that undergo post-translational modification such that four polypeptides are seen in a short-pulse period and two appear over a long period of labeling (6a). ICSP 10, 11, 18, and 32, as well as 5–8, are early polypeptides that belong to the kinetic class A described by Powell and Courtney (18). ICSP 9 is the major capsid protein of the virus particle and belongs to the kinetic class C. The remaining polypeptides—ICSP 12, 13, 15, 22, 34, 35, and 47—all belong to the intermediate kinetic class B (18). The polypeptides labeled in Fig. 2 as a, b, h, c, and d are minor species of DNA-binding proteins consistently found in 0.3 M salt eluates from denatured DNA columns loaded with infected-cell extracts. The two polypeptides labeled h are probably of host origin and will be discussed later. Polypeptides a and b are two previously unidentified polypeptides in infected-cell extracts, presumably observed now because of concentration by this procedure relative to ICSP 9, the major capsid protein. The complex designated c represents a group of poorly resolved polypeptides present in trace amounts in the eluate. Polypeptide d is also a low-molecular-weight minor species of DNA-binding proteins detected consistently, but in variable amounts in 0.3 M eluates.

Densitometric tracings of autoradiographs of 8% polyacrylamide slab gels from the experiment of Fig. 2 are shown in Fig. 3. Such tracings allow quantitation of the polypeptides present in the crude high-salt extract, the 0.05 M wash, and the 0.1, 0.3, 0.5, and 1.0 M KCl eluates. It can be seen from this figure that most of the major DNA-binding proteins are quantitatively removed by passage over a denatured DNA-cellulose column and are present in relatively small amounts in the 0.05 M wash. The 0.1 M eluate contains a small amount of many polypeptides, but the major one detected

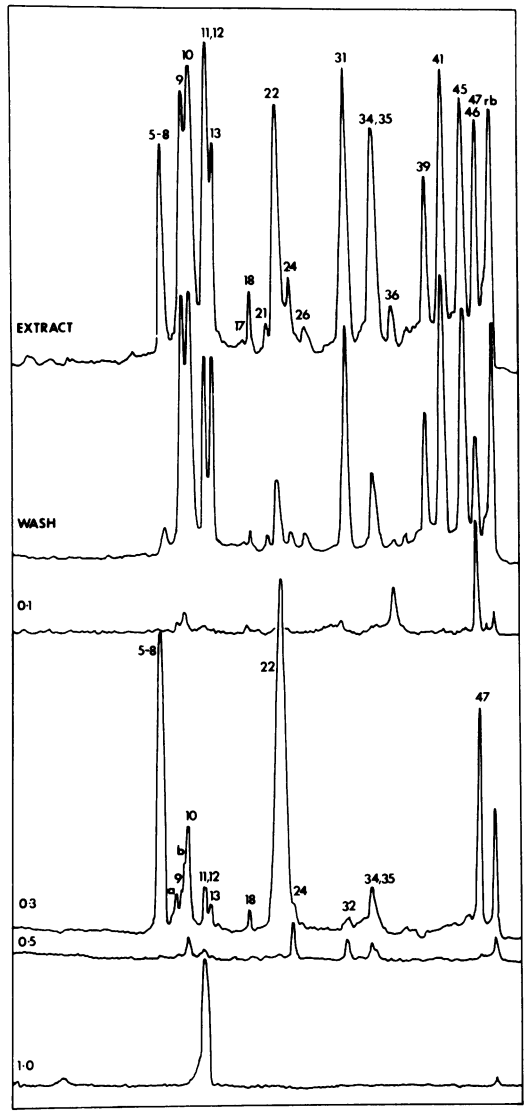

FIG. 3. *Densitometric tracings of the autoradiogram shown in Fig. 2 of DNA-binding proteins from HSV-2-infected cells chromatographed on denatured DNA-cellulose. Extract is the sample loaded onto the column, wash designates the 0.05 M KCl wash and void volume fraction, and numbers 0.1, 0.3, 0.5, and 1.0 refer to the molarity of KCl resulting in elution.*

is ICSP 36, which is completely eluted from DNA-cellulose at this low salt concentration. The 0.3 M eluate contains the majority of the DNA-binding proteins, with the major species being ICSP 5–8, 10, 22, and 47. A subsequent 0.5 M elution contains a much simpler profile, with ICSP 10, 24, 32, 34, and 35 being the major

species, with traces of ICSP 9, 11, 12, and 22. The only ICSPs remaining after a 0.5 M elution and removed by a subsequent 1.0 M elution are ICSP 11 and 12.

The polypeptides were quantified from densitometric tracings as described previously (18), and the proportion of each polypeptide retained on the columns is shown in Table 2. The major species of polypeptide bound to denatured DNA-cellulose were ICSP 5-8, 11, 12, 22, 34, 35, and 47. The proportion of individual polypeptides retained by denatured DNA from the original samples applied ranged from 6.1% (ICSP 13) to 80.5% (ICSP 5-8). For most DNA-binding species, over 50% of the polypeptide originally in the extract was bound by the denatured DNA-cellulose column, except ICSP 13 (6.1%), 9 (9.05%), and 10 (22.1%).

Several DNA-binding species present special problems with regard to their quantification in that either (i) they cannot be detected in infected-cell extracts (e.g., a, b, c, d, and h), (ii) they are present in such extracts in amounts too small to be accurately quantitated (ICSP 15), or (iii) they migrate too closely to a major polypeptide species to be differentiated from it accurately from densitometric tracings (ICSP 32). It should be emphasized that Table 2 gives percentages bound to DNA-cellulose from one experiment. The polypeptide composition of various eluates is consistent in different experiments; however, the proportion of individual polypeptides that bind does vary somewhat from experiment to experiment.

Virus specificity of the DNA-binding proteins. Figures 2 and 3 show that certain polypeptides with identical electrophoretic mobility to ICSPs previously identified in HSV-2-infected HEp-2 cells bind to denatured DNA-cellulose with considerable affinity. However, in no case is a polypeptide completely removed by passage through the column as described. It is possible that certain host DNA-binding proteins of identical electrophoretic mobility to ICSP are selected by this chromatography. Indeed, two new polypeptides, a and b, are apparent in Fig. 2 and 3 in 0.3 M eluates which are presumably masked in infected-cell extracts by the large amounts of ICSP 9 present.

Two types of experiments were done to determine the viral specificity of the observed DNA-binding proteins. First, mock-infected cell DNA-binding proteins were concentrated from 0.1, 0.3, 0.5, and 1.0 M NaCl eluates from a denatured DNA-cellulose column and subjected to electrophoresis in adjacent wells of an 8% polyacrylamide slab gel with analogous eluates from an infected-cell extract that had been chromatographed in parallel. Figure 4 compares the DNA-binding proteins of mock-infected and infected cells present in 0.3 and 0.5 M eluates, containing all the infected cell-specific DNA-binding proteins of interest. Immediately apparent is the gross simplification of the profile of DNA-binding proteins upon infection with HSV-2. The synthesis of most host DNA-binding proteins is switched off rapidly after infection, as is the case with other proteins (18). The mock-infected profile is of such complexity that there is at least a minor band from mock-infected cells with a similar mobility to several infected-cell-specific DNA-binding proteins. It is clear that certain polypeptides, ICSP 9, 10, 11, 12, 22, 32, and 35, have no counterpart in mock-

TABLE 2. *Major HSV DNA-binding proteins*

ICSP	Mol wt $\times 10^{-3}$	Kinetic class	Molarity of salt for elution	% Extracted cpm bound to DNA-cellulose		Amt precipitated relative to ICSP 9 immunoprecipitation	Mock-infected species of similar mol wt
				Denatured	Native		
5-8	182-186	A	0.3	80.50	79.50	0.24	+
9	157	C	0.3 to 0.5	9.05	6.35	1.00	-
10	153	A	0.3 to 0.5	22.10	8.90	1.19	-
11	146	A	0.3 to 1.0	62.80	47.60	1.46	-
12	143	B	0.3 to 1.0	62.80	47.60	1.46	-
13	140	B	0.3	6.10	ND[a]	1.46	-
15	126	B	0.3	CD[b]	CD	0.82	+ (Major)
18	110	A	0.3	53.90	ND	0.28	+ (Minor)
22	94	B	0.3 to 0.5	75.70	68.60	1.48	-
24	87	B	0.3 to 0.5	61.90	6.00	0.60	-
32	62	A	0.3 to 0.5	CD	CD	1.05	-
34	54	B	0.3 to 0.5	50.90	42.60	1.18	+
35	54	B	0.3 to 0.5	50.90	42.60	1.18	-
47	28	B	0.3	55.20	27.20	0.81	-

[a] ND, Not detected.
[b] CD, Cannot quantitate.

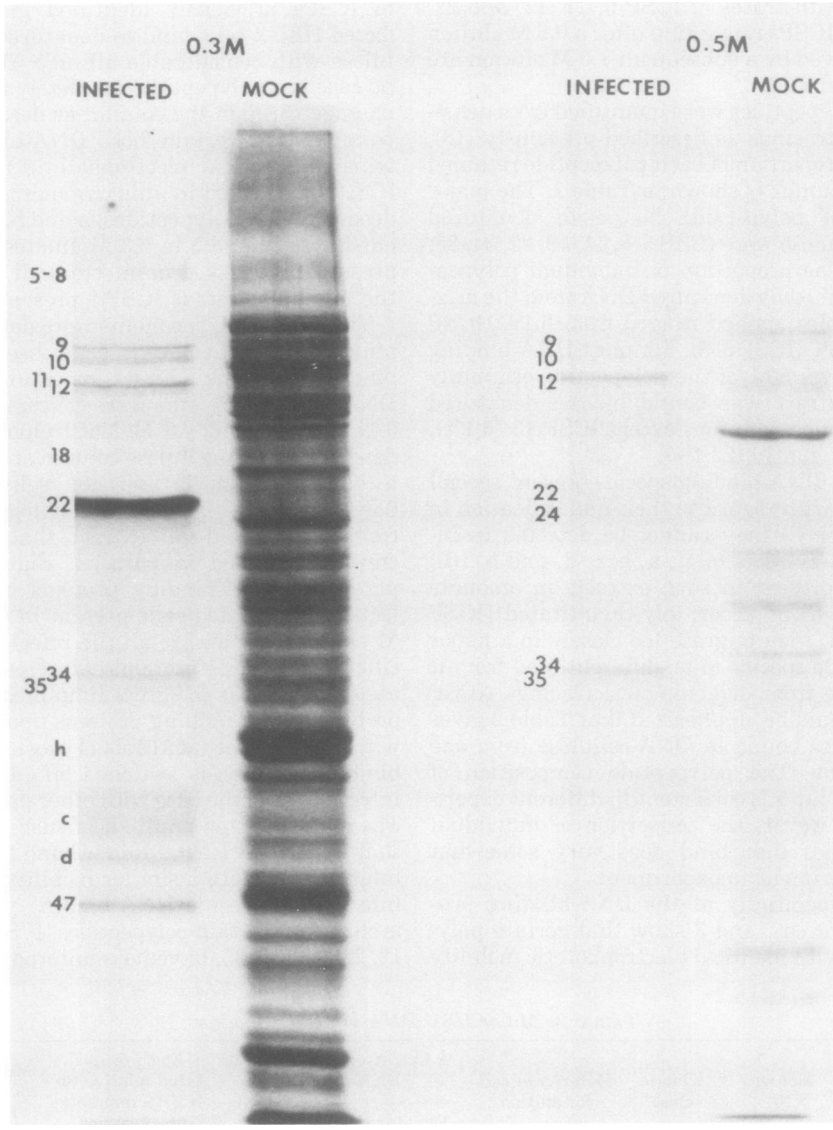

FIG. 4. *Autoradiographic comparisons of electrophoretically separated ^{14}C-amino acid-labeled infected-cell and ^{14}C-amino acid-labeled mock-infected cell DNA-binding proteins present in 0.3 and 0.5 M NaCl eluates from a denatured DNA-cellulose column. Solubilized proteins were subjected to electrophoresis in parallel in adjacent wells of an 8% polyacrylamide slab gel. The 1.0 M eluates of infected cells contained ICSP 11 and 12, and no bands were detected from such eluates of mock-infected proteins except for refractile boundary.*

infected cells. Other polypeptides (those designated h and ICSP 15) have identical mobility to major DNA-binding proteins of the host. The synthesis of host DNA-binding proteins of these mobilities were greatly reduced after infection. For other infected-cell-specific DNA-binding proteins, the co-migrating host counterpart is a minor species of the host extracts, and for these species to be seen in the infected cell a selective increased rate of synthesis would be required. As seen in Fig. 2, the only infected-cell-specific proteins present in 1.0 M eluates were ICSP 11 and 12. We have been unable to observe any distinct polypeptides in 1.0 M eluates from DNA-cellulose chromatography of mock-infected cell extracts, even with 28-day exposures of slab gels loaded with five times the radioactivity used with infected cell 1.0 M eluates. We

conclude that the host cell DNA-binding proteins eluting with 1.0 M NaCl probably represent multiple species.

Second, infected-cell DNA-binding proteins concentrated from 0.3, 0.5, and 1.0 M salt eluates were pooled, dialyzed, and precipitated with antiserum specific for viral antigens as detailed in the Materials and Methods. In this experiment, 54.4% of the acid-insoluble radioactivity in the pool was precipitated with antiserum, compared with 3.2% with preimmune serum from the same rabbit. Similar experiments with mock-infected cell controls failed to precipitate significant quantities of radioactivity with either serum. To quantify the precipitation of individual polypeptides, the pool used for precipitation and the immunoprecipitate were subjected to electrophoresis in parallel on an 8% polyacrylamide gel slab. The resulting autoradiograms were quantified as described and the quantity of each polypeptide present and precipitated was obtained. To relate the quality of immunoprecipitation to that of a known virus structural protein, the percentage of each protein precipitated was related to the percentage of the major capsid protein of the virus, ICSP 9 precipitated. These data are given in Table 2. Most infected-cell-specific DNA-binding proteins are precipitated with similar efficiency to that of ICSP 9, with the vivid exceptions of ICSP 5–8 and 18.

Relative binding strengths of DNA-binding proteins. Figures 2 through 4 illustrate that herpesvirus ICSPs bind with different affinities to denatured DNA-cellulose. Numerous experiments have shown the polypeptide species represented in the 0.3, 0.5, and 1.0 M eluates to be remarkably reproducible. Additional experiments were done to confirm that these observations are indeed a reflection of different affinities of various ICSPs for denatured DNA-cellulose. The 0.3, 0.5, and 1.0 M NaCl eluates from denatured DNA-cellulose were dialyzed to remove the salt and individually reapplied to new denatured DNA-cellulose columns. Each column was then washed with 0.05 M NaCl and eluted in stepwise fashion with 0.1, 0.3, 0.5, and 1.0 M NaCl. The results, shown in Fig. 5, confirm the affinity of binding of individual ICSPs. Radiolabeled proteins initially present in a 0.3 M NaCl eluate were retained by the column throughout washing and lower-molarity salt. Most of this material was eluted at 0.3 M, but significant amounts required 0.5 and 1.0 M salt for elution. Proteins present initially at 0.5 M eluted at 0.5 and at 1.0 M. Upon rechromatography, proteins initially present in 1.0 M fractions remained bound until a 1.0 M elution.

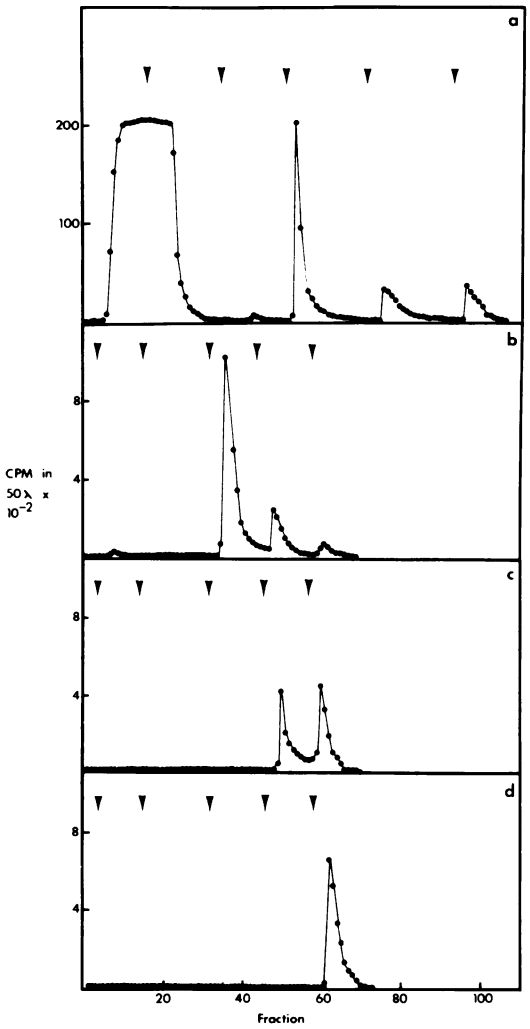

FIG. 5. *Additional chromatography of peak fractions of infected-cell proteins eluted with various molarities of NaCl from denatured DNA-cellulose. The arrows indicate, from left to right, the position where 0.05, 0.1, 0.3, 0.5, and 1.0 M NaCl-containing buffer was loaded. (a) ^{14}C-amino acid-labeled infected-cell extracts chromatographed on denatured DNA-cellulose. The peak fractions eluting with each of 0.3, 0.5, and 1.0 M NaCl were collected, pooled separately, and dialyzed against 0.05 M NaCl. (b) Subsequent chromatography of the 0.3 M peak fractions. (c) Chromatography of the original radiolabel eluting with 0.5 M NaCl. (d) Subsequent chromatography of the initial 1.0 M NaCl eluate.*

Thus, upon rechromatography proteins were eluted only by the same or higher salt concentrations, indicating that the molarity of salt required for elution was determined by their affinity for DNA.

Peak fractions from the columns shown in Fig. 5b, c, and d were concentrated and analyzed by polyacrylamide gel electrophoresis. The autoradiograms are too faint for reproduction, but indicate further separation of polypeptides present in mixtures. When 0.5 M eluates of the initial column were rechromatographed, the major ICSPs present at the subsequent 0.5 M elution were ICSP 34 and 35, whereas material that was bound more firmly and eluted with 1.0 M NaCl contained ICSP 11 and 12 as a major peak. A similar separation was achieved by rechromatography of the 0.3 M eluate. In this case, major peaks eluting at 0.5 M were ICSP 34 and 35, and at 1.0 M, ICSP 11, and 12.

A second experiment to ascertain if the elution profiles of various ICSPs represent their different affinities for denatured DNA-cellulose was done as follows. Extracts were prepared from infected cells as previously described, applied to a denatured DNA-cellulose column, and after washing with 0.05 M NaCl, eluted with a linear continuous gradient of 0.1 to 1.0 M NaCl. The results are shown in Fig. 6. The elution profile observed differed considerably from that seen with stepwise elution (Fig. 1). Portions of individual fractions were subjected to polyacrylamide gel electrophoresis. The diagram beneath the elution profile illustrates fractions in which various ICSPs were found. The dotted lines represent (from left to right) 0.1, 0.3, 0.5, and 1.0 M salt, respectively. These results coincide closely with the ICSP profiles of batch eluates; e.g., ICSPs found between 0.1 and 0.3 M do appear in a 0.3 M batch eluate. ICSP 9, 10, 11, 12, 22, 24, 34, and 35 require higher salt concentrations for complete elution. The only ICSPs observed to be eluted at NaCl concentrations above 0.7 M were ICSP 11 and 12.

From batch elution, numerous polypeptides appear in the 0.3 M eluate in trace amounts: e.g., see Fig. 2 (ICSP 36, c, d, h). By gradient elution these are observed between 0.1 and 0.15 M NaCl and may be retained by the column due to weak ionic exchange interactions. Collectively, these results indicate that HSV-2 ICSPs bind with different affinities to denatured DNA-cellulose. The relative binding strength of these proteins can be estimated from the

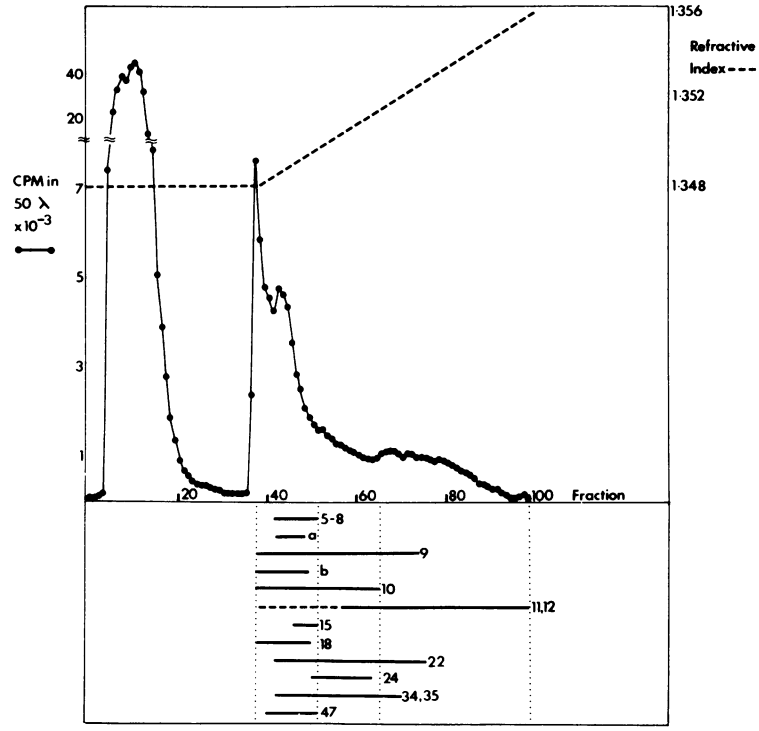

FIG. 6. *Chromatography of infected-cell extracts on denatured DNA-cellulose with elution by a 70-ml continuous linear gradient of 0.1 to 1.0 M NaCl. Individual fractions (every second one) were collected, and the polypeptides present were analyzed by electrophoresis on 8% polyacrylamide slab gels. The dotted lines below the elution profile represent, from left to right, 0.1, 0.3, 0.5, and 1.0 M NaCl, and the diagram illustrates fractions in which individual polypeptides were observed.*

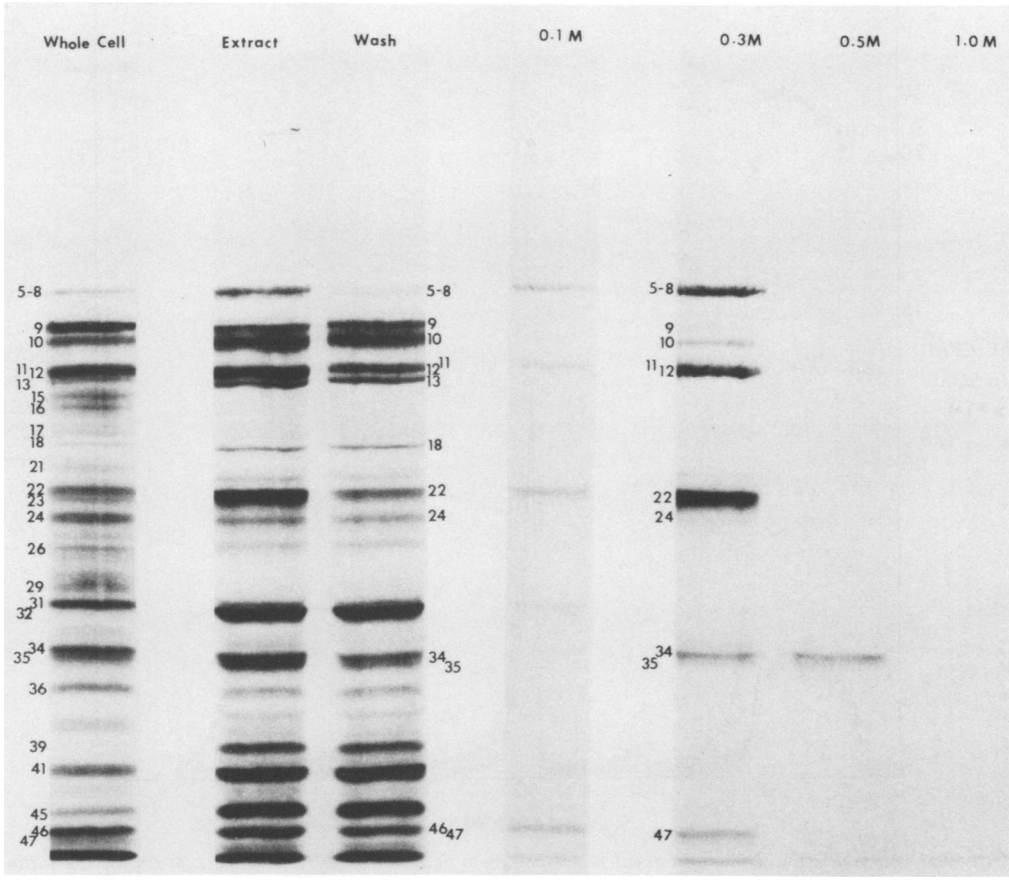

FIG. 7. *Autoradiograph of DNA-binding proteins from infected-cell extracts eluted from a native DNA-cellulose column with KCl and subjected to electrophoresis in 8% polyacrylamide slab gels. Designations are as in Fig. 2.*

molarity of NaCl required for their elution. These proteins are listed according to decreasing electrostatic binding strength: ICSP 11, 12>9, 10, 13, 22, 24, 34, 35>a, b, c, d, h, 5–8, 15, 18, 32, and 47.

Binding of HSV ICSP to denatured and native DNA. To compare the binding of HSV-2 ICSPs to native and denatured DNA-cellulose, proteins eluted from native DNA-cellulose were analyzed on polyacrylamide gels. All ICSPs found binding to denatured DNA also bound to native DNA-cellulose columns, except for ICSP 13 and 32, which are minor species from denatured DNA-cellulose columns. Figure 7 shows the infected-cell polypeptides eluted from a native DNA-cellulose column. The major polypeptides binding to native DNA-cellulose are the same ones we detected binding to denatured DNA-cellulose; i.e., ICSP 5–8, 11, 12, 22, 34, 35, and 47. The proportion bound is given in Table 2 and in each case is less than that bound to denatured DNA. Additional experimentation is required to assess accurately whether preferential binding occurs.

Binding of HSV-induced alkaline DNase to denatured DNA-cellulose. Since a major objective in analyzing DNA-binding proteins of HSV-2-infected cells was to discover HSV gene products that may be important in DNA replication, recombination, and/or repair, we examined the ability of HSV-induced alkaline DNase to bind to denatured DNA-cellulose columns. This enzyme is induced by HSV and is probably viral coded (16). The high-salt extraction using KCl recovered virtually 100% of the alkaline DNase activity present in HSV-2-infected-cell extracts. Figure 8 shows the behavior of the enzyme activity on chromatography on denatured DNA-cellulose columns. A large proportion of the enzyme activity remained bound to the column and was eluted with 0.3 M KCl.

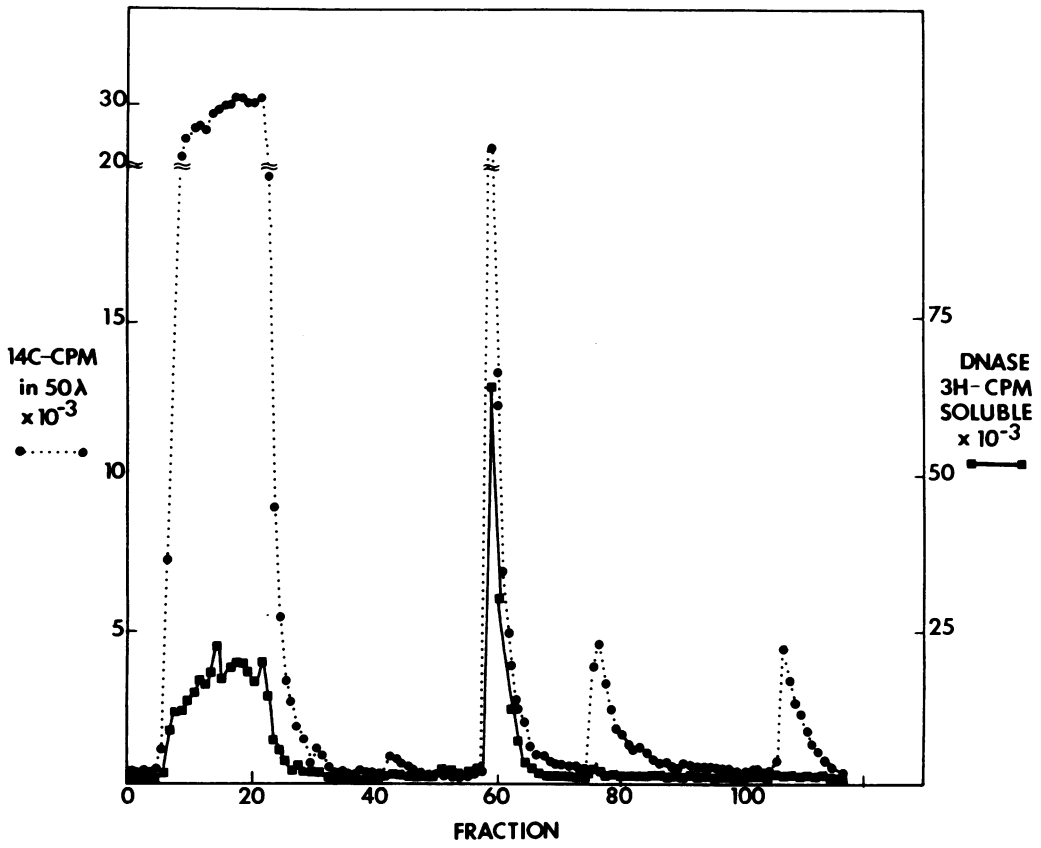

FIG. 8. *Elution of HSV-2-induced alkaline DNase from denatured DNA-cellulose.* ^{14}C*-amino acid-labeled infected-cell extracts were chromatographed as previously described using 0.1, 0.3, 0.5, and 1.0 M KCl for elution. Samples (50 μl) were assayed for DNase activity as described in Materials and Methods. The* ^{3}H*-labeled HEp-2 cell DNA solubilized per 50 μl of each fraction is given by (■).*

DISCUSSION

The use of HSV as a model to study the replication of large DNA molecules is especially attractive, since genetic studies in several laboratories have indicated that a large portion of the HSV genome apparently codes for proteins required for DNA synthesis (23, 25). Except for a few viral enzymes (14), the identity or role of such HSV proteins is unknown. We have used DNA-cellulose chromatography to identify 17 proteins in HSV-2-infected cells which interact with and presumably function with DNA.

A recent report by Bayliss et al. (3) describes the isolation of 16 DNA-binding proteins from HSV-1-infected BHK cells. The presence of numerous DNA-binding proteins from HSV-1- and HSV-2-infected cells is not too surprising, since 19 DNA-binding proteins of the T-even bacteriophage, whose genome is only slightly larger than that of HSV, have recently been isolated by a similar technique (13). The T-even bacteriophage forms an excellent model for a large DNA virus infecting a cell whose replicative machinery is more complex than its own. Although Alberts et al. (2) were the first to demonstrate DNA-binding proteins of T4, Huang and Buchanan (13) have recently reexamined these proteins of T4 using high-resolution polyacrylamide gel electrophoresis. In remarkable similarity to our results, they found (i) a large number of T4-infected cell-specific proteins bound to denatured DNA-cellulose columns (19 out of 51); (ii) most of the DNA-binding proteins could be detected in infected-cell extracts before chromatography; and (iii) most were only removed in part by chromatography on a DNA-cellulose column.

The mechanism whereby the HSV ICSPs bind to DNA is not understood, but the binding is specific for DNA inasmuch as no radiolabeled proteins bind to cellulose columns devoid of

DNA. Although proteins do interact with DNA to be retained on the column, this may not reflect a functional interaction since two other explanations, in addition to a high affinity for DNA per se, are possible. (i) At this pH most proteins are negatively charged, but weak ionic binding between certain proteins and DNA may explain the weak binding of a small proportion of certain polypeptides which elute between 0.1 and 0.15 M NaCl. Basic proteins would bind to DNA, but, to date, there have been no confirmed reports of histone-like basic proteins synthesized in HSV-2-infected cells. (ii) A protein may bind to another protein that is bound specifically to DNA. Such a synergistic interaction has been observed with T4 DNA-binding proteins (13) wherein two bacteriophage-specified proteins are bound with high affinity to DNA-cellulose if, and only if, gene 43 product (DNA polymerase) is present.

In this study it has been possible to distinguish between weakly and strongly binding proteins of HSV-2-infected-cell extracts for denatured DNA-cellulose. Results from batch elution with various molarities of salt, both KCl (Fig. 2) and NaCl (Fig. 4), and elution by a linear continuous gradient (Fig. 5), support the tentative order given in Results. Clearly, ICSP 11 and 12 have the highest affinity for denatured DNA-cellulose. Similarities between the binding of ICSP 11 and 12 and that of gene 32 product of T-even phage (2) include the following. (i) Both bind to native and denatured DNA-cellulose. (ii) ICSP 11 and 12 have the highest affinity for denatured DNA of all the herpesvirus-infected cell-specific polypeptides that bind to DNA. (iii) ICSP 11 and 12 and gene 32 product all bind to denatured DNA with higher affinity than they do to native DNA-cellulose, as evidenced by a requirement for higher-molarity salt for elution from the former. Of additional interest is the behavior of ICSP 11 and 12 during recycling on denatured DNA-cellulose, as was shown in Fig. 5. Most of ICSP 11 and 12 originally eluted at 0.3 or 0.5 M NaCl required higher-molarity salt for elution upon rechromatography. This behavior is similar to that reported for gene 32 product; i.e., when gene 32 product was applied at low concentration, it was eluted with 0.6 M NaCl, but when applied at higher concentrations, 2.0 M NaCl was required for elution (2). Since synergistic interactions of proteins can affect their binding to DNA, purified individual polypeptides will be required for exact delineations of relative binding strengths, as well as for determination of preferential binding to native or denatured DNA. In addition, purified HSV DNA should be used to determine whether the polypeptides isolated by DNA affinity chromatography or other HSV-2-infected cell-specific polypeptides bind specifically or selectively to HSV DNA.

Of the polypeptides found in this study, 17 are HSV-2 ICSPs which have been described previously and are detected in infected cells before DNA synthesis (18). The earliest kinetic class proteins, class A (18), whose rate of synthesis is maximal by 3 to 4 h after infection, make up a higher proportion of DNA-binding proteins than they do of total ICSP of HSV-infected cells. Especially interesting are the very early ICSP 5–8, which have been previously shown to be overproduced when DNA synthesis is inhibited (20). Analogous polypeptides of HSV-1 (designated VP175 [6] or ICP 4 [10]) are overproduced after infection with DNA-negative temperature-sensitive mutants (20) or with defective virions (7, 17). These early proteins are the major HSV-1- and HSV-2-infected cell-specific polypeptides found after reversal of a cycloheximide block added at the time of infection (12, 18). The DNA-binding properties of analogous polypeptides indicate that they may function directly with DNA.

Polypeptides which have DNA-binding properties and which are structural components of virions are of interest, since at least one protein of the herpes virion was reported to be associated with nucleic acid in the nucleocapsid (8) and others may interact with HSV DNA in the course of its packaging into particles. In this study the DNA-binding proteins which have identical electrophoretic mobility to structural polypeptides of HSV-2 strain 186 are ICSP 9, 13, 24, and 47 (K. L. Powell, manuscript in preparation). The finding that the major capsid ICSP 9 appears to have a DNA-binding form is analogous to the situation recently reported for the capsid protein of λ bacteriophage (4), which may relate to some stage of virus particle assembly.

By far, the majority of the DNA-binding proteins of HSV-2-infected cells are not structural components of purified virus. Nonstructural proteins include the major species ICSP 5–8, 11, 12, 22, 34, and 35. Some of these proteins can be expected to function in DNA replication, recombination, repair, or degradation, as inferred from work with other systems (1, 2, 13). We have found that two HSV-2-induced enzymes, DNA polymerase (assayed as described previously [21] and alkaline DNase [Fig. 8]), bind to denatured DNA-cellulose columns and are eluted with 0.3 M KCl. These are probably herpesvirus-specified enzymes, since the antiserum described in Materials and Methods neutralizes both activities. Bayliss et al. (3) re-

ported a similar observation for HSV-1 DNA polymerase and, in addition, reported that HSV-1-induced thymidine kinase was not retained. Collectively, these results are as expected; HSV enzymes that interact directly with DNA are retained, whereas other HSV enzymes are not.

The DNA-binding proteins that are minor species present special problems of quantitation and require some additional comment. These include ICSP 15, 18, 32 and a, b, c, d, and h. Polypeptides designated h co-migrate with major DNA-binding proteins of host cells, as does ICSP 15. In the latter case, the relatively good immunoprecipitation of ICSP 15 from a pool of DNA-binding protein (Table 2) suggests that additional studies of its binding to DNA should be attempted when conditions for its efficient extraction are devised (Fig. 3). ICSP 18, although poorly immunoprecipitated, is bound in large amounts to denatured DNA (Table 2). ICSP 32 cannot be quantified due to its proximity to ICSP 31; however, it is efficiently immunoprecipitated, binds with high affinity (0.3 to 0.5 M salt is required for elution), and constitutes approximately 14% of the total 0.5 M eluate of Fig. 2. Polypeptides a, b, c, and d require further study to determine their origin, since they are either viral-coded or host DNA-binding proteins that are selectively synthesized in infected cells. Minor species might be expected to have a catalytic function, and may have great importance in virus replication, whether of host or viral origin.

At this point it is difficult to compare DNA-binding proteins of HSV-2-infected cells with those found by Bayliss et al. in HSV-1-infected cells. In general, more of the HSV-2 DNA-binding proteins were of higher molecular weight than those found with HSV-1. Major species with similar molecular weights include HSV-1 BP2 (molecular weight, 136×10^3), which may be analogous to ICSP 11 and 12, and HSV-1 BP-3 (molecular weight, 87×10^3), which may be analogous to ICSP 22 or 24. Such comparisons are premature and may be misleading, since no detailed comparisons of HSV-1- and HSV-2-infected cell-specific polypeptides are available. Furthermore, (i) since Bayliss et al. (3) employed the polyethylene glycol procedure of Alberts and Herrick (1) for extraction, which in our hands results in very low recovery of protein (<5%), (ii) since the total proportion of their extract which bound to DNA-cellulose was only 1 to 2% (3), and (iii) since the DNA-binding efficiencies of individual polypeptides were not considered by Bayliss et al. (3), any comparison of our results is fraught with difficulties. One additional point to be made is that the different systems used for polyacrylamide gel electrophoresis differ in resolving capabilities for certain molecular-weight ranges.

From among the 50 odd HSV-2-infected cell-specific polypeptides, DNA-cellulose chromatography allows a selection in a native state of 17, some of which probably have a DNA-related function. Further purification is being attempted to determine their function. DNA-cellulose chromatography is a useful step in their purification. For example, ICSP 11 and 12 (Fig. 2) are almost radiochemically pure upon elution with 1.0 M NaCl. Temperature-sensitive mutants of HSV-2 (23) are being tested for alterations in the induction of DNA-binding proteins. Recent evidence of the DNA-binding of T antigens of SV40 (5) and adenovirus (9) predicts an additional importance of DNA-binding proteins in herpesvirus-transformed cells. We are currently examining transformed cells for HSV-2 DNA-binding proteins.

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