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The 65,000-M_r DNA-Binding and Virion *trans*-Inducing Proteins of Herpes Simplex Virus Type 1

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The possible identity of the herpes simplex virus type 1 (HSV-1) 65K (65,000-M_r) virion protein which stimulates transcription from immediate-early genes with the HSV-1 65K DNA-binding protein was investigated. The two proteins were found to be distinct by the three separate criteria of immunological reactivity, tryptic peptide fingerprinting, and mobility in two-dimensional gels. Using HSV-1/HSV-2 intertypic recombinants and a serotype-specific antiserum, we located the gene encoding the 65K DNA-binding protein between coordinates 0.574 and 0.682 on the HSV-1 genome. The protein is posttranslationally modified by phosphorylation. In crude extracts of HSV-1-infected cells the 65K *trans*-inducing protein did not detectably bind to double-stranded calf thymus DNA under the conditions of our assay.

Expression of herpes simplex virus (HSV) genes is temporally regulated and is considered to occur in at least three phases termed immediate-early (IE), early, and late or α, β, and γ (13, 27, 28, 31, 50). IE genes are expressed in the absence of de novo protein synthesis (27) but their expression is stimulated by a component of the virus particle (5, 42). This *trans*-inducing factor was identified as polypeptide Vmw65 (11) and is designated $65K_{TIF}$ in the present study.

The mechanism by which $65K_{TIF}$ stimulates transcription from IE genes is not understood; however, one possibility is that it interacts with some IE-gene-regulatory DNA sequence. Interestingly, a responder element located several hundred bases upstream of the IE mRNA 5′ terminus has been identified and corresponds to the consensus sequence TAATGARATTC (R is purine) (10, 35, 44, 53).

Previously, we identified a major DNA-binding protein in HSV type 1 (HSV-1)-infected cells with an apparent M_r of ca. 62,000 (6). This DNA-binding protein and $65K_{TIF}$ have the same electrophoretic mobilities in sodium dodecyl sulfate–5 to 12.5 polyacrylamide gels, and so we designated the DNA-binding protein $65K_{DBP}$. It seemed important to establish whether $65K_{DBP}$ and $65K_{TIF}$ were one and the same protein and whether $65K_{TIF}$ can bind to DNA. This study addresses these questions. We characterized the two proteins and show that $65K_{DBP}$ and $65K_{TIF}$ are distinct and that $65K_{TIF}$ does not detectably bind to DNA under the conditions of our assay.

MATERIALS AND METHODS

Cells. BHK21 clone 13 cells (34) were used throughout.

Viruses. HSV-1 strain 17syn$^+$ (9) and HSV-2 strain HG52 (51) were used in this study. The isolation of HSV-1/HSV-2 intertypic recombinants and the determination of their genome structures have been previously reported (12, 15, 37, 46, 54).

Radioactive labeling. Confluent monolayers in 50-mm-diameter dishes or roller bottles were infected at a multiplicity of infection of 5 to 20 PFU of HSV-1 per cell. After 1 h, unadsorbed virus was removed and the infected cell monolayer was radiolabeled. [^{35}S]methionine (specific activity, >1,000 Ci/mmol; Amersham International) was used at a concentration of 20 μCi/ml for most experiments or 280 μCi/ml for two-dimensional (2D) gel electrophoresis; labeling was performed in Eagle medium containing one-fifth the normal concentration of methionine and 2% calf serum. Label was added 5 h after the end of the absorption period, and cultures were harvested after an additional 30 min (pulse) or 18 h. To chase the radioactivity in pulse-labeled cells, monolayers were washed with complete medium and incubated for an additional 6 h in that medium. [^{3}H]mannose (specific activity, 10 to 20 Ci/mmol; Amersham) was used at a concentration of 100 μCi/ml in Eagle medium supplemented with 2% calf serum. (The ethanol in which the mannose was supplied was removed before use.) Label was added 5 h after the end of absorption, and cultures were harvested about 18 h later. To label with [^{32}P]P_i, uninfected cell monolayers were grown for 3 h in Eagle medium containing 10 μM P_i (one-tenth the normal concentration) and serum which had previously been dialyzed against 0.9% (wt/vol) NaCl. After virus absorption, infected cells were washed with and labeled in the phosphate-reduced medium with 140 μCi of carrier-free [^{32}P]P_i (Amersham) for 22 h.

In vitro protein synthesis. RNA samples were translated in a fractionated rabbit reticulocyte system treated with micrococcal nuclease (41, 43).

Hybrid arrest of in vitro protein synthesis. Hybrid arrest was performed as described previously (36, 45) with 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 $65K_{TIF}$ (11).

Purification of DNA-binding proteins. A high-salt extract of 10^9 BHK cells infected with 12.5 PFU of HSV-1 per cell was prepared as described (14). To reduce the salt concentration, the extract (210 ml) was then dialyzed at 4°C against three changes (2 liters each) of B2 buffer (50 mM NaCl, 20 mM Tris hydrochloride [pH 8.2], 1 mM EDTA, 1 mM 2-mercaptoethanol, 10% glycerol). The extract was dialyzed for a total of 20 h. The dialysate was centrifuged at 10,000 × *g* for 1 h to remove the slight precipitate which formed, and

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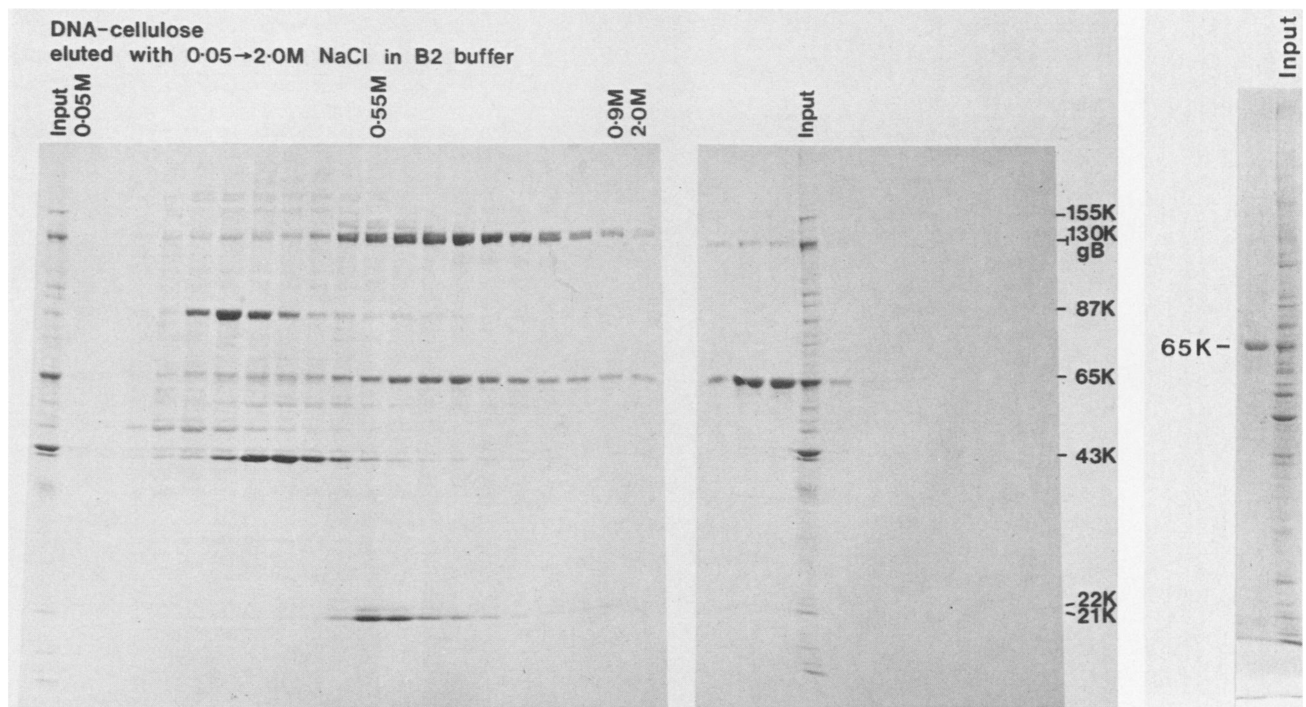

FIG. 1. Purification of $65K_{DBP}$ by DNA-cellulose chromatography. A high-salt extract of BHK cells infected with HSV-1 strain 17 and labeled with [^{35}S]methionine was dialyzed against B2 buffer containing 0.05 M NaCl and then applied to a DNA-cellulose column. Bound proteins were eluted with a linear NaCl gradient (from 0.05 to 0.90 M) and then a step to 2 M NaCl. Fractions were collected and analyzed by SDS-PAGE. (Left panel) Autoradiograph of two gels. The left shows polypeptides eluted by the salt gradient and the first fraction of the 2 M NaCl step (as indicated above the tracks), and the right gel shows polypeptides in the subsequent fractions of the 2 M NaCl step. A sample of the extract loaded on the column (input) was also analyzed on each of the gels. (Right panel) Polypeptides in the third and fourth tracks of the right-hand gel (of the left panel) stained with Coomassie brilliant blue.

the supernatant was loaded onto a double-stranded DNA—cellulose column (1) which had been equilibrated first with B2 buffer, then with B2 buffer containing 2 M NaCl, and finally with B2 buffer again. DNA-cellulose was made with calf thymus DNA (D-1501; Sigma Chemical Co.) and constituted a 100-ml bed volume packed in a 26-mm-diameter column. A flow rate of 1 ml/min was used throughout, and 10-ml fractions were collected. After the input was loaded, the column was washed with 350 ml of B2 buffer (about twice the volume necessary to remove unbound proteins). Bound proteins were eluted first with a 200-ml salt gradient in B2 buffer, starting at 0.05 M NaCl and rising to 0.9 M NaCl followed by a 2.0 M NaCl step in B2 buffer. All fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

In experiments designed to test whether $65K_{TIF}$ binds to calf thymus DNA, the procedure was scaled down to the use of an extract of 2×10^7 infected cells, and the extract (in B2 buffer containing 0.05 M NaCl as above) was loaded onto a 1-ml DNA-cellulose column which had been equilibrated as described above (14). Bound proteins were eluted sequentially with B2 buffer containing 0.15, 0.6, and 2.0 M NaCl.

Purification of virions. Cell-released virions labeled with [^{35}S]methionine were prepared from BHK cells infected with 5 PFU of HSV-1 per cell as described previously (18, 49). For two-dimensional (2D) gel electrophoresis, the virions were purified further by centrifugation through 54% Percoll (in 0.025 M sucrose, 0.01% bovine serum albumin, and 5 mM Tris hydrochloride [pH 7.5]) for 30 min at 74,000 × *g* (35,000 rpm; Beckman 50 Ti rotor). The virus-containing band was collected, diluted in 1 mM EDTA–1 mM Tris hydrochloride (pH 7.5) to a total volume of 4.1 ml, and layered onto a 1-ml 60% (wt/vol) sucrose cushion in 10 mM Tris hydrochloride (pH 7.5), and centrifugation was performed at 84,000 × *g* for 90 min (30,000 rpm; Sorvall AH650 rotor). Purified virus was collected from the interphase. The whole procedure was carried out at 4°C.

NP-40 extraction of virions. Virus particles were suspended in 50 mM NaCl–10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA at 1.6×10^{10} particles per ml and adjusted to a concentration of 0.03% in Nonidet P-40 (NP-40). The mixture was allowed to stand for 1 h at 4°C and then centrifuged at 100,000 × *g* for 1 h at 5°C. The supernatant was stored at −70°C.

Immunoprecipitation. Extracts for immunoprecipitation were precleared by centrifugation at 11,600 × *g* for 5 min (13,000 rpm; MSE Microfuge). A 50-μl portion of precleared virion extract or partially purified $65K_{DBP}$ was incubated for 3 h at 4°C with (i) 10 μl of LP1 monoclonal antibody (39) plus 10 μl of rabbit anti-mouse immunoglobulin G (Cedarlane Laboratories), (ii) 10 μl of nonimmune ascites fluid, or (iii) 10 μl of 13809 or 13810 rabbit serum. Antibody-polypeptide complexes were precipitated by binding to protein A-Sepharose (1 h at 4°C) and washed several times with 0.6 M LiCl containing 0.1 M Tris hydrochloride (pH 8.0) and 1% 2-mercaptoethanol, and bound protein was eluted with 0.125 M Tris hydrochloride (pH 6.8) containing 2% SDS, 2% glycerol, and 5% 2-mercaptoethanol.

Gel electrophoresis. Samples were prepared, and SDS-PAGE was carried out by using the buffer system of

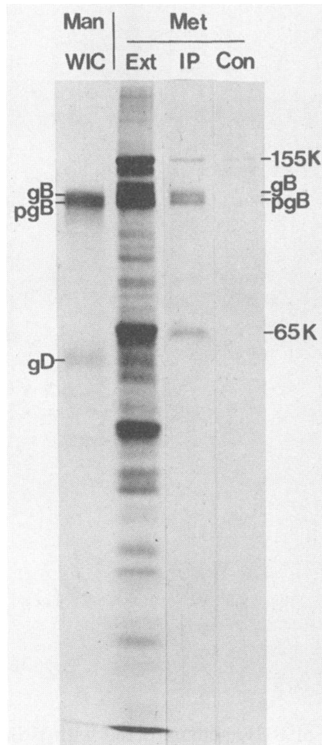

FIG. 2. Immunoprecipitation of proteins from an extract (Ext) of [^{35}S]methionine-labeled HSV-1-infected BHK cells with rabbit antiserum 13810 (IP) or nonimmune rabbit serum (Con). A sample of HSV-1-infected BHK cells labeled with [^{3}H]mannose (Man WIC, i.e., mannose, whole infected cell) was also analyzed to identify the position to which HSV glycoproteins had migrated. Polypeptides were separated by SDS-PAGE.

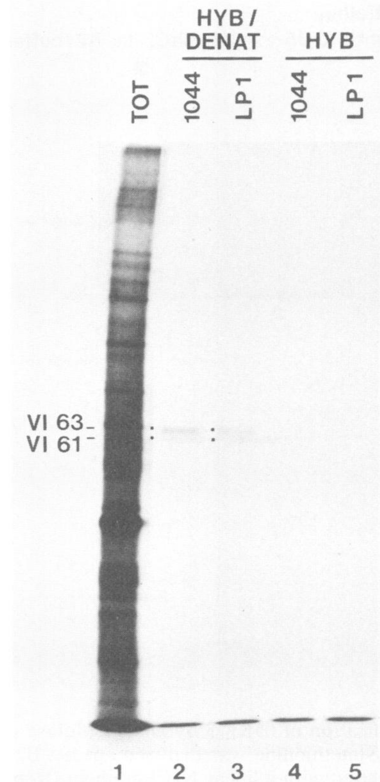

FIG. 3. Specificity of monoclonal antibodies MA1044 and LP1 for $65K_{TIF}$. Total cytoplasmic RNA extracted from BHK cells at 7 h after infection with 20 PFU of HSV-1 was translated in vitro (TOT, track 1). Other aliquots of the RNA were first hybridized to plasmid pMC6 (HYB, tracks 4 and 5) or to pMC6 and the hybrid denatured (HYB/DENAT, tracks 2 and 3) before in vitro translation. Monoclonal antibodies MA1044 and LP1 were used to immunoprecipitate the in vitro translation products (tracks 2 to 5). Polypeptides were resolved by SDS-PAGE.

Laemmli (33) and either 5 to 12.5% gradient gels cross-linked with 1/20 (wt/wt) N,N'-methylenebisacrylamide or 7.5% gels cross-linked with 1/40 (wt/wt) N,N'-methylene bisacrylamide. 2D nonequilibrium pH gradient gel electrophoresis was performed essentially as described by O'Farrell et al. (40) with minor modifications (23). The second dimension was a 9% polyacrylamide gel cross-linked with 1/40 (wt/wt) N,N'-diallyltartardiamide.

Treatment with alkaline phosphatase. Bacterial alkaline phosphatase (EC 3.1.3.1; Sigma) was diluted in water and incubated at a final concentration of 15 U/ml for 30 min at 37°C with proteins extracted as for 2D gel electrophoresis.

Autoradiography and fluorography. Slab gels from 2D gel electrophoresis were fixed, infused with 2,5-diphenyloxazole as described by Bonner and Laskey (8), dried, and exposed to Kodak XAR-5 film at −70°C. Other gels were treated with En^{3}Hance (New England Nuclear Corp.), dried, and exposed to Kodak X-Omat XS1 film at −70°C.

Western blotting (immunoblotting). Proteins were electrophoretically separated by SDS-PAGE and transferred to nitrocellulose strips (15 by 0.6 cm) with a Trans-Blot apparatus (Bio-Rad Laboratories, Inc.). Antigens immobilized on the nitrocellulose and reacting with antiserum were detected with ^{125}I-labeled protein A iodinated by the method of Hunter and Greenwood (29) as described by Towbin et al. (52) with minor modifications (23).

Tryptic peptide mapping. Both the 2 M NaCl fraction from the DNA-cellulose column containing partially purified $65K_{DBP}$ and a 0.03% NP-40 virion extract containing $65K_{TIF}$

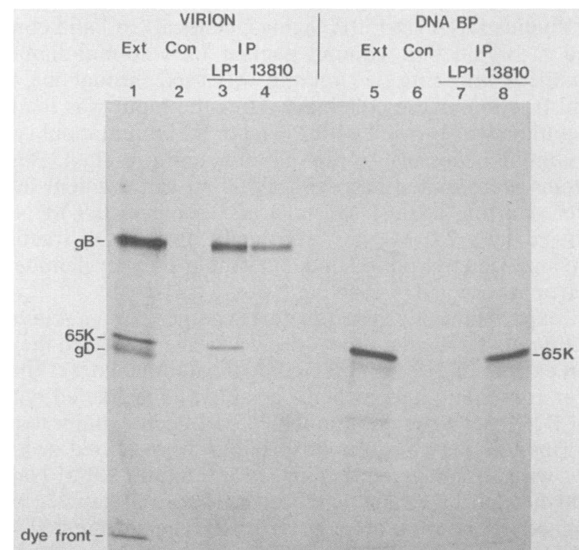

FIG. 4. Antigenic distinction between $65K_{DBP}$ and $65K_{TIF}$. [^{35}S]methionine-labeled NP-40 extracts of virions and DNA-binding proteins (DNA BP) in the 2 M NaCl eluate shown in Fig. 1 were immunoprecipitated (IP) with monoclonal antibody LP1 or rabbit antiserum 13810. Nonimmune ascites fluid was used as a control (Con). Proteins were analyzed by SDS-PAGE.

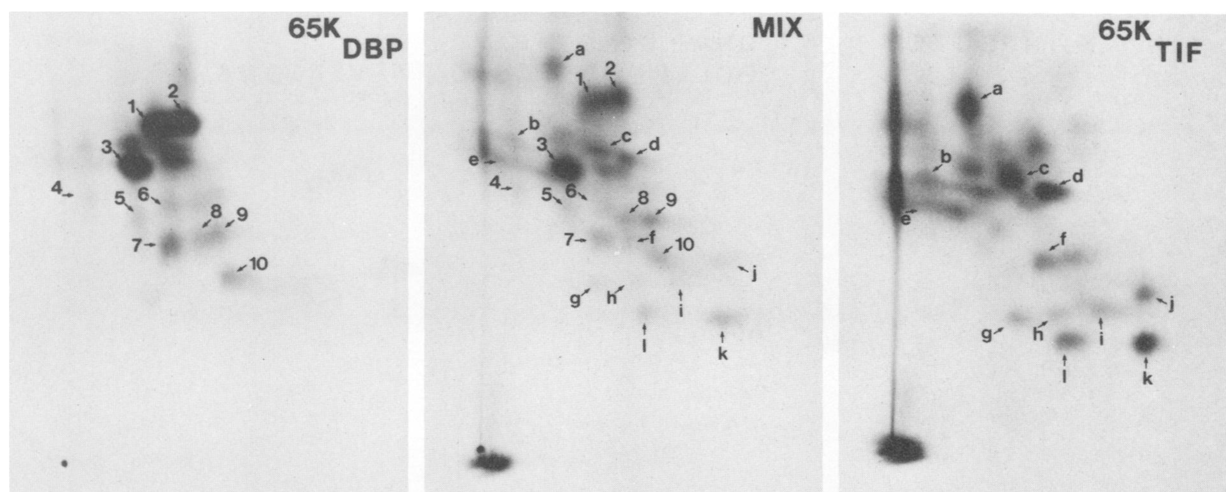

FIG. 5. Fluorographs showing 2D tryptic peptide maps of $65K_{DBP}$ (left panel), $65K_{TIF}$ (right panel), and a mix of $65K_{DBP}$ and $65K_{TIF}$ (middle panel). Extracts of DNA-binding proteins and virions prepared as shown in Fig. 4 were individually separated on SDS–10% polyacrylamide gels, and the relevant proteins were excised. Tryptic peptides were prepared from each protein and applied to thin layers of cellulose: the point of application (origin) is shown by the black dot at the bottom left of each panel. Peptides were separated first by electrophoresis (vertical direction of the fluorograph) and then by ascending chromatography (horizontal direction of the fluorograph). Some tryptic peptides have been designated with a number or a letter to facilitate comparison.

were subjected to SDS–10% PAGE. The gels were dried immediately after electrophoresis. Four spots of radioactive ink were placed at the corners of the gel to facilitate alignment with the autoradiographs. Slices of the gel containing the desired polypeptides were cut out, and the polypeptides were eluted by the method of Anderson et al. (2). They were desalted by passage through Sephadex G25, and the SDS was removed by the method of Henderson et al. (25). The pellet obtained from this procedure was suspended in 100 μl of a 1% solution of ammonium bicarbonate containing 10 μg of L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin. After 16 h at 37°C, an additional 1 μg of treated trypsin was added and incubation was continued for 4 h. The peptides were lyophilized, oxidized with performic acid (26), and then diluted 50-fold with water and lyophilized. Peptides in pH 2.1 electrophoresis buffer (acetic acid-formic acid-water, 8:2:90) were applied to a spot 4 cm from each of two adjacent edges of cellulose chromatogram sheets (20 by 20 cm; no. 13255; Eastman Chemical Products, Inc.). After electrophoresis for 45 min at 600 V, the chromatogram was dried in a current of cold air and peptides were separated in the second dimension by ascending chromatography in water–butan-1-ol–pyridine–acetic acid (24:30:20:6). Dried chromatograms were sprayed with En^{3}Hance and exposed at −70°C to Kodak XS1 film.

RESULTS

Purification of $65K_{DBP}$. DNA-binding proteins from BHK cells infected with HSV-1 (strain 17) were prepared and analyzed by SDS-PAGE. Figure 1 shows the proteins in the extract which was loaded onto the column (input) and in the relevant fractions eluted from the column by increasing concentrations of NaCl. $65K_{DBP}$ eluted across the whole range of salt concentrations with two peaks, one around 0.7 M NaCl and one in the 2 M NaCl step. The $65K_{DBP}$ eluting in the 2M NaCl step had been substantially purified as judged by both autoradiography (Fig. 1, left) and Coomassie brilliant blue staining (Fig. 1, right); the fraction did contain, in addition to $65K_{DBP}$, small amounts of the major capsid protein (155K), glycoprotein B or the major DNA-binding protein (130K), and some lower-molecular-weight components.

Preparation of an anti-$65K_{DBP}$ serum. The 2 M NaCl fraction containing $65K_{DBP}$ was emulsified with an equal volume of Freund complete adjuvant. Rabbits were immunized with three intramuscular injections at 10-day intervals. Each injection contained about 20 μg of $65K_{DBP}$, as judged by the intensity of Coomassie brilliant blue staining of the electrophoretically separated protein compared to that of known amounts of β-galactosidase, ovalbumin, and bovine serum albumin. Ten days after the third injection serum samples were collected. To test the specificity of the two antisera (designated 13809 and 13810), immunoprecipitations were performed with an extract of infected BHK cells labeled with [^{35}S]methionine. The immunoprecipitates were analyzed by SDS-PAGE (Fig. 2). Infected cells labeled with [^{3}H]mannose were also run on the same gel as the markers. The 13810 serum selectively precipitated from the extract a number of proteins, including a 65K protein and proteins comigrating with gB and pgB. The serum may also immunoprecipitate the major capsid protein (155K); however, the presence of a minor comigrating band in the control immunoprecipitation raises doubts on this point. A similar specificity was seen with serum 13809 (data not shown).

Immunological evidence that $65K_{DBP}$ and $65K_{TIF}$ are distinct. Previous experiments (11), which established that the virion factor responsible for *trans*-activation of IE genes was $65K_{TIF}$, used the technique of hybrid arrest of translation and used monoclonal antibody MA1044. This antibody specifically precipitated two polypeptides, VI63 and VI61, which were recognized as the in vitro translation products corresponding to $65K_{TIF}$. As demonstrated by the experiment shown in Fig. 3, in which plasmid pMC6 was used to arrest specifically the translation of $65K_{TIF}$, monoclonal antibody LP1 (39) also gives a specific reaction with $65K_{TIF}$. In the experiments described below we used LP1 to identify $65K_{TIF}$.

The antigenic relatedness of $65K_{DBP}$ and $65K_{TIF}$ was investigated by immunoprecipitation experiments with

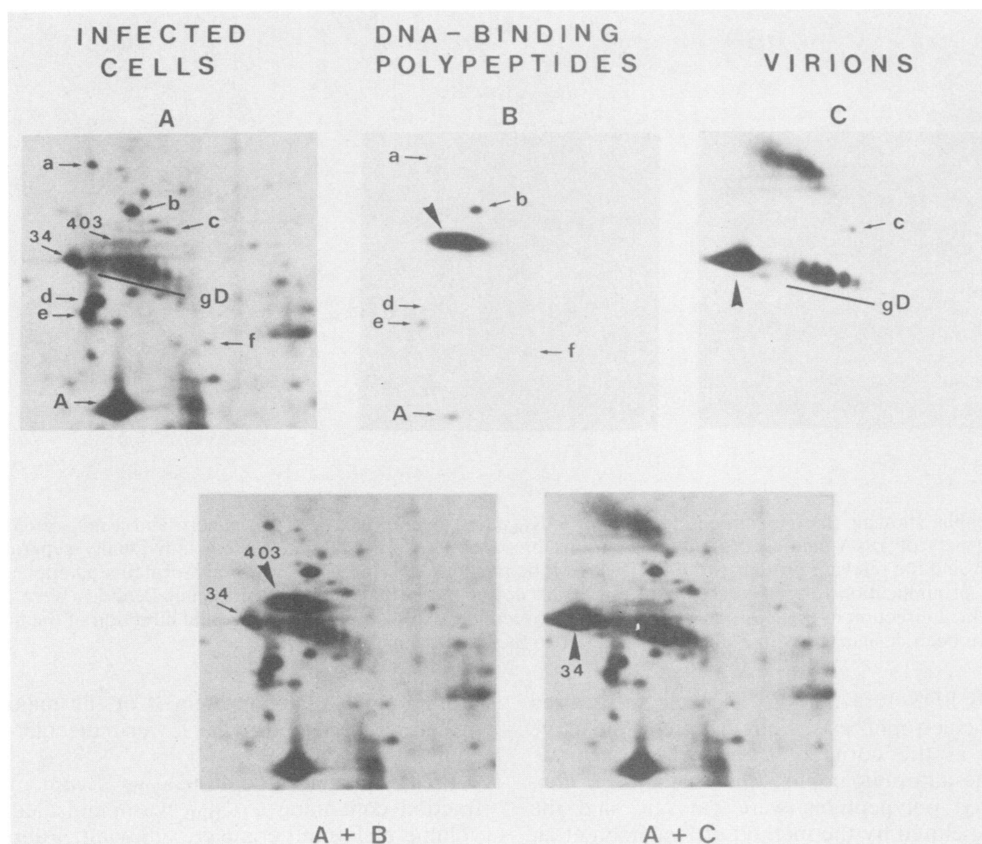

FIG. 6. Differential mobility on 2D gels of $65K_{DBP}$ and $65K_{TIF}$. DNA-binding proteins and extracts of BHK cells infected with HSV-1 strain 17 and purified virions were resolved by 2D gel electrophoresis and visualized by autoradiography. Here and in the 2D gels shown in other figures, ampholines (pH 3.5 to 10) were used for electrophoresis in the first dimension and 9% gels cross-linked with *N,N'*-diallyltartardiamide for the second dimension. Samples were run individually (A, B, and C) and are displayed as individual autoradiographs and as the superposition of autoradiographs (A+B and A+C). Spots 34 and 403 were previously described in the numbering scheme of Marsden et al. (36). Spots A (actin) and a, b, c, d, e, and f were used to align the autoradiographs. Only the relevant portion of the gels is shown and includes polypeptides with apparent molecular weights of between about 43,000 and 85,000 and relative mobilities in the nonequilibrium pH gradient gel (36) between about 0.1 and 0.5.

monoclonal antibody LP1 and rabbit antiserum 13810 (Fig. 4). The source of $65K_{TIF}$ was an NP-40 extract of virions (produced as described in Materials and Methods) known to contain the activity stimulating IE transcription (Fig. 4, lane 1), whereas the source of $65K_{DBP}$ was the DNA-binding proteins in the 2 M NaCl eluate shown in Fig. 1 (Fig. 4, lane 5). Antibody LP1 precipitated a 65K protein from the virion extract (lane 3) but not the DNA-binding proteins (lane 7), whereas antiserum 13810 precipitated a 65K protein from the DNA-binding proteins (lane 8) but not from the virion extract (lane 4). Detergent treatment with NP-40 at concentrations of up to 3% did not extract from virions any 65K protein which was precipitable by antibody 13810 (data not shown). No proteins were detected in immunoprecipitations with control nonimmune sera (lanes 2 and 6). This experiment demonstrated that the 65K protein in the virion extract is distinct from that in the purified DNA-binding proteins.

The presence of gB in the immunoprecipitate of the virion extract with antiserum 13810 (Fig. 4, lane 4) was expected, since the serum contains antibodies specific for gB (Fig. 2). However, the presence of gB in the immunoprecipitate with LP1 (Fig. 4, lane 3) was unexpected. This observation has been consistently made and suggests either that there might be a physical association between $65K_{TIF}$ and gB or that the epitope recognized by LP1 is common to both $65K_{TIF}$ and gB.

Biochemical evidence that $65K_{DBP}$ and $65K_{TIF}$ are distinct. Two biochemical tests, tryptic peptide fingerprinting and mobility on 2D gels, were used to investigate the relatedness of $65K_{DBP}$ and $65K_{TIF}$. For tryptic peptide fingerprinting it was necessary to purify the proteins. This was done by first preparing extracts of virions and DNA-binding proteins as described above. These extracts were separately subjected to SDS-PAGE, and then the 65K bands were excised and fingerprinted. Figure 5 shows the pattern obtained for $65K_{DBP}$ (left panel), $65K_{TIF}$ (right panel), and a mixture of $65K_{DBP}$ and $65K_{TIF}$ peptides (middle panel). To facilitate comparison, 10 $65K_{DBP}$ peptides (1 to 10) and 12 $65K_{TIF}$ peptides (a to l) were identified in both the patterns of the individual proteins and the mixture. This analysis of the patterns revealed that they were different and thus demonstrated that the two proteins are distinct.

To compare the mobilities of $65K_{DBP}$ and $65K_{TIF}$ by 2D nonequilibrium pH gradient gel electrophoresis (40), infected-cell extracts were made by the method of Haarr and Marsden (22), whereas DNA-binding protein and virion extracts were prepared as described above. The extracts were subjected to 2D electrophoresis, and the relevant

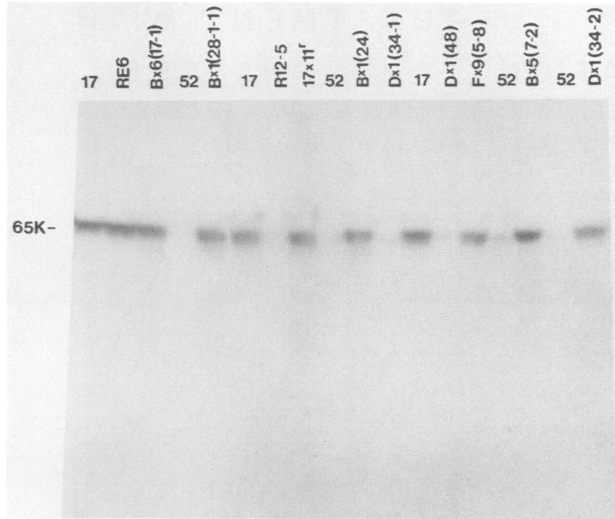

FIG. 7. Physical mapping of $65K_{DBP}$. BHK cells were infected with HSV-1 (17), HSV-2 (52), and the intertypic recombinants RE6, Bx6(17-1), Bx1(28-1-1), R12-5, $17^{+}x11^{r}$, Bx1(24), Dx1(34-1), Dx1(48), Fx9(5-8), Bx5(7-2), and Dx1(34-2). Proteins were denatured and analyzed by electrophoresis in 5 to 12.5% gradient SDS-polyacrylamide gels. Separated polypeptides were transferred electrophoretically to nitrocellulose membranes and incubated with antiserum 13810. Bound antiserum was visualized by ^{125}I-protein A and autoradiography.

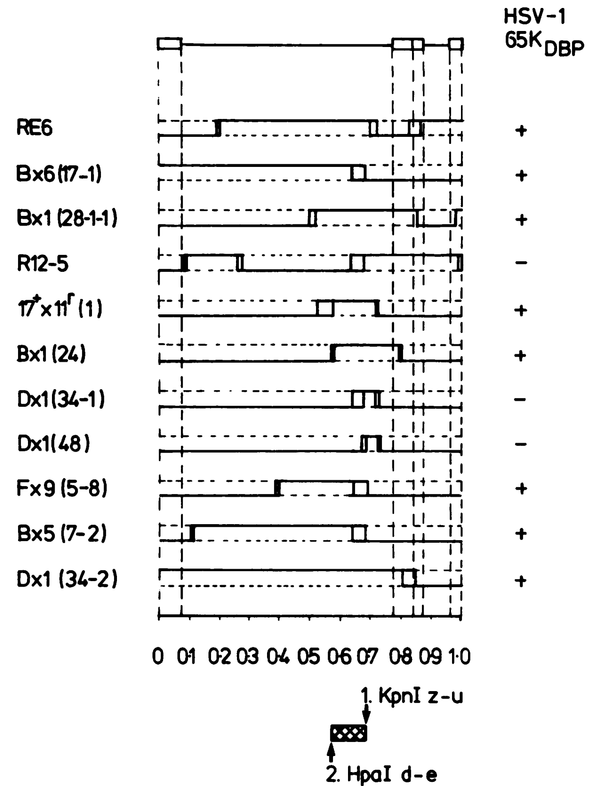

FIG. 8. Summary of the genome structures of the eleven recombinants used for the experiment shown in Fig. 7. The genome arrangement of HSV DNA is illustrated in the prototype configuration at the top of the figure, showing the long and short repeat sequences and the long and short unique regions. Vertical dotted lines correspond to the limits of the long and short repeat sequences. Those sequences of the recombinant derived from the type 1 and type 2 parent are represented by a thick continuous line superimposed respectively on the upper and lower of the two horizontal dotted lines. Crossover regions are indicated by one or two vertical lines between the thick continuous horizontal lines. The distance between two vertical lines indicates the remaining region of uncertainty for that crossover event. When the region of uncertainty was small, the crossover appears as a single vertical line. The scale at the bottom is expressed as fractions of the genome length. The induction or lack of induction of $65K_{DBP}$ by each recombinant is indicated on the right-hand side of the figure (+ or −, respectively). The deduced map location of $65K_{DBP}$ is shown by the cross-hatched area, and delimiting restriction sites in the genome in HSV-1 (↓) and HSV-2 (↑) are *Kpn*I *z-u* and *Hpa*I *d-e*, respectively.

regions of the autoradiographs obtained are shown in Fig. 6. Panel A shows the infected-cell extract, in which gD and spots 34 and 403 are indicated. The nomenclature used is that of Marsden et al. (36), and spots A (actin) and a to f serve to align the autoradiographs. Panels B and C show $65K_{DBP}$ and $65K_{TIF}$ (arrowheads), respectively. Superposition of the autoradiographs of the infected-cell and DNA-binding protein extracts (A+B) shows that $65K_{DBP}$ corresponds to spot 403, whereas superposition of the autoradiographs of the infected-cell and virion extracts (A+C) shows that $65K_{TIF}$ corresponds to spot 34. This result also demonstrates that $65K_{DBP}$ and $65K_{TIF}$ are distinct.

Physical mapping of $65K_{DBP}$. We next attempted to locate the region of the HSV-1 genome which encodes $65K_{DBP}$. A set of HSV-1/HSV-2 intertypic recombinants could be used since the 13810 antiserum was type specific for $65K_{DBP}$. BHK cells were infected with these recombinants and the parental strains 17 (HSV-1) and 52 (HG52, HSV-2). The infected-cell proteins were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and probed with antiserum 13810, and bound antibody was visualized with ^{125}I-protein A. Figure 7 shows that the antiserum reacted with HSV-1- (e.g., lane 1) but not HSV-2-infected cells (e.g., lane 4) and that it reacted with cells infected with recombinants RE6, Bx6(17-1), BX1(28-1-1), $17^{+}x11^{r}$(1), Bx1(24), Fx9(5-8), Bx5(7-2) and Dx1(34-2) but not R12-5, Dx1(34-1), or Dx1(48). Correlation of these data with the genome structure of the recombinants (Fig. 8) gives a map location for $65K_{DBP}$ between approximate coordinates 0.574 and 0.682 with all data consistent. These coordinates are delimited on the left by the HSV-2 *Hpa*I *d-e* restriction enzyme site and on the right by the HSV-1 *Kpn*I *z-u* restriction enzyme site.

Posttranslational processing of $65K_{DBP}$. The data presented in Fig. 6 identified $65K_{DBP}$ as spot 403 defined previously in a 2D gel analysis of HSV-1-induced polypeptides (36) and classified as a processed polypeptide. We therefore decided to investigate the processing events involved in its genesis. Infected cells were pulse-labeled for 30 min at 5 h after absorption with [^{35}S]methionine and either harvested immediately or chased in unlabeled medium for 6 h. An extract of the infected cells was made, and proteins from aliquots of the extract were immunoprecipitated with antiserum 13809. The extracts and immunoprecipitates were analyzed by 2D gel electrophoresis, and relevant regions of the autoradiographs are shown in the left four panels of Fig. 9. The data show that spots 39, 40, 41, 42, and 315 (previously identified as short-lived-precursor or transient-intermediate polypeptides [36]) were labeled during the pulse and that during the chase the more basic of these spots disappeared and the more acidic 403 spot appeared.

We next investigated whether $65K_{DBP}$ was phosphorylated. Infected cells were labeled with [^{32}P]P_i, and extracts

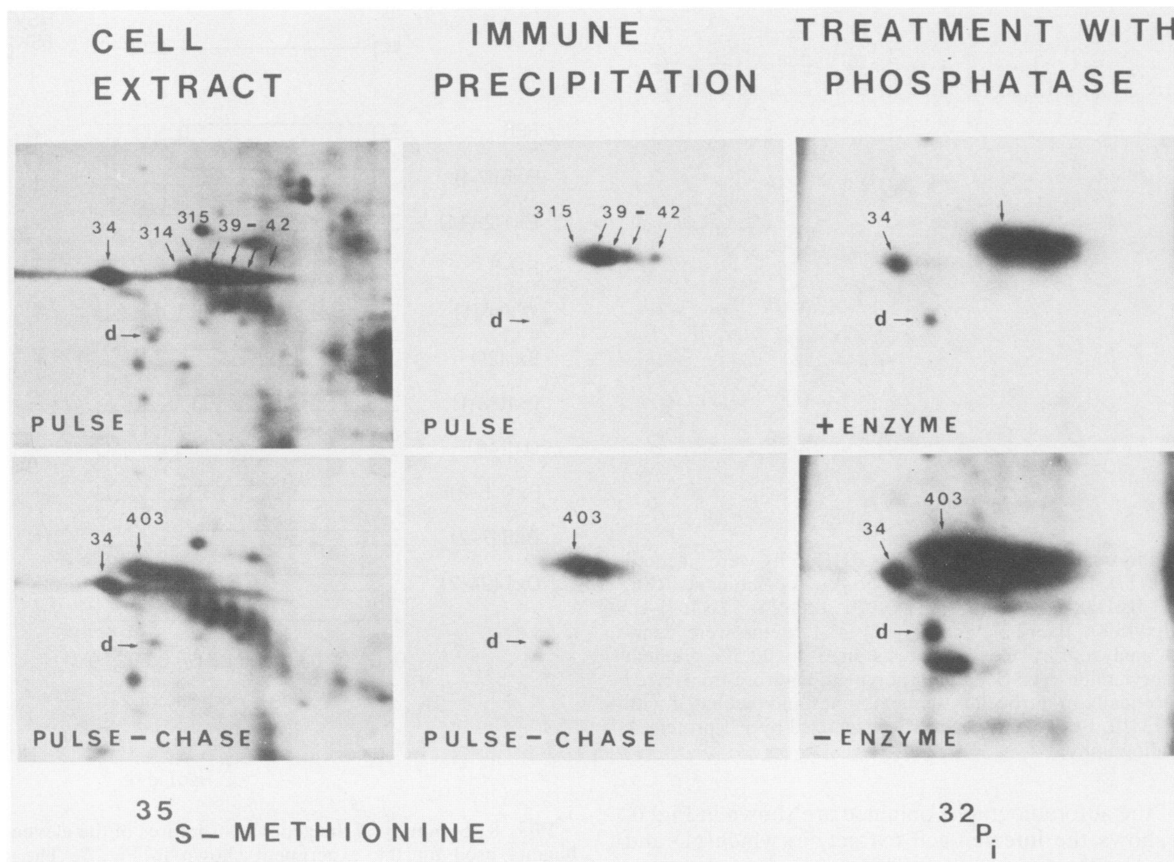

FIG. 9. Posttranslational modification of $65K_{DBP}$. Infected cells were pulse-labeled with [^{35}S]methionine (upper left and middle panels), and then the label was chased (lower left and middle panels). Cell extracts were made (left panels), and aliquots were immunoprecipitated with antiserum 13809 (middle panels). Phosphorylation of $65K_{DBP}$ was evaluated by electrophoresis of ^{32}P-labeled infected-cell proteins after digestion with alkaline phosphatase (+enzyme; upper right panel) or treatment with H_2O as a control (−enzyme; lower right panel). Numbered arrows show previously identified virus-induced polypeptides. Spot d is a cellular polypeptide used here to align the autoradiographs. The acidic end of the gel is on the left of the figure.

for 2D gel electrophoresis were prepared. One portion was incubated with alkaline phosphatase and the other with H_2O as a control. The autoradiographs show that spot 403 ($65K_{DBP}$) was heavily phosphorylated and that digestion with alkaline phosphatase caused much of the label to be lost, particularly from the more acidic forms of the protein.

$65K_{TIF}$ does not bind detectably to calf thymus DNA. To address the question whether $65K_{TIF}$ binds to DNA an extract of HSV-1-infected BHK cells was chromatographed on double-stranded calf thymus DNA-cellulose; we then tested by immunoprecipitation for the presence of $65K_{TIF}$ in the input to the column and in the various fractions eluting from the column. Figure 10 shows that $65K_{TIF}$ was precipitated with the monoclonal antibody LP1 from the material loaded onto the column (Input) and also from the material which did not bind to the column (flow thr.). It was barely detectable in the material eluted by 0.15 M NaCl and was not detectable in the 0.6 M NaCl elution. In this experiment no proteins were eluted from the column with 2.0 M NaCl. That the column was able to bind proteins was demonstrated by the similarity of the polypeptide profiles of the 0.15 and 0.6 M NaCl eluates containing the major DNA-binding protein, 87K and 43K to those observed in our previous experiments (6) and also by the presence of immunoprecipitable $65K_{DBP}$ in the eluates (data not shown). We conclude that $65K_{TIF}$ does not bind detectably to double-stranded calf thymus DNA under the conditions of our assay.

DISCUSSION

The experiments reported here establish that there are two distinct proteins induced by HSV-1 which have very similar electrophoretic mobilities in SDS-polyacrylamide gels. One, $65K_{TIF}$, is a component of purified virions and is responsible for the stimulation of transcription from IE genes (5, 11, 42). The other, $65K_{DBP}$, is a major DNA-binding protein (6) which might be expected to play some regulatory or catalytic role.

DNA-cellulose chromatography yielded a preparation of $65K_{DBP}$ containing only minor amounts of other proteins. This protein has unusual chromatographic properties in that it elutes across the entire salt gradient, with a gradual peaking around 0.65 M followed by a second peak in the 2 M NaCl wash. The possibility that this behavior was due to the presence of 65K proteins other than $65K_{DBP}$ was examined by subjecting every alternate fraction to analysis by 2D gel electrophoresis. No protein other than $65K_{DBP}$ was detected and no difference in the distribution of radioactivity within $65K_{DBP}$ was apparent in any of the fractions (data not shown). Thus the reason why $65K_{DBP}$ is eluted over such a

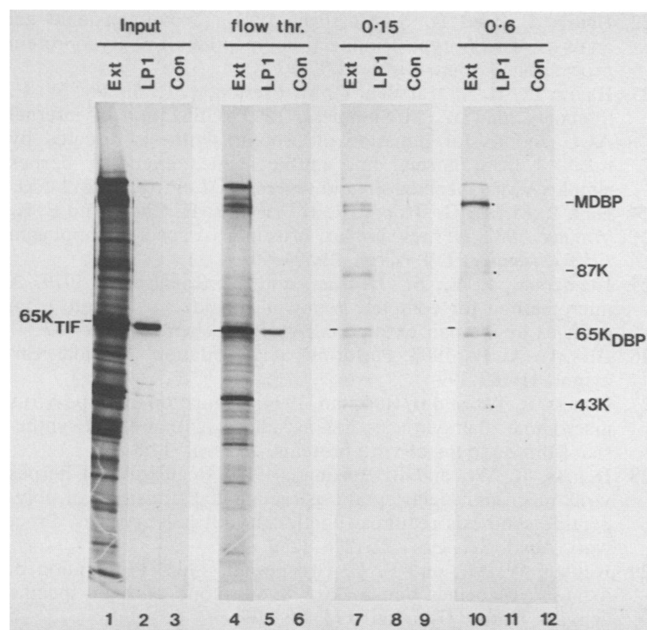

FIG. 10. Failure of $65K_{TIF}$ to bind to double-stranded calf thymus DNA-cellulose. A high-salt extract of BHK cells infected with HSV-1 and labeled with [^{35}S]methionine (Input) was loaded onto a 1-ml DNA-cellulose column. Proteins not binding to the column (flow thr.) were collected, as well as proteins eluted with 0.15, 0.6, and 2.0 M NaCl. These various fractions (Ext; tracks 1, 4, 7, and 10) were precipitated with the anti-$65K_{TIF}$ monoclonal antibody LP1 (LP1; tracks 2, 5, 8, and 11) and a control nonimmune ascites fluid (Con; tracks 3, 6, 9, and 12). Proteins were analyzed by SDS-PAGE. DNA-binding proteins eluted with 0.15 and 0.6 M NaCl (tracks 7 and 10) include the major DNA-binding protein (MDBP) and the 87K, $65K_{DBP}$, and 43K proteins. No proteins were detected in the 2.0 M eluate (not shown). All tracks received equal exposures and were from the same gel though they have been realigned to facilitate comparison. All the extracts were diluted 10-fold before electrophoresis.

broad range of salt concentrations remains obscure but might reflect its differential affinity for subsets of DNA sequences in calf thymus DNA or interactions involving other proteins on the column. These possibilities remain to be tested.

We have now performed six separate purifications of $65K_{DBP}$. In only three of these was the protein so cleanly and preferentially eluted in the 2 M NaCl step. In other preparations it eluted before the end of the 0.05 to 0.90 M NaCl salt gradient. Again the basis for this variability is not known.

The absence of $65K_{TIF}$ in any of the fractions eluted from the DNA-cellulose column (Fig. 4 and 10) demonstrates that $65K_{TIF}$ does not detectably bind to calf thymus DNA under the conditions of this assay. We have furthermore been unable to detect binding of $65K_{TIF}$ isolated from partially purified virions to DNA fragments which contain the TAATGARATTC element (unpublished data).

It is possible that $65K_{TIF}$ could bind to DNA by association with other viral or cellular proteins. Precedents for this possibility exist. Purified adenovirus type 2 E1A protein, a *trans*-activating protein which induces efficient transcription of early adenovirus genes (7, 30) does not bind DNA (16). However E1A contained in crude extracts of infected cells does associate with DNA, suggesting that E1A interacts indirectly with DNA (32). Another example is the HSV IE regulatory protein IE175 (or ICP4), which does not by itself

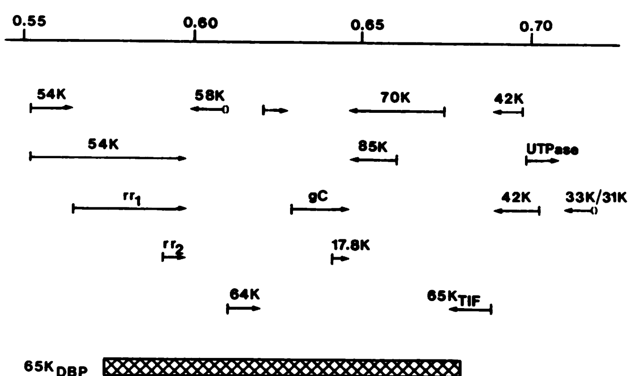

FIG. 11. Transcripts and proteins mapping within and close to the map location of $65K_{DBP}$. The scale of the genome has been expanded to allow individual transcripts and proteins mapping between approximate coordinates 0.55 and 0.73 to be depicted. The hatched bar at the bottom of the figure shows the limits for the map location of $65K_{DBP}$.

bind to DNA but appears to require a cell protein for binding (19). So far we have made no attempt to identify conditions under which $65K_{TIF}$ might bind to DNA. Our observations suggest, however, that $65K_{TIF}$ does not stimulate IE gene expression by direct interaction with naked DNA and therefore that recognition of IE-gene-specific transcription signals such as TAATGARATTC may involve cell proteins.

By means of HSV-1/HSV-2 intertypic recombinants and a type-specific antiserum, the gene encoding $65K_{DBP}$ was mapped to between restriction enzyme sites HSV-2 *Hpa*I *d-e* and HSV-1 *z-u* (approximate coordinates of 0.574 and 0.682, respectively). Figure 11 shows the transcripts and proteins mapping within and close to this region. The map is based on data from several sources (2, 3, 11, 20, 21, 24; P. A. Schaffer, E. K. Wagner, G. B. Devi-Rao, and V. G. Preston, *in Genetic Maps 1986*, in press). For the following reasons, many of the transcripts can be excluded as potentially encoding $65K_{DBP}$. (i) Some encode a different protein, e.g., gC (21), rr_1 (the large subunit of ribonucleotide reductase [48], rr_2 (the small subunit of ribonucleotide reductase [4, 17, 38]), $65K_{TIF}$ (11; this manuscript), and dUTPase (47). (ii) The two 5′ coterminal transcripts specifying the 54K protein (24) probably share the same coding region, which is to the left of 0.574. (iii) The 17.8K protein (21) is too small to be $65K_{DBP}$ (Fig. 3). Thus only five identified transcripts remain which might potentially encode $65K_{DBP}$. These specify in vitro translation products of 58, 64, 85, and 70 kilodaltons and the protein mapping between the 64 kilodalton product and gC.

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