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Identification of the Gene Encoding the 65-Kilodalton DNA-Binding Protein of Herpes Simplex Virus Type 1

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Hybrid arrest of in vitro translation was used to localize the region of the herpes simplex virus type 1 genome encoding the 65-kilodalton DNA-binding protein ($65K_{DBP}$) to between genome coordinates 0.592 and 0.649. Knowledge of the DNA sequence of this region allowed us to identify three open reading frames as likely candidates for the gene encoding $65K_{DBP}$. Two independent approaches were used to determine which of these three open reading frames encoded the protein. For the first approach a monoclonal antibody, MAb 6898, which reacted specifically with $65K_{DBP}$, was isolated. This antibody was used, with the techniques of hybrid arrest of in vitro translation and in vitro translation of selected mRNA, to identify the gene encoding $65K_{DBP}$. The second approach involved preparation of antisera directed against oligopeptides corresponding to regions of the predicted amino acid sequence of this gene. These antisera reacted specifically with $65K_{DBP}$, thus confirming the gene assignment.

Many proteins which bind to DNA play some role in regulation of transcription or DNA replication. Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) induce the expression of up to 20 DNA-binding proteins (DBPs) as determined by their affinity for DNA-containing matrices (3, 36). Only a few have been studied in detail: the most abundant of these is the major DBP, infected-cell polypeptide 8 (ICP8), or ICSP11,12 (8, 36, 45), of M_r 128,000 (1,196 amino acids [37]). ICP8 binds preferentially to single-stranded DNA (18, 32), is able to destabilize poly(dA-dT) helices (31), and is required for the synthesis of HSV DNA (11, 20). The HSV DNA polymerase and the alkaline exonuclease also bind to DNA, and analysis of temperature-sensitive mutants with lesions in these genes demonstrates that they too are essential for viral DNA replication (16, 28, 35). ICSP34,35 is another distinct protein which binds to DNA and copurifies with the HSV-2 DNA polymerase (43). The function of ICSP34,35, however, is not known. Interestingly, these four DBPs may interact in vivo, since immunoabsorbent columns containing monoclonal antibodies (MAbs) directed to the major DBP, the alkaline exonuclease, or ICSP 34,35 bind at least two of these four proteins. These interactions have been postulated to reflect the existence of replication complexes (42).

Recently, we partially characterized a 65-kilodalton DBP ($65K_{DBP}$) of HSV-1, which was the major protein species in high-salt eluates from double-stranded DNA-cellulose columns used to fractionate HSV nuclear proteins (22). Whether the protein is intrinsically DNA binding or whether its binding is mediated by some other protein is not known. We demonstrated that it was distinct from the 65-kilodalton virion *trans*-inducing factor ($65K_{TIF}$), which stimulates transcription from immediate-early genes (2, 5, 30), and showed that its gene mapped to between coordinates 0.574 and 0.682 on the HSV-1 genome (22). These coordinates were based on published restriction enzyme and transcript maps (collated in reference 38). Determination of the DNA sequence of the entire HSV-1 genome (D. J. McGeoch et al., manuscript in preparation) permits absolute coordinates for HSV-1 to be calculated, although interpolation is still required to determine coordinates for HSV-2. Throughout this manuscript we use these more accurate values. For $65K_{DBP}$ the revised coordinates are 0.580 to 0.687.

The strong affinity of $65K_{DBP}$ for DNA suggested to us that it might play an important role in HSV replication and prompted us to further characterize it. In this communication we describe the isolation of a MAb which specifically reacts with $65K_{DBP}$ and its use to identify the in vitro translation products of HSV mRNA encoding $65K_{DBP}$. In addition, hybrid-arrested translation experiments have allowed us to further localize the region encoding $65K_{DBP}$ to within the *Hin*dIII L fragment. The sequence of this region (24) was used in two different and independent methods, hybrid selection of mRNA with specific nucleotide probes and reaction with sera directed against oligopeptides corresponding to regions of the predicted amino acid sequence, to unambiguously identify the gene encoding $65K_{DBP}$.

MATERIALS AND METHODS

Cells, virus, and radioactive labeling. Baby hamster kidney (BHK) clone 21 cells were used throughout. Stocks of the HSV-1 17syn$^+$ strain (4) and the HSV-2 HG52 strain (40) were prepared as described previously. To label viral proteins with [^{35}S]methionine, we infected BHK cells with HSV-1 at a multiplicity of infection of approximately 10 to 20 PFU/cell and grew them at 37°C in Eagle medium containing 20% the normal concentration of methionine and supplemented with 2% calf serum. At 4 h postinfection, [^{35}S]methionine (specific activity, >1,000 Ci/mmol; Amersham International plc) was added to yield a final concentration of 20 μCi/ml, and incubation continued at 37°C until 18 to 24 h postinfection. High-salt extracts of labeled cells for the preparation of DBPs were made as described previously (22).

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Preparation of MAbs and antipeptide sera. Previously we reported the production of an antiserum, 13809, in rabbits by using a preparation of DBPs highly enriched for $65K_{DBP}$ (22). To produce a MAb we emulsified the same preparation in an equal volume of Freund complete adjuvant and used it to immunize mice with three injections intraperitoneally at 7-day intervals. At 5 weeks after the last injection the mice were boosted with the same DBP preparation diluted threefold in phosphate-buffered saline and then bled 10 days later from the retro-orbital sinuses. Serum samples were tested for their ability to immunoprecipitate a 65-kilodalton polypeptide (a 65K polypeptide) from HSV-1-infected cells. Mice giving a positive response were sacrificed, their spleens were removed, and the cells were fused with P3-X67 Ag8 myeloma cells as described previously (9). Culture supernatants from clones were screened for their ability to immunoprecipitate a 65K HSV-1 protein from infected cells, one reactive clone (MAb 6898) was inoculated intraperitoneally into mice, and ascites fluid was harvested.

The MAb LP1 directed against $65K_{TIF}$ was a gift from A. C. Minson, Cambridge University, Cambridge, England, and has been described elsewhere (22, 27).

Peptides were obtained from Cambridge Research Biochemicals, Ltd., Cambridge, England. Peptide 1 (Table 1) was coupled to bovine serum albumin via the tyrosine residue with bisdiazobenzidine (1), whereas peptide 2 (Table 1) was coupled to bovine serum albumin with glutaraldehyde (17, 44). Antipeptide sera were produced in rabbits by immunization with an emulsion consisting of 1.5 mg of conjugated peptide and 1.5 ml of Freund adjuvant (complete for the primary injection and incomplete for subsequent booster injections). Rabbits were inoculated at four sites subcutaneously for all injections. Booster injections were given 10, 35, and 40 days after the primary injection. At 10 days after the last boost, rabbits were sacrificed and exsanguinated. Serum was passed through a bovine serum albumin-Sepharose affinity column to remove bovine serum albumin-specific antibodies and stored in aliquots at −20°C.

Preparation of DNA and RNA. Plasmid pMC6, which contains 2.4 kilobase pairs of the HSV-1 sequence representing most of the genome region specifying the 1.9-kilobase mRNA that encodes $65K_{TIF}$ (5), was kindly provided by C. M. Preston. Plasmid pGX80, which contains the HSV-1 *Hin*dIII L fragment (genome coordinates 0.592 to 0.649), was produced by B. Matz and kindly provided by V. G. Preston. Plasmid preparations were purified in CsCl-ethidium bromide gradients. Single-stranded M13 recombinant bacteriophage were prepared by polyethylene glycol precipitation of infected *Escherichia coli* JM101 culture supernatants. Phage were subsequently extracted with phenol to purify the DNA. Total cytoplasmic RNA was prepared from infected and mock-infected BHK cells as described previously (33). Synthetic oligonucleotides were prepared on a BioSearch 8600 DNA synthesizer (New Brunswick Scientific Co., Inc., Edison, N.J.). They were then purified by electrophoresis on 15% polyacrylamide–7 M urea gels, excised, eluted in buffer containing 0.5 M ammonium acetate, 1 mM EDTA, and 0.1% sodium dodecyl sulfate (SDS), ethanol precipitated, suspended in sterile water, and stored at −20°C.

Gel electrophoresis. SDS-polyacrylamide gel electrophoresis (PAGE) of proteins was conducted with 5 to 12.5% gradient gels cross-linked with 5% (wt/wt) *N,N'*-methylenebisacrylamide (BIS), except for analysis of proteolytic cleavage products, for which 15% gels cross-linked with 2% (wt/wt) BIS was used. The buffer system used was that of Laemmli (19). Gels were routinely fixed, treated with En^{3}Hance (New England Nuclear Corp., Boston, Mass.), dried, and exposed to X-ray film (XS-1 or XAR-5; Eastman Kodak Co., Rochester, N.Y.) at −70°C.

Partial proteolysis of polypeptides. Analysis of partial proteolytic cleavage products by using V8 protease (Sigma Chemical Co., St. Louis, Mo.) was performed essentially as described previously (7).

In vitro translation. Micrococcal nuclease-treated rabbit reticulocyte lysates (Amersham) were incubated at 30°C with RNA, isolated and purified as described above, and [^{35}S]methionine as specified by the manufacturer.

Immunoprecipitation. Cells labeled with [^{35}S]methionine were prepared for immunoprecipitation by lysis in extraction buffer (0.1 M Tris hydrochloride [pH 8.0], 10% glycerol, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.2 mM phenylmethylsulfonyl fluoride), and the lysate was clarified by centifugation for 10 min at 13,500 × *g* in a microcentrifuge (39). The products of in vitro translation were first diluted in an equal volume of 2×-concentrated extraction buffer containing 0.2% SDS, whereas DBPs were first diluted in 0.2 M Tris hydrochloride (pH 8.0)–1 mM EDTA–1 mM 2-mercaptoethanol to reduce the salt content to below 300 mM and then diluted in an equal volume of 2X-concentrated extraction buffer containing 0.2% SDS. All immunoprecipitations were performed as described previously (47).

Western Blotting. Western immunoblotting was performed essentially as described previously (41) with some modifications (12, 15). Antigen-MAb complexes were detected with horseradish peroxidase-linked goat anti-mouse immunoglobulin G (Bio-Rad Laboratories, Richmond, Calif.) as specified by the manufacturer. Antigen-antipeptide antibody complexes were detected with ^{125}I-labeled protein A.

Hybrid selection of RNA. Hybrid selection was performed essentially as described previously (21) with a few modifications. Synthetic oligonucleotides or recombinant M13 single-stranded DNA was heated at 100°C for 5 min, cooled quickly, and spotted onto nitrocellulose to yield 4 to 5 μg per filter. Filters were air dried for at least 3 h and baked for 2 h at 80°C in a vacuum oven. They were then heated at 100°C for 1 min in sterile water and washed one additional time in water. Total cytoplasmic RNA from HSV-1–infected cells was diluted to contain 20 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (pH6.4), 0.2% SDS, 0.4 M NaCl, and 65% freshly deionized formamide (Fluka Chemical Co., Neu-Ulm, Federal Republic of Germany), heated to 70°C for 10 min, added to filters, and hybridized as described in the figure legends. Filters were washed 10 times in 0.15 M NaCl–

TABLE 1. Synthetic peptides used for antiserum production

Peptide	Sequence	Relative location[a]	Antiserum
1	Arg-Pro-Ala-Ala-Pro-Asp-Ala-Arg-Ser-Gly-Ser-Arg-Tyr	443–445	18823, 18824
2	Arg-Gly-Gly-Pro-Gln-Thr-Pro-Tyr-Gly-Phe-Gly-Phe	476–487	18825, 18826

[a] Amino acid number is based on the first ATG in the HLORF B being designated as number 1. The total number of amino acids in this open reading frame is 488.

1 mM EDTA–0.5% SDS and 10 times in the same buffer without SDS. Selected RNA was eluted by boiling for 1 min in sterile water containing 15 μg of *E. coli* rRNA (kindly provided by C. M. Preston), quick-frozen in a dry ice-ethanol bath, extracted with phenol-chloroform, and ethanol precipitated. RNA was suspended in sterile water and either used immediately for in vitro translations or stored at −70°C.

RESULTS

Characterization of MAb 6898. MAb 6898 immunoprecipitates a 65K polypeptide from a high-salt extract of the nuclei of HSV-1–infected BHK cells (Fig. 1, lane 3), as well as from a fraction which eluted at high salt concentration from a DNA-cellulose column (the DBP fraction [lane 4]). To ascertain whether MAb 6898 recognized an epitope on $65K_{DBP}$ or another protein of apparent M_r 65,000, we compared the partial proteolytic digestion products of $65K_{DBP}$ with the 65K species immunoprecipitated by MAb 6898. The sources of protein were a high-salt extract of the nuclei of infected BHK cells immunoprecipitated with either serum 13809 or MAb 6898 and the DBP fraction, either not further purified or immunoprecipitated with MAb 6898. Proteins were separated by SDS-PAGE, and the 65K polypeptides were excised. The HSV-1 thymidine kinase polypeptide (apparent M_r 43,000) was also excised from the gel containing high-salt extracts of nuclear proteins.

One-dimensional SDS-PAGE analysis of proteolytic degradation products with V8 protease is shown in Fig. 2. Some degradation of both the 43K (lanes 1) and 65K (lanes 2, 3, 4, and 5) species occurred even in the absence of V8 protease. However, the fragments produced in the presence of 25 and 125 ng of protease were similar for all of the purified 65K species, but differed from those produced by digestion of the control 43K thymidine kinase polypeptide. We conclude from this experiment that MAb 6898 and the 13809 serum recognize the same $65K_{DBP}$.

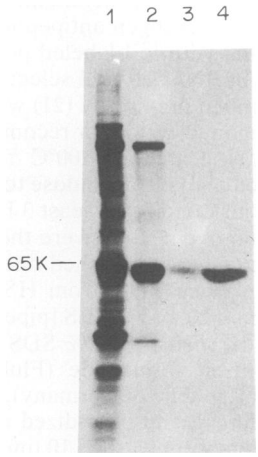

FIG. 1. Immunoprecipitation of HSV-1 polypeptides with MAb 6898. BHK cells were infected with the HSV-1 $17syn^+$ strain and labeled with [^{35}S]methionine from 4 to 24 h postinfection. Lanes: 1, high-salt extract of nuclear proteins; 2, DBPs eluted at high salt concentration from a double-stranded DNA-cellulose column; 3, high-salt extract of nuclear proteins immunoprecipitated with MAb 6898; 4, DBPs immunoprecipitated with MAb 6898. In this and subsequent experiments, labeled polypeptides were separated by electrophoresis in SDS–5 to 12.5% polyacrylamide gels unless otherwise specified.

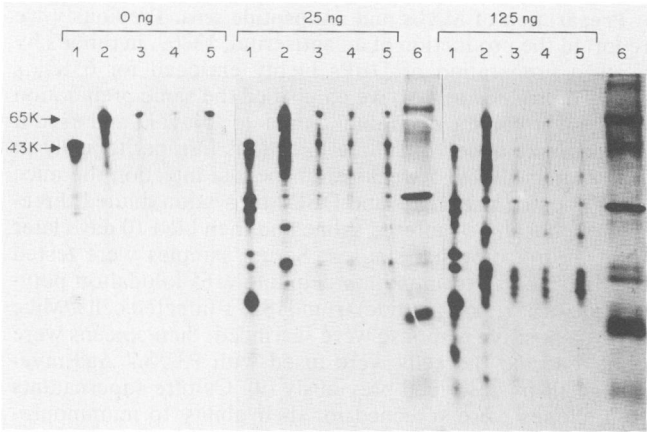

FIG. 2. Partial proteolysis of purified HSV-1 polypeptides. Preparative SDS–5 to 12.5% polyacrylamide gels were used to separate high-salt extracts of nuclear proteins, DBPs, and immunoprecipitated polypeptides. Polypeptides were excised from wet gels following autoradiography, and partial proteolytic fragments were separated in SDS–15% polyacrylamide gels. Lanes: 1, 43K thymidine kinase species from the high-salt extract of nuclear proteins; 2, 65K polypeptide from DBP fractions; 3, 65K polypeptide immunoprecipitated from the high-salt extract of nuclear proteins with rabbit polyclonal antiserum 13809; 4, 65K polypeptide immunoprecipitated from the high-salt extracts of nuclear proteins with MAb 6898; 5, 65K polypeptide immunoprecipitated from DBPs with MAb 6898; lane 6, M_r markers.

Localization of the genomic region encoding $65K_{DBP}$. We previously mapped the location of the gene encoding $65K_{DBP}$ to between coordinates 0.574 and 0.682 (22). To further localize the gene, we used plasmids containing DNA sequences representing parts of this region to inhibit the in vitro translation of the protein from mRNA isolated from HSV-1–infected cells (34). The products of in vitro translation were analyzed by two-dimensional gel electrophoresis as previously described (22, 23). Hybridization of plasmid pMC6 (map coordinates 0.681 to 0.693) to HSV-1 RNA arrested the translation of spot 34, previously identified as $65K_{TIF}$ (22), whereas spots 39 and 40, previously identified as precursors of $65K_{DBP}$ (22, 23), were not arrested. Upon denaturation of the hybrid before translation, the $65K_{TIF}$ spot reappeared as a translation product. Plasmid pGX80, containing the HSV-1 *Hin*dIII L fragment (map coordinates 0.592 to 0.649), completely arrested translation of the $65K_{DBP}$ spots, whereas denaturation of the hybrid allowed their translation (data not shown). These results demonstrate that at least a portion of the gene encoding $65K_{DBP}$ is contained within the *Hin*dIII L region.

Gene organization within the *Hin*dIII L region. The sequence of the HSV-1 $17syn^+$ strain *Hin*dIII L fragment was determined and will be reported elsewhere (McGeoch et al., in preparation). Figure 3 summarizes the organization of predicted coding regions within this 8,684-base-pair sequence. Identification of the open reading frames as putative genes was consistent with normal codon usage for HSV and with transcript termination and polyadenylation at sites corresponding to the consensus sequence AATAAA. At the left of the *Hin*dIII L region is the downstream portion of the gene encoding the small subunit of the ribonucleotide reductase (Fig. 3, RR_2 [26]), whereas at the right is the entire open reading frame encoding glycoprotein C (Fig. 3, gC) and a smaller open reading frame (14). The latter species is probably too small to encode $65K_{DBP}$. Thus, the three internal

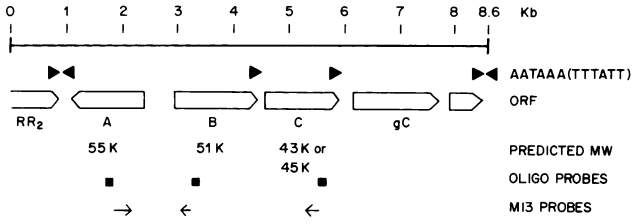

FIG. 3. Organization of the 8,685-base-pair HSV-1 *Hin*dIII L fragment. The figure shows the location of the canonical recognition site AATAAA for polyadenylation (◀ or ▶) and open reading frames (ORF). The relative locations of the synthetic 50-mers of DNA and the M13 cloned sequences used for hybrid selection are also indicated.

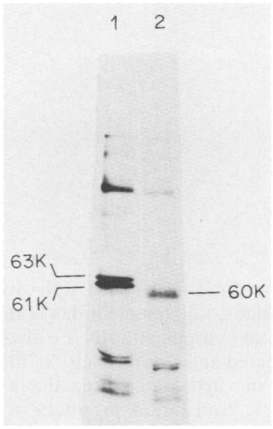

FIG. 4. Immunoprecipitation of in vitro translation products of HSV-1 mRNA with MAbs 6898 and LP1. mRNA was isolated at 6 h postinfection from cells infected at a multiplicity of infection of 20 PFU per cell and translated in vitro in the presence of [^{35}S]methionine. Translation products were immunoprecipitated with LP1 (lane 1) or MAb 6898 (lane 2).

open reading frames, designated HLORF A, HLORF B, and HLORF C, were considered the best candidates for the gene encoding 65K$_{DBP}$. In vitro translation products of M_r 58,000 and 64,000 have previously been shown to be produced by transcripts mapping in the regions of HLORF A and HLORF B, respectively (13).

Translation in vitro of mRNA corresponding to *Hin*dIII L open reading frames. To identify which of these three genes encodes 65K$_{DBP}$, we immunoprecipitated the in vitro translation products of total cytoplasmic RNA from HSV-1–infected cells with the MAb LP1, specific for 65K$_{TIF}$, and with MAb 6898, specific for 65K$_{DBP}$. As previously reported (5, 22), two proteins of apparent M_r 63,000 and 61,000 corresponding to the two in vitro translation products of 65K$_{TIF}$ (Fig. 4, lane 1) were immunoprecipitated by LP1, and a single species of apparent M_r 60,000 was immunoprecipitated by MAb 6898 (lane 2).

We determined the kinetics of 65K$_{DBP}$-specific mRNA accumulation by immunoprecipitation with MAb 6898 of in vitro translation products. Maximum levels were found 11 h after infection with a multiplicity of 5 PFU per cell or at 5 h after infection with a multiplicity of 20 PFU per cell (L. D. Goodrich et al., manuscript in preparation).

On the basis of the sequence of the *Hin*dIII L fragment and that of the gene encoding 65K$_{TIF}$ (10, 29), we synthesized 50-mers of DNA complementary to the predicted mRNA for each of HLORF A, HLORF B, and HLORF C, as well as 65K$_{TIF}$. Sequences were selected to have approximately the same G+C content. Table 2 lists these sequences and gives their relative map locations. In initial experiments, we used the synthetic oligonucleotides in an attempt to arrest the translation of the 65K$_{DBP}$ mRNA. Although the 50-mer corresponding to HLORF B reduced the translation of the polypeptide reactive with MAb 6898 by up to 75%, it was never possible to completely arrest translation, and the level of inhibition was variable (results not shown). Therefore, we used the oligonucleotides to specifically select mRNA corresponding to each of the open reading frames.

Cytoplasmic RNA was hybridized at 40°C to nitrocellulose filters to which each of the oligonucleotides was bound. After 3 h, filters were washed and the hybridized mRNA was eluted. The in vitro translation products of each RNA were analyzed by SDS-PAGE (Fig. 5). The oligonucleotide complementary to the gene encoding 65K$_{TIF}$ selected an RNA which could be translated into the characteristic doublet of 63 and 61 kilodaltons (Fig. 5, lane 5). The slower-migrating species (shown by an arrow in Fig. 5) is a commonly observed background of the reticulocyte lysate system, which is present even when water is substituted for exogenous RNA (lane 6). Only oligonucleotide B selected an RNA which could be translated into a polypeptide of 60 kilodaltons (lane 3), compatible with its corresponding to the 65K$_{DBP}$ primary translation product.

Because of the low efficiency with which the oligonucleotides appeared to select mRNA, we decided to use longer fragments for mRNA selection. M13 DNAs containing inserts of HSV-1 DNA complementary to predicted mRNAs

TABLE 2. DNA molecules used for hybrid selection of RNA

Oligonucleotide or clone	Sequence	Gene	Location[a]
Oligonucleotides			
A	5′-GTCGGTGTGGCAGCGGACAAAGAGGGCCAGGAACTGGGGGTAGCTCATCT-3′	HLORF A	1711–1760
B	5′-CTGGTTGGCGCGAAACACGCTCAGGAGGGAGCGCTTCTGGTCCACGAGAG-3′	HLORF B	3316–3267
C	5′-AAGCGCCTGACACAGCTCCGTCAGGGCCACCGCCACATGGCCTCCGAGAA-3′	HLORF C	5608–5559
65K$_{TIF}$	5′-AAGAAACGAGAGAGCGCTTCGTAGTAGAGCCCGAGGCCGTCGCGGGTGGC-3′	65K$_{TIF}$	
Clones[b]			
HL 448		HLORF A	1850–2222
HL 593		HLORF B	3230–3036
HL 548		HLORF C	5540–5324
BF 631		65K$_{TIF}$	

[a] Nucleotide number from the 5′ to 3′ end within the *Hin*dIII L sequence.
[b] Recombinant M13 clones.

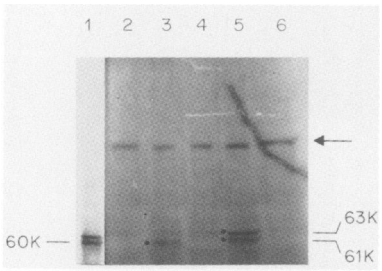

FIG. 5. In vitro translation of HSV-1 RNA hybrid selected with synthetic oligonucleotides. Oligonucleotides bound to nitrocellulose were hybridized to total cytoplasmic RNA isolated at 5 h postinfection from HSV-1–infected cells (multiplicity of infection, 20). Eluted RNA was translated in vitro, and the products were analyzed by SDS-PAGE. Lanes: 1, translation products of total cytoplasmic HSV-1–infected cell RNA; 2, translation products of mRNA selected with oligonucleotide A; 3, oligonucleotide B; 4, oligonucleotide C; 5, oligonucleotide from the gene encoding $65K_{TIF}$; 6, translation products obtained by the addition of water instead of exogenous RNA. Lanes 2 to 6 were exposed to film for 18 times longer than lane 1.

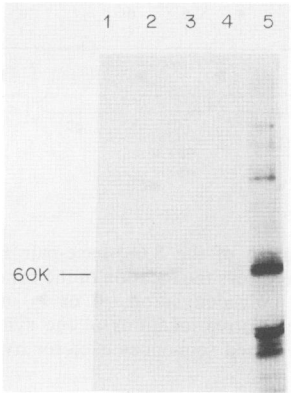

FIG. 7. Immunoprecipitation of in vitro translation products with MAb 6898. Translation products of mRNA selected by the M13 clones were immunoprecipitated with MAb 6898 and analyzed by gel electrophoresis. The figure shows immunoprecipitable products translated from mRNA from HLORF A (lane 1), HLORF B (lane 2), HLORF C (lane 3), and the $65K_{TIF}$ gene (lane 4). Lane 5, immunoprecipitable products from total cytoplasmic HSV-1 mRNA.

were bound to nitrocellulose. Table 2 and Fig. 3 indicate the locations of the inserts in the *Hin*dIII L fragment. Following hybridization at 50°C for 3 h and stringent washing at 65°C, mRNA was eluted, extracted, and translated in vitro (Fig. 6). The HLORF B-specific M13 clone was bound to three different filters, and the mRNA was eluted. Each of the filters selected an RNA which translated into a 60K polypeptide (Fig. 6, lanes 2 to 4), as expected for the primary translation product corresponding to $65K_{DBP}$, thus confirming the previous result. However, the amount of this product remained low. With these longer fragments, the amount of material translated from mRNA selected with the M13 clone complementary to the $65K_{TIF}$ coding strand improved considerably over the previous procedure (compare Fig. 6, lane 6, with Fig. 5, lane 5). Clones complementary to HLORF A and HLORF C again failed to select mRNA yielding detectable translation products (Fig. 6, lanes 1 and 5).

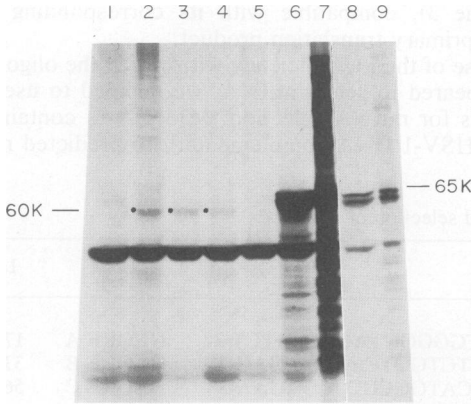

FIG. 6. In vitro translation of HSV-1 mRNA selected with recombinant M13 DNA. Single-stranded phage DNA was bound to nitrocellulose and used to select mRNA as described in the legend to FIG. 5. Translation products from mRNA were selected by using clones hybridizing to mRNAs from HLORF A (lane 1), HLORF B (lanes 2 to 4), HLORF C (lane 5), and $65K_{TIF}$ (lanes 6 and 8). Lanes 7 and 9 show translation products from the unselected mRNA. Lanes 6 and 7 were exposed to film for 16 times longer than lanes 8 and 9.

To confirm that the polypeptide translated from the RNA selected with the HLORF B-specific clone was the primary translation product corresponding to $65K_{DBP}$, we immunoprecipitated the translation products from each selection with MAb 6898 (Fig. 7). Owing to the low abundance of polypeptide translated from clone B RNA, all three replicate samples were pooled and a single species was immunoprecipitated (Fig. 7, lane 2). This species was identical in apparent M_r to that precipitated from the translation products of total RNA (lane 5). That this precipitation was specific is confirmed by the fact that although over 30 times the radioactivity was present in the clone BF631 ($65K_{TIF}$)-selected material than in the clone B-selected material, no polypeptide was observed following immunoprecipitation with MAb 6898 (lane 4). Not surprisingly, no polypeptide was precipitated by MAb 6898 from clone A- or C-selected in vitro translation products (lanes 1 and 3).

Antipeptide sera. Elucidation of the DNA sequence of *Hin*dIII-L provides the opportunity to use synthetic oligopeptides in an independent approach to identifying the viral gene product predicted to be encoded by HLORF B. Antisera against each of the two synthetic peptides shown in Table 2 were raised in two rabbits and designated by the numbers shown. These sera were screened by immunoblotting (Fig. 8). MAb 6898 was used to identify the position of $65K_{DBP}$. All sera reacted specifically with a 65K polypeptide (Fig. 8). Sera 18823 and 18826 were the most potent, reacting strongly when used at a 100-fold dilution (lanes 6 and 14). A 40K polypeptide was consistently recognized by serum 18825 when diluted only 4-fold (lane 11) and by the weaker18824 serum at a 10-fold dilution (lane 8). Similarly, a 121K polypeptide was consistently recognized by the 18823 sera at a fourfold dilution (lane 4). The relationship of the 121K and 40K polypeptides to $65K_{DBP}$ has not been investigated. Other minor bands were not consistently seen. None of the sera reacted with proteins from uninfected cells, nor did nonimmune sera react with proteins from infected cells (data not shown).

To confirm that the reactive 65K species was in fact the DBP under investigation, we used each serum to immunoprecipitate a preparation of HSV-1 DBPs (Fig. 9). The mixture of DBPs contained predominantly species of apparent M_r 130,000, 65,000, and 43,000. Antisera 18823, 18825,

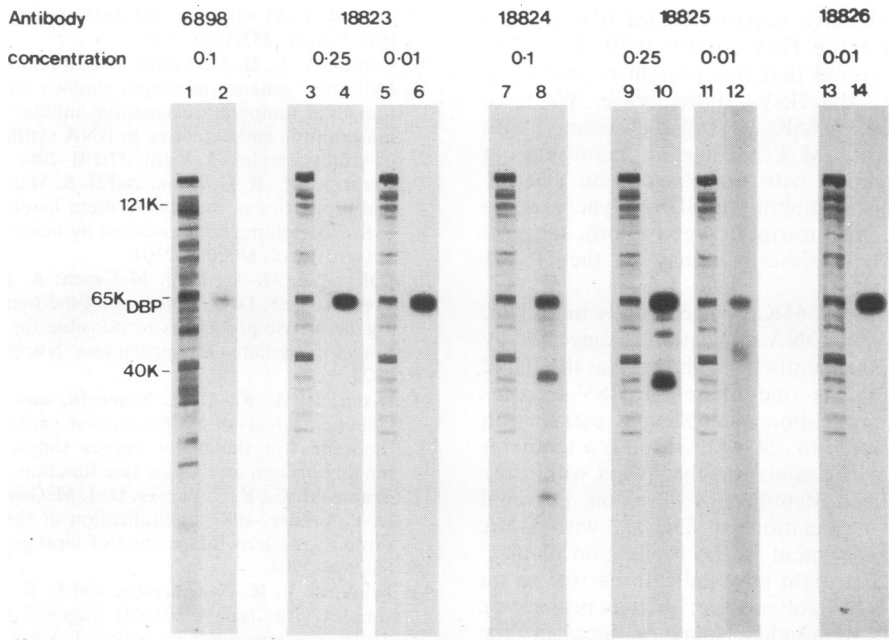

FIG. 8. Western blotting with antisera directed against oligopeptides corresponding to regions of HLORF B. BHK cells infected with HSV-1 and labeled with [^{35}S]methionine were separated by SDS-PAGE and blotted onto nitrocellulose membranes (lanes 1, 3, 5, 7, 9, 11, and 13). The membranes were probed with rabbit antisera 18823, 18824, 18825, and 18826 and MAb 6898 at the concentrations shown. Bound antibody was visualized with ^{125}I-labeled protein A (lanes 2, 4, 6, 8, 10, 12, and 14) and autoradiographs were aligned as described previously (15).

and 18826 precipitated a polypeptide of M_r 65,000. When immunoprecipitations were carried out in the presence of competing homologous peptide, the appearance of the 65,000-M_r species was either eliminated or severely reduced in intensity, demonstrating the specificity of the reaction. The polypeptide precipitated with the antipeptide sera was identical in mobility to that precipitated with MAb 6898 (data not shown).

Our previous analysis of the two-dimensional patterns of DBPs demonstrated that 65K$_{DBP}$ is the only abundant 65K species in the preparation used (22). Therefore, these results independently confirm that the gene encoding the HSV-1 65K$_{DBP}$ corresponds to HLORF B.

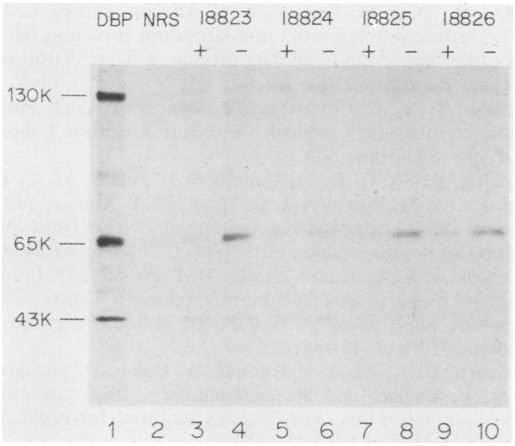

FIG. 9. Immunoprecipitation of HSV-1 DBPs with antipeptide sera. DBPs (lane 1) prepared as described in Materials and Methods were immunoprecipitated with normal rabbit serum (lane NRS) or with each of the antipeptide sera in the presence of 50 μg of homologous peptide (lanes +) or with a comparable amount of water (lanes −). Precipitates were analyzed by SDS-PAGE.

DISCUSSION

By using two independent approaches we have unambiguously identified the gene encoding 65K$_{DBP}$ to be that corresponding to the region we have designated HLORF B (UL42 in the complete DNA sequence of HSV [24; D. J. McGeoch et al., manuscript in preparation]). In the first method, we used hybrid selection of mRNA followed by in vitro translation in a rabbit reticulocyte lysate system. Synthetic 50-mers, hybridizing to regions within the predicted amino acid coding regions for HLORF B and 65K$_{TIF}$, selected mRNAs which were translatable into polypeptides of the sizes anticipated from experiments involving immunoprecipitation of in vitro translation products with MAbs to 65K$_{DBP}$ and 65K$_{TIF}$, respectively. Sequences hybridizing to longer stretches of HLORF B and the gene encoding 65K$_{TIF}$ selected the mRNAs much more efficiently, and the translation product of only the HLORF B-selected mRNA was immunoprecipitable with MAb 6898. The inability to detect translatable mRNA by using sequences which could hybridize to HLORF A or HLORF C possibly reflects the relative low abundance or poor stability of these species.

The second method which we used to identify the gene for 65K$_{DBP}$ also relied on the availability of the sequence of the *Hin*dIII L region. Antipeptide sera to two distinct peptides, predicted from the sequence encoding HLORF B, reacted with a polypeptide of apparent M_r 65,000. This is greater than the M_r 51,156 predicted for the HLORF B gene product. Such discrepancies frequently occur between estimates of M_rs of HSV proteins from polyacrylamide gels and the M_rs determined by DNA sequence analysis (see, for example, reference 25).

The map location we have determined for $65K_{DBP}$ lies within that obtained for the HSV-2 DBP ICSP34,35 (43). These authors demonstrated that this protein is physically closely associated with the HSV-2 polymerase. We have found that, like ICSP34,35, $65K_{DBP}$ is also associated with the HSV DNA polymerase (M. L. Gallo et al., manuscript in preparation). This, together with the observation that the two proteins have the same mobility in SDS-polyacrylamide gels (M. Murphy et al., manuscript in preparation), suggests that the $65K_{DBP}$ we have described may be the HSV-1 counterpart of ICSP34,35.

The function of the HSV-1 $65K_{DBP}$ is currently unknown. That it is important in HSV DNA replication is suggested by two findings. First, it has recently been shown that the UL42 gene, encoding $65K_{DBP}$, is one of seven HSV-1 genes necessary to support replication of a plasmid carrying an HSV-1 origin of replication (6, 24, 46). Second, a temperature-sensitive mutant with a mutation that maps within the HLORF B gene has been identified (V. Preston, personal communication). The association of $65K_{DBP}$ with DNA polymerase and its involvement in the replication of plasmids containing an HSV origin may indicate its role as an accessory protein for DNA polymerase, such as primase or helicase, or it may indicate a tight association necessary for the integrity of replication complexes. Our identification of the nucleotide sequences encoding $65K_{DBP}$ will facilitate site-directed mutagenesis of this gene and should enable us to better understand the role of this protein in HSV DNA replication.

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