Interaction of Cell and Virus Proteins with DNA Sequences Encompassing the Promoter/Regulatory and Leader Regions of the Herpes Simplex Virus Thymidine Kinase Gene*

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During the course of a productive infection with herpes simplex virus (HSV), gene expression is coordinately regulated in a cascade fashion. Three major kinetic classes of genes, termed α, β, and γ, are sequentially activated. The mechanism responsible for repression and subsequent activation of β and γ genes is not known. A mobility-shift electrophoresis assay was used to examine DNA fragments containing the promoter/regulatory and the mRNA leader regions of the thymidine kinase gene (TK, a model β gene) for their ability to bind proteins present in nuclear extracts prepared from uninfected and infected cells. Specific complexes unique to each extract were formed. Using a monoclonal antibody specific for ICP4 (the major regulatory protein of HSV) we demonstrated that this protein is present in the complexes formed between probes encompassing either the promoter/regulatory or leader sequence DNAs and proteins in infected-cell extracts. These complexes formed despite the lack of a high affinity binding site for ICP4 in either of these regions. The stability of complexes formed in infected-cell extracts with DNA probes containing the promoter/regulatory, leader region, and a high affinity ICP4-binding site were compared by dissociation analysis. The relative k_diss for these DNA-protein complexes was in the order TK-leader region >> TK-promoter/regulatory region ≈ high affinity ICP4-binding site. Cu^{2+}/1,10-phenanthroline footprinting revealed that infected-cell complexes which form on a probe containing a high affinity ICP4-binding site generate a protection pattern, whereas those formed on a probe containing the TK-leader sequence do not. In contrast, complexes formed with the latter probe in extracts from uninfected cells are kinetically stable and refractile to cleavage. A model for activation of the TK gene which incorporates these results is presented.

The linear, double-stranded genome of herpes simplex virus (HSV) has the capacity to encode at least 75 genes (1, 2). Three temporally regulated gene families, termed α, β, and γ, on the basis of their temporal order of synthesis, are expressed during the course of a lytic infection (3, 4). Regulated expression of these genes results from an interactive control network comprised of cis- and trans-acting elements present in the virus chromosome (5–12). However, the relationships between these factors and the sequences with which they interact are not well understood.

The five members of the α gene family are the first to be expressed (3, 13). During the infectious cycle, transcription from all α genes except α0 (14, 15) is turned off in response to the synthesis of α gene products (11, 16–19). One of these α gene products, ICP4, is the major regulatory protein of the virus (17, 20, 21). β genes are transcriptionally silent until functional ICP4 is synthesized (15, 22). Finally, after the initiation of virus DNA replication, the γ genes are expressed (23). Their transcription also requires functional ICP4 and is regulated by the presence of ICP27, another α gene product (24–28).

The promoter for the thymidine kinase (TK) gene, a prototype of the β kinetic class, has been extensively mutated and these mutants have been exhaustively studied. These analyses revealed that the promoter is composed of overlapping elements that affect both constitutive and regulated expression of this gene (29–31). Extensive transcriptional analyses of mutants with alterations in the sequences which comprise the promoter/regulatory and adjacent downstream region of the gene have shown a requirement for conservation of the nucleotides within this region for both basal level expression and transcriptional activation by virus-specified proteins (30–34).

We previously suggested that ICP4 may regulate transcription of HSV genes by multiple independent pathways (35). This model envisioned ICP4 interacting with sequences other than the defined high affinity binding site (36–38), or working via interactions with other cellular regulatory factors and/or virus-specified proteins. Recently, ICP4 was shown to be able to interact and bind sequences which lack or contain degenerate homologues of the high affinity binding site (36, 39–41). Despite several elegant studies, no specific target sequences in the TK promoter have been identified as ICP4-responder elements. Binding experiments, where sequences spanning the TK promoter/regulatory and the mRNA leader regions were incubated in crude nuclear extracts from infected cells, suggested that ICP4 is a component of a complex formed under these conditions (42).

In this study, we demonstrate that sequences which compose both the promoter/regulatory (nucleotides -197 to -11) and leader regions (nucleotides 16 to 165) of the HSV TK gene form specific complexes in extracts from both uninfected and infected cells, and that ICP4 is a component of the latter complexes. Analysis of the stability of complexes formed in extracts from infected cells reveals that those formed with the promoter/regulatory region are stable, whereas those formed with the leader region are kinetically extremely labile. In support of these results, we demonstrate that the complexes
formed with the leader region of the TK gene generate a chemical nuclelease protection pattern that is indistinguishable from that obtained with unbound DNA. These data have been incorporated in a model which suggests that a part of the pathway for activation of transcription of the TK gene results from decreasing the affinity with which cellular negative regulatory factors bind virus DNA.

**MATERIALS AND METHODS**

Cells, Viruses, and Preparation of Extracts—HeLa cells were propagated in filter-sterilized Dulbecco’s modified Eagle’s medium, supplemented with 5% calf serum and 2% fetal bovine serum (Hyclone). Monolayers (90% confluence) were placed at 37°C in a humidified CO2 (5%) incubator. To prepare extracts, 5 × 10^6 actively growing cells were seeded in 150-mm culture dishes in 20 ml of Dulbecco’s modified Eagle’s medium, supplemented as described above. The medium was replaced every 24 h and when the monolayer was ~90% confluent, the cells were either mock-infected or infected at a multiplicity of infection of 5 plaque-forming units/cell with HSV-1 strain F (43) or 17 (44). Virus was adsorbed for 30 min at 37°C, after which fresh medium was added. Infections were allowed to proceed for an additional 4.5 h at 37°C before the cells were harvested for extract preparation. Nuclear extracts were prepared by the procedure of Dignam et al. (45) with the following modifications: 1) pelleted cells were washed three times in 5 volumes of 4°C phosphate-buffered saline before suspension in 5 packed cell-pellet volumes of buffer A; 2) all of the buffers employed after the phosphate-buffered saline washes contained 0.2 mM L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanoic acid and 2 mM l-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone (Boehringer Mannheim) which were added immediately before use as additional protease inhibitors; and 3) buffer D (used in the final dialysis step) contained 0.1 M NaCl rather than 0.1 M KCl as originally specified. The nuclear extracts were frozen as aliquots in liquid nitrogen and stored at −70°C. The protein concentration varied between 5 and 8 mg/ml and was determined using a Bio-Rad protein assay kit.

Cloning and Preparation of Probe and Competitor DNAs—The HSV-1 thymidylate kinase (TK) gene fragments used as either probes or competitors in this study were derived from a wild-type TK gene or “linker scanning” (LS) mutants cloned in pBR322 and generously provided by S. McKnight (31, 46). All probe and competitor DNAs were isolated by digesting plasmids with the appropriate restriction enzymes and subcloned in suitable sites within the polylinker region of the vector pIBI31 (International Biotechnologies, Inc.) according to standard protocols (47). A schematic description of each TK probe and competitor DNA is found in Fig. 1. The high affinity ICP4 binding site (ICP4 BS) probe contained a 49-bp AuaI to BamHI fragment from the (~4 gene obtained from HSV-1 strain F (43) or 17 (44). All DNA probes were 3'-end-labeled using either [α-32P]dATP or [32P]dCTP (3000 Ci/mmol, 1 Ci = 37 GBq, Du Pont-Nuclear) and the Klenow fragment of DNA polymerase 1. Additional DNA probes were prepared using a Nick-Tag protein assay kit.

DNA-Protein Interactions at the HSV TK Locus 9403

**Mobility-shift Electrophoresis Assays—** Samples of nuclear extracts (5 μg) were mixed with 2 μg of the nonspecific carrier poly(dl-dc)-poly(dl-dc) (Pharmacia LKB Biotechnology Inc.) for 5 min prior to addition of 0.5–1 ng of the 32P-labeled DNA probe. Poly(dl-dc)-poly(dl-dc) was dissolved in 10 mM Tris- HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, and treated with the factor derived by the transcription reactions of poly(dl-dc)-poly(dl-dc). Electrophoretic separations were performed at room temperature (25°C) for 30 min in a final volume of 20 μl containing 10 mM Tris- HCl (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, 5% (v/v) glycerol, 2% polyvinyl alcohol, and 0 or 45 mM (depending on the extract) NaCl. Following binding, the reaction mixtures were assayed for complex formation by electrophoresis on a 6% nondenaturing polyacrylamide gel. Probe DNAs were located after a brief treatment of the gels with 1% acetic acid and visualized by autoradiography.

**Chemical Nuclease Cleavage Analysis—** Binding reactions were set up 5-fold and fractionated by the addition of 0.1M cupric sulfate (CuSO4) solution (1:200, 5 mM cupric sulfate (CuSO4)) in 100 mM Tris. HCl (pH 7.5), 1 mM EDTA, and electrophoresed as described above. To ensure that the dissociation rates followed first-order kinetics, the possible dependence of the rates on the concentrations of poly(dl-dc)-poly(dl-dc), 32P-labeled probe DNA, unlabeled ICP4 BS DNA, and extract was monitored. The rates were independent of all these parameters (data not shown).

Antibodies and Supershift Assays—Monoclonal antibodies (500 ng of antibody/1 μg of nuclear extract) were added to the preformed complexes and incubated for an additional 40 min prior to mobility-shift electrophoresis analysis (49). A monoclonal antibody specific for ICP4 was the generous gift of Dr. L. Pereira (University of California, San Francisco), and HC-1, specific for glycoprotein C (gC) was provided by Dr. P. G. Spear (Northwestern University).

**GTC**

GGGCGG) found within the 77-bp “GC box” of SV40 (nucleotides 37–114).

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Maxum-Gilbert sequencing ladder of the labeled DNA, were loaded immediately on a standard 15% polyacrylamide, 8.3 M urea sequencing gel in TBE buffer and electrophoresed at constant power (90 watts). Following electrophoresis, the gel was fixed in a 10% acetic acid, 10% methanol solution for 20 min, transferred to two pieces of Whatman 3MM paper, vacuum-dried, and exposed to Kodak XAR-5 film at -70 °C with an intensifying screen for 3-7 days.

RESULTS

Recognition of TK DNA by Cell and Infected-cell Factors—A DNA fragment containing the promoter/regulatory and leader sequences from the TK gene was previously shown to form a complex after incubation in extracts from infected cells (42). To investigate the sequence requirements for complex formation, the 5′ 33 bp of the TK gene were cloned as three separate regions after dissection at conveniently located restriction endonuclease sites. These regions, termed TK A, B, and C, contain 181, 72, and 84 bp, respectively, and constitute the known promoter/regulatory domain, the mRNA leader, and the translation initiation site for the gene (Fig. 1). Each region was cloned into pIBI31, end-labeled at the HindIII site, and then excised by digestion with either BamHI (TK A) or EcoRI (TK B and C) and tested for its ability to interact with proteins present in crude nuclear extracts prepared from either uninfected or infected cells.

DNA-protein interactions were monitored by assaying for the presence of discrete complexes in native polyacrylamide gels. The results of these analyses are shown in Fig. 2. TK A and B probes each formed complexes after incubation with either extract, whereas the C fragment failed to form a detectable complex under the same conditions. The TK A and B complexes which form in extracts from uninfected cells can be differentiated on the basis of their salt sensitivity. Thus, formation of the complexes with the A region probe occurred at both 5 (Fig. 2, lane c) and 50 (Fig. 2, lane b) mM NaCl, whereas formation of complexes with the B probe was sensitive to the lower salt concentration (Fig. 2, lanes d and g). By contrast, complex formation in infected-cell extracts with either fragment only occurred at 5 mM NaCl (Fig. 2, lanes d and h) (51). The middle band which formed with the TK B probe in extracts from uninfected cells at 5 mM NaCl (Fig. 2, lane g) was not reproducible and was deemed to be nonspecific. Complexes formed in extracts from infected cells migrated with a different mobility from those detected after incubation with extracts from uninfected cells. This experiment demonstrated that the promoter/regulatory region and DNA sequences from which the mRNA leader is transcribed are recognized and bound by both cell and infected-cell proteins. Moreover, there are distinct differences in the rate of migration of the complexes which form after incubation in the two extracts and the TK A and B sequences are differentially recognized by cell factors as determined by their salt requirements for binding. Although there was precedence for a role of leader sequences in regulation of HSV γ genes (53-55), complex formation with DNA from this region of a β gene was unexpected.

Specificity of Complex Formation—Competition experiments were performed to ask if complex formation with either region resulted from sequence-specific binding. Only homologous competitor DNA was effective at inhibiting TK A complex formation in extracts from uninfected cells (Fig. 3A, b, c, and j) or 5-h-infected bovine serum albumin. The reaction mixtures were loaded onto 4% low ionic strength polyacrylamide gels, and analyzed for complex formation by the mobility-shift electrophoresis assay.

![Fig. 1. Schematic representation of the 5' end of the HSV-1 TK gene. The region of the TK gene from -197 to +140 (relative to the transcription start site) was dissected by restriction endonuclease digestion, and subcloned as PvuII to MluI (-197 to -11). TK A, MluI to BglII (-16 to +56, TK B), and BglII to MluI (+53 to +140, TK C) fragments into suitable sites within the polylinker region of the vector pIBI31. Each sequence was excised and used as probe in a mobility-shift electrophoresis assay. The TK B region (a) was excised by digestion with either BamHI (TK A) or EcoRI (TK B and C) and tested for its ability to interact with proteins present in crude nuclear extracts prepared from either uninfected or infected cells.](http://www.jbc.org/doi/suppl/10.1074/jbc.270.21.9404/suppl_file/1)

![Fig. 2. Identification of protein-binding sequences within the 5' end of the HSV-1 TK gene. TK A, B, and C regions were cleaved at the EcoRI site within the polylinker of the vector pIBI31, end-labeled, digested with HindIII to release the virus insert, and incubated with 50 mM NaCl (lanes b, f, j, and k) or 5-h-infected HeLa cells, as described under "Materials and Methods." Binding reactions contained either 50 mM NaCl (lanes b, f, and j) or 5 mM (contributed by the extract) NaCl (lanes c, d, g, h, k, l). Lanes a, e, and i contain probe DNAs incubated with 5 μg of bovine serum albumin. The reaction mixtures were loaded onto native, 4% low ionic strength polyacrylamide gels, and analyzed for complex formation by the mobility-shift electrophoresis assay.](http://www.jbc.org/doi/suppl/10.1074/jbc.270.21.9404/suppl_file/2)
FIG. 3. Competition experiments with TK DNAs. Nuclear extracts prepared from uninfected (lanes a–j) or 5-h-infected (lanes k–t) HeLa cells were preincubated in the presence of varying molar equivalents of unlabeled competitor DNAs, and then labeled TK A (A) or TK B (B) DNA probes were added and the incubation continued for 30 min. The reaction mixtures were analyzed for complex formation by the mobility-shift electrophoresis assay, and dried gels were exposed to x-ray film. Only the regions of the gels containing complexes are shown. The position of bands that are shifted are indicated by solid circles, and ICP4-specific bands are indicated by arrowheads.

lanes b and c). Fragments containing either the B or C regions, TK B LS+16/+36 sequence (Fig. 1b), a high affinity ICP4-binding site (ICP4 BS, Fig. 8, schematic), or a tandem repeat of Sp-1-binding sites (derived from SV40) had no effect on complex formation even at high molar ratios of competitor to probe (Fig. 3A, lanes d–f). The competition profile in infected-cell extracts was different. The B, TK B LS+16/+36, and ICP4 BS fragments all inhibited complex formation when present at a 20-fold molar excess (Fig. 3A, lanes o, p, and q), although none was as effective as the homologous competitor (Fig. 3A, lanes l and m). Neither the C fragment or the Sp1-binding sites affected complex formation (Fig. 3A, lanes g and t).

The TK B sequence was studied in an identical manner. As before, homologous DNA was an effective competitor in extracts from uninfected cells (Fig. 3B, lanes b and c), whereas heterologous sequences, with the exception of the TK B LS+16/+36 and ICP4 BS fragments, did not compete for complex formation (Fig. 3B, lanes f, h, and i). When the B probe was analyzed in infected-cell extracts, the results were very similar to those found with the A probe (Fig. 3B, lanes k–t). That is, both homologous (TK B, TK B LS+16/+36) and heterologous (TK A, ICP4 BS) fragments competed, whereas TK C or the Sp1-binding sites fragments did not. These experiments demonstrate that binding to the A and B probes is specific and reveal that there are distinct differences in recognition of these sequences by extracts prepared from uninfected or infected cells. These differences may represent the interaction of unique factors specific for each region. Moreover, the demonstration that A region DNA competes with the B probe and vice versa only in extracts from infected cells suggests that a novel, infected-cell specific factor is utilized. Competition by a high affinity ICP4-binding site in extracts from uninfected cells suggests that common factors recognize the TK B and ICP4 BS regions. Inhibition of A and B complex formation after incubation in infected-cell extracts by DNA containing a well characterized ICP4-binding site indicates that ICP4 participates in complex formation with both of these regions.

ICP4 is a Component of the Complexes—The observation (42) that ICP4 is present in a complex formed with a 253-bp fragment spanning the TK promoter and cap site (–197 to +56), and the ability of a sequence containing a high affinity ICP4-binding site to inhibit complex formation after incubation of either the A or B probe with extracts from infected cells prompted us to further define the regions required for ICP4 to participate in complex formation. Accordingly, the sequences within the TK gene were probed using a monoclonal antibody specific for ICP4 to tag ICP4-containing complexes and the mobility-shift electrophoresis assay to separate complexes containing ICP4 from those that do not. This "supershift" assay was first used to explore complex formation with the A probe. Incubation of the A probe with uninfected-cell extracts in the presence or absence of antibody revealed no difference in the mobility of the complexes which form (Fig. 4A, lanes a and b). When this probe is incubated with extracts from infected cells complexes with altered mobilities form (Figs. 2, lane d, and 4A, lane c). Addition of antibody specific for ICP4 after these complexes are formed results in the loss of bands and the appearance of novel, slower-migrating species (Fig. 4A, lane d). The fidelity of this reaction was tested by probing with an antibody specific for gc (a virus glycoprotein) which has no effect on the rate of migration of these complexes (Fig. 4A, lane e).

We next asked if ICP4 participated in complex formation with the TK B DNA probe. Two B region probes were used in this experiment. The wild-type probe contains the sequences from –16 to +56, while the other was derived from the ψ gene linker-scanning mutant described by McKnight et al. (31). In the latter probe (TK B LS+16/+36, Fig. 1b) 20 bp of TK DNA between +16 and +36 were deleted and replaced with a decanucleotide containing a BamHI restriction site. These two probes allowed us to ask if specific sequences within the TK B region were required to form complexes which

FIG. 4. Autoradiographic images of complexes formed in the presence of antibodies that recognize HSV-1-encoded proteins. Binding reactions with the TK A (A), TK B and γ gene B (B) regions were performed with nuclear extracts prepared from uninfected (lanes a, b, f, and g) or 5-h-infected (lanes c, d, e, h, i, and j) HeLa cells, as described under "Materials and Methods." Monoclonal antibodies specific for ICP4 (lanes b, d, g, and i) or glycoprotein C (lanes e and j) were added after incubation of the probes with extract. Samples in lanes a, c, and h were postincubated with a preimmune rabbit serum. The reaction mixtures were loaded onto native, 4% low ionic strength polyacrylamide gels, and complex formation was monitored by the mobility-shift electrophoresis assay. Only the regions of the gels containing complexes are shown. The position of bands that disapper or decrease in intensity after incubation with antibody is noted by solid circles, and ICP4-specific bands are indicated by arrowheads.
Each probe formed specific complexes with identical mobility patterns in extracts from uninfected cells and, as expected, their mobility was unaltered after incubation with antibody to ICP4 (Fig. 4B, lanes a, b, f, and g). Both probes formed again complexes with indistinguishable migration profiles when incubated with extracts from infected cells, and several of these migrated with a reduced mobility after incubation with antibody specific for ICP4 (Fig. 4B, lanes c and d, h, and i), but not when reacted with the gC-specific antibody (Fig. 4B, lanes e and f).

Thus, ICP4 is present in the infected-cell complexes formed when either the A or B region is used as probe. The data also reveal that the sequence between +16 and +36 in the B region is not required for complex formation with uninfected-cell extracts or for interaction of ICP4, which was previously suggested by the competing behavior of the TK B LS+16/+36 DNA (Fig. 3B, lanes f and p). However, much less of the slowly-migrating species is detected after incubation of the TK B LS+16/+36 probe in either extract.

Sequence Requirements for TK B Complex Formation.—To determine whether discrete sequences within the B region were required for complex formation, probes encompassing nucleotides −3 to +56, −16 to +14, −16 to +38, and +19 to +56 (Fig. 1, c, d, e, and f) were prepared and analyzed for their ability to form complexes after incubation in the two extracts. Each probe gave rise to specific complexes after incubation in extracts from uninfected cells, although their abundance was low and variable and the slower-migrating species were difficult to detect (Fig. 5, lanes c, e, g, and i). The most abundant complexes were detected with the +19 to +56 probe (Fig. 5, lane i). After incubation in extracts from infected cells these probes, with the exception of that spanning the −16 to +14 sequence, formed novel migrating species that were more abundant than those formed in extracts from uninfected cells (Fig. 5, lanes d, f, h, and j). However, the yield was uniformly greater with those probes which contain the 3′ half of the B sequence.

These results suggest that complex formation occurs as a consequence of recognition of multiple sites scattered throughout the B region. To further probe these interactions, the cytosome residues at −6 in the noncoding and −7 in the coding strand and the adenines at +18 in the noncoding and +15 in the coding strand were methylated using HaeIII and PstI methylases, respectively. These DNAs were then extensively digested with the corresponding restriction enzyme, and the uncleaved fragments representing a homogenous population of fully methylated DNAs were isolated from nondenaturing polyacrylamide gels. Complex formation with each of the three methylated probes in uninfected-cell extracts was greatly decreased (Fig. 5, lanes k, m, and o), whereas it was only marginally reduced when infected-cell extracts were used for the binding reactions (Fig. 5, lanes l, n, and p).

From these two experiments we infer that nucleotides throughout the B sequence are required to generate abundant specific complexes with extracts from uninfected cells. Methylation of even 2 bases at divergent sites results in decreased complex formation. Although the 5′ region (−16 to +14) did not form abundant complexes in either extract, it seems that its presence promotes complex formation about the entire TK B region (perhaps by providing stabilizing nonspecific contacts) because, perturbation of the sequence by the addition of methyl residues at either −6 and −7 (both of which protrude in the major groove of the double helix) decreases complex formation in extracts from uninfected cells. In marked distinction to these results we find that the methylated DNAs barely affected complex formation in extracts from infected cells. Thus, the interactions which occur in infected-cell extracts are distinct from those that take place in uninfected-cell extracts and, the presence of infected-cell proteins alters the sequence requirements for complex formation.

To further verify that the sequence requirements for complex formation in the two extracts were different, competition experiments with DNA fragments encompassing various parts of the B region were performed (Fig. 6). Complex formation in extracts from uninfected cells, with the wild-type B probe, was more efficiently competed by DNA encompassing the 3′ portion of this sequence (+19 to +56) than by its 5′ DNA (−16 to +14) counterpart (Fig. 6, lanes a, b, and c). However, this was not true when the TK B LS+16/+36 probe was tested (Fig. 6, lanes d, e, and f). Although binding was weak to begin with, each competitor inhibited complex formation. Because the yield of complex with the 5′ sequence is exceedingly low, we can only say that the rapidly migrating complex is not competed by the 3′ sequence (Fig. 6, lanes g and h). By comparison, complex formation with the 3′ sequence is readily competed by the 5′ sequence (Fig. 6, lanes i and j). These data demonstrate that both the 5′ and 3′ regions of the TK B sequence are recognized by some shared cellular factors, whose binding to the corresponding sequences leads to cooperative interactions that enhance TK B complex formation resulting in its increased abundance (Fig. 6, lane a).

The competition profile in extracts from infected cells...
f, h, m, p, and r) from the TK B region. The indicated probes were preincubated in the presence or absence of a 20-fold molar excess of fragments from the TK B region. Nuclear extracts prepared from uninfected (lanes a-j) or 5-h-infected (lanes h-t) HeLa cells were the unlabeled competitor DNAs encompassing either the -16/+14 sequence (lanes c, f, h, m, p, and r) or the +19/+56 sequence (lanes c, d, e, h, i, j, l, m, n, o, r, s, and t) HeLa cells, as described under "Materials and Methods," and then incubated in the presence of monoclonal antibodies specific for ICP4 (lanes b, d, g, i, l, n, q, and s), glycoprotein C (lanes c, e, j, o, and t) or a nonspecific rabbit antiserum (lanes a, f, h, k, m, p, q, and r). The reaction mixtures were loaded onto native, 4% low ionic strength polyacrylamide gels, and complex formation was monitored by the mobility-shift electrophoresis assay. Only the regions of the gels containing complexes are shown. The following probes were used: wild-type TK B region (lanes a, b, c, k, l, and m), LS/+16/+36 TK B region (lanes d, e, f, n, o, and p), -16/+14 DNA (lanes g, h, q, and r), and +19/+56 DNA (lanes i, j, s, and t).

revealed that the 3' portion of the B region (+19 to +56) inhibited binding of both the wild-type and the TK B LS+16/+36 probes (Fig. 6, lanes k and p), whereas the 5' sequence (-16 to +14) failed to inhibit complex formation (Fig. 6, lanes l and o). Furthermore, complex formation with the 3' sequence was refractile to inhibition by the 5' portion of the B region (Fig. 6, lanes s and t). These data support a hypothesis in which common and disparate proteins recognize the same sequences within the B region and virus gene expression modifies interactions between the B region and cellular proteins. They also emphasize the importance of the 3' sequence for complex formation in extracts from infected cells.

Sequences Required for Participation of ICP4 in TK B Complex Formation—The supershift assay revealed that ICP4 is present in complexes formed after incubation of the TK B probe with extracts from infected cells. Complexes formed with the subregion probes described in Fig. 1 were examined for the presence of ICP4 by this assay. This analysis demonstrated that infected-cell complexes formed with the -3 to +36, -16 to +38, and +19 to +56 probes all contained ICP4 (Fig. 7, lanes d, n, and s). Although the slower migrating of the low abundance complexes formed after incubation with the -16 to +14 probe appears to be disrupted after incubation with the antibody, no novel migrating species was detected (Fig. 7, lanes h and i). Both the -16 to +38 and +19 to +56 probes efficiently form ICP4-containing complexes (Fig. 7, lanes n and s). Control experiments with nonspecific antibody demonstrated the fidelity of this assay (Fig. 7, lanes e, o, and r). As expected, incubation of the complexes formed in infected-cell extracts with the antibody to ICP4 resulted in no change in their mobility (Fig. 7, lanes a, b, k, l, p, q). These results, together with the observation that ICP4 is present in the TK B LS+16/+36 complex (Fig. 4B, lane i) suggest that ICP4 interactions occur at the 3' end of the B region and that there are at least two distinct domains within this region that can promulgate these interactions. Moreover, there appears to be redundancy built into the B sequence and only a portion of it is required for ICP4 to be present in the complexes.

Complex Formation with a Sequence Containing a High Affinity ICP4-binding Site—ICP4 binds to various regions of the HSV genome with different affinities (36, 38-30, 56). A high affinity binding site overlapping the cap site of the a4 gene inhibits complex formation with the B region probe in extracts from both uninfected and infected cells (Fig. 3B, lanes h and r). Furthermore, our results indicate that common host factors recognize and form complexes with the ICP4-binding sequence (ICP4 BS) and the complex B region. The ICP4 BS sequence inhibited TK A complex formation only in extracts from infected cells (Fig. 3A, lanes h and i versus r and s). Therefore, we asked if complexes could be formed using the ICP4 BS probe in both extracts and, if either TK A or B DNAs inhibited formation of these putative complexes. Therefore, a high affinity ICP4-binding site probe (ICP4 BS) was incubated with extracts from uninfected and infected cells under conditions that elicited the A and B complexes. Complexes which differed in their mobility and abundance were formed in each extract (Fig. 8). Multiple species were observed in extracts from uninfected cells, whereas only a single abundant complex was present in extracts from infected cells. Moreover, complexes formed in each extract at both 50 and 50 mm NaCl (Fig. 8, lanes a, b, k, and l) distinguishing them from those formed with the TK B probe (51). Competition experiments revealed that these interactions were specific (Fig. 8, lanes i and s), and demonstrated that the TK B region was an efficient competitor in either extract (Fig. 8, lanes g, h, q, and r). As predicted, TK A DNA failed to inhibit complex formation in extracts from uninfected cells (Fig. 8, lanes e and f), but did so in infected-cell extracts (Fig. 8, lanes a and p). Furthermore, incubation of the complex formed in extracts from infected cells with the anti-ICP4 monoclonal antibody revealed the presence of ICP4 in the complex (Fig. 8, lane t).

These results complement those obtained with the TK A and B regions and demonstrate that common cellular proteins recognize and interact with both the ICP4 BS and the TK B DNAs, despite any apparent homology between these sequences. In addition, complex formation with these two regions is differentially affected by changes in ionic strength. Moreover, it is clear that some of the host factors bound to the TK A region are distinct from those recognizing ICP4 BS and TK B region sequences. In all instances ICP4 was identified as a complex participant in extracts from infected cells.

**Fig. 6.** Complexes formed in the presence of competitor fragments from the TK B region. Nuclear extracts prepared from uninfected (lanes a-j) or 5-h-infected (lanes h-t) HeLa cells were preincubated in the presence or absence of a 20-fold molar excess of the unlabeled competitor DNAs encompassing either the -16/+14 sequence (lanes b, e, j, l, o, and t) or the +19/+56 sequence (lanes c, f, h, m, p, and r) from the TK B region. The indicated probes were then added and the incubation continued for 30 min. The reaction mixtures were analyzed for complex formation by the mobility-shift electrophoresis assay, and dried gels were exposed to x-ray film. Only the regions of the gels containing complexes are shown. The following probes were used: wild-type TK B region (lanes a, b, c, k, l, and m), LS+16/+36 TK B region (lanes d, e, f, n, o, and p), -16/+14 DNA (lanes g, h, q, and r), and +19/+56 DNA (lanes i, j, s, and t). Complexes which differed in their mobility and abundance were formed in each extract (Fig. 8). Multiple species were observed in extracts from uninfected cells, whereas only a single abundant complex was present in extracts from infected cells. Moreover, complexes formed in each extract at both 50 and 50 mm NaCl (Fig. 8, lanes a, b, k, and l) distinguishing them from those formed with the TK B probe (51). Competition experiments revealed that these interactions were specific (Fig. 8, lanes i and s), and demonstrated that the TK B region was an efficient competitor in either extract (Fig. 8, lanes g, h, q, and r). As predicted, TK A DNA failed to inhibit complex formation in extracts from uninfected cells (Fig. 8, lanes e and f), but did so in infected-cell extracts (Fig. 8, lanes a and p). Furthermore, incubation of the complex formed in extracts from infected cells with the anti-ICP4 monoclonal antibody revealed the presence of ICP4 in the complex (Fig. 8, lane t).

**Fig. 7.** Autoradiographic images of complexes formed with fragments from the TK B region in the presence of antibodies that recognize HSV-1-encoded proteins. Binding reactions with the -3/+56 (lanes a-o), -16/+14 (lanes f-j), -16/+36 (lanes k-o), and +19/+56 (lanes p-t) probes were performed using nuclear extracts prepared from uninfected (lanes a, b, f, g, k, l, p, and q) or 5-h-infected (lanes c, d, e, h, i, j, l, m, n, o, r, s, and t) HeLa cells, as described under "Materials and Methods," and then incubated in the presence of monoclonal antibodies specific for ICP4 (lanes b, d, g, i, l, n, q, and s), glycoprotein C (lanes e, j, o, and t) or a nonspecific rabbit antiserum (lanes a, c, f, h, k, m, p, and r). The reaction mixtures were loaded onto native, 4% low ionic strength polyacrylamide gels, and complex formation was monitored by the mobility-shift electrophoresis assay. Only the regions of the gels containing complexes are shown. The position of bands that disappear or decrease in intensity after incubation with antibody is noted by solid circles, and ICP4-specific bands are indicated by arrowheads.
amined their relative dissociation rates. For each of the three probes (TK A, TK B, and ICP4 BS), complexes were allowed to form for 30 min (51) and then chased with a 250-fold molar excess of unlabeled ICP4 BS DNA was added to each reaction and samples were withdrawn at 1, 5, 10, and 20 min after the chase, and applied directly to a running 4% low ionic strength polyacrylamide gel.

**FIG. 8.** Complex formation with DNA containing a high affinity ICP4-binding site. A 49-bp Aul/BamHI fragment from the ori gene (schematic) was end-labeled at the BamHI site and incubated in reaction mixtures containing extracts from uninfected (lanes a–j) or 5-h-infected (lanes k–t) HeLa cells, in the presence or absence of various unlabeled competitor DNAs. The binding reactions contained either 5 mm (contributed by the extract) NaCl (lanes a, b, and m–l), or 50 mm NaCl (lanes b–j and l). The competitor DNAs and their molar ratios relative to probe were: lanes c, m and d, n, ICP4 BS at 5- and 10-fold molar excess, respectively; lanes e, o and f, TK A region DNA at 10- and 20-fold molar excess, respectively; lanes g, q and h, r, TK B region DNA at 10- and 20-fold molar excess, respectively; and lanes i and s, each contain a 50-fold molar excess of the ICP4 BS probes are shared (Figs. 4A, lane d, 4B, lane d, and i, and 8, lanes g and i, and 9B, lanes f and g). This chemical nuclease cleavage analysis further differentiates the interactions which occur when the 3’ end of the TK B region probe is incubated in uninfected- or infected-cell extracts. Specifically, these results would be expected given the kinetically labile nature of the complexes formed in infected-cell extracts and suggest that host factors stably interact with this sequence in the absence of virus-specified proteins. In contrast, the ICP4 BS complexes which form in...
FIG. 10. Chemical nuclease cleavage pattern of complexes formed with the TK B region or high affinity ICP4-binding site DNAs. Complexes were formed with the TK B region (A) or ICP4 BS (B) DNAs and nuclear extracts prepared from uninfected or 5-h-infected HeLa cells in standard binding reactions that were scaled up 10-fold. Reaction products were then fractionated by a preparative mobility-shift electrophoresis assay. Immediately after electrophoresis, the gels were subjected to chemical nuclease treatment as described under "Materials and Methods," and free and bound DNAs were then transferred to an NA45 membrane. Bands corresponding to the most abundant DNA-protein complexes (uninfected-cell extracts) and ICP4-containing complexes (infected-cell extracts) were located, eluted, denatured, and displayed on a standard 15% polyacrylamide sequencing gel. A, lanes a and f contain Maxam-Gilbert G+A sequencing reactions; lanes b and e contain cleaved DNAs isolated from bands corresponding to free DNAs from reactions containing extracts from uninfected and infected cells, respectively; and lanes c and d contain cleaved DNAs isolated from complexes formed in reactions containing extracts from uninfected and infected cells, respectively. B, lanes a and e contain Maxam-Gilbert G+A sequencing ladders; lanes b and f contain cleaved DNAs isolated from bands corresponding to free DNAs from reactions containing extracts from uninfected and infected cells, respectively; lanes c and g contain cleaved DNAs isolated from complexes formed in reactions containing extracts from uninfected and infected cells, respectively; and lane d depicts the cleavage pattern of DNA isolated from a nonspecific, fast-migrating complex formed in extracts from uninfected cells. Arrowheads connected by a line in both panels indicate the protected regions.

either extract are stable and appear to assemble on the same core sequence.

DISCUSSION
Regulated expression of the HSV thymidine kinase gene involves the interaction of trans-acting factors with cis-acting sequences. Genetic and biochemical studies have demonstrated that functional ICP4 is required for transcriptional activation of TK and other β genes during the course of a lytic infection (21, 35, 57, 58). To identify regions within the TK gene that interact with cell- and virus-specified proteins, we prepared nuclear extracts from uninfected and 5-h-infected HeLa cells and, using a mobility-shift electrophoresis assay and footprinting with a chemical nuclease, examined their ability to form complexes with the DNA sequences that compose the promoter/regulatory and leader regions of the gene, in comparison with the binding properties of a DNA fragment which overlaps the cap site for the α4 gene and contains a well characterized high affinity binding site for ICP4.

In this study, we report that all of these regions form specific complexes in extracts from uninfected cells which differ in their sensitivity to ionic strength and kinetic stability. These interactions are differentially altered after expression of at least one virus-specified protein, ICP4, as determined by both the above criteria as well as chemical nuclease cleavage analysis.

To date, the association of ICP4 with a high affinity binding site or a number of different DNA sequences that deviate from it has been demonstrated in five HSV genes encoding gD (16, 38, 39, 59), ICPs 0 (19, 36, 42, 60), 4 (37, 41, 42, 56), 25 and 42 (40). Previous attempts to map an ICP4-binding site within the TK promoter did not localize the interaction. Our present analyses demonstrate that complexes which form with the TK sequences in either extract are specific and in each instance their mobility is altered by the presence of at least ICP4. The complexes formed with the A and B region probes in extracts from uninfected cells were competed by low concentrations of homologous DNAs. Furthermore, the B but not the A probe was inhibited from forming specific complexes in both extracts by the ICP4 BS. The complexes which formed with the A and B probes in extracts from infected cells were each competed by the other DNA and by the fragment containing the ICP4-binding site. Most of the complexes formed in extracts from infected cells with each of the TK probes contained ICP4, as determined by supershift
experiments. Thus, the adjacent A and B regions appear to interact with both common and unique factors. The complexes which they form in the two extracts are differentiated by their physical and biochemical properties and are distinct from one another.

The TK A region includes sequences recognized by well characterized cellular transcription factors (61-63) and gives rise to specific complexes in binding reactions with extracts from HSV-1-infected cells. Some of these complexes may represent the interaction of ICP4 and/or other α gene products with the cellular transcription factors that occupy this region. In addition, CCAAT, ATG, and perhaps Sp1 binding activities might be modified after infection, leading to altered mobilities of complexes that either contribute to trans-induction per se or increase promoter strength, perhaps by stabilizing or making more accessible the transcription machinery.

There are several examples from other systems where trans-induction appears to be mediated via modulation of known binding activities. Abmayr et al. (64) have reported that an immediate-early protein from another herpes virus, pseudorabies, facilitates the interaction of the ATA box binding factor TFIID with promoters. Wu et al. (65) and Simon et al. (66) have implicated ATA box recognition in the induction of the adenovirus E1b and the hsp70 genes by adenovirus E1a.

The efficient competition by A region DNA for the TK R probe only in extracts from infected cells, implies that the two DNA fragments bind a common infected-cell specific factor(s). The binding protein(s) might contain independent DNA-binding domains, each capable of interacting with a different sequence element or, a single domain of the protein(s) could be capable of recognizing divergent DNA sequences as demonstrated for the octamer-binding proteins (67). This may be accomplished by recognition of a common DNA structure or conformation, as proposed for the yeast transcription activator, HAP1, which interacts with different sequence elements upstream of the CYC1 and CYC7 genes (68). Alternatively, diversity may be generated by the participation of other cell- and/or virus-specified non-DNA-binding proteins in the complex. The biologic consequences of these multiple interactions are at present unknown, but they might potentiate the induction or repression of TK gene expression.

The TK B region was more thoroughly analyzed. The binding properties of subsets of sequences that encompassed this region were examined. Our results demonstrate that this region is bound by cell factors and that common cellular factors recognize this sequence and the ICP4 BS. The appearance of multiple species in the mobility-shift analysis predicts that, despite its small size, the TK B region is probably bound by more than one cell factor. The 5' end of this region (-16 to +14) does not efficiently form complexes in either extract, while probes containing all or portions of the 3' half (+19 to +56) do. Moreover, it is the complexes formed with the 3' end of the B region with which ICP4 strongly interacts. The TK R I, S4-16/4-38, -16/4-38, and +19/ +56 probes all form complexes that contain ICP4. Therefore, we posit that ICP4 can interact with complexes formed at either of two or both sets of sequences between +19 to +38 and +38 to +56 within the TK B region. The dissociation analysis and competition experiments with the high affinity ICP4-binding site indicate that this interaction is mediated through protein-protein and not DNA protein contacts (see also Ref. 51).

The kinetic experiments with extracts from infected cells and the TK B region probe suggest a biochemical basis for our inability to generate a chemical nuclease cleavage pattern that differed between free and bound DNAs. The very short lifetimes of these complexes (≤2 min) predict that bound proteins can rapidly detach from the protected DNA fragment, allowing the chemical nuclease to gain access and cleave it. Thus, "stable" virus-specific complexes should have the same cleavage pattern as the corresponding free DNA. It is the exclusion volume of the gel which increases the local concentration of the reactants shifting the equilibrium toward reassociation that permits these kinetically labile complexes to be detected as stable species by the mobility-shift electrophoresis assay (69, 70).

The experiments with the ICP4 BS were designed to monitor the fidelity of ICP4 binding. We demonstrated that this sequence was bound by extracts from uninfected cells, and that the complexes which formed with this probe utilized the same, or a subset of core proteins used to form complexes with the TK B-region probe. These sequences originate from genes which are expressed at two different times postinfection. An efficient mechanism for differential regulation of the α and β kinetic classes of HSV genes might utilize the same or a subset of core trans-acting cell factors which are subsequently altered by virus-specified or induced proteins, thus assuring temporal regulation of the various kinetic classes.

A potential advantage to using common factors and multiple sites to control transcription is that small changes in the concentration of transcription factors can be amplified, leading to large differential effects on RNA synthesis. The regulatory effects could be positive or negative in nature. Thus, fluctuations in the concentrations of ICP4 and other α gene products could have major effects on factor and/or RNA polymerase II binding to viral genes and the efficiency of the DNA as templates for RNA synthesis. Recent studies showed that the virus-associated transactivator α-TIF (71-73) and the E1a protein from adenovirus (74) recognize DNA-bound proteins. How they are targeted to the appropriate sequences and activate transcription is still unclear. If ICP4 uses this alternative mechanism of activation then it alone, or in conjunction with other α gene products, could differentially affect the arrangement of cell factors along the corresponding promoter/regulatory domains of a virus gene.

The complexity of the interactions we observed suggest that sequences throughout the promoter/regulatory and mRNA leader regions of the TK gene interact with an array of polypeptides. Therefore, recognition results from the summation of multiple independent protein-DNA contacts rather than solely from essential contacts within a core binding site. Accordingly, modification to or removal of any sequence, except a core element that is obligatorily required for transcription is unlikely to completely inhibit activation or repression of the TK gene.

To accommodate the experimental data we propose that virus genes are regulated as a consequence of a series of discrete interactions which initially are between cis-acting regulatory elements and trans-acting cell factors. This interaction results in activation or repression of transcription depending on the kinetic class of the gene. Thus, α genes are transcriptionally active at early times postinfection and their level of activity is enhanced by the interaction of α-TIF with OTF-1 (72, 75). Subsequently, when sufficient ICP4 is synthesized, it can bind to high affinity sites which initially are occupied by cell factors. Substitution of cell factors by ICP4 can now result in repression of transcription (19). Regulation of β gene expression, which is of necessity different, is readily accommodated by the model. Early after infection there is no detectable expression from the TK gene (14, 16, 22, 30). We envision that at these very early times the promoter/regula-
DNA-Protein Interactions at the HSV TK Locus

Proteins that cover the defined promoter of the virus TK gene pass through the leader region and transcribe the gene (Fig. 1). The effect of this interaction is unknown. We have constructed a model for DNA-protein interactions at the HSV TK locus. A schematic representation of the interactions which occur between extracts prepared from uninfected (A) and 5-h-infected (B) HeLa cells, and the 5' end of the HSV-1 TK gene is shown. The number of cell-specific factors which interact with the TK B region probe is unknown and has been arbitrarily assigned. Similarly, the location and number of ICP4 molecules bound to the TK A region have not been determined. The altered configuration of cell-specific factors on the TK B region template is based on the data presented in Figs. 9 and 10A, which show that ICP4, for complexes formed in extracts from infected cells is greater than that for complexes formed in extracts from uninfected cells. Accordingly, the proteins which interact with the TK B region are depicted as being more loosely associated with the DNA. ICP4 appears to be able to interact with complexes which form either of two sequences within the TK B region (see Figs. 4B and 7). Evidence for an infected-cell-specific factor is based on comparison experiments with the A and B region probes in extracts from infected cells. We postulate that it is the sum of these interactions that weakens the association of cell-specific negative regulators to allow RNA polymerase II to pass through this region, resulting in activation of transcription.

A consequence of this alteration is that infected-cell extracts dissociate much more readily than their counterparts which form in uninduced state (51), may represent potential routes for posttranslational modification and regulation. Additionally, accumulation of TK mRNA by the deletion mutant can be attributed to the effect of ICP4 on the upstream sequence. Experiments to test the validity of our model are in progress. We are currently mutating and deleting sequences in and around the leader and rebuilding these in the virus chromosome to ascertain the contribution of the TK B region to regulated expression of the TK gene.

In conclusion, our data suggest that ICP4, the major regulatory protein of herpes simplex virus, interacts with cell proteins that cover the defined promoter of the virus TK gene and a region adjacent to and downstream of it. The biological effect of this interaction is unknown. We have constructed a model wherein cellular factors interacting with the A and B promoter-leader sequences maintain or repress basal level expression. The model predicts that these interactions negatively regulate transcription of this gene. ICP4 (alone or in conjunction with other α gene products (e.g. ICPO)), through its destabilizing effect at the TK B site, may increase the frequency of transcription initiation, leading to an increased transcription rate and possibly an increased probability that transcripts will be converted to stable RNA. In this model, the presence of functional ICP4 affects the frequency with which the transcription machinery (which forms over the promoter/regulatory region, TK A) bypasses the downstream DNA-host-protein complexes. There is precedence for a role for DNA encoding mRNA leader sequences in herpes simplex virus gene expression. These sequences affect regulation and stabilization of the virus RNAs encoding α-TIF and VP5 (54, 55) and the TK gene (32). Moreover, cis-acting regulatory signals in 5'-transcribed noncoding sequences have also been reported in other systems (77-80). The nature of the contribution to regulation provided by the 3' end of the TK B region has been probed in a single instance (81). Here, the region from -12 to +189 was deleted in vitro and the deleted sequence restored to the intact virus genome. The TK gene in this virus was actively transcribed. The findings from this study are supported by our model which envisions that transcriptional activation of the TK gene results from the action of ICP4 at both upstream (TK A) and downstream (TK B) sites. Action at the upstream site activates transcription, whereas the downstream site is not obligatorily required for transcriptional activation but may regulate the activation process. Therefore, accumulation of TK mRNA by the deletion mutant can be attributed to the effect of ICP4 on the upstream sequence. Experiments to test the validity of our model are in progress. We are currently mutating and deleting sequences in and around the leader and rebuilding these in the virus chromosome to ascertain the contribution of the TK B region to regulated expression of the TK gene.

From these studies we infer that ICP4 may have dual regulatory functions and that these are reflected in different binding activities. If high affinity binding sites are used for negative regulation by direct binding of ICP4 (19, 37), then alternate binding sites (39-41), or modulation of DNA-protein or protein-protein interactions which normally maintain the uninduced state (51), may represent potential routes for positive regulation by this protein. In this context, the function of ICP4 may well be determined by the nature and extent of the posttranslational modification to which it has been subjected.

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