JOURNAL OF VIROLOGY, Mar. 1989, p. 1059–1068
0022-538X/89/031059-10$02.00/0

Vol. 63, No. 3

Characterization of DNA Sequence-Common and Sequence-Specific Proteins Binding to *cis*-Acting Sites for Cleavage of the Terminal *a* Sequence of the Herpes Simplex Virus 1 Genome

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Received 13 September 1988/Accepted 12 November 1988

The terminal 500-base-pair *a* sequence of the herpes simplex virus 1 genome contains signals for cleavage (*Pac1* and *Pac2*) of unit-length DNA molecules from concatemers in unique stretches of sequences designated U_b and U_c, respectively, and a *cis* site for cleavage designated DR1. We report that nuclear extracts from infected cells contain factors which form two DNA-virus-specific protein complexes with components of the *a* sequence. Purification of the factors forming the V2 complex yielded a protein with an apparent molecular weight of 82,000 binding to DNA in a non-sequence-specific manner. Addition of Mg^{2+} to the purified protein-DNA probe mixture resulted in exonucleolytic degradation of the DNA. The protein was identified as the virus-specific DNase with monoclonal antibody specific for the viral enzyme. The purification of the proteins forming the V4 complex yielded two proteins with molecular weights of >250,000 and 140,000 corresponding to infected cell protein 1 and to an as yet unidentified protein, respectively. These proteins formed two DNA sequence-common bands with a number of DNA probes and one sequence-specific band with probes containing both *Pac2* and DR1 but not with probes containing either site alone or *Pac1* and DR1. Since the DNA probe containing *Pac2* and DR1 inserted into viral genome or into amplicons induced specific cleavage of the DR1 sequence whereas the nonreactive probes failed to induce the cleavage, the formation of this sequence-specific DNA-protein complex is significant and may reflect a DNA-protein interaction essential for cleavage. The possible role of the proteins identified in this study for the cleavage-packaging of viral DNA into capsids is presented.

The terminal sequence, designated sequence *a*, of the herpes simplex virus 1 (HSV-1) genome (Fig. 1) encodes several *cis*-acting sites involved in (i) the circularization of viral DNA after infection, (ii) the inversion of the two covalently linked components of HSV-1 DNA, long (L) and short (S), relative to each other, (iii) the cleavage of genomic-length DNA from circles or concatemers and packaging of the excised molecules into capsids, and (iv) the expression of an mRNA extending from the *a* sequence into the reiterated sequence of the L component (2–7, 21–23, 25, 26, 28, 29). In the expectation that specific viral or host proteins or both interact with the DNA at the specific *cis* sites, we constructed several viral DNA probes encompassing most of the *a* sequence and tested these for reactivity with proteins contained in nuclear extracts of mock-infected and infected cells. We report that two DNA-protein complexes formed by the probes with nuclear extracts of infected cells each contain a virus-specified protein. The properties of these proteins are described.

Relevant to this report are the structure and nucleotide sequence arrangement of the *a* sequence. In HSV-1 strain F [HSV-1(F)], the *a* sequence situated at the junction between the L and S components and repeated in an inverted orientation at the termini of the genome consists of a 20-base-pair (bp) terminal repeat designated DR1, a 64-bp unique sequence designated U_b, 22 repeats of a 12-bp sequence designated direct repeat 2 (DR2), three repeats of a 37-bp sequence designated direct repeat 4 (DR4) and containing 11 of the 12 nucleotides of DR2, a unique 58-bp sequence designated U_c, and a second copy of DR1 (23). Tandem repeats of the *a* sequence share the intervening DR1. The number of copies of DR2 and DR4 vary, and partial copies of these sequences may also be repeated. This variability accounts for most of the observed *a* sequence polymorphism (4–6, 21, 23, 28).

The observations that the terminal DR1 of the L component consists of 18 bp plus a single nucleotide extended 3′ and that the terminal DR1 of the S component consists of 1 bp plus a single nucleotide extended 3′ producing, when ligated, an intact DR1 led to the conclusion that the genomic-length DNA is excised from concatemers by cleavage within DR1 sequences (23). Recent studies have demonstrated that the signals for the cleavage and packaging of the DNA designated *Pac2* and *Pac1* (Fig. 1) are located in the U_c and U_b sequences, respectively, of the *a* sequence (6, 28).

(These results were presented at the International Herpesvirus Workshop, Irvine, Calif., August 1988.)

MATERIALS AND METHODS

Cells, viruses, and protein extracts. HeLa cells grown to confluency in 850-cm^2 roller bottles were either mock infected or infected with 5 PFU of HSV-1(F) or herpes simplex virus (HSV-2) strain G [HSV-2(G)] per cell. Cells were harvested 20 to 24 h after infection or mock infection, and nuclear extracts were prepared in 0.42 M KCl as described previously (8).

Preparation of DNA probes and competitor DNA and DNA band shift assays for DNA-binding proteins. DNA probes used in the text were prepared by digesting the plasmids with

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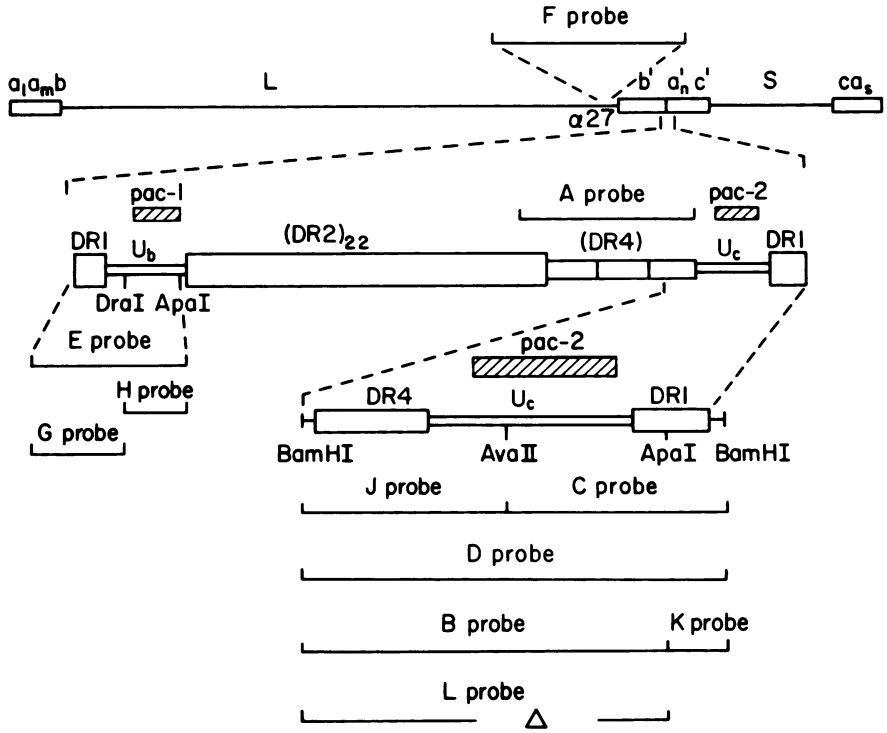

FIG. 1. Schematic diagram of the sequence arrangements in the HSV-1 genome and in the *a* sequence and the position in the genome of the DNA probes used in this study. Top line: Sequence arrangement of the HSV-1 genome. a_1 and a_s refer to the terminal *a* sequences of the L and S components. Subscript n, One or more *a* sequences; subscript m, none to more than one *a* sequences. The insert indicates the position of the F probe consisting of the 48 bp of the regulatory domain of the HSV-1(F) α27 gene from pRB606 (13). Second line: Sequence arrangement of the *a* sequence. The wild-type *a* sequence contains a 20-bp directly repeated element (DR1) flanking the *a* sequence; a 64-bp unique sequence (U_b); 22 copies of the 12-bp directly repeated element (DR2); 3 copies of the 12-bp directly repeated element (DR4); and a 58-bp unique sequence (U_c). The A probe encompassing three copies of DR4 and one copy of DR2 was derived from the plasmid A-1 and generated by *Bss*HII and *Apa*I restriction digest of the A-1 clone (6). The D and E probes derived from pRB3389 and pRB3387, respectively, were generated by cleaving the parent plasmid with *Bam*HI for probe D and *Bam*HI to *Apa*I for probe E. The pRB plasmids have been previously described (2). The L probe was generated by *Bam*HI and *Apa*I cleavage of a plasmid which contains a *Bss*HII deletion of pRB3389 as shown; the C, J, B, and K probes were generated from pRB3389 by taking the *Bam*HI-to-*Ava*II subfragment, the *Ava*II-to-*Bam*HI fragment, the *Bam*HI-to-*Apa*I fragment, and the *Apa*I-to-*Bam*HI fragment, respectively, as indicated. The G and H probes were generated from pRB3387 by taking the *Bam*HI-to-*Dra*I fragment and the *Dra*I-to-*Apa*I fragment, respectively.

appropriate restriction enzymes. The DNAs were then dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and loaded on a 5% nondenaturing polyacrylamide gel. The fragments of interest were then purified from polyacrylamide gels and 5′ end labeled with [^{32}P]ATP (>7,000 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) and T4 polynucleotide kinase (United States Biochemical Corp., Cleveland, Ohio) to an activity of approximately 30,000 cpm/ng of DNA fragment (17).

The standard competitor DNA used in these assays was synthetic poly(dI-dC) · poly(dI-dC) (Pharmacia, Molecular Biology Division, Piscataway, N.J.). Competitor polymers were used at 2 to 3 μg per reaction of standard nuclear extract and 0.5 μg per reaction of purified proteins in gel binding retardation assays.

Binding reaction mixtures (12 μl) contained approximately 0.1 ng of labeled DNA, 2 to 3 μg of competitor DNA, 9 μl of binding reaction buffer (20 mM Tris hydrochloride [pH 7.4], 50 mM KCl, 0.05% Nonidet P-40, 5% glycerol, 50 μg of bovine serum albumin per ml, 5 mM β-mercaptoethanol, 1 mM EDTA), and 2 to 3 μg of extract protein. After incubation at room temperature for 30 min, reaction mixtures were loaded on a 5% polyacrylamide gel in 50 mM Tris-borate–1 mM EDTA buffer (pH 8.3) prerun at 230 V for 2 h. Gels were run at 230 V until bromophenol blue dye reached the gel bottom; then they were dried and autoradiographed (27). When monoclonal antibody was used in the binding reaction, 1 μl of specific diluted monoclonal antibody was added to the reaction mixture after the incubation period in the standard reactions and the reaction was allowed to incubate for additional 30 min before loading onto gels.

Chromatographic purification of proteins. The purification scheme used in these studies is shown in Fig. 2. For P11 phosphocellulose chromatography, 50 to 70 mg of nuclear extract stored at 12.9 mg/ml in buffer D (20 mM Tris hydrochloride [pH 7.9], 0.42 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol) was diluted with buffer C (20 mM Tris hydrochloride [pH 7.9], 0.2 mM EDTA, 0.5 mM dithiothreitol, 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) to a final KCl concentration of 0.125 M. The diluted extract was loaded onto a preequilibrated P11 phosphocellulose column (Whatman, Inc., Clifton, N.J.) prepared according to the instructions of the manufacturer. The column was washed with 2 column volumes of buffer C containing 0.15 M KCl. A step gradient in buffer C containing KCl ranging from 0.15 to 0.6 M in steps of 0.05 M was then applied, and the fractions were collected. The fractions were then analyzed for the

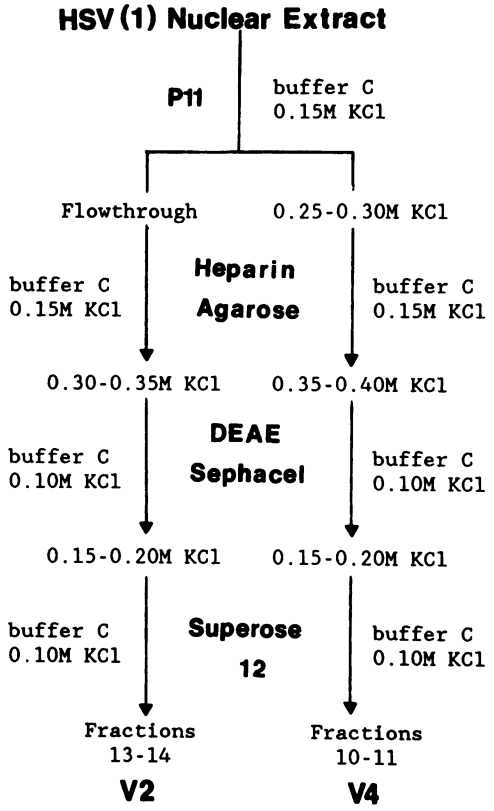

FIG. 2. Flow diagram of the procedures for purification of the factors forming the V2 and V4 DNA-protein complexes. HSV-1 infections in HeLa cells, preparation of the nuclear extracts, and chromatographic purification of the proteins are described in Materials and Methods. Column sizes used in these purifications procedures are 6 ml of P11 phosphocellulose, 3 ml of heparin-agarose, 1 ml of DEAE-Sephacel, 24 ml of Bio-Gel A-0.5M (0.8 by 47 cm), and 25 ml of fast protein liquid chromatography Superose 12.

ability to form complexes with probe DNA in gel retardation assays. The active fractions were pooled.

For heparin-agarose column chromatography, active fractions eluted from the P11 column were pooled and diluted with buffer C to a KCl concentration of 0.15 M and loaded on a preequilibrated heparin-agarose column (Sigma Chemical Co., St. Louis, Mo.). After the column was washed with 2 column volumes of buffer C–0.15 M KCl, a step gradient consisting of buffer C with a salt concentration from 0.2 to 0.5 M KCl was developed and fractions were collected and assayed by the binding gel retardation assay. Active fractions were pooled and processed.

For DEAE-Sephacel column chromatography, protein fractions pooled from the preceding column were diluted with buffer C to a KCl concentration of 0.1 M and loaded onto a preequilibrated DEAE-Sephacel column (Pharmacia). Elution procedures were the same as above.

For Bio-Gel A-0.5M (25 ml; Bio-Rad Laboratories, Richmond, Calif.) or fast-protein liquid chromatography Superose 12 (24 ml; Pharmacia) chromatography, protein fractions were concentrated to less than 200 μl, 1% of the column bed volume, with a Centricon 30 concentration (Amicon Corp., Danvers, Mass.) prior to loading. After loading, proteins were eluted with buffer C containing 0.1 M KCl and fractions were collected on a fraction collector and assayed for binding activity.

Gel electrophoresis of proteins. Electrophoretic separations of denatured proteins were done in 9.25% polyacrylamide gels cross-linked with *N,N'*-diallyltartardiamide containing sodium dodecyl sulfate as described by Morse et al. (24). The gels were stained with silver for visualization (20) and mounted for photography.

RESULTS

Experimental design. Preliminary experiments established that nuclear extracts of infected cells contained factors capable of binding to DNA fragments derived from the *a* sequence. In the experiments we report here, labeled DNA fragments were used as probes to monitor the purification of these factors, to determine the specificity of binding of the viral proteins recognizing DNA sequences contained in the terminal *a* sequence, and last, to identify the proteins involved in forming the complexes. As shown in Fig. 1, probe A contained the reiterated sequence DR4 and one copy of the DR2 sequence. Probes B, D, C, and J contained all or portions of the DR4, U_c, and DR1 domains of the *a* sequence. Probes G, H, and E similarly covered the U_b and DR1 domains of the *a* sequence. Last, probe F containing unique sequences of the L component was used as the representative of non-*a* sequence domains of the HSV-1 genome. Probes B and C were used for detecting and monitoring the purification of viral proteins capable of binding to the *a* sequence. These probes overlap; whereas the C probe contains most if not all of the *Pac2* sequence required for cleavage recognition and the cleavage site, the B probe contains only the cleavage recognition sequence. All the probes were used for defining the specificity of binding.

During preliminary experiments involving DNA band shift assays, we identified in the nuclear extracts of infected and mock-infected cells several factors capable of binding the labeled DNA probes used in these studies. Of particular interest were two factors designated V2 and V4 detected only in extracts of infected cells. To differentiate between these factors and to study their properties, we used the concentration and partial purification scheme shown in Fig. 2.

Demonstration of factors from infected cells capable of binding to the DNA probe sequences. Figure 3 shows DNA band shift assays utilizing probes B (panels A and B) and C (panels C and D) and both crude and P11-fractionated (Fig. 2) nuclear extracts of infected and mock-infected cells. The results were as follows.

(i) Both labeled DNA probes formed multiple bands containing DNA-protein complexes when reacted with nuclear extracts of mock-infected and HSV-2(G)-infected cells. In the experiments shown, the competitor DNA was poly(dI-dC) · poly(dI-dC), but substantially identical results were obtained with other synthetic polymers [poly(dI-dC); data not shown).

(ii) Several of the DNA-protein complexes (e.g., the band designated as H1 in Fig. 3A and B) were detected in DNA band shift assays with extracts of nuclei from mock-infected and from HSV-1- or HSV-2-infected cells. In contrast, the DNA-protein complexes designated V2 and V4 were detected only in assays done with extracts of infected cells. In general, the host protein-DNA complexes tended to obscure the infected cell-specific protein-DNA complexes formed by crude extracts of infected cells (for example, compare the lanes marked M [mock] and G [HSV-2] shown between panels with the lanes showing the reactions of fractionated proteins in extracts of mock-infected [panels B and D] and

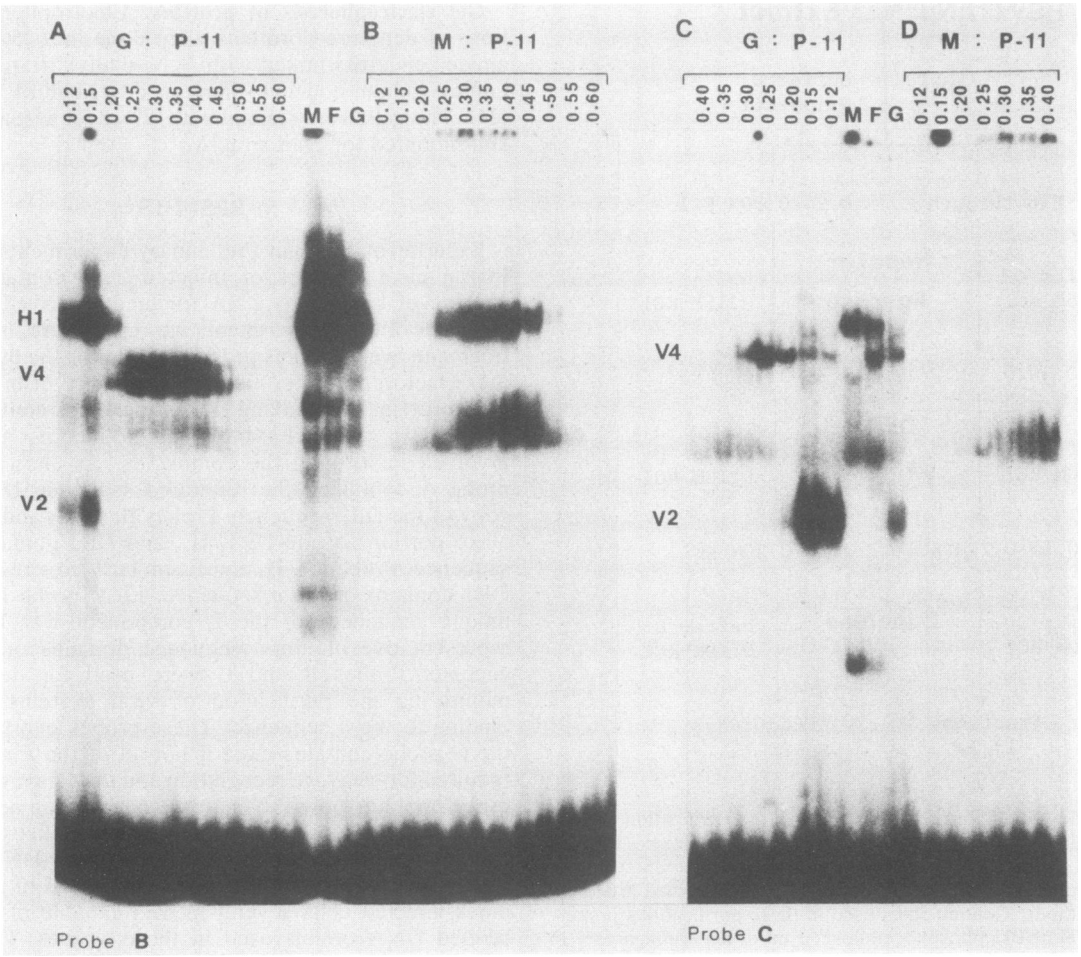

FIG. 3. Autoradiographic images of labeled DNA-protein complexes electrophoretically separated in nondenaturing gels. DNA probe fragments (Fig. 1) generated by digestion of the plasmids with the appropriate enzymes were dephosphorylated with calf intestinal alkaline phosphatase, purified from polyacrylamide gels, and 5′ end labeled with [γ-^{32}P]ATP (>7,000 Ci/mmol) by T4 polynucleotide kinase to an activity of approximately 30,000 cpm/ng of DNA fragment (17). (A and B) Reaction of probe B DNA with chromatographic fractions of nuclear extract prepared from HSV-2(G)-infected cells (A) and mock-infected cells (B). (C and D) Reaction of probe C DNA with the same chromatographic fractions as in panels A and B. Lanes M, F, and G, Reactions of nuclear extracts of mock-, HSV-1(F)-, and HSV-2(G)-infected cells. Numbers at the top of the gel indicate the KCl concentration in buffer C used to elute the protein fractions from column. V2 and V4 designate the viral protein-DNA complexes. H1 is discussed in the text.

infected [panels A and C] cells). Moreover, the infected cell-specific protein-DNA complexes formed by extracts of HSV-2(G)-infected cells were stronger and more prominent than those formed by extracts of HSV-1(F)-infected cells.

(iii) The properties and the specificity of binding of the factors responsible for the V2 and V4 DNA-protein complexes are dealt with in the text below. Of the host factors, the H1 factor defined by P11 column chromatography (Fig. 3) was shown in other experiments described later in the text to bind strongly to probes B, J, and D but not to the C probe. The designation of H1 assigned to the comigrating designated bands in Fig. 3A and B is provisional; either the host DNA-binding proteins in the mock-infected cell extract are different from those detected in the infected cell extracts or their properties have been altered during infection inasmuch as their elution profiles were reproducibly different.

Concentration and partial purification of V2 and V4 factors from nuclear extracts of infected cells. The protocol for the purification of the proteins involved in the formation of V2 and V4 DNA-protein complexes is shown in Fig. 2 and is described in Materials and Methods and in the legend to Fig. 2. The purification was monitored by DNA band shift assays (Fig. 4) and by silver staining of proteins electrophoretically separated in denaturing 9.3% polyacrylamide gels (Fig. 5A and B). In each step of the purification procedure, the fractions containing the activity detected by DNA band shift assays were pooled and used as starting material in the next step.

As noted earlier in the text, activity of the factors in the V2 and V4 DNA-protein complexes were barely detectable in crude nuclear extracts of HSV-1-infected cells but were more abundant in those of HSV-2(G)-infected cells. The factors forming the V2 DNA-protein complex eluted in the flowthrough fraction from P11 column chromatography and at a slightly lower salt concentration than V4 from heparin-agarose chromatography (Fig. 4). The Superose 12 column fraction containing most of the V2 activity material yielded on electrophoresis in a denaturing sodium dodecyl sulfate-polyacrylamide gel a single major silver-stained polypeptide band with an apparent molecular weight of 85,000 for HSV-

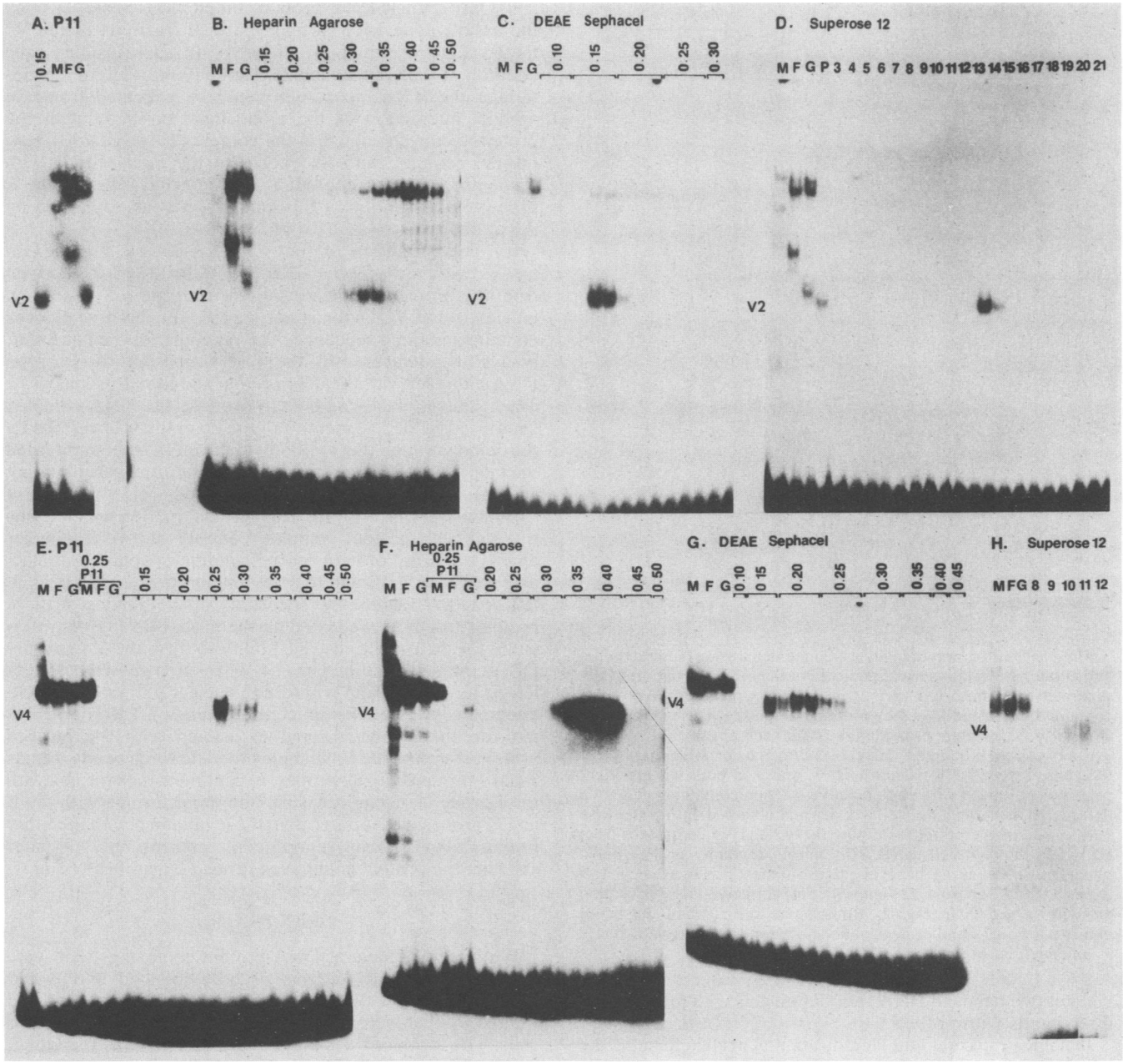

FIG. 4. Autoradiographic images of labeled DNA-protein complexes electrophoretically separated in nondenaturing gels. (A to D) The autoradiograms show the binding activity of the V2 protein fractionated in sequential order by (A) P11 phosphocellulose, (B) heparin-agarose, (C) DEAE-Sephacel, and (D) Superose 12 columns. The chromatographic procedures were as described in the legend to Fig. 2. Lanes M, F, and G containing reaction mixtures of nuclear extracts of mock-, HSV-1(F)-, and HSV-2(G)-infected cells, respectively, with probe C DNA indicate the position of V2 protein-DNA complexes. Fractions collected from each column were identified by the KCl content (P11, heparin-agarose, and DEAE-Sephacel) or serially (Superose 12). (E to H) Same as panels A and D except that probe B DNA was used to probe V4 DNA-protein complexes. Lanes M, F, and G, Reaction mixtures of nuclear extracts of mock-, HSV-2(F)-, and HSV-2(G)-infected cells with probe DNAs. The letters V2 and V4 mark the position of the corresponding bands; as noted in the text, these bands are frequently not seen in assays of crude nuclear extracts.

1(F) (Fig. 5, right panel) or 82,000 for HSV-2(G) (data not shown) and a very faint band with an apparent molecular weight of 60,000. The faint band was not always present; it is seen in lane 4 but not in lane 3 of Fig. 5 and may be a degradation product of the higher-molecular-weight protein.

The material eluting from the Superose 12 column and forming the V4 DNA-protein complex formed two, apparently equimolar, major silver-stained bands with apparent molecular weights of >250,000 and 140,000 (Fig. 5, left panel), referred to as proteins A and B, respectively. Similar bands were obtained from extracts of HSV-2(G)-infected cells, and analyses done with monoclonal antibodies reactive with infected cell protein 1 (ICP1) suggest that protein A is ICP1 (data not shown). It should be stressed, as will be shown below, that at all stages of purification of the proteins forming the V4 complex, the fractions containing the activity

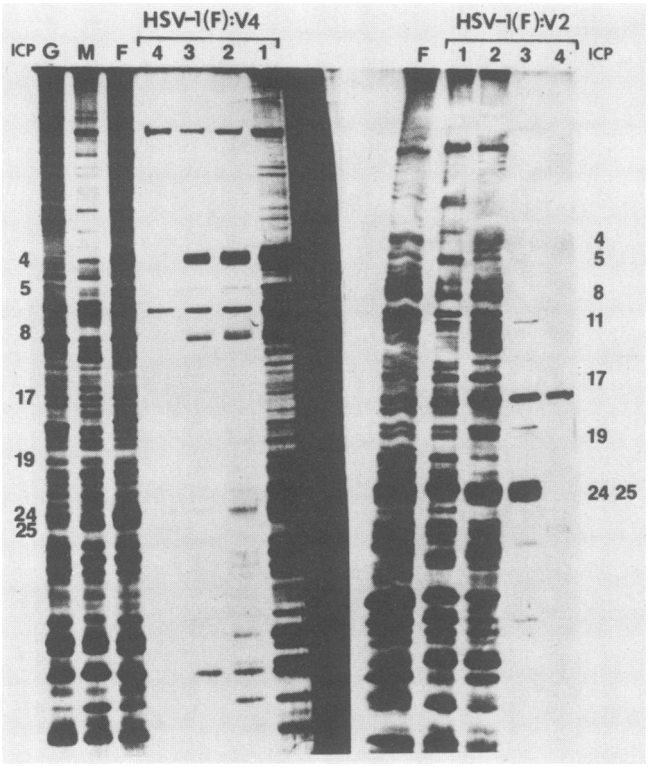

FIG. 5. Photograph of a silver-stained denaturing (sodium dodecyl sulfate) 9.3% polyacrylamide gel containing electrophoretically separated proteins from various stages of purification of proteins contained in V2 and V4 DNA-protein complexes. Lanes M, F, and G were loaded with approximately 20 μg each of the crude nuclear extracts of mock-infected, HSV-1(F)-infected, or HSV-2(G)-infected cells, respectively. Lanes 1, 2, 3, and 4 in both left and right panels were loaded with pooled active fraction containing either V4 (left panel) or V2 (right panel) activity and obtained during stepwise purification of the proteins forming the V2 and V4 complexes from HSV-1(F)-infected cell extracts by chromatography on P11 phosphocellulose (lanes 1), heparin-agarose (lanes 2), DEAE-Sephacel (lanes 3), and Superose 12 (lanes 4), respectively. The purification protocol is shown in Fig. 2. Numbers to the right of the figure indicate the ICP number designations of Honess and Roizman (12) as amended by Morse et al. (24).

yielded two bands in DNA retardation gels with probe B and three bands with probe C.

Biologic properties and identification of protein forming the V2 DNA-protein complex. During studies of the biologic properties of the proteins forming the V2 complex, we noted that addition of the purified V2 (Fig. 5, right panel, lane 4) to the labeled DNA probes in the presence of 10 mM Mg^{2+} resulted in the loss of the terminal ^{32}P label from the probe DNAs (Fig. 6, right panel). One hypothesis that could account for the apparent loss of the labeled DNA probes is that addition of Mg^{2+} activated a DNase. To test the possibility that the protein contained in the V2 DNA-protein complex is the viral DNase, we performed band shift assays in the presence and absence of monoclonal antibody Q1 obtained from K. L. Powell of the Wellcome Research Laboratories and directed against the HSV-1 and HSV-2 enzymes. Earlier we showed that antibody specific to the DNA-binding proteins frequently binds the protein in the DNA-protein complex and further retards the mobility of the DNA (14). The results shown in Fig. 6, left panel, and other studies not shown indicated the following.

(i) Only a single DNA-protein complex was observed in all the assays done with partially purified fractions of the V2 factor (Fig. 5, right panel, lane 4). In each instance, the addition of the Q1 monoclonal antibody retarded the migration of the V2 complex, as would be expected from the specific interaction of the monoclonal antibody with the protein factor contained in the complex (14). A faint DNA-protein complex was also noted in the assays done with crude nuclear extracts of HSV-2(G)-infected cells and the E probe. The migration of this complex was also retarded by the antibody.

(ii) The formation of the V2 DNA-protein complex in assays with the probe F DNA, a 48-bp fragment derived from the promoter-regulatory domain of the α27 gene (13), indicated that V2 factor is not specific for DNA sequences contained in the *a* sequence. The reliability of the band shift assays is underscored by the observation that the F probe DNA did react with the αH1 protein and the virus-specific α *trans* inducing factor (αTIF) to produce the DNA complex shown in Fig. 6. The identity of the complex was verified by the demonstration that antibody to the αTIF protein retarded the migration of the complex in nondenaturing gels (data not shown), as was previously demonstrated (19).

Binding specificity of V4. The purified fraction which forms the V4 DNA-protein complex consists of two equimolar amounts of viral proteins corresponding in apparent molecular weight to ICP1 and to a yet unidentified protein (Fig. 5, left panel). To define the specificity of the binding of these proteins to DNA, we tested all the probes generated in this study for their reactivity and ability to form the V4 protein-DNA complex with the purified V4 fraction shown in Fig. 5, left panel, lane 4, and with crude nuclear extracts of mock-infected cells and cells infected with HSV-1(F). The results indicate that probes D and C reacted with the purified proteins forming the V4 complex to yield three bands. Bands 2 and 3 formed with all probes, except the bands formed with the J probe were barely visible and those formed with the L and G probes were fainter than those obtained with the other probes. Band 1 was reproducibly present only in lanes showing the protein complexes formed by probes D and C.

DISCUSSION

We identified several DNA-protein complexes that were formed by nuclear extracts of both infected and mock-infected cells and two DNA-protein complexes designated V2 and V4 that were specific for nuclear extracts of infected cells. The focus of the studies reported here was the identification of viral proteins which reacted with the *a* sequence of HSV-1. It is convenient to consider the identity and significance of the proteins forming the various complexes separately.

V4 protein-DNA complex. The most purified preparation which formed the probe C DNA-V4 protein complex contained two viral proteins in approximately equimolar amounts. Protein A was identified as ICP1 by its apparent molecular weight and its reactivity with a monoclonal anitbody (9). Protein B comigrated with a protein of a molecular weight predicted for ICP7, but its identity is not known. Of the three bands formed with probe C, the two designated bands 2 and 3 (Fig. 7) were also formed with other probes, whereas the complex designated as band 1 formed only with probes D and C, i.e., the probes that contain at least a portion of the U_c sequence and DR1. We may conclude from these observations that either the specificity of binding requires the interaction of both proteins or one of the two

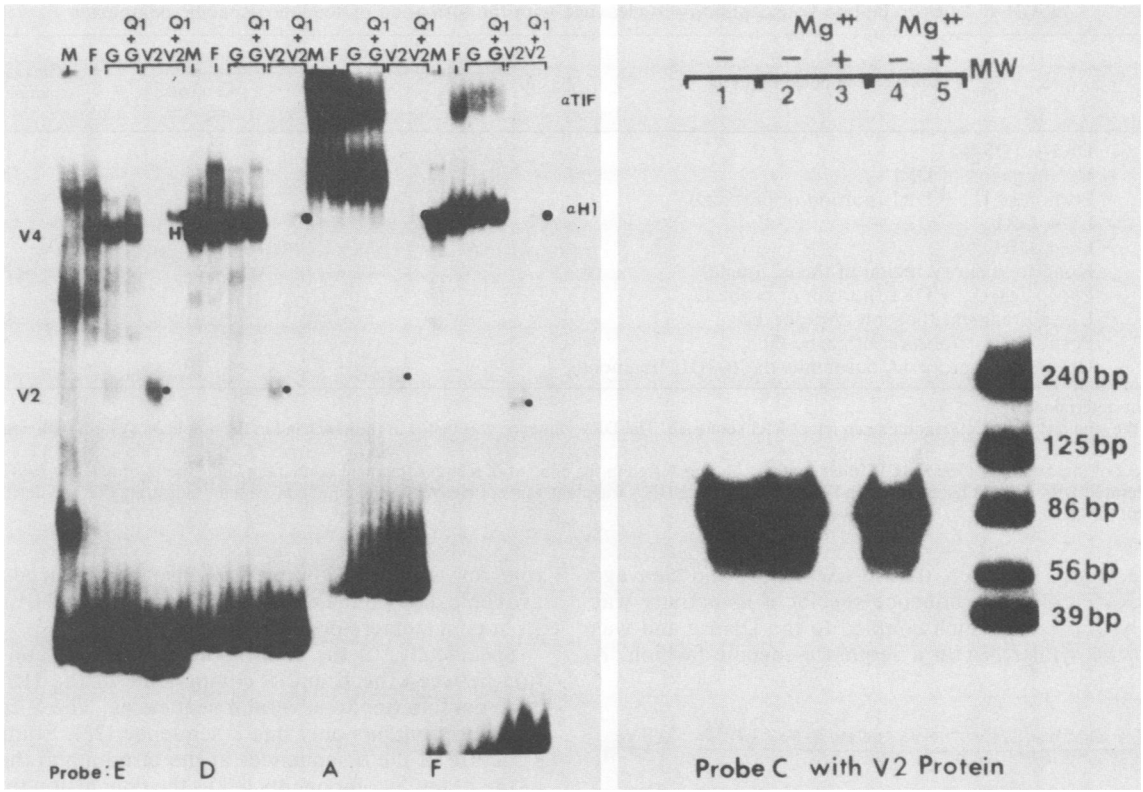

FIG. 6. Autoradiographic images of labeled DNA electrophoretically separated in nondenaturing gels. Right panel: Images of purified V2 protein-labeled probe C DNA complexes formed in the presence or absence of Mg^{2+} in the binding reaction. Lane 1, Labeled probe without protein added; lane 2, probe DNA and 0.2 μl of V2 fraction containing no Mg^{2+}. Lane 3, Same as lane 2 except that the binding buffer contained 10 mM Mg^{2+}. Lanes 4 and 5, Same as lanes 2 and 3, respectively, except that the concentration of V2 protein was 0.5 μl per binding reaction. The V2 preparation is the one shown in Fig. 5, right panel, lane 4. The probe C DNA and the V2 fraction in a total of 20 μl were reacted at 37°C for 1 h. The reaction was terminated by the addition of sodium dodecyl sulfate and proteinase K to final concentrations of 0.4% and 100 μg/ml, respectively. The DNA was extracted with phenol, ethanol precipitated, dried, and subjected to electrophoresis on a 5% nondenaturing gel as described in the Results. Left panel: Reactivity of V2 protein-DNA complexes with monoclonal antibody Q1 specific for HSV DNase. Lanes M, F, and G, Reactions of nuclear extracts of mock-infected, HSV-1(F)-infected, and HSV-2(G)-infected cells described above with various probe DNAs; lanes designated V2, reaction of labeled DNA probes identified on the bottom of the lanes with purified V2 protein shown in Fig. 5, right panel, lane 4. In lanes designated +Q1, the reaction mixture contained in addition to the stated ingredients 1 μl of monoclonal antibody to HSV-1 DNase. The dot to the right of the band identifies the V2 protein-DNA complexes. Note that probe F contains the *cis* site for the induction of α genes by the virion αTIF (19). With HSV-1(F)- or HSV-2(G)-infected cell extracts and the competitor DNAs, probe F DNA forms complexes with a host protein designated as αH1 (15) and with both αH1 and αTIF. The Q1 antibody has no effect on the formation of αH1 or αTIF bands (compare lane G, probe F, with lane G + Q1, probe F). V2 forms a complex with probe F, and this complex is retarded in its electrophoretic mobility by antibody Q1 (rightmost two lanes). MW, Molecular weight.

proteins was copurified adventitiously and binds to DNA nonspecifically.

Proteins A (ICP1) and B have not been extensively characterized. ICP1 is a structural protein (VP1); it is located in the tegument and is not readily removed from virions deenveloped by treatment with nonionic detergents (16). Fewer than 150 molecules of ICP1 per virion have been estimated to be present based on its then estimated molecular weight of 260,000 to 270,000 (9). Since its translated molecular weight is currently predicted to be 336,000 (18), the actual number of molecules per virion might be significantly smaller.

Independently derived data link ICP1 with encapsidated DNA and more specifically with the release of viral DNA from capsids (1). Thus, in cells infected with HSV-1(HFEM)*ts*B7 at the nonpermissive temperature, the capsids are transported to the nuclear pore, but viral DNA is not released into the nucleus. The DNA is released upon shift down to the permissive temperature. The mutation in HSV-1(HFEM)*ts*B7 was mapped by marker rescue techniques to the domain of the ICP1 gene. It is conceivable that ICP1 may be involved in the shuttling of the DNA in and out of the capsids, but at least the packaging function remains to be proved.

The gene specifying protein B has not been identified. In earlier studies, protein B would have been obscured by other virion proteins with similar electrophoretic mobilities (9).

V2 protein-DNA complex. The most purified V2 protein preparation contained a single polypeptide. The electrophoretic mobility of the protein, its enzymatic activity, and its reactivity with monoclonal antibody are consistent with its being the virally encoded DNase. The binding of the DNase to DNA, although not unexpected, has not been previously reported. Although the virally encoded DNase has strong exonucleolytic activity, it has been reported to exhibit non-sequence-specific endonucleolytic activity in the presence of Mg^{2+} (10, 11). The binding of the DNase to the viral DNA fragments does not appear to be sequence specific. It

TABLE 1. Correlation of susceptibility to cleavage with the formation of sequence-specific complexes

Probe	*a* sequence component present	Sequence present			Cleavage function in amplicon or virus
		Pac1	*Pac2*	Cleavage *cis* site	
A	DR2 + $(DR4)_3$	−	−	−	ND[a]
B	U_c + portion of DR1	−	+	−	ND
C	Portion of U_c + DR1 (portion of probe D)	−	+	+	ND
D	U_c + DR1	−	+	+	+ (6)
E	U_b + DR1	+	−	+	− (6)
F	None (regulatory region of the α27 gene)	−	−	−	ND
G	Portion of U_b + DR1 (portion of probe E)	−	−	+	−[b]
H	U_b subfragment (portion of probe E)	+	−	−	ND
J	Portion of U_c (portion of probe D)	−	−	−	ND
L	U_c, same as probe D, but minus the *Bss*HII fragment	−	−	−	−[c]

[a] ND, Not determined.
[b] This is the smallest fragment containing an intact DR1 sequence. The DR1 sequence was tested in the virus and in the amplicon system with negative results (6).
[c] The effect of the deletion of the *Bss*HII fragment from U_c was tested in the context of a larger fragment containing DR1, the U_c minus the *Bss*HII fragment, and the reiterated DR4 and DR2 sequences and U_b sequence. The DNA fragment was not cleaved in the amplicon system, indicating that the deleted sequence was essential for cleavage (6).

should be noted, however, that DNA binding and cleavage by the DNase could be sequence specific if its activity was directed by a protein which coupled to the DNase and was bound to the viral DNA in a sequence-specific fashion. A role for the viral DNase may also be deduced from the available data on the cleavage of unit-length DNA molecules from concatemers or circles.

Specifically, (i) the L component terminus and the junction between the L and S components of the HSV-1 DNA each contain one to several *a* sequences. The S component terminus contains only one *a* sequence (23). Studies on the structure of the *a* sequences at the termini and the junction of the L and S components led to the conclusion that the site of the cleavage of concatemers or circles into linear unit-length DNA molecules is within the DR1 sequence shared by two adjacent *a* sequences and results in single-base 3′ extensions in the ends of the linear molecules (23). The endonuclease responsible for the cleavage of the DNA has not been identified. Although the cleavage and packaging appear to be related (7), it is not inconceivable that viral DNase in connection with other site-directed proteins is responsible for this cleavage. The HSV DNase can act as an endonuclease.

(ii) A key question regarding cleavage-packaging of viral DNA was the fate of newly synthesized molecules with a single *a* sequence at the component junctions. Insertion of a single *a* sequence into virus resulted in one of two events. In some instances, the *a* sequence was reduplicated in tandem, yielding upon cleavage two identifiable ends each containing an *a* sequence. When the *a* sequence was not amplified, the cleavage event occurred in the distal (post-U_c) DR1 and the terminus containing solely the residual portion of DR1 but not the remaining components of the *a* sequence was no longer detectable and was presumed to be degraded (28). If the same sequence of events was to occur in cleavage-

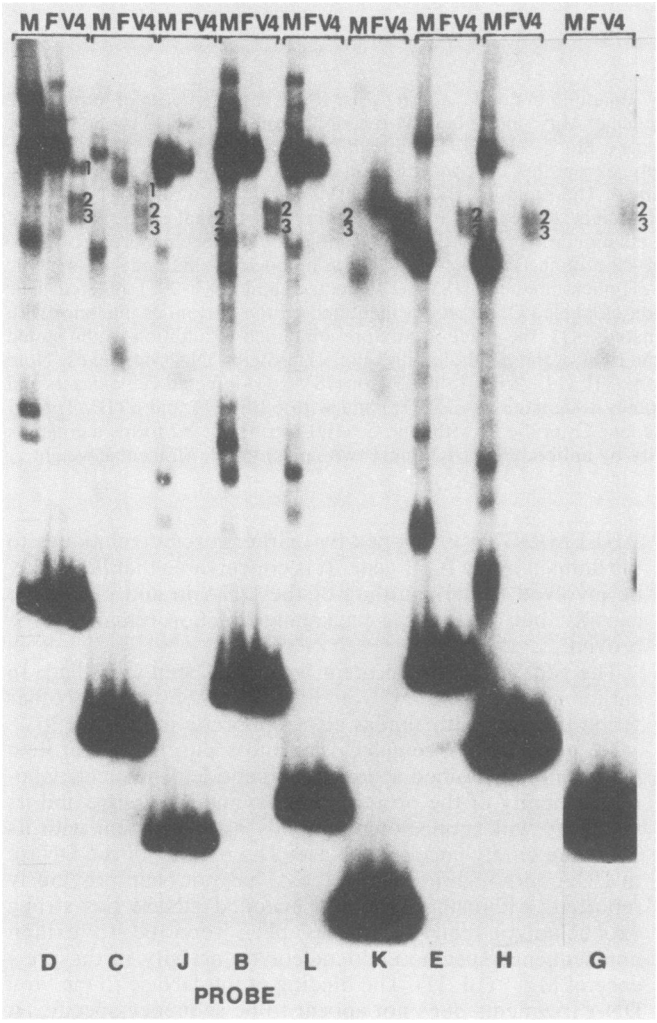

FIG. 7. Autoradiographic images of the complexes formed by labeled DNA probes with crude extracts and purified fractions containing the proteins forming the V4 complex. The complexes formed by each of the probes shown on the bottom of the figure with nuclear extracts of mock-infected cells (M) or HSV-1(F)-infected cells (F) or with purified V4 (V4) are shown in the three corresponding lanes of each triplet. The V4 bands unique to probes D and C are identified as band 1. Bands 2 and 3 appear to form with most probes tested in this study. The purified V4 is that shown in Fig. 5, left panel, lane 4. The complexes were electrophoretically separated on nondenaturing gels. The bands formed with extracts of infected and mock-infected cells and migrating slightly slower than band 1 correspond to those designated H1 in Fig. 3.

packaging of viral genomes from concatemers, it would be expected that the DNA molecule lacking an *a* sequence at its terminus would be degraded exonucleolytically until a component junction containing a competent *a* sequence would be reached. The HSV DNase can act as an exonuclease.

Possible role of proteins forming the V4 complex in cleavage-packaging of HSV-1 DNA. The significant aspects of the interaction of the proteins in the purified preparation with the DNA probes rest on the correlation between the formation of the sequence-specific band 1 in Fig. 7 and the in vivo ability of the probe sequences to act as targets for cleavage-packaging in viral DNA or in amplicons (Table 1). Previous studies have shown that the relevant *cis*-acting sites for bona fide cleavage are in the sequences designated *Pac1* in U_b and *Pac2* in U_c and, in intact *a* sequences, the intervening DR1 sequence which contains the actual site of cleavage of viral DNA from circles or concatemers into unit-length molecules and for packaging into capsids (6, 7, 28). The results reported here indicate that the complex exemplified by band 1 in Fig. 7 is formed only with probes D and C which contain the *Pac2* and DR1 sequences but not with probe E containing *Pac1* and the DR1 sequence. The upper band (band 1) did not form with DNA fragments containing only DR1 or *Pac2* (e.g., probes B and G). The concordant data are that insertion of probe D into amplicons or into viral DNA results in the cleavage of the DR1 of the probe sequence, whereas the insertion of probe E or of the appropriate DNA fragments with a deletion identical to that in probe L does not result in cleavage (6). These observations predict that the V4 proteins form part of the complex recognizing the DR1-U_c sequence required for the cleavage-packaging of the viral DNAs from concatemers. The identification of the viral DNase as one of the proteins binding to our DNA fragments, albeit nonspecifically, may be fortuitous, but at this time we cannot reject the possibility that cleavage of the DR1 sequence by an endonuclease is specifically directed by the V4 protein complexes and, as noted above, that viral DNase has the appropriate attributes to fulfill this role.

ACKNOWLEDGMENTS

We thank K. L. Powell, Wellcome Research Laboratories, for the monoclonal antibody Q1 to the viral DNase and J. Wesley, the University of Chicago, for use of the Superose 12 column for purification of proteins in this work.

These studies were aided by Public Health Service grants CA08494, CA19264, and CA47451 from the National Cancer Institute and AI124009 from the National Institute for Allergy and Infectious Diseases and by grant MV2X from the American Cancer Society.

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