A Host Cell Protein Binds to a Highly Conserved Sequence Element (pac-2) within the Cytomegalovirus *a* Sequence

GEORGE W. KEMBLE AND EDWARD S. MOCARSKI*

Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305

Received 21 April 1989/Accepted 20 July 1989

The human cytomegalovirus (CMV) *a* sequence has significant homology to two regions, pac-1 and pac-2, within the *a* sequence of herpes simplex virus type 1 (HSV-1). Both regions have been shown to be important *cis*-acting signals in HSV-1 genome maturation. We have demonstrated that a small fragment from within the CMV *a* sequence, containing the pac-1 and pac-2 motifs, carries all of the signals necessary for generation of genomic termini and for inversion. These observations indicated that the function of these highly conserved sequence motifs was similar in CMV and HSV-1. We have identified and partially purified a host cell protein with affinity for the sequence 5'-GGCGGGCGGCATAAAA-3' within CMV pac-2. This partially purified protein has an apparent molecular weight of 89,000 under denaturing conditions and could be renatured after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, suggesting that the capacity to bind DNA was the property of a single polypeptide chain. This activity was found in a wide variety of human cell lines, including those that are permissive as well as those that are nonpermissive for CMV growth, but not in cell lines from monkey, mouse, or drosophila origins. Our work implicates a host cell protein in *a* sequence function.

Human cytomegalovirus (CMV), the cause of severe disease in immunocompromised individuals and in the developing fetus, is a prototype member of the betaherpesvirus subfamily of herpesviruses (13, 35, 36). The DNA genome (230 kilobase pairs) contains long (L) and short (S) components with unique sequences (U_L and U_S , respectively) bracketed by inverted repeats (designated *ba* and *ca*, respectively). The genome can be depicted schematically as *ab-U_L-b'a'c'-U_S-ca* (21), an arrangement that is similar to the herpes simplex virus type 1 (HSV-1) genome (35). In both CMV- and HSV-1-infected cells, DNA replication results in the production of concatamers that subsequently mature by being cleaved and packaged into progeny capsids (8, 14, 15, 22, 49). The L and S components invert relative to each other by a localized recombination involving the repeat sequences (2, 28-31, 37). In HSV-1, and to a more limited extent in CMV, the *a* sequence has been shown to play a central role as the *cis* signal in genome maturation and inversion (2, 7, 8, 28-31, 37-40, 43, 46, 49); in addition, the HSV-1 *a* sequence contains a promoter-regulator (3). In HSV-1, the *a* sequence has the structure $DR1 = U_c-(DR4)_n-(DR2)_m-U_b-DR1$ and ranges in overall size from 220 to 500 base pairs (bp), depending on the particular viral strain (7, 27, 29). In CMV, the *a* sequence is larger, lacks DR1-like sequences, and consists of central repeats flanked by unique sequences in the arrangement U_b -(tandem repeats)- U_c (40). The extent of sequence homology between CMV and HSV-1 *a* sequences is very small (40, 45).

The portion of the *a* sequence that is required in *cis* for HSV-1 genome maturation contains two short sequence motifs (7) called pac-1 (within HSV-1 U_b) and pac-2 (within HSV-1 U_c) that are the only sequences highly conserved in the CMV *a* sequence (Fig. 1) (40, 45). In HSV-1, these elements are juxtaposed at an *a-a* junction, which is the cleavage signal (33); deletion of either pac-1 or pac-2 destroys the ability of an *a* sequence to function in genome maturation (7, 46). The occurrence of these elements near the CMV genomic termini and the previous observation that

the CMV *a* sequence can substitute as a cleavage/package signal for HSV-1 (40) suggest the involvement of pac-1 and pac-2 in CMV cleavage/package. The mutant which we describe in this paper demonstrated that this region functions as a cleavage/package signal in the CMV genome.

One potentially powerful approach to understanding the role of conserved sequence motifs involves the isolation of proteins that bind to them. Previously, Dalziel and Marsden identified two small polypeptides found in infected cells, with molecular weights (MW) of approximately 20,000 that bound specifically to the HSV-1 *a* sequence (6), but further work on the identity of these proteins or a definition of the binding site has not been reported. More recent work on the HSV-1 *a* sequence has demonstrated two viral proteins, ICP1 and ICP7, that form sequence-specific DNA protein complexes with a fragment that encompasses both the pac-2 and DR1 sequence motifs (4). We constructed a recombinant CMV (RC2063) and determined that a small fragment of the CMV *a* sequence containing the pac-1 and pac-2 motifs carried all the necessary *cis* elements to serve as both a cleavage/package and recombination signal. Since we demonstrated that these conserved motifs were indeed functional, we set out to identify and characterize viral or host cell proteins that interacted in a sequence-specific manner to this region. Here, we demonstrate that a host cell protein with an apparent MW of 89,000 specifically bound to the CMV pac-2 motif. Notably, this sequence-specific binding protein, referred to as pac-2 DNA binding protein (pac2BP), was found only in cells of human origin. We purified pac2BP 300-fold from high-salt nuclear extracts and established its binding characteristics.

MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblasts (HF) and Hep-2 (ATCC CCL 23) cells were grown in Dulbecco minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% NuSerum (Collaborative Research, Inc., Waltham, Mass.).

Plasmid construction and oligonucleotide synthesis. All restriction enzymes were purchased from New England Bio-

* Corresponding author.

FIG. 1. The structure of the CMV *a* sequence and localization of conserved sequence elements within the region. (A) The top line depicts the CMV genome. The thick black line represents the U_L and U_S regions each bordered by the *b* and *c* repeats (■), respectively. At both ends of the genome as well as at the L-S junction is the *a* sequence (□). The plasmid clone pON208 (40) carries one L-S junction *a* sequence with small amounts of flanking *b* and *c* sequences. The arrows denote the family of direct repeats within the *a* sequence (40). The position of the pac-2 homology (▨) and the position of the pac-1 homology within the *a* sequence (▩) are shown. pON297 carries *b* sequences adjacent to the *a* sequence (■) extending from a *KpnI* site in the *b* sequence to an *NaeI* site at nucleotide 184 within the *a* sequence (40). The plasmid pON2027 carries sequences from the *AvaI* site at nucleotide 754 in the *a* sequence to the *XhoI* site at nucleotide 961 in the *c* sequence. This region contains both the pac-2 and pac-1 homologies (40). Restriction sites in parentheses have been deleted during construction of the plasmids. The *TaqI* site shown in pON208 (not all *TaqI* sites are marked in pON208) is within the *XhoI*-*SalI* fusion point of that plasmid. (B) The dotplot on the top depicts the regions of homology between one entire *a* sequence of CMV (horizontal axis) and HSV-1 (vertical axis). The dotplot on the bottom depicts the regions of homology between the complementary strand of the CMV *a* sequence (horizontal axis) and HSV-1 (vertical). The large arrows indicate the largest regions of homology after the alignments are refined with the BestFit program. Other areas of homology are exclusively G-C rich and of lower significance. The numbers on the outside of the box refer to nucleotide numbers from published sequence (29, 40). (C) Comparison of the pac-1 and pac-2 motifs of CMV (Towne) (40) and HSV-1(F) (29) (large arrows in Fig. 1B). The sequences are gapped and aligned for maximal homology, and the lowercase letters represent mismatches between the two sequences.

Labs, Inc. (Beverly, Mass.) and used according to the instructions of the manufacturer. Nucleotide numbering is as previously reported (40); plasmid pON208. An *AvaI* (nucleotide 754)-to-*PstI* (at nucleotide 967 within the polylinker sequences) fragment from pON208, containing the pac-1 and pac-2 regions of CMV (Towne), was cloned into pUC19 to generate pON2027 (Fig. 1); a *BamHI* (at nucleotide 1, the *BamHI*-*KpnI* fusion point)-to-*NaeI* (nucleotide 184) fragment from pON208 was cloned into *BamHI*-*SmaI*-digested pUC9 to generate pON297 (Fig. 1). A *TaqI* fragment from pON2027, containing pUC19 polylinker sequences adjacent to nucleotides 754 to 962 of the *a* and *c* sequences, was ligated into *ClaI*-digested pON256 (42) to yield pON2063 (destroying the *ClaI* site in the process).

The plasmid pON2 was generated by inserting the *BamHI*-*PstI* *lacZ*-containing fragment from pON1 (41) into *BamHI*-*PstI* digested pMT11 (41). The *BamHI*-*HindIII* *lacZ* fragment from pON1 was ligated into *BamHI*-*HindIII* pGEM-2 to yield pON4.

A series of BAL 31 (23) deletion mutants (pON2059, pON2060, pON2083, pON2084, pON2090, pON2091, and pON2092) were isolated from *HindIII* (nucleotide 976)- or *MluI* (nucleotide 779)-digested pON208, and an *XbaI* linker (5'-CTCTAGAG-3') (New England BioLabs) was inserted to mark the deletion site. Precise deletion endpoints were determined by dideoxy sequencing of double-stranded plasmid DNA with Klenow polymerase (Bethesda Research Laboratories, Gaithersburg, Md.) at 42°C with the substitution of 7-deaza-2'-deoxy-guanosine-5'-triphosphate (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for ddGTP (20).

Single-stranded oligonucleotides prepared on a DNA syn-

thesizer (Applied Biosystems, Inc., Foster City, Calif.) were purified by electrophoresis on a 20% polyacrylamide gel containing 7 M urea and were eluted (26). The two oligonucleotides 5'-GATCCGCTTTTATGCGCCGCCGCCGTCCC AACCA-3' and 5'-GATCTGGTTGGGACGGCGCGGCGG CATAAAAGCG-3' were annealed in 65 mM Tris (pH 7.5)-13 mM $MgCl_2$ by gradually decreasing the temperature as follows: 90°C for 2 min, 68°C for 10 min, 37°C for 20 min, and 22°C for 30 min; the double-stranded oligonucleotide was designated OLpac2_{CMV} (see Fig. 4). The double-stranded oligonucleotide containing one copy of the 18-bp repeat (OL18R_{CMV}) (5'-GATCTCCCATTTGACGTCAATGGG-3') and (5'-GATCCCCATTGACGTCAATGGGA-3') of the CMV enhancer, described previously (1), was used as control.

Blot analysis. Viral DNA was prepared from cytoplasmic extracts of RC256-infected HF as described (40), and DNA from RC2063-infected cells was isolated as described (38). DNA was digested with restriction enzymes, separated on 0.7% agarose gels, transferred to nitrocellulose, and hybridized to the indicated probes. DNA fragments containing *lacZ* to be used as probes were generated by digesting pON2 with either *KpnI* and *ClaI* (5' probe) or *ClaI* and *EcoRI* (3' probe) and isolated from low-melting-point agarose (Bethesda Research Laboratories) by extraction with an Elutip (Schleicher & Schuell, Inc., Keene, N.H.). These fragments were radiolabeled with Klenow polymerase and synthetic hexamers (Pharmacia, Inc., Piscataway, N.J.) (23).

Construction of recombinant virus. CMV (Towne) was used to infect roller bottles at a multiplicity of infection of 0.01, and infected cell DNA was harvested 5 days post-100%

pac -1

CMV(TOWNE)	CgGGGGGTGTtTTTaGcGGGGGG
HSV-1(F)	CcGGGGGTGTgTTTcGgGGGGGG

pac-2

CMV(TOWNE)	CGCCGCaCGCcgcCTTTTaTGCGCcGCCGCC
HSV-1(F)	CGCCGcCGC CTTTTtTGCGC GCCGCC

FIG. 1—Continued.

cytopathic effect. For each roller bottle, the medium was removed and the monolayer was rinsed twice with 140 mM NaCl–2.7 mM KCl–8.3 mM Na_2HPO_4 –1.5 mM KH_2PO_4 . The cells were lysed in 10 mM Tris (pH 7.5)–0.1 M NaCl–0.001 M EDTA–0.6% sodium dodecyl sulfate (SDS)–0.1 mg of proteinase K (Boehringer Mannheim Biochemicals) per ml for 2 h at 37°C. The solution was sequentially extracted twice with phenol-chloroform and ether. The DNA was precipitated by the addition of 2 volumes of isopropanol, collected, desiccated, and suspended in 1 ml of 10 mM Tris (pH 7.5)–1 mM EDTA. Shear forces were kept to a minimum at all points during the extraction procedure. CMV (Towne) DNA (10 μg) was coprecipitated with *Bst*EII-digested pON2063 (1 μg) by the CaPO_4 method and transfected into cells as described (40). Four days after 100% cytopathic effect, virus stocks were made. Serial 10-fold dilutions of the stock were plated into each well of a 96-well dish. Several days after the higher dilutions reached 100% cytopathic effect, the supernatant was placed into an identical position in another 96-well dish and frozen at -70°C . The cells were lysed by the addition of 0.2 ml of 0.2 M NaOH at 22°C for 20 min and added to a dot blot apparatus in which each well contained 0.2 ml of 5 M NaCl (11). After the solution was drawn through the nitrocellulose with vacuum, the filter was removed and rinsed in $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After baking, the filter was hybridized to a ^{32}P -labeled RNA probe specific for *lacZ* sequences generated by the addition of T7 RNA polymerase (Promega Biotec, Madison, Wis.) to *Pvu*II-digested pON4 according to conditions recommended by the manufacturer. Hybridization and washing were performed as described (40), followed by washing the filter in $2\times$ SSC containing 10 μg of RNase A per ml at 22°C for 10 min. The RNase A was removed, and the filter was further washed in $0.2\times$ SSC at 50°C for 30 min. The filter was subjected to autoradiography, and *lacZ*-positive wells present at the highest dilutions were identified. Supernatant from these positive wells was thawed and plated onto monolayers of HF cells in 25-cm² dishes to produce viral stocks. These stocks were serially diluted and plated onto HF cell monolayers in 100-mm tissue culture dishes, and 6 days postinfection, medium containing 0.5% agarose (at 42°C) was overlaid onto the cell monolayer. After 72 h, individual plaques were picked and inoculated onto fresh HF cells (48). After development of 100% cytopathic effect, the media was added to an equal volume of sterile skim milk and frozen at -70°C and the DNA from the infected cells was harvested (38). A virus carrying a disrupted copy of *lacZ* sequences and having the appropriate structure, as determined by Southern blot hybridization analysis with various restriction enzymes, was designated RC2063.

Nuclear extracts. Extracts were prepared from either roller bottle cultures of CMV-infected HF (multiplicity of infection, 10), uninfected HF, or uninfected HEP-2 cells. Cells were rinsed twice with 10 ml of cold phosphate-buffered saline and either scraped from the bottle with a rubber policeman (CMV-infected or uninfected HF) or incubated in 10 ml of phosphate-buffered saline–0.5 mM EDTA at 37°C and dislodged (HEP-2 cells). For each roller bottle, the cells were collected by centrifugation at $1,400\times g$ for 5 min, suspended in 10 ml of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.5)–0.5 mM phenylmethylsulfonyl fluoride–1.0 mM dithiothreitol (DTT)–2 μg of leupeptin per ml–0.1 mM EDTA–10 mM sodium bisulfite (NaHSO_3) (buffer A), collected again, suspended in 2.5 ml of buffer A, and lysed with 10 (CMV-infected or uninfected HF) or 25 (HEP-2) strokes in a Dounce homogenizer (B

pestle). The nuclei were suspended in buffer A (10 ml), collected by low-speed centrifugation, washed in 10 ml of buffer A, and pelleted again. The nuclei were suspended at 1 ml per roller bottle in buffer A–10% sucrose, frozen in liquid N_2 , and stored at -70°C . Nuclear pellets were lysed by a modification of the procedure of Ohlsson and Edlund (34). Briefly, the stored pellets were thawed slowly on ice and the following reagents were added to the slurry to produce the following concentrations: KCl to 15 mM, MgCl_2 to 2 mM, and phenylmethylsulfonyl fluoride to 1 mM. A 4 M concentration of $(\text{NH}_4)_2\text{SO}_4$ was added dropwise to a final concentration of 0.3 M, and the lysate was incubated on ice for 30 min. The lysate was centrifuged at $120,000\times g$ at 4°C for 2 h, the supernatant was decanted, and solid $(\text{NH}_4)_2\text{SO}_4$ was added to a final concentration of 0.2 g/ml. The precipitate was left on ice for 10 min and collected by centrifugation at $18,000\times g$ at 4°C for 20 min. The pellet was suspended at 0.4 ml per roller bottle in 20 mM HEPES (pH 7.5)–0.5 mM phenylmethylsulfonyl fluoride–1 mM DTT–2 μg of leupeptin per ml–10 mM NaHSO_3 –10% glycerol (buffer B)–50 mM NaCl and dialyzed extensively against the same solution. The resulting protein solution was cleared by centrifugation at $10,000\times g$ at 4°C for 10 min, and the supernatant was either used directly or frozen in portions in liquid nitrogen. All protein assays were performed with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, Calif.) or, when Nonidet P-40 (NP40) (BDH, Poole, England) was present, the *o*-phthalaldehyde assay (Pierce Chemical Co., Rockford, Ill.) (50).

Gel mobility shift assay. ^{32}P -labeled probes were generated by filling in the 5' extensions of gel-purified restriction fragments in the presence of Klenow polymerase and [^{32}P]dCTP (Amersham Corp., Arlington Heights, Ill.) or by filling in the 5' extensions of OLpac2_{CMV} in the presence of [^{32}P]dATP and subsequently removing the unincorporated label with a P-10 (Bio-Rad Laboratories) spin column equilibrated in 10 mM Tris (pH 7.5)–1 mM EDTA. The binding reaction (44) contained protein (between 0.02 and 10 μg , as indicated) and poly(dA-dT)-poly(dA-dT) (Pharmacia) (10:1 ratio of protein:nucleic acid) in 30 μl of 20 mM HEPES (pH 7.5)–4% glycerol–90 mM NaCl–1 mM DTT–1 μg of bovine serum albumin. The reaction was incubated for 15 min at 22°C, 5 to 50 fmol of the ^{32}P -labeled probe was added, and incubation was continued for an additional 25 min at 22°C. A 5- μl portion of 0.05% bromophenol blue in 20% Ficoll (Pharmacia) was added to the reaction and loaded onto a 4% polyacrylamide (30:1 ratio of acrylamide:*N,N*-methylenebisacrylamide) gel containing 45 mM Tris hydrochloride (pH 8.3)–45 mM boric acid–1 mM EDTA and electrophoresed. The gels were dried and subjected to autoradiography on Kodak X-ray film with Cronex intensifying screens (Du Pont Co., Wilmington, Del.).

Purification of pac2BP. Nuclear extract was prepared from 126 g of HEP-2 cells as described above. The protein (185 mg) was loaded onto a 50-ml P-11 phosphocellulose column (Whatman, Inc., Clifton, N.J.), equilibrated with buffer B–50 mM NaCl. After washing with 5 column volumes of buffer B–50 mM NaCl, 111 fractions (5 ml each) were collected by using a linear gradient of 0.1 to 0.8 M NaCl in buffer B. The active fractions (0.33 to 0.45 M NaCl) were pooled, dialyzed against buffer B–50 mM NaCl, and loaded onto a 5-ml heparin-agarose column equilibrated in buffer B. The column was washed with 5 column volumes of buffer B–100 mM NaCl, and 48 fractions (1 ml each) were collected by using a linear gradient of 0.1 to 1.0 M NaCl in buffer B. The active fractions (0.5 to 0.6 M NaCl) were pooled, adjusted to 0.1%

NP40, and dialyzed against buffer B–0.1% NP40. The protein solution (0.50 mg) was incubated with 100 μ g of poly(dA–dT)–poly(dA–dT) for 20 min at 4°C. The pac2BP was purified by passing the pooled heparin-agarose fractions through two oligonucleotide affinity (OAF) columns made by conjugating OLPac2_{CMV} to Sepharose as described by Kadonaga and Tjian (17). First, pooled heparin-agarose fractions were added to a 2-ml OAF column and allowed to absorb for 30 min at 4°C, and the unbound material was collected. This OAF column (OAF1) was washed with 5 volumes of buffer B–0.1% NP40–50 mM NaCl and then eluted stepwise with 3 column volumes each of 0.25 M NaCl, 0.35 M NaCl, 0.50 M NaCl and 0.75 M NaCl in buffer B–0.1% NP40. The protein from each fraction was concentrated and desalted with buffer B–0.1% NP40 with a Centricon-30 as described by the manufacturer (Amicon Corp., Danvers, Conn.). The unbound fraction of this column (OAF2) was then reappplied to a second 4-ml OLPac2_{CMV}-conjugated Sepharose column in the same fashion as above (OAF2). After extensively washing the column with buffer B–0.1% NP40–100 mM NaCl, OAF2 was eluted with 3 column volumes of 0.20 M NaCl, 0.35 M NaCl, 0.50 M NaCl, and 0.75 M NaCl in buffer B–0.1% NP40. The 0.35 M NaCl fraction of OAF2 contained the majority of pac2BP activity, and this fraction was combined with the 0.25 M NaCl and 0.35 M NaCl fractions from OAF1 to generate the protein pool OAFP.

DNase I protection. The plasmid pON2027 was digested with *Eco*RI and end labeled with [α -³²P]dATP (Amersham) and Klenow polymerase. DNA purified by phenol extraction was digested with *Ava*II; the released fragment was purified by nondenaturing polyacrylamide gel, electrophoresed, and extracted (26). Approximately 7 fmol of this DNA was incubated with the indicated amount of OAFP for 25 min at 22°C in buffer A; MgCl₂ was then added to a final concentration of 6 mM, and 0.5 U of DNase I (Promega Biotec) was added to the reaction and incubated at 22°C for 1 min. An equal volume of stop buffer was added (2% SDS–0.1 mg of protease K [Boehringer] per ml–20 mM EDTA–0.65 mg of yeast tRNA per ml) and incubated at 68°C for 30 min, and the DNA was extracted once with phenol–chloroform and precipitated with ethanol. The pellet was suspended in 2 μ l of loading solution (80% deionized formamide–10 mM NaOH–10 mM EDTA–0.05% xylene cyanol–0.05% bromophenol blue) and electrophoresed on an 8% polyacrylamide sequencing gel containing 7 M urea. The gels were subjected to autoradiography on Kodak X-ray film with an intensifying screen.

Renaturing the pac2BP activity. By a modified protocol described by Hager and Burgess (12), 800 μ g of nuclear lysate from HEp-2 cells was denatured in SDS sample buffer (2% SDS–3% sucrose–50 mM Tris (pH 7.5)–0.8 M 2- β -mercaptoethanol) by immersion in a boiling water bath for 3 min. The denatured polypeptides were separated by SDS–polyacrylamide gel electrophoresis on a 9.25% (28:0.735 ratio of acrylamide:*N,N'*-diallyltartardiamide) gel with a 3% stacking gel as described (32). Strips were cut at 1-cm intervals with a razor blade, crushed, and eluted in 1.0 ml of 50 mM Tris (pH 7.5)–150 mM NaCl–5 mM DTT–0.1% SDS–0.1 mM EDTA overnight at room temperature. The fluid was collected, and proteins were precipitated by the addition of 5 volumes of cold acetone (–20°C), incubation for 30 min on dry ice, and centrifugation at 10,000 $\times$ *g* for 30 min at 4°C. The pellet was suspended in a small volume (50 μ l) of 20 mM HEPES (pH 7.5)–100 mM NaCl–4% glycerol–0.1% NP40–1 mM DTT (buffer C) containing 6 M guanidine

hydrochloride. After 30 min at room temperature, the guanidine hydrochloride was removed by a P-10 spin column equilibrated with buffer C. The resultant solution was adjusted to 0.2 ml with buffer C, and the proteins were allowed to renature for 60 min on ice. To detect binding activity using the mobility shift assay, 1/10 of this solution was used.

Computer-aided sequence analysis. We used the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package (version 5.3) installed on a Digital Equipment Co. VAX to analyze the sequence data (9). The HSV-1 (29) and CMV (Towne) (40) *a* sequences were aligned by using the Compare program with a window/stringency setting of 18/13, and the results of this analysis were represented with DotPlot. The Bestfit program was used to refine alignments between regions of significant homology.

RESULTS

Conservation of the pac-1 and pac-2 motifs. We focused our attention on a small region from within the CMV *a* sequence carrying both the pac-1 and pac-2 motifs, a 129-bp *Mlu*I-to-*Ava*II fragment of pON2027 (Fig. 1). These motifs were the only regions of striking similarity between HSV-1 and CMV *a* sequences, although their relative position varies (Fig. 1B). In HSV-1, pac-1 and pac-2 are located within U_B and U_C, respectively; however, in CMV both motifs are found in U_C. An alignment of these motifs indicates this high level of sequence similarity (Fig. 1C).

Because experiments with HSV-1 indicated that pac-1 and pac-2 motifs were essential elements in the cleavage/packaging of that virus, we tested whether a small region of the CMV *a* sequence containing these motifs could function as a signal for cleavage/packaging or recombination in the CMV genome. A 220-bp *Taq*I fragment (containing nucleotides 754 to 962 from within the CMV *a* and *c* sequences) was cloned into the *lacZ* open reading frame of pON256, as indicated in Fig. 2, and inserted into the viral genome by homologous recombination after cotransfection of the modified plasmid and intact CMV (Towne) DNA (Fig. 2A). The predicted structure of the recombinant (RC2063) should have the inserted *a* sequence fragment in an inverted orientation relative to the nearest authentic copy of the *a* sequence at the end of the *b* repeat (Fig. 2A). Hybridization of restriction digests of RC2063 with either 5' or 3' *lacZ* probes demonstrated that the insert had the appropriate structure, as indicated in Fig. 2. The *Hind*III patterns of RC2063 and RC256 were similar; the small 220-bp difference between these two viruses was not resolved because of the large size of fragments generated from this region. As expected for an insertion into the *b* sequence, hybridization with the 5' *lacZ* probe identified one terminal and two junction fragments in both RC256 and RC2063 (Fig. 2) (42). The intensity of the terminal fragment in RC256 was greater than that of the two junction fragments. This observation was consistent with the relative abundance of novel termini (0.5 M with respect to fragments from unique sequences) and unique junction fragments (0.25 M) in a preparation of CMV DNA from packaged material (21, 22). In RC2063, however, the apparent reversal of these intensities was caused by the use of DNA prepared from both cytoplasm and nuclei together, in which termini are normally underrepresented because of the presence of endless concatamers in the population (21, 22). The destruction of the *Cl*aI site at the point of insertion was observed (Fig. 2B), and the presence of a novel *Kpn*I site was detected (data not shown), confirming the structure of this recombinant virus. Interestingly, longer exposure of the

FIG. 2. Analysis of RC2063. (A) Diagram of RC2063 and RC256 genomes. The top line represents the position of the *lacZ* insert in the genome of RC256 (42). The *a* (□) and *b* (■) repeats of the virus are expanded, and the 5' (▨) and 3' (▩) *lacZ* probes are indicated in RC256 and RC2063 as well as the remaining *lacZ* and simian virus 40 sequences (▤). The small *a* sequence insert present in RC2063 is expanded, and the locations of the *pac*-1 and *pac*-2 motifs are indicated along with the position of the predicted cleavage site (↖). The *b* repeat

FIG. 3. Sequence-specific binding of pac2BP in nuclear extracts of infected and uninfected cells. Mobility shift (44) assay was performed with 2 μ g of extract from CMV-infected HF (A), uninfected HF (B), or uninfected HEp-2 (C) cells. In each experiment, the nuclear extract was incubated with the 129-bp *MluI*-*AvaII* fragment from pON2027 either in the absence (0) or in the presence of the indicated molar excess of the unlabeled 129-bp *MluI*-*AvaII* competitor fragment from pON2027 (2027) or the unlabeled 106-bp *HindIII*-*AvaII* competitor fragment from pON297 (297) prior to electrophoresis.

autoradiograms revealed a 2.4-kilobase-pair fragment in the *ClaI*-*EcoRI* digest of RC2063, which indicated the presence of a novel terminus at the predicted point (Fig. 2A and B). Besides the data shown in Fig. 2, additional digests (*KpnI*, *ClaI*) were used to confirm the generation of genomic termini from the insert. Moreover, two large 6.5- and 12.0-kilobase-pair novel junction fragments were present, consistent with recombination between the inserted *a* sequence and the *a* sequence at the end of the *b* repeat. This data indicated that the small 220-bp *TaqI* fragment from pON2027 functioned as both a cleavage/packaging signal and recombination signal, consistent with a significant volume of work in HSV-1 and with the previous hypothesis that the pac-1 and pac-2 motifs are functionally conserved between HSV-1 and CMV (40).

Binding specificity of pac2BP. Because of their conservation in many herpesviruses and the indication of their functional importance, we considered the pac-1 and pac-2 motifs likely sites of interaction with proteins. After incubation of the *MluI*-*AvaII* fragment with CMV-infected HF nuclear extract in the presence of poly(dA-dT), DNA-protein complexes were resolved electrophoretically by mobility shift assay (44). Several complexes were detected, most of which were judged to be non-sequence specific because of

their ability to bind several different nonoverlapping DNA probes; however, one was clearly sequence specific on the basis of its inability to bind heterologous probes (data not shown) and competition analysis (Fig. 3A). This specific complex was competed by a 5- to 50-fold excess of cold *MluI*-*AvaII* fragment in the reaction but not by similar amounts of control DNA fragments (a 106-bp *HindIII*-*AvaII* fragment from pON297 [Fig. 3A] carrying *b* sequences as well as other unrelated fragments such as a 102-bp *EcoRI*-*AvaII* fragment from pON297 or a 188-bp *BamHI*-*PvuII* fragment from pUC8 [47] [data not shown]). Thus, this complex was sequence specific, indicating the presence of pac2BP in infected cells.

Host cell origin of pac2BP. pac2BP was present in uninfected HF cells as well as in CMV-infected cells (Fig. 3) and represented a protein of cellular origin. When infected cells were investigated further, we could not detect any increase or decrease in the activity over uninfected cells or any difference in mobility (data not shown). After analysis of a number of human (HEp-2, HF, 143, 293) and nonhuman (VERO [monkey], NIH3T3 [mouse] and Schneider [*Drosophila*]) cell types, we found the activity only in human cells.

at the bottom of the diagram depicts the predicted structure of the RC2063 *b* repeat after recombination between the inserted *a* and the authentic *a* sequence at the terminus of the *b* repeat. (B) DNA blot analysis of RC2063. RC256 or RC2063 DNA was cleaved with the indicated restriction enzyme (*HindIII* [H], *EcoRI* [RI], *KpnI* [K], or *ClaI* [C]), separated on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to either the 5' or 3' *lacZ* probe as indicated. T, Migration of the novel terminus generated by the insert in RC2063; J, Migration of the novel junction fragments generated in the RC2063 genome. The lines at left indicate the migration of λ *HindIII* in the same gel.

FIG. 4. Deletion analysis of the binding site. (A) The sequence of the *MluI*-*AvaII* fragment from the CMV *a* sequence and the regions of significant homology to the *pac-1* and *pac-2* motifs (overlined). The bars below the sequence indicate the sequences carried in BAL 31 deletion mutants. For each clone, the ability to bind *pac2BP* is indicated to the right. The sequence shown at the bottom depicts the position of the double-stranded OL*pac2*_{CMV} oligonucleotide after filling in of the 5' extensions. Uppercase letters represent those regions derived from the *a* sequence and lowercase letters represent those that were added. (B) Autoradiogram of the mobility shift assay of various BAL 31 mutations. Approximately 3 fmol of each ³²P-labeled probe was incubated either with (+) or without (-) 2 μ g of HEP-2 nuclear extract and subjected to the mobility shift assay. The arrow at the left of the figure marks the migration of the sequence specific complex. The probe is identified by the number above each set of lanes and corresponds to the plasmid clone. The specific fragments are as follows: pON2027, *AvaII*-*MluI* fragment; pON2083, pON2084, pON2059, pON2060, *XbaI*-*AvaII* fragment; pON297, *HindIII*-*AvaII* fragment; pON2092, pON2091, pON2090, *XbaI*-*MluI* fragment.

Notably, permissive as well as nonpermissive human cell types contained *pac2BP* (Fig. 3; data not shown).

The site for binding of *pac2BP*. In order to localize the binding site of *pac2BP* within this 129-bp fragment, a series of deletion mutations was constructed and tested by mobility shift assay. BAL 31 deletions were introduced from the *MluI* and *HindIII* sites flanking the binding site, and an *XbaI* linker was inserted to mark the deletion endpoint. DNA fragments were prepared from the resultant clones by diges-

tion with *XbaI* and either *MluI* or *AvaII*. The DNA was gel purified, radiolabeled, and used as a probe in the mobility shift assay. The fragment from pON2092 did not bind as efficiently as other probes from that BAL 31 series; yet, it competed for binding with the intact *MluI*-*AvaII* probe (data not shown). However, the fragment from pON2060, in which the *pac-2* motif was disrupted, failed to bind (Fig. 4B) and failed to compete for binding to the intact fragment (data not shown). Notably, the fragment from pON2060 was only 9 bp

FIG. 5. The minimal pac2BP site. Nine femtomoles of ^{32} P-labeled OLpac2_{CMV} was incubated with 10 μ g of HEp-2 nuclear extract and 1 μ g of poly(dA-dT)-poly(dA-dT) in the presence of an excess of the indicated fragment competitor. Nonlabeled competitor OLpac2_{CMV} was present at a 10-, 25-, 50-, or 100-fold molar excess with respect to the probe as indicated at the top of each lane. pac2_{HSV-1} is a 45-bp *SmaI-AvaII* fragment from the plasmid pON114 (33), which spans the pac-2 region of HSV-1(F). Ub_{HSV-1} is a 41-bp *SmaI-SmaI* fragment from pON114 which encompasses a region within Ub of the HSV-1 *a* sequence. OL18R_{CMV} is a double-stranded, 23-bp synthetic oligonucleotide that spans one 18-bp repeat from the CMV immediate-early enhancer and shares no similarity with OLpac2_{CMV} (1). The arrow locates the level of migration of the pac-2 sequence-specific protein-DNA complex.

shorter than that of pON2059, which readily bound pac2BP, suggesting that the A-T-rich region within the pac-2 homology was important for recognition. We do not believe that pac2BP simply bound to A-T-rich blocks of sequence because neither poly(dA-dT)-poly(dA-dT) nor control fragments (including HSV-1 pac-2) with blocks of A-T-rich sequence competed significantly with this probe. Furthermore, as described below, sequences adjacent to this A-T-rich sequence are important for binding.

With the data from this set of experiments to predict a minimal binding site, two complementary oligonucleotides were synthesized (Fig. 4A), representing sequences that included the A-T-rich element as well as flanking sequences within pac-2. This double-stranded oligonucleotide (OLpac2_{CMV}) both bound the protein efficiently and competed for pac2BP-specific binding (Fig. 5). Heterologous sequences, such as a 45-bp *SmaI-AvaII* fragment from the pac-2 region of HSV-1, a 41-bp *SmaI* fragment from within the *a* sequence of HSV-1, or a 23-bp double-stranded oligonucleotide containing an 18-bp repeat element from the CMV enhancer were not able to compete for the complex even when present in a 100-fold excess over the probe. Since OLpac2_{CMV} could bind efficiently and specifically to the pac2BP, we used this property to purify the protein from uninfected HEp-2 cells.

Partial purification of the pac2BP. Approximately 190 mg of nuclear protein extracted from HEp-2 cells was loaded onto a phosphocellulose column and eluted with a linear gradient of NaCl, as described in Materials and Methods. The fractions were individually tested for binding activity

(Fig. 6), and pac2BP was eluted between 0.33 and 0.45 M NaCl. Active fractions were pooled, desalted, and loaded onto a heparin-agarose column which was eluted with a linear gradient of 0.1 to 1.0 M NaCl. The peak fractions were eluted between 0.50 and 0.60 M NaCl as evident in the mobility shift assay (Fig. 7). This material was subjected to two independent cycles of OAF chromatography (17). Active fractions (0.50 mg total) were pooled, dialyzed, and then incubated with 0.10 mg of poly(dA-dT)-poly(dA-dT) and loaded onto a 2.0 ml OLpac2_{CMV}-Sepharose oligoaffinity column (OAF1). The column was washed extensively with 100 mM NaCl and then eluted with steps of 0.25, 0.35, 0.50, and 0.75 M NaCl in buffer B-0.1% NP40. A significant amount of the binding activity was eluted in the 0.25 and 0.35 M fractions; yet a majority of the protein was found in the flow through of this column (data not shown). To determine whether this protein represented inactive protein or overloading of the matrix, we reapplied the flowthrough from OAF1 to a second 4.0-ml OLpac2_{CMV}-Sepharose column (OAF2). In this case, after washing the column with 100 mM NaCl, we eluted the protein with 0.20, 0.35, 0.50, and 0.75 M NaCl in buffer B-0.1% NP40. A majority of pac2BP was found in the 0.35 M NaCl fraction of OAF2, and less than 50% was found in the flowthrough (Fig. 8). The active fractions from OAF1 and OAF2 were pooled. pac2BP activity had been purified approximately 300-fold over the starting material with this procedure (Table 1). Taking into account both the overall yield of the protein and purity (approximately 20%) of this step, pac2BP represents 0.1% of the total nuclear protein in the cell (Table 1; data not shown).

pac2BP DNase I footprint. In order to determine the binding domain, pac2BP (OAFP) was used in a DNase I footprint analysis on the *EcoRI-AvaII* fragment from pON2027 (Fig. 9). Protected nucleotides extended over the region 5'-GGCGGCGGCGCATAAA-3' in agreement with the BAL 31 deletion analysis. Thus, the A-T stretch and flanking G-C-rich sequences of the pac-2 region interact with pac2BP. The T residue highlighted by an arrow in Fig. 9 was hypersensitive to DNase I even at low concentrations of pac2BP, suggesting that the protein interaction resulted in structural changes near the binding site.

The apparent MW of pac2BP. We determined the MW of pac2BP by SDS-polyacrylamide gel electrophoresis. Binding activity was renatured from electrophoretically separated HEp-2 nuclear proteins. After cutting the gel into 11 sections, eluted and renatured protein was tested by mobility shift assay (Fig. 10). The pac2BP was eluted in a fraction (MW, 75,000 to 93,000) with an overall recovery of 5%. A silver stain of the fractions from OAF2 is presented beneath the gel shift autoradiogram. Two proteins were evident in the stained gel of the affinity-purified material; one with an MW of 60,000 which was present in all of the fractions from this column and one with an apparent MW of 89,000 which was present in only the 0.35 M NaCl fraction, the only fraction with binding activity (Fig. 8). These data taken together demonstrate pac2BP to be a monomeric protein with an MW of 89,000.

DISCUSSION

We have constructed the recombinant CMV RC2063 to address the location of the functional regions within the CMV *a* sequence. We demonstrated that the region of the *a* sequence containing the pac-1 and pac-2 motifs apparently functions as both a cleavage/packaging signal and recombination signal in CMV-infected cells, a result consistent with

FIG. 6. The pac2BP elution profile after phosphocellulose column chromatography. (A) The protein profile (◇) and ionic strength (NaCl (◆)) of column fractions. The bar indicates the position in the gradient of the pac2BP activity as determined by the mobility shift assay. (B) Mobility shift assay of fractions. ^{32} P-labeled OLpac_{CMV} was added to 10 µg of the HEp-2 extract in the first three lanes. Sequence specificity of binding was determined by including a 50-fold excess of unlabeled OLpac_{CMV} in the reaction (+ Sp. Oligo) or a 50-fold excess of unlabeled OL18R_{CMV} in the reaction (+ N.S. Oligo). The arrow at the left marks the migration of the sequence-specific complex. Lane 4 contains 1 µg of the crude material as a reference, and the other lanes contain 1 µg of various fractions indicated by the number above the lane. The bar (fractions 54 to 70) indicates peak fractions of pac2BP activity that were pooled for further purification.

the established role of pac-1 and pac-2 motifs in HSV-1 genome maturation (2, 7, 46). We have identified a heretofore unrecognized host cell polypeptide that binds in a sequence specific manner to the pac-2 homology within the CMV *a* sequence. This recognition site does not show significant homology to sites for other known DNA-binding proteins. The binding site has separate A-T- and G-C-rich regions that are not characteristic of known sites for other proteins. pac2BP binds as a single polypeptide chain to the DNA based on two observations. First, a prominent species (MW, 89,000) was detected in active fractions from OAF2, and second, only one size (MW, 75,000 to 93,000) fraction of separated nuclear proteins was sufficient to reconstitute binding activity. This fraction very likely contains the protein (MW, 89,000) found in OAF2. This method provides a useful way to determine an MW range for proteins that can be renatured after SDS-polyacrylamide gel electrophoresis.

The pac2BP did not bind as well to the pac-2 region of HSV-1 as did CMV, as judged by competition and direct binding analyses. A 100-M excess of cold HSV-1 pac-2 competed no better than a 10-fold excess of CMV pac-2 in

the binding reaction (Fig. 5); therefore, pac2BP binds at least 10 times less efficiently to the HSV-1 pac-2 sequence. Furthermore, with the HSV-1 pac-2 sequence used as a probe in the binding assay, a complex with pac2BP was not observed. Although similar, the pac-2 motifs of CMV and HSV-1 are not identical, and gaps must be introduced to achieve maximal alignment of the two sequences (Fig. 1). These gaps are likely to influence the binding specificities and affinities of different proteins. Preliminary results indicated that a DNA fragment encompassing the pac-2 region of HSV-1 binds a protein, distinct from pac2BP, from crude extracts of HEp-2 cells. Similarly, Chou and Roizman identified a protein in uninfected HeLa cells that bound the region of the HSV-1 pac-2 sequence in an assay that used poly(dI-dC)-poly(dI-dC) (designated H1 in reference 4). pac2BP may represent a family of proteins that recognize related but different sequences and carry out similar functions. The fact that pac2BP described here recognizes the CMV motif and is present only in human cells could be important in CMV biology. CMV is highly species specific and replicates well only in differentiated human cells (13).

FIG. 7. The elution profile of pac2BP after heparin (Hep.)-agarose chromatography. (A) The protein profile ($\diamond$) and ionic strength (NaCl [$\blacklozenge$]) of column fractions. The bar (fractions 22 to 34) indicates the position in the gradient of the pac2BP activity as determined by the mobility shift assay. (B) Mobility shift assay of the fractions. ^{32} P-labeled OLpac2_{CMV} was added to 1 μ g of the peak fractions from the phosphocellulose column, and specificity of binding was determined as described in the legend to Fig. 6. Approximately 1/200 of the column flowthrough (Flo-Thru) and every second fraction from the heparin-agarose column were assayed for activity. The bar (fractions 22 to 34) indicates peak fractions that were pooled for further purification.

pac2BP alone could not cause this restriction, although it could be one of a number of human proteins required for CMV growth.

Insight into the function of pac2BP during the viral life cycle can be gained by examining the role of the *cis* signal to which it binds. The pac-2 signal is a highly conserved sequence occurring near the termini in all herpesviruses (7, 24; this paper). Genetic characterization of this signal in HSV-1 has revealed its role in both genome maturation and site-specific recombination (2, 7, 8, 28–31, 37–40, 43, 46, 49). Deletion mutants within the HSV-1 *a* sequence that destroy the pac-2 motif are not efficient at the cleavage/packaging process (7, 46). Furthermore, although pac-2 alone cannot function as a cleavage/packaging signal, it can direct the insertion of an intact *a* sequence to restore this function (7). Site-specific recombination plays a role in the inversion of L and S components in HSV-1, and the *a* sequence carries important *cis*-acting elements for inversion (2, 28–31, 37). Also, in concordance with the observation, recent studies

FIG. 8. Affinity purification of pac2BP. Material from OAF2 was concentrated in a Centricon-30, and approximately 1/400 of each fraction was tested for the ability to bind ^{32} P-labeled OLpac2_{CMV} (45 fmol total). A significant amount of pac2BP is present in both the flowthrough fraction (Flo), and the 350 mM NaCl fraction of this column. LOAD represents 1/800 of the material that was loaded onto this column. Specificity of binding was determined as described in the legend to Fig. 6.

indicate that the U_c region of the HSV-1 *a* sequence contains a signal essential for site-specific recombination between *a* sequences (J. Smiley, personal communication).

Host cell proteins play essential roles in bacteriophage genome recombination and packaging (for reviews, see references 5 and 10). For example, integration host factor, a sequence-dependent DNA-binding protein of *Escherichia coli*, is required in combination with terminase for lambda phage genome maturation (10). Integration host factor also is involved in site-specific recombination that gives rise to integration and excision of lambda (10). Similarly, the *fis*

TABLE 1. The purification of pac2BP from uninfected HEP-2 cells

Material	Total mg of protein	Sp act (U/mg) ^a	Total no. of units	% Recovery	Purification factor
HEP-2 crude nuclear protein	184	100	18,400	100	1
Phosphocellulose column active fractions	21.84	250	5,460	30	2.5
Heparin-agarose active fractions	0.425	5,263	2,237	12	53
OAF pooled active fractions	0.018	27,222	490	3	272

^a A unit is defined as the amount of protein necessary to shift 20 fmol of OLpac2_{CMV} specifically in the standard gel mobility shift assay containing 45 fmol of probe.

FIG. 9. DNase I footprint analysis of pac2BP. Seven femtomoles of an *EcoRI-AvaII* from pON207 uniquely labeled at the *EcoRI* site was incubated with various amounts of OAF2 and digested with DNase I. After phenol-chloroform extraction, the DNA was separated on an 8% polyacrylamide-urea gel. The region of protection from DNase I is indicated by a stippled bar at the left of the sequence, and the arrow at the left indicates the hypersensitivity of the DNA to the nuclease. The Maxam and Gilbert sequence ladders were run in parallel to the DNase I digestion products as markers.

gene product is important in Mu phage site-specific recombination (5, 16, 18, 19). The involvement of either integration host factor in the packaging of certain phage genomes or *fis* in the site-specific recombination in Mu may provide insight into the importance of pac2BP in the life cycle of CMV. Work is proceeding in our laboratory to analyze pac2BP binding site deletions for a role in the packaging of viral DNA or inversion of genome components. The information gained from the experiments to define the role of the binding site and identify pac2BP through sequence analysis should lead us to a better understanding of the role of the pac-2 motif and the pac2BP in the genome inversion and maturation mechanisms in CMV and other herpesviruses.

FIG. 10. Molecular weight determination of the pac2BP. (A) ^{32} P-labeled OLpac2_{CMV} (45 fmol) was incubated with 10 μ g of crude HEP-2 extract (LOAD), and the specificity of binding was determined as described in the legend to Fig. 6. The arrow indicates the position of the sequence-specific complex. One-tenth of each renatured fraction (indicated by the number above the lane) from SDS-polyacrylamide gel electrophoresis was used in the subsequent lanes. The position of the molecular weight markers is indicated above the fraction numbers. (B) Silver stain (25) of pac2BP active material after SDS-polyacrylamide gel electrophoresis of a 9.25% gel. Approximately two binding units of each pac2BP pool was denatured and loaded onto the gel. The arrows mark the migration of the material (molecular weight, 89,000 and 60,000) in the affinity-purified fraction. Abbreviations: Hep-2 crude; crude nuclear extract; P-cell Pool, fractions 54 to 70 from phosphocellulose column pooled; Ha-pool, fractions 22 to 34 from heparin-agarose chromatography pooled; OAF2, material from the second oligonucleotide affinity column; FLO, flowthrough fraction.

ACKNOWLEDGMENTS

We greatly appreciate advice and materials provided by I. R. Lehman, J. J. Crute, and G. W. G. Wilkinson and the technical

assistance of M. Kirichenko. We thank W. Manning and A. Geballe for the gifts of pON2 and pON4, respectively.

This work was supported by a Public Health Service grant (AI20211) from the National Institutes of Health. George Kemble was supported by a Public Health Service Training Grant (AI07328) from the National Institutes of Health.

LITERATURE CITED

- Cherrington, J. M., and E. S. Mocarski. 1989. Human cytomegalovirus iel transactivates the α promoter-enhancer via an 18-base-pair repeat element. *J. Virol.* **63**:1435-1440.
- Chou, J., and B. Roizman. 1985. Isomerization of herpes simplex virus 1 genome: identification of the cis-acting and recombination sites within the domain of the α sequence. *Cell* **41**:803-811.
- Chou, J., and B. Roizman. 1986. The terminal α sequence of the herpes simplex virus genome contains the promoter of a gene located in the repeat sequences of the L component. *J. Virol.* **57**:629-637.
- Chou, J., and B. Roizman. 1989. Characterization of DNA sequence-common and sequence-specific proteins binding to cis-acting sites for cleavage of the terminal α sequence of the herpes simplex virus 1 genome. *J. Virol.* **63**:1059-1068.
- Craig, N. L. 1985. Site-specific inversion: enhancers, recombination proteins, and mechanism. *Cell* **41**:649-650.
- Dalziel, R. G., and H. S. Marsden. 1984. Identification of two herpes simplex virus type 1-induced proteins (21K and 22K) which interact specifically with the α sequence of herpes simplex virus DNA. *J. Gen. Virol.* **65**:1467-1475.
- Deiss, L. P., J. Chou, and N. Frenkel. 1986. Functional domains within the α sequence involved in the cleavage-packaging of herpes simplex virus DNA. *J. Virol.* **59**:605-618.
- Deiss, L. P., and N. Frenkel. 1986. Herpes simplex virus amplicon: cleavage of concatemeric DNA is linked to packaging and involves amplification of the terminally reiterated α sequence. *J. Virol.* **57**:933-941.
- Devereux, J., P. Haeblerli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387-395.
- Friedman, D. I. 1988. Integration host factor: a protein for all reasons. *Cell* **55**:545-554.
- Fung, M.-C., K. Y. M. Chiu, T. Weber, T.-W. Chang, and N. T. Chang. 1988. Detection and purification of a recombinant human B lymphotropic virus (HHV-6) in the baculovirus expression system by limiting dilution and DNA dot-blot hybridization. *J. Virol. Methods* **19**:33-42.
- Hager, D. A., and R. R. Burgess. 1980. Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: results with sigma subunit of Escherichia coli RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. *Anal. Biochem.* **109**:76-86.
- Ho, M. 1982. Cytomegalovirus: biology and infection. Plenum Publishing Corp., New York.
- Jacob, R. J., L. S. Morse, and B. Roizman. 1979. Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatamers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of virion DNA. *J. Virol.* **29**:448-457.
- Jean, J. H., N. Yoshimura, T. Furukawa, and S. A. Plotkin. 1978. Intracellular forms of the parental human cytomegalovirus genome at early stages of the infective process. *Virology* **86**:281-286.
- Johnson, R. C., M. F. Bruist, and M. I. Simon. 1986. Host protein requirements for in vitro site-specific DNA inversion. *Cell* **46**:531-539.
- Kadonaga, J. T., and R. Tjian. 1986. Affinity purification of sequence-specific DNA binding proteins. *Proc. Natl. Acad. Sci. USA* **83**:5889-5893.
- Kahmann, R., F. Rudt, C. Koch, and G. Mertens. 1985. G inversion in bacteriophage Mu DNA is stimulated by a site within the invertase gene and a host factor. *Cell* **41**:771-780.
- Koch, C., J. Vandekerckhove, and R. Kahmann. 1988. Escherichia coli host factor for site-specific DNA inversion: cloning and characterization of the fis gene. *Proc. Natl. Acad. Sci. USA* **85**:4237-4241.
- Kornelok, R. G., F. Quan, and R. A. Gravel. 1985. Rapid and reliable dideoxy sequencing of double-stranded DNA. *Gene* **40**:317-323.
- LaFemina, R. L., and G. S. Hayward. 1980. Structural organization of the DNA molecules from human cytomegalovirus, p. 39-55. In B. N. Fields, R. Jaenisch, and C. F. Fox (ed.), *Animal virus genetics*, vol. 28. Academic Press, Inc., New York.
- LaFemina, R. L., and G. S. Hayward. 1983. Replicative forms of human cytomegalovirus DNA with joined termini are found in permissive human cells but not in non-permissive Balb/c-3T3 mouse cells. *J. Gen. Virol.* **64**:373-389.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Marks, J. R., and D. H. Spector. 1988. Replication of the murine cytomegalovirus genome: structure and role of the termini in the generation and cleavage of concatamers. *Virology* **162**:98-107.
- Marshall, T. 1984. Detection of proteins in polyacrylamide gels using an improved silver stain. *Anal. Biochem.* **136**:340-346.
- Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* **65**:499-560.
- Mocarski, E. S., L. P. Deiss, and N. Frenkel. 1985. Nucleotide sequence and structural features of a novel US-a junction present in a defective herpes simplex virus genome. *J. Virol.* **55**:140-146.
- Mocarski, E. S., L. E. Post, and B. Roizman. 1980. Molecular engineering of the herpes simplex virus genome: insertion of a second L-S junction into the genome causes additional genome inversions. *Cell* **22**:243-255.
- Mocarski, E. S., and B. Roizman. 1981. Site-specific inversion sequence of the herpes simplex virus genome: domain and structural features. *Proc. Natl. Acad. Sci. USA* **78**:7047-7051.
- Mocarski, E. S., and B. Roizman. 1982. Herpesvirus-dependent amplification and inversion of cell-associated viral thymidine kinase gene flanked by viral α sequences and linked to an origin of viral DNA replication. *Proc. Natl. Acad. Sci. USA* **79**:5626-5630.
- Mocarski, E. S., and B. Roizman. 1982. Structure and role of the herpes simplex virus DNA termini in inversion, circularization and generation of virion DNA. *Cell* **31**:89-97.
- Morse, L. S., L. Pereira, B. Roizman, and P. A. Schaffer. 1978. Anatomy of herpes simplex virus (HSV) DNA. X. Mapping of viral genes by analysis of polypeptides and functions specified by HSV-1 X HSV-2 recombinants. *J. Virol.* **26**:389-410.
- Nasseri, M., and E. S. Mocarski. 1988. The cleavage recognition is contained within sequences surrounding an α - α junction in herpes simplex virus DNA. *Virology* **167**:25-30.
- Ohlsson, H., and T. Edlund. 1986. Sequence-specific interactions of nuclear factors with the insulin gene enhancer. *Cell* **45**:35-44.
- Roizman, B. 1980. Genome variation and evolution among herpesviruses. *Ann. N.Y. Acad. Sci.* **354**:472-483.
- Roizman, B., L. E. Carmichael, F. Deinhardt, G. de The, A. J. Nahmias, W. Plowright, F. Rapp, P. Sheldrick, M. Takahashi, and K. Wolf. 1981. Herpesviridae. Definition, provisional nomenclature, and taxonomy. *Intervirology* **16**:201-217.
- Smiley, J. R., B. S. Fong, and W. Leung. 1981. Construction of a double-jointed herpes simplex virus DNA molecule: inverted repeats are required for segment inversion, and direct repeats promote deletion. *Virology* **113**:345-362.
- Spaete, R. R., and N. Frenkel. 1982. The herpes simplex virus amplicon: a new eucaryotic defective-virus cloning-amplifying vector. *Cell* **30**:295-304.
- Spaete, R. R., and N. Frenkel. 1985. The herpes simplex virus amplicon: analyses of cis-acting replication functions. *Proc. Natl. Acad. Sci. USA* **82**:694-698.
- Spaete, R. R., and E. S. Mocarski. 1985. The α sequence of the cytomegalovirus genome functions as a cleavage/package sig-

- nal for herpes simplex virus defective genomes. *J. Virol.* **54**: 817–824.
41. Spaete, R. R., and E. S. Mocarski. 1985. Regulation of cytomegalovirus gene expression: α and β promoters are *trans* activated by viral functions in permissive human fibroblasts. *J. Virol.* **56**:135–143.
  42. Spaete, R. R., and E. S. Mocarski. 1987. Insertion and deletion mutagenesis of the human cytomegalovirus genome. *Proc. Natl. Acad. Sci. USA* **84**:7213–7217.
  43. Stow, N. D., E. C. McMonagle, and A. J. Davison. 1983. Fragments from both termini of the herpes simplex virus type 1 genome contain signals required for the encapsidation of viral DNA. *Nucleic Acids Res.* **11**:8205–8220.
  44. Strauss, F., and A. Varshavsky. 1984. A protein binds to a satellite DNA repeat at three specific sites that would be brought into mutual proximity by DNA folding in the nucleus. *Cell* **37**:889–901.
  45. Tamashiro, J. C., and D. H. Spector. 1986. Terminal structure and heterogeneity in human cytomegalovirus strain AD169. *J. Virol.* **59**:591–604.
  46. Varmuza, S. L., and J. R. Smiley. 1985. Signals for site-specific cleavage of HSV DNA: maturation involves two separate cleavage events at sites distal to the recognition sequences. *Cell* **41**:793–802.
  47. Vieira, J., and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**:259–268.
  48. Villarreal, L. P., and P. Berg. 1977. Hybridization in situ of SV40 plaques: detection of recombinant SV40 virus carrying specific sequences of nonviral DNA. *Science* **196**:183–185.
  49. Vlazny, D. A., A. Kwong, and N. Frenkel. 1982. Site-specific cleavage/packaging of herpes simplex virus DNA and the selective maturation of nucleocapsids containing full-length viral DNA. *Proc. Natl. Acad. Sci. USA* **79**:1423–1427.
  50. Weidekamm, E., D. F. H. Wallach, and R. Fluckiger. 1973. A new sensitive, rapid, fluorescence technique for the determination of proteins in gel electrophoresis and in solution. *Anal. Biochem.* **54**:102–114.