The EMBO Journal vol.10 no.2 pp.397-406, 1991

The interaction of ICP4 with cell/infected-cell factors and its state of phosphorylation modulate differential recognition of leader sequences in herpes simplex virus DNA

Athanasios G.Papavassiliou[2], Kent W.Wilcox[1] and Saul J.Silverstein

Department of Microbiology, College of Physicians and Surgeons, Columbia University, 701 W 168th Street, New York, NY 10032 and [1]Department of Microbiology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA

[2]Present address: European Molecular Biology Laboratory, Differentiation Programme, Postfach 10.2209, Meyerhofstrasse 1, 6900 Heidelberg, Germany

Communicated by I.W.Mattaj

Regulation of herpes simplex virus (HSV) gene expression requires the synthesis of functional ICP4, a phosphoprotein that binds to several specific sites in virus DNA and acts in *trans* either to activate or to repress transcription of the three major kinetic classes of virus genes. Binding of ICP4 to specific sites in α genes (which are the first to be transcribed) causes repression of α-gene expression. ICP4 also indirectly participates in the formation of DNA–protein complexes with sequences present in the promoter/regulatory and leader regions of the β and γ genes that are sequentially activated later in infection. Here we demonstrate that the extent of phosphorylation of ICP4 contributes to its ability to participate differentially in complex formation with *cis*-acting elements present in β and γ genes. Dephosphorylated ICP4 retains its binding properties for the high affinity sites present in α promoters, whereas only phosphorylated forms of the protein are able to participate in complex formation with model β and γ sequences. These studies also reveal a requirement for cell and infected-cell factors to recognize the β and γ sequences. Our data suggest that the state of phosphorylation and concentration of ICP4 within the nucleus of infected cells determine the extent to which ICP4 interacts with these other factors.

Key words: DNA binding/herpes simplex virus/ICP4/phosphorylation

Introduction

Herpes simplex virus (HSV) has a double-stranded DNA genome of ~152 kilobase pairs (kbp), with the capacity to encode at least 75 polypeptides (McGeoch *et al.*, 1986, 1988). Gene expression in cells infected with HSV is coordinately regulated and sequentially ordered in a cascade fashion (Honess and Roizman, 1974, 1975). Three major kinetic classes of genes termed α, β, and γ are temporally expressed during the course of a productive infection (Honess and Roizman, 1975). Transcriptional regulation of the promoters for these genes requires functional ICP4 (Infected-Cell Protein 4), an α-gene product that is the major regulatory protein of the virus, throughout the replication cycle (Dixon and Schaffer, 1980; Preston, 1979a,b; Watson and Clements, 1980).

ICP4 is predicted to have a molecular mass of 132 835 daltons (McGeoch *et al.*, 1986) and localizes to the nucleus of infected cells (Courtney and Benyesh-Melnick, 1974; Pereira *et al.*, 1977). The protein is ADP-ribosylated (Preston and Notarianni, 1983) and phosphorylated to different extents (Pereira *et al.*, 1977). The phosphates bound to ICP4-cycle during the lytic infection and particular species predominate at different stages (Wilcox *et al.*, 1980). Some of these phosphates were shown to contribute to the transactivation properties of ICP4 (DeLuca and Schaffer, 1987). Native ICP4 isolated from HSV-infected cells exists as a highly elongated homodimeric complex which is phosphorylated at several serine and threonine residues (Faber and Wilcox, 1986b; Metzler and Wilcox, 1985). The purified protein binds with high affinity to DNA containing the sequence 5′-ATCGTC-3′ (Faber and Wilcox, 1986a; Kattar-Cooley and Wilcox, 1989; Kristie and Roizman, 1986; Muller, 1987). Recently residues between amino acids 170 and 250, which include a contiguous tract of serines, as well as other unidentified residues carboxy-terminal to amino acid 309 have been genetically implicated as sites of ICP4 phosphorylation (DeLuca and Schafer, 1988). Residues 262–490 of ICP4 are sufficient for direct DNA binding to a high affinity site (Wu and Wilcox, 1990).

ICP4 acts as a negative regulator of α-gene expression and as a transcriptional activator of β and γ genes. Viruses with temperature-sensitive, deletion, or nonsense mutations in the gene encoding ICP4 overproduce α- and fail to synthesize β- and γ-gene products (DeLuca *et al.*, 1985; De Luca and Schaffer, 1988; Dixon and Schaffer, 1980. Marsden *et al.*, 1976; Preston, 1979a,b). A variant of ICP4 that lacks amino acids 143–210 retains its ability to bind a high affinity site and repress transcription of α genes; however, it fails to activate expression driven by a β-gene promoter (Shepard *et al.*, 1989). The DNA-binding domain is required for repression of α-gene transcription (Paterson and Everett, 1988. Roberts *et al.*, 1988; Shepard *et al.*, 1989; Wu and Wilcox, 1990). ICP4 also participates in the formation of DNA–protein complexes with fragments containing the promoter/regulatory and leader regions of many β and γ genes (Michael *et al.*, 1988; Papavassiliou and Silverstein, 1990a,b; Tedder *et al.*, 1989). Analysis of the protein-binding sites in these β and γ genes revealed that their sequences deviate substantially from the high affinity sites defined for ICP4. Furthermore, when DNA fragments from β and γ genes are incubated with extracts from infected cells several ICP4-containing complexes form indicating that additional proteins are involved in recognition of these sequences (Flanagan *et al.*, 1991; Papavassiliou and Silverstein, 1990a,b).

These properties of ICP4 prompted us to investigate the molecular nature of its interaction with DNA probes derived from representative α, β and γ genes that include

the noncoding leaders and a region that is between the cap site and TATA box from each gene. The leader sequences contained in these α and γ probes are important for regulated expression of the genes from which they are derived (Blair *et al.*, 1987; Flanagan *et al.*, 1991; Homa *et al.*, 1986; Resnick *et al.*, 1989; Roberts *et al.*, 1988). There is as yet no known role for the leader sequences in controlling expression of β genes. The leader from the virus thymidine kinase gene (nts -12 to $+189$) has been deleted (Halpern and Smiley, 1984) and transposed to other virus promoters without affecting inducibility (Mavromara-Nazos and Roizman, 1989). Here we demonstrate that the extent of phosphorylation of ICP4 differentially affects the interactions that occur with each of these sequences and that an infected-cell factor (ICF) is required to form complexes between the β-leader probe and ICP4, whereas only uninfected cell factors and ICP4 are required to generate the complexes seen with the γ-leader probe in extracts from infected cells.

Results

Correlation between the phosphorylation state of ICP4 and its binding to a high affinity site in DNA

We examined the relationship between the state of phosphorylation of ICP4 and its ability to participate in formation of complexes with representative leader sequences from α, β and γ genes by treating nuclear extracts from cells infected with HSV-1 for 5 h with potato acid phosphatase (AP) for various periods of time and terminating the reactions by the addition of AP inhibitors. The effect of AP treatment (dephosphorylation) on ICP4 was monitored by immunoblot analysis and the DNA-binding properties of the treated extracts were tested by a mobility-shift electrophoresis assay. Figure 1A (lanes 1–3) demonstrates that dephosphorylation results in loss of the slowest-migrating electrophoretic from and the appearance of four novel species of ICP4 which differ in their abundance. Increased incubation time (lane 4) or higher concentrations of AP (data not shown) did not alter the electrophoretic mobility of these species. None of these four novel species of ICP4 were observed when infected-cell extracts were incubated with AP for 30 min in the presence of AP inhibitors and, therefore, they are not proteolytic degradation products (Figure 1A, lane 1).

Gel-retardation analysis of extracts treated with AP for the same periods of time revealed that they all retained the capacity to form complexes with a high affinity ICP4-binding site (Figure 1B). The more rapid mobility of the complexes correlated with the increased rate of migration of dephosphorylated ICP4.

Effect of dephosphorylation of infected-cell extracts on the formation of complexes with representative leader sequences from HSV-1 genes

A mobility-shift electrophoresis assay was used to examine the effects of dephosphorylation on the ability of ICP4-containing extracts to form complexes with DNA probes containing leader sequences from each of the three major kinetic classes of HSV-1 genes. Incubation of each probe with extracts from mock-infected cells gave rise to specific complexes (Figure 2 lane 1). Treatment of these extracts with AP had no effect on complex formation (Figure 2, lane 4).

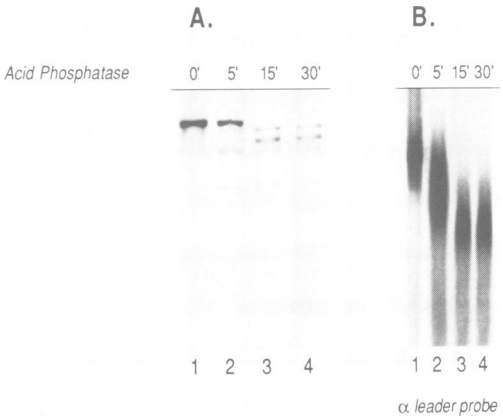

Fig. 1. Kinetics of acid phosphatase treatment. **A** Western blots of acid phosphastase-treated extracts using an ICP4-specific monoclonal antibody as a probe. Samples of nuclear extracts (28 μg) prepared from 5 h-HSV-1-infected HeLa cells were mixed with 8 μg of acid phosphatase (AP) and incubated at RT for the indicated periods of time. Immediately after treatment, reaction mixtures were chased with AP inhibitors, electrophoresed through a linear gradient SDS–PAGE, and the separated proteins were then subjected to Western immunoblot analysis as detailed in Materials and methods. Lane 1 contains a control sample where the extracts were treated with AP for 30 min in the presence of AP inhibitors. **B.** Mobility-shift electrophoresis assay of an α-leader probe after incubation with AP-treated nuclear extracts. The probe was incubated with aliquots (7 μg) of nuclear extracts from infected cells that were treated with 2 μg of acid phosphatase for the indicated periods of time. The acid phosphatase reactions were terminated as in A before assaying each sample for DNA binding. Only the region of the gel containing complexes is shown.

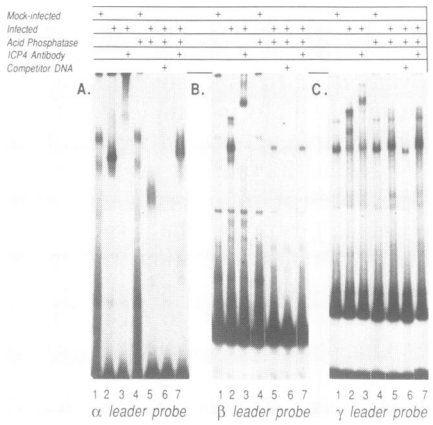

Fig. 2. Mobility-shift electrophoresis assays showing binding of untreated and acid phosphatase-treated nuclear extracts to the α-, β-, and γ-leader probes (panels **A**, **B**, and **C**, respectively). Samples of nuclear extracts (7 μg) prepared from mock-infected or infected cells were preincubated with (lanes 4–7) or without (lanes 1–3) 2 μg of acid phosphatase (AP) for 15 min at RT. Reaction mixtures were subsequently chased with AP inhibitors and assayed directly for DNA binding, as described in Materials and methods. The binding reaction in lane 6 was performed in the presence of a 5-fold molar excess of unlabeled self competitor DNA, whereas that in lane 7 was further incubated with a monoclonal antibody specific for ICP4. The final NaCl concentration of the binding reactions was 50 mM for lanes 1 and 4 and 5 mM (contributed by the extract) for all other lanes.

Novel DNA–protein complexes formed when these probes were incubated in extracts from infected cells and some of these complexes contain ICP4, as shown by the ability of an ICP4-specific monoclonal antibody to retard

their mobility further (Figure 2, lanes 2 and 3). Treatment of extracts from infected cells with AP altered the pattern of the complexes that formed with each probe to different extents. As previously shown (Figure 1B), the α-leader probe formed faster-migrating complexes with the AP-treated extracts (Figure 2A, lane 5). Binding of the β-leader probe in AP-treated infected-cell extracts resulted in the appearance of a single, low abundance complex that migrated to the same position as the most abundant ICP4-containing species formed in untreated extracts (Figure 2B, lanes 2 and 5). However, its mobility was not altered after incubation with the ICP4-specific monoclonal antibody (Figure 2B, lane 7). In these extracts the binding pattern of the γ-leader probe was indistinguishable from that generated after incubation in extracts from mock-infected cells (Figure 2C, compare lane 5 with lanes 1 and 2). The specificity of complex formation in AP-treated extracts from infected cells was shown by competition analysis (Figure 2, lane 6). Probing of the complexes formed in AP-treated extracts from infected cells with a monoclonal antibody specific for ICP4 revealed that only the α-leader complexes contained ICP4 (Figure 2, lane 7). Thus, phosphates on ICP4 and/or other proteins present in infected-cell extracts differentially contribute to complex formation with the α, β-, and γ-leader probes.

Effect of acid phosphatase treatment on the mobility of equilibrated complexes

To determine if ICP4-containing complexes have exposed phosphates whose removal might affect their equilibria, α-, β-, and γ-leader complexes were allowed to form under standard reaction conditions and then incubated with AP. This treatment did not alter the abundance or migration profile of any of the complexes formed between proteins in extracts from infected cells and the β- or γ-leader probes (Figure 3, lanes 3–6). AP treatment of the α-leader complex increased its mobility and yielded a pattern similar to that obtained with extracts from infected cells treated for 5 min with AP (Figure 3, lane 2, compare with Figure 1B, lane 2). These findings suggest a differential arrangement of ICP4 in the complexes formed with each target DNA.

Cell proteins make different contributions to DNA–protein complex formation between purified ICP4 and α-, and β- and γ-leader probes

The molecular details of the ICP4 complex-forming ability with different DNA targets were further studied by examining the interaction of affinity-purified ICP4 with either the α-, β-,and γ-leader probes or with equilibrated α-, β-, and γ-leader complexes formed in extracts from mock-infected cells. ICP4 forms a single abundant complex with the high affinity site in the α-leader probe (Figure 4A, lane 1). Higher concentrations of ICP4 drive more probe into this complex and slower-migrating species which most likely arise as a result of the binding of higher oligomeric forms of ICP4, are generated as the protein concentration increases (Figure 4A, lane 2). The specificity of this interaction was demonstrated by competition analysis with a 5-fold molar excess of unlabeled homologous HSV-1 DNA (Figure 4A, lane 3). Addition of a μg equivalent (1 μg equivalent is that amount of ICP4 normally found in 1 μg of infected-cell extract) of ICP4 to equilibrated complexes formed in extracts from mock-infected cells alters the mobility-shift profile to that seen in extracts from infected cells, resulting in the

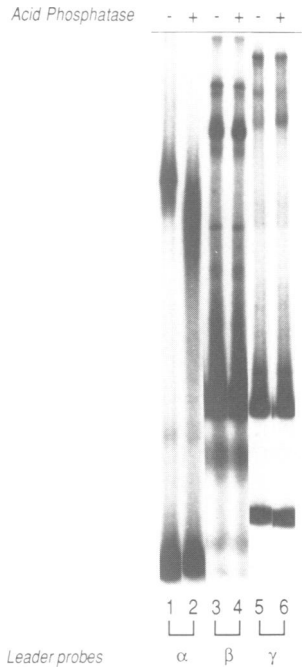

Fig. 3. Effect of acid phosphatase on equilibrated α-, β- and γ-leader complexes. Aliquots of nuclear extracts (7 μg) prepared from infected cells were incubated with the α-(lanes 1 and 2), β-(lanes 3 and 4), or γ-(lanes 5 and 6) leader probes for 30 min at RT ($NaPO_4$ was omitted from the binding buffer in this experiment). At the end of the incubation period, binding reactions were treated with either 2 μg of BSA (lanes 1, 3 and 5) or 2 μg of acid phosphatase (lanes 2, 4 and 6) for 15 min at ambient temperature. They were then chased with AP inhibitors and analyzed for complex formation by the mobility-shift electrophoresis assay as described in Materials and methods. The final NaCl concentration in all binding reactions was 5 mM (contributed by the extract).

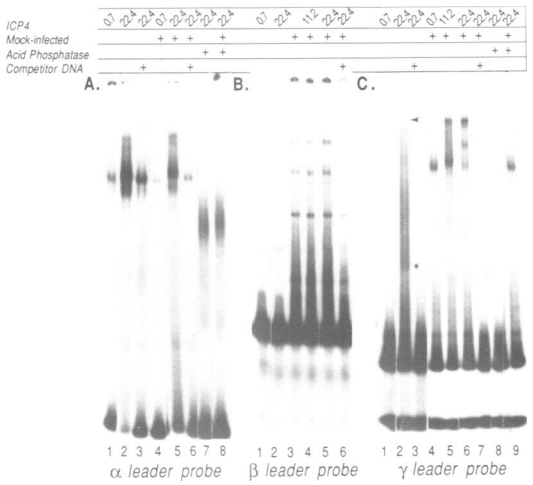

Fig. 4. Interaction of untreated or acid phosphatase-treated purified ICP4 with the α-, β-, and γ-leader probes and complexes equilibrated in extracts from mock-infected cells. α-, β-, and γ-leader probes (Panels **A**, **B** and **C**) were incubated under binding conditions with the amount of ICP4 indicated above each lane. In some instances untreated ICP4 (A and B, lanes 4–6 and C, lanes 4–7) or AP-treated ICP4 (A, lanes 7 and 8, and C, lanes 8 and 9) was added to equilibrated complexes formed in extracts from mock-infected cells. A five-fold molar excess of competitor DNA was added to the appropriate reactions, as indicated above the lanes, to demonstrate specificity. The arrowhead in panel C points to the specific low-abundance complex discussed in the text.

displacement of cell proteins by ICP4 (compare Figure 2A, lanes 1 and 2 with Figure 4A, lanes 4 and 5) and the appearance of a single abundant species with the expected specificity (Figure 4A, lane 6). The migration profile of the complexes formed between purified ICP4 treated with AP and the α-leader probe was indistinguishable from the pattern seen after AP treatment of nuclear extracts from infected cells (Figure 4A, lane 7 and Figure 1B, lane 3). When AP-treated ICP4 is restored to complexes formed in nuclear extracts from mock-infected cells a specific heterogenous band of ICP4-containing complexes is detected (Figure 4A, lane 8). These data demonstrate that both native and dephosphorylated forms of ICP4 bind directly to high affinity sites on the α-leader template.

The same analysis was performed with the β- and γ-leader probes. Purified ICP4 does not form a detectable DNA−protein complex with the β-leader probe at either low or high concentrations of the protein (Figure 4B, lanes 1 and 2). Addition of purified ICP4 to β-leader complexes formed in extracts from mock-infected cells does not alter the binding profile (Figure 4B, compare lane 3 with lanes 4 and 5). The specificity of these interactions was verified by competition analysis using homologous DNA (Figure 4B, lane 6). Moreover, treatment of ICP4 with AP did not affect these results (data not shown). Therefore, addition of either form(s) of ICP4 to equilibrated complexes formed in extracts from mock-infected cells does not generate the mobility pattern obtained in infected-cell extracts. From these results we infer that complexes formed with the β-leader probe require a factor(s) which is present only in infected cells (ICF). We note that addition of purified ICP4 to dephosphorylated extracts prepared from infected cells results in a binding profile that is indistinguishable from that of the untreated extracts (data not shown). Thus, dephosphorylation of infected-cell extracts does not alter the ability of the infected-cell factor(s) to interact with exogenously added ICP4 and generate the mobility-shift pattern seen in untreated extracts.

Incubation of the γ-leader probe with high concentrations of purified ICP4 results in formation of a low-abundance complex (Figure 4C, lane 1 and 2, arrowhead). This reaction is sequence specific as demonstrated by competititon analysis (Figure 4C, lane 3). Addition of increasing amounts of purified ICP4 to equilibrated binding reactions in extracts from mock-infected cells reconstituted the infected-cell profile (Figure 4C, lanes 4−6, compare with Figure 2C, lane 2). We note that the abundant, rapidly-migrating complex (lane 4) decreases as the concentration of ICP4 increases. The mobility of this complex was unaltered after incubation with the ICP4 monoclonal antibody and therefore does not contain ICP4 (Figure 2C, lane 3).In concert with the loss of this species are the appearance of a novel complex of intermediate mobility and two slowly-migrating species, one of which has the same electrophoretic mobility (but not abundance) as the one that appears after incubation with high concentrations of purified ICP4 (Figure 4C, lane 2, arrowhead). Closer inspection of Figure 2C (lanes 2 and 3) reveals that a fraction of the slowest-migrating of these two species persists after further incubation of the complexes with the ICP4-specific monoclonal antibody (regardless of the amount of antibody added; data not shown). This species is likely to be composed of two bands with very similar electrophoretic mobilities. One contains at least ICP4, while the other contains only cell factors. Because the latter complex is not generated in reactions containing extracts from mock-infected cells we postulate that it arises as a result of the presence of ICP4 in the extracts. The fidelity of reconstitution was demonstrated by competition analysis (Figure 4C, lane 7). Treatment of ICP4 with AP reveals that some of the phosphates on the molecule are required for interaction of the protein with the γ-leader probe (Figure 4C, compare lane 2 with lane 8) and with γ-leader complexes formed in extracts from mock-infected cells (Figure 4C, lane 9).

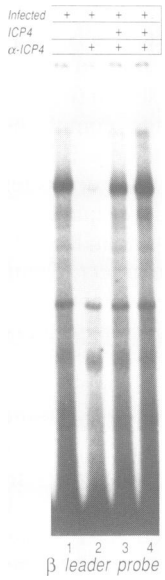

Fig. 5. Effect of depletion and reconstitution of ICP4 from infected-cell extracts on complex formation with the β-leader probe. Infected-cell extracts were incubated with monoclonal antibodies specific for gC (lane 1) or ICP4 (lanes 2−4) as described in Materials and methods. The β-leader probe was then incubated with 7 μg of the supernatant from the treated extracts supplemented with 0 (lane 2), 0.7 (lane 3) or 11.2 (lane 4) ng of purified ICP4 and binding reactions were analyzed by the mobility-shift electrophoresis assay. α-ICP4 denotes preincubation of the extracts with a monoclonal antibody specific for ICP4.

An infected-cell factor (ICF) is required to form ICP4-containing complexes with the β-leader probe

If formation of ICP4-containing complexes is dependent on ICF, then removal of ICP4 should permit us to ask if specific complexes still form and whether addition of purified ICP4 would reconstitute the binding profile seen after incubation of the β-leader probe in extracts from infected cells. After antibody-mediated depletion of ICP4, infected-cell extracts form a single, low abundance complex with the β-leader probe (Figure 5, lane 2). Addition of a μg equivalent (Kattar-Cooley and Wilcox, 1989), or a 16-fold excess of purified ICP4 to the depleted extract restored the infected-cell binding pattern (Figure 5, lanes 3 and 4). This result demonstrates that formation of ICP4-containing complexes with the β-leader probe requires a factor(s) which is present only in infected cells (ICF) and that ICF is unlikely to be tightly bound to ICP4, or in vast excess, as evidenced by the ability to reconstitute binding of extracts depleted of ICP4.

Protein kinase A restores the mobility-shift patterns obtained in AP-treated extracts from infected cells

We next asked if treatment of dephosphorylated extracts with protein kinase A (PKA, a serine−threonine kinase) would

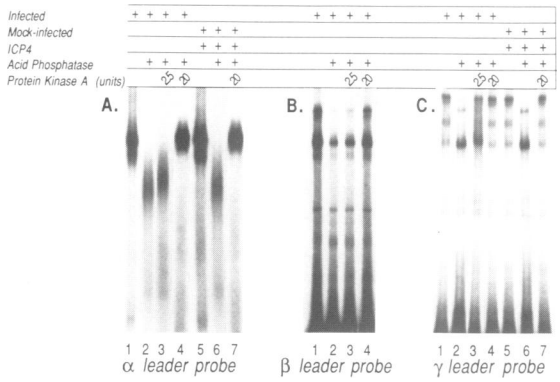

Fig. 6. Binding profiles of acid phosphatase-treated, extracts or purified ICP4, after incubation with protein kinase A. Aliquots of nuclear extacts (7 μg, lanes 1−4) prepared from infected cells, or affinity-purified ICP4 (22.4 ng, lanes 5−7) were preincubated with (lanes 2, 3,4, 6 and 7) or without (lanes 1 and 5) 2 μg of acid phosphatase (AP) for 15 min at RT, chased with AP inhibitors, and subsequently treated with either BSA (lanes 1, 2, 5 and 6), 2.5 (lane 3) or 20 (lanes 4 and 7) picomolar units of protein kinase A per μl for 15 min at RT. Reaction mixtures containing nuclear extracts from infected cells were then analyzed for binding to the α-, β-, or γ-leader probes (panels **A**, **B** and **C**, respectively), whereas those containing the ICP4 preparation were mixed and incubated with equilibrated α- or γ-leader complexes formed in extracts from mock-infected cells. Binding reactions were then examined for complex formation by the mobility-shift electrophoresis assay. The final NaCl concentration of the binding reactions was 5 mM (contributed by the extract) for those containing nuclear extracts from infected cells (lanes 1−4) and 50 mM for those containing nuclear extracts from mock-infected cells and ICP4 (lanes 5−7). These salt concentrations were chosen to optimize binding of each probe (Flanagan *et al.*, 1990; Papavassiliou and Silverstein, 1990a,b).

restore their ability to form specific complexes with the three leader probes. Accordingly, AP-treated extracts from infected cells were incubated with 2.5 or 20 pmolar units of PKA in the presence of ATP and then assayed for their ability to form complexes with each of the three leader probes. After dephosphorylation, the binding pattern of each probe was differentially affected (Figure 6, lanes 1 and 2) as described above. Addition of PKA restored the mobility-shift profiles seen with infected-cell extracts and each of the probes (Figure 6, lanes 3 and 4). We next examined if incubation of AP-treated purified ICP4 with PKA would alter the migration of the α- and γ-leader complexes formed in extracts from mock-infected cells. As expected, the binding patterns of these probes were restored (Figure 6A and C, lanes 5 and 7). While it is clear that PKA is able to restore properties associated with phosphorylated ICP4, we do not know if it is the actual phosphate donor *in vivo*.

The state of phosphorylation of ICP4 does not alter its DNA-binding specificity for a high affinity site

The results presented in Figure 6 prompted us to examine the forms of ICP4 in AP-treated infected-cell extracts after exposure to different amounts of PKA. AP-treated extracts prepared from 5 h-infected cells were incubated with either BSA or 20, 40 or 80 pmolar units of PKA and then subjected to Western blot analysis. ICP4 in untreated extracts migrates predominantly as a single abundant species (Figure 7A, lane 1). After treatment, four novel bands were detected and the slowest-migrating species disappeared (lane 3). Incubation of the AP-treated extracts with increasing amounts of PKA gradually regenerated the ICP4 profile seen in the untreated

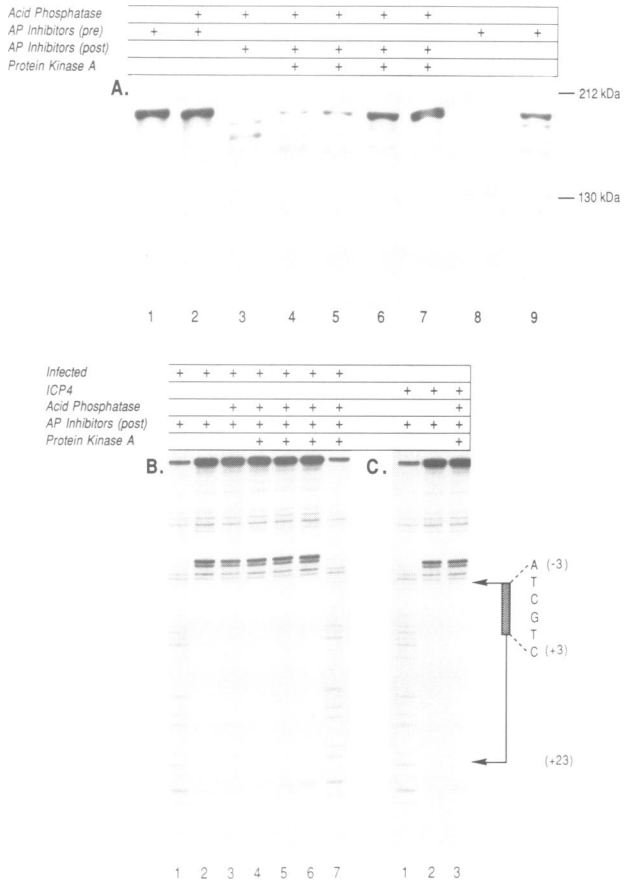

Fig 7. A. Western immunoblot analysis of ICP4 in dephosphorylated and protein kinase A-treated extracts. Samples of nuclear extracts (28 μg) prepared from 5 h- (lanes 1−7) or 2 h- (lane 9) infected cells were incubated with (lanes 2−7) or without (lanes 1 and 9) 8 μg of acid phosphatase (AP) for 15 min at RT, in the presence (lanes 1, 2 and 9) or absence (lanes 3−7) of AP inhibitors (pre). Reaction mixtures except those in lanes 1, 2 and 9 were subsequently chased with AP inhibitors (post) and then treated with either BSA (lanes 1, 2, 3 and 9) or 20 (lanes 4 and 5), 40 (lane 6) or 80 (lane 7) picomolar units of protein kinase A per μl for 15 min (lanes 4, 6 and 7) or 30 min (lane 5) at RT in the presence of 1 mM (lanes 1−4 and 6−9) or 100 μM (lane 5) ATP. Immediately after treatment, reaction mixtures were subjected to SDS−PAGE and the separated proteins were transferred to a nitrocellulose filter and probed with an ICP4-specific monoclonal antibody, as detailed in Materials and methods. Lane 8 contains prestained protein molecular weight markers treated exactly as the samples in lanes 1 and 9. **B**, **C**. Footprint analysis of complexes formed with the α-leader probe and acid phosphatase/protein kinase A-treated extracts or purified ICP4. Aliquots of nuclear extracts (14 μg) prepared from 5 h-infected cells (**B**), or affinity-purified ICP4 (44.8 ng, C) were incubated with (B lanes 3−6 and C lane 3) or without (B and C lane 2) 4 μg of acid phosphatase (AP) for 15 min at RT. Reaction mixtures were subsequently chased with AP inhibitors (post) and then treated with either BSA (B lanes 2 and 3, and C lane 2) or 10 (B lane 4), 20 (B lane 5, and C lane 3), or 40 (B lane 6) picomolar units of protein kinase A per μl for 15 min at RT. Immediately after treatment, reaction mixtures were transferred to tubes containing 2 volumes of binding buffer and the α-leader probe, incubated for 30 min at RT, and subjected to DNase I treatment as described in Materials and methods. After separation in a nondenaturing gel, DNA was eluted from the bound (B lanes 2−6 and C lanes 2 and 3) and the corresponding free (B lanes 1 and 7 and C lane 1) fractions, deproteinized, electrophoresed in a 12% sequencing gel, and autoradiographed. Arrows connected with a line demarcate the boundaries of the protected region and the dot-filled bar denotes the high affinity ICP4-binding sequence.

extracts (Figure 7A, lanes 4 to 7). This effect was dependent on the ATP concentration and time of incubation (Figure 7A, compare lanes 4 and 5).

ICP4 is synthesized at very early times post infection and continues to accumulate in the nucleus until ~8 h post-infection (Davison *et al.*, 1984; Metzler and Wilcox, 1985). Therefore we also examined the forms of ICP4 present at 2 h post infection by immunoblot analysis. ICP4 in this extract migrates as five specific bands, each of which has a counterpart in the 5 h AP-treated extracts (Figure 7A, compare lane 3 with lane 9).

To test if the DNA-binding specificity of AP/PKA-treated extracts of purified ICP4 might differ from that of the native protein, DNase I footprinting patterns of the complexes formed with the α-leader probe and either dephosphorylated extracts or ICP4 (Figure 6A) were compared. Figure 7B and C demonstrate that the DNase protection pattern was unaltered by either AP or subsequent PKA treatment. Thus, native ICP4 and species of the protein that differ in their extent of phosphorylation (and possibly ADP ribosylation) bind and protect the same site on the high affinity substrate.

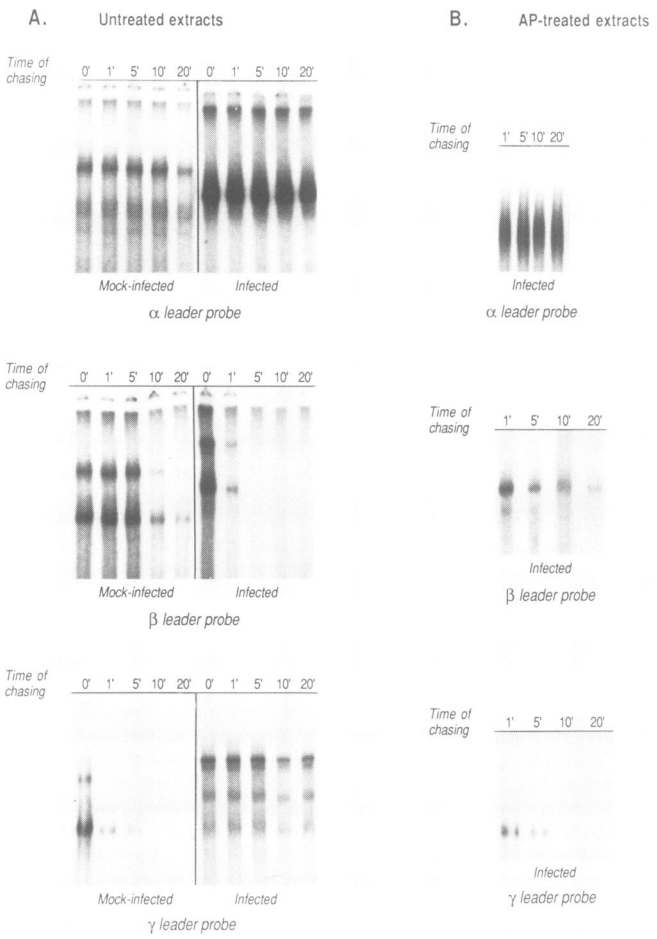

Fig. 8. Relative dissociation kinetics of complexes formed with the α-, β-, and γ-leader probes and untreated or acid phosphatase-treated extract. **A.** 60 fmol of each probe was mixed with 7 μg of nuclear extracts from mock-infected or infected cells that were preincubated in the presence of AP inhibitors only (Untreated). **B.** The same amount of each probe was mixed with 7 μg of nuclear extracts from infected cells that were treated with acid phosphatase and subsequently chased with AP inhibitors (AP-treated). In each instance, complexes were allowed to form under standard reaction conditions (30 min at RT) and at the end of the incubation period, a 250-fold molar excess of unlabeled α-leader DNA was added to each reaction and samples were withdrawn after 1, 5, 10 and 20 min, and applied directly to a running, low ionic-strength 4% polyacrylamide gel. Lanes denoted with 0′ represent control reactions, not chased with unlabeled α-leader sequence. Only the regions of the gels containing complexes are shown.

Phosphorylation governs the stability of ICP4-containing complexes formed with β- and γ- but not α-leader sequences

The requirement of ICP4 throughout the early and late phases of a productive infection suggests that it interacts in different ways with the regulatory sequences present in genes from each of the three major kinetic classes. We qualitatively examined the relative dissociation rates of complexes formed between leader probes and proteins in both mock-infected and infected-cell extracts and what effect AP treatment of infected-cell extracts had on the stability of complexes formed with each of the three probes. Complexes formed with the α-leader probe and nuclear proteins from mock-infected or infected cells are stable for at least 20 min (Figure 8A). AP treatment of infected-cell extracts results in the appearance of heterogeneously migrating α-leader complexes that are also stable for at least 20 min (Figure 8B). Therefore, these complexes have a very low $K_{d(obs)}$ which is unaffected by the state of phosphorylation of ICP4. The β-leader complexes formed in extracts from infected cells are much more labile than those formed in extracts from mock-infected cells (Figure 8A; Papavassiliou and Silverstein, 1990b). However, after AP treatment of the infected-cell extract the stability of the β-leader complex that formed, and which lacked ICP4 (Figure 2B, lane 7), was like that of complexes formed in untreated extracts from mock-infected cells (Figure 8B). Therefore, it is the presence of highly phosphorylated ICP4 interacting with infected cell-specific factor(s) that renders the β-leader complexes kinetically labile. The γ-leader complexes which formed in extracts from mock-infected cells were kinetically extremely labile, whereas those formed in extracts from infected cells were stable (Figure 8A). After AP treatment, the mobility-shift profile and stability of the γ-leader complexes were the same as in extracts from mock-infected cells (Figure 8B), demonstrating that highly phosphorylated ICP4 stabilizes an otherwise weak interaction between cellular factors and their target in the γ-leader sequences.

Discussion

We have demonstrated that ICP4 differentially participates in complex formation with leader sequences present in each of the three major kinetic classes of HSV-1 genes. These interactions are specific and result in the displacement of cellular factors from the α leader and alterations in the properties of complexes that form with the β- and γ-leader sequences and cell or infected-cell proteins. These latter effects are governed by a requirement for phosphorylated ICP4.

When infected-cell extracts or purified ICP4 were treated with AP the major immunoreactive species was converted to a family of discrete bands (Figures 1A and 7A). The abundance and migration of these bands were unchanged by extended digestion or exposure to higher concentations of AP. Therefore, the multiple species represent the limit products of dephosphorylation of a family of modified core molecules whose phosphates are not equally accessible to

AP. These studies and others (Shepard *et al.*, 1989; Wu and Wilcox, 1990) demonstrate that the phosphates are not required for direct binding of ICP4 to a high affinity site.

Repression of the α4 and α0 genes occurs as a result of direct binding of ICP4 to a region that includes the sequence ATCGTC in the cap site and promoter, respectively, of each of these genes (Resnick *et al.*, 1989; Roberts *et al.*, 1988). ICP4 also acts as an activator of transcription and is required throughout the virus replication cycle to turn on β and γ genes. Activation occurs at the initiation step of transcription (Beard *et al.*, 1986; Harris-Hamilton and Bachenheimer, 1985; Tedder and Pizer, 1988). Our data suggest that formation of complexes which contain ICP4, with leader sequences found in model β and γ genes, occurs predominantly as a consequence of its interaction with infected-cell and/or cell factors. AP treatment does not alter the migration pattern or abundance of equilibrated β- and γ-leader complexes (Figure 3). Thus, the phosphate moieties on ICP4 that participate in these interactions are inaccessible to AP and are most likely lying on the surface of the molecule that interacts with these other factors. Prior dephosphorylation of infected-cell extracts results in alteration of the mobility-shift profile of both β- and γ-leader complexes (Figure 2B and C). The β-leader complex that formed did not contain ICP4 and was kinetically more stable than its mobility counterpart formed in untreated extracts from infected cells (Figure 8). For both β- and γ-leader probes, PKA treatment of dephosphorylated extracts from infected cells results in restoration of the mobility-shift profiles to those seen in infected-cell extracts (Figure 6).

Addition of ICP4 to α- and γ-leader complexes formed in mock-infected cell extracts results in mobility-shift profiles that are indistinguishable from those seen in extracts from infected cells. In contrast to these results, there was no change in the binding pattern when the β-leader probe was incubated under the same conditions. Whereas the α-leader probe formed an abundant complex with highly purified ICP4, and a small fraction of the γ-leader probe formed a stable complex after addition of 22.4 ng of ICP4, the β-leader probe was not able to bind ICP4 (Figure 4). From these experiments we hypothesized that an infected-cell factor(s) (ICF) was necessary for formation of β-leader complexes containing ICP4. To test for the presence of ICF we devised the ICP4 depletion/reconstitution assay (Figure 5). This analysis demonstrated the existence of ICF and showed that it is required to promulgate formation of β-leader complexes that can subsequently interact with ICP4.

The DNA-binding studies presented here correlate with the continuous requirement for ICP4 throughout the replication cycle. ICP4 appears to repress α-gene expression by binding to its cognate site in these genes. The next function for this protein is to activate β genes. Because β-promoters are transcriptionally silent at very early times postinfection, we view this event as a two-part reaction involving both release from repression and transactivation. Genetic analyses demonstrated that it is not possible to dissociate the sequences present in the promoter/leader region of the thymidine kinase gene (the model β sequence used in this study) which are required for basal level expression from those needed to activate transcription of the gene during virus infection (Coen *et al.*, 1986; Eisenberg *et al.*, 1985; Zipser *et al.*, 1981). Failure to detect direct binding of ICP4 to the β-promoter (nts −197 to −11, data not shown) and leader (Figure 4B) probes, and the demonstration that the same host proteins are utilized for complex formation in extracts from mock-infected and infected cells (Papavassiliou and Silverstein, 1990a), suggests that activation of transcription from this gene occurs as a consequence of the interaction of ICP4 with cell and infected-cell factors. Thus, the requirement for an infected cell-specific factor(s) to form ICP-4-containing complexes with the β-promoter (Papavassiliou and Silverstein, 1990b) and leader (Figure 4B) probes supports this mode of action.

The dissociation analysis of the β-leader complexes (Figure 8A) suggests that those which contain ICP4 are kinetically labile. In contrast, ICP4 interactions with the upstream promoter region of this β- gene further stabilize the preexisting complexes (Papavassiliou and Silverstein, 1990b). We propose that it is the interaction of ICP4 with known transcription factors and the infected-cell factor(s) bound to the β-gene promoter region which signals transactivation. ICP4 interactions at the leader region modulate activation by lowering the affinity of infected-cell proteins for DNA, resulting in formation of kinetically labile β-leader complexes which permit RNA polymerase II to transit the gene. Therefore, the leader sequence is not obligatorily required for activation. While effective interaction of ICP4 with the α-leader probe does not require posttranslational modification of the protein (Figure 1B and 2A; Wu and Wilcox, 1990), complex formation in extracts from infected cells with the β promoter (data not shown) and leader (Figure 2B) is highly dependent on phosphorylated species of the molecule. In support of this, we note that the slowest-migrating and presumably most highly phosphorylated form of ICP4 predominates at early and late stages of the virus replication cycle (Figure 7A).

Although the γ-gene promoters appear to be composed of little more than a TATA box (Homa *et al.*, 1988; Johnson and Everett, 1986), their leaders are important for transcription and stabilization of their mRNAs (Blair *et al.*, 1987; Homa *et al.*, 1986). These downstream regulatory elements contain both low affinity ICP4-binding sites (Michael *et al.*, 1988; Tedder *et al.*, 1989) and sites that form complexes which contain ICP4 (Michael *et al.*, 1988). Both types of sequences are present in the γ-gene leader used in this study and these sites are important for *trans*activation of this gene (Flanagan *et al.*, 1990). γ-gene activation requires DNA replication, functional ICP4, and also ICP27, another α-gene product (McCarthy *et al.*, 1989; Rice and Knipe, 1990.; Sacks *et al.*, 1985; Sekulovich *et al.*, 1988; Su and Knipe, 1989). Late in infection, replication factories containing virus DNA and high concentrations of ICPs 8 (a single-stranded DNA-binding protein) and 4 are formed as discrete subnuclear bodies (Knipe *et al.*, 1987; Randall and Dinwoodie, 1986). This change in distribution of ICP4 from a diffuse nuclear to a highly focal one results in a local increase in concentration of the protein which may trigger γ-gene transcription. Only at high concentrations of purified ICP4 was it possible to: (i) form one of the complexes seen with the γ-leader probe in extracts from infected cells (Figure 4C, lane 1) and (ii) generate the infected-cell binding profile (Figure 4C, lane 5). However, unlike its effect on β-leader complexes, ICP4 appears to stabilize the interactions between cell factors and the γ-leader probe (Figure 8A). Furthermore, while the ability to form β-promoter and leader complexes in extracts from infected cells diminishes as a function of the time postinfection (Papavassiliou and Silverstein, 1990a), the yield of ICP4-containing complexes formed with the γ-

leader probe reaches its maximum levels at late stages postinfection (Flanagan *et al.*, 1991). As with formation of the β-leader complexes, the highly phosphorylated form of ICP4 is required to generate the stable γ-leader complexes (Figure 8).

Although PKA can serve as a phosphate donor to restore the kinetic class-specific complex-forming ability of ICP4, we do not know if this is the kinase which is utilized *in vivo*. However, PKA accumulates in the nucleus where it is associated with the transcriptional machinery (Nigg *et al.*, 1985; Sikorska *et al.*, 1988) and modifies the activity of proteins which bind to DNA (Cherry *et al.*, 1989; Ghosh and Baltimore, 1990; Luscher *et al.*, 1990; Poteat *et al.*, 1990; Riabowol *et al.*, 1988; Yamamoto *et al.*, 1988). Accordingly, the altered binding properties of ICP4, which correlate with changes in the state of phosphorylation of this protein, could conceivably play an important role in regulating its diverse biologic functions. Another virus regulatory protein with diverse functions is the adenovirus E1A protein which, although not a DNA-binding protein, serves to redirect the hosts transcriptional machinery through interactions with a TATAA-specific factor (Wu *et al.*, 1987), TFIIIC (Yoshinaga *et al.*, 1986) and E2F (Raychaudhuri *et al.*, 1990). The latter effect is regulated by the state of E4F phosphorylation (Raychaudhuri *et al.*, 1989).

Isolation of the cell and infected-cell factor(s) and *in vitro* reconstitution of the system will further clarify the role of the participants in this complex regulatory network.

Materials and methods

Cells, viruses and preparation of extracts

HeLa cells were propagated as previously described (Gelman and Silverstein, 1985). Nuclear extracts from mock-infected and 5 h HSV-1 (strain F)-infected cells were prepared by a modification of the procedure of Dignam *et al.* (Dignam *et al.*, 1983; Papavassiliou and Silverstein, 1990b) and frozen as aliquots at −80°C. Protein concentration was determined by the method of Bradford (Bradford, 1976).

Purification of ICP4

Fraction IX of affinity DNA-cellulose purified ICP4 (>90% of the total protein mass) was used in our assays (Kattar-Cooley and Wilcox, 1989). It contained 1.2 mg/ml of ICP4 (3.4 pmol of the dimeric form/μl) in 50% glycerol, 10 mM Tris−HCl (pH 8.0), 1 mM EDTA, 10 mM 2-mercaptoethanol, and was diluted (from a fresh aliquot each time) into DNA-binding buffer to the desired concentration just prior to assembling the reaction mixtures.

Preparation of probe and competitor DNAs

The α4- (nts −17/+32 relative to the cap site), βTK- (nts −16/+56), and γ38- (nts −16/−103) leader probe and competitor DNAs were prepared as previously described (Papavassiliou and Silverstein, 1990b; Flanagan *et al.*, 1991).

Acid phosphatase and protein kinase A treatment

Acid phosphatase from potato (AP: Grade I, Boehringer Mannheim Biochemicals) was centrifuged out of $(NH_4)_2SO_4$ suspension, dissolved in 20 mM PIPES−KOH (pH 6.0), and dialyzed against two changes of Dignam's buffer D/PIPES (20 mM PIPES−KOH, pH 6.0, 0.1 M NaCl, 0.2 mM Na−EDTA, 20% glycerol, 0.5 mM dithiothreitol, 0.5 mM PMSF, 0.2 mM TPCK and 0.2 mM TLCK). Approximately 2 μg (0.12 U) of enzyme was used for every 7 μg of nuclear extracts or 22.4 ng of affinity-purified ICP4, and incubation was perfomed at RT (25°C) for 15 min in the presence or absence of AP inhibitors [5 mM NaH_2PO_4/Na_2HPO_4 (pH 7.9), 100 mM NaF; (Hollander, 1971 and references therein)], as indicated in the figure legends. AP inhibitors were subsequently added to reaction mixtures lacking them to block phosphatase action, and the samples were either assayed directly for DNA binding, or treated with the catalytic subunit (CS) of Protein kinase A (PKA). For this purpose, the dephosphorylated extracts or the purified ICP4 preparations were brought to 1 mM ATP and immediately treated with 2.5−20 picomolar units (U) of PKA-CS per μl (scaled up accordingly, as indicated in the appropriate figure legends) for 15 min at RT. Equal amounts of acetylated DNase-free bovine serum albumin (BSA; Promega) were employed in control reactions run in parallel. Bovine PKA-CS was purchased from Sigma (P-2645) and prepared in Dignam's buffer D supplemented with 3 mM-mercaptoethanol, 4 mM $MgCl_2$, 2 mM $MnCl_2$, 0.2 mM TPCK, and 0.2 mM TLCK [modified from Cherry *et al.* (Cherry *et al.*, 1989)]. Reaction mixtures were then assayed for DNA binding as described below.

DNA−protein binding and mobility-shift electrophoresis assays

Samples of nuclear extracts (1 μl = 7 μg) were treated according to the description in each figure legend and mixed with 2.5 μg of nonspecific competitor salmon-sperm DNA (Worthington; sonicated to an average chain length of 250 bp) for 2 min prior to adding 60 fmol of the ^{32}P-labeled DNA probe. Incubations were performed at RT for 30 min in a final volume of 20 μl containing 10 mM Tris−HCl (pH 7.9), 2 mM dithiothreitol, 1 mM EDTA, 10% v/v glycerol, 2% polyvinyl alcohol, 0.1 mM TPCK and TLCK, 5 mM NaH_2PO_4/NaH_2PHO_4 (pH 7.9) [to ensure inhibition of acid phosphatase (see above)] and 0 or 45 mM NaCl (as indicated in the figure legends). Binding reactions with purified ICP4 (0.7−22.4 ng, 2−64 fmol of the dimeric form) were performed in a similar manner, except that preincubation with the nonspecific carrier DNA was omitted, and in addition to the components described above, they contained 50 μg/ml BSA to maintain a constant concentration of the protein. Following binding, the reaction mixtures were assayed for complex formation by electrophoresis through a native, low ionic-strength 4% polyacrylamide gel (acrylamide:biscarylamide weight ratio of 29:1) containing 6.7 mM Tris−HCl (pH 7.8) 3.3 mM sodium acetate, and 1 mM Na−EDTA (Strauss and Varshavsky, 1984). Prior to loading the samples, the gel was run for 90 min at 5 V/cm, and electrophoresis was carried out at 35 mA for 3−4 h at RT with constant buffer recirculation. After soaking the gel for 20 min in 5% glycerol to retard cracking, it was transferred to Whatman 3MM paper, dried under vacuum, and autoradiographed with an intensifying screen at −70°C. Resolution of the complexes obtained when the β-leader probe was incubated with extracts from mock-infected cells (Figure 2B) required that the gel be preelectrophoresed for no more than 45 min before loading the binding reactions (Figures 4B and 8; Papavassiliou and Silverstein, 1990b). For competition experiments the conditions were exactly as above, except that competitor DNAs (5-fold molar excess over the probe) were included in the mixture at the same time as the DNA probe.

Supershift assays

ICP4-specific monoclonal antibody (750 ng of antibody diluted in DNA-binding buffer per 1 μg of nuclear extract) was added to the equilibrated complexes, and reaction mixtures were incubated for an additional 20 min at ambient temperature prior to mobility-shift electrophoresis analysis. The monoclonal antibody recognizing ICP4 (H640) was kindly provided by Dr L.Pereira (UCSF).

ICP4 depletion and reconstitution assays

Nuclear extracts (42 μg) from infected cells were incubated at 4°C for 1.5 h with 1 μg of monoclonal antibody specific for either gC (a virus glycoprotein) or ICP4 per μg of extract, in the presence of 1/10 volume of protein A−Sepharose (1:1 slurry in Dignam's buffer D) and 1 μg of rabbit polyclonal antimouse IgG Fc per μg of extract. The mixture was spun for 1 min at 4°C and 7 μg of the supernatant was then either incubated directly with the β-leader probe or, first supplemented with 0.7 or 11.2 ng of purified ICP4 and then reacted with the probe. After 30 min, binding reactions were tested for complex formation by the mobility-shift electrophoresis assay. Western blot analysis of the depleted extracts failed to detect ICP4 (data not shown).

DNase I footprinting of ICP4−DNA complexes

The coding strand of the α-leader DNA was 3′ end-labeled with [α-^{32}P]dATP and binding reactions with this probe were scaled up 2-fold from the standard reactions with nuclear extracts or affinity-purified ICP4 treated as described in the legend to Figure 7B. After incubation for 30 min at RT, $MgCl_2$ was added to 2.5 mM, and the samples were digested with DNase I (Promega) at a final concentration of 5 μg/ml for 2.5 min at ambient temperature. Reactions were terminated by the addition of EDTA (pH 8.0) to 5 mM and the complexes separated from free DNA by native polyacrylamide gel electrophoresis as detailed above. DNA was eluted from the free and bound fragment bands (Maxam and Gilbert, 1980) and precipitated with 2 volumes of ethanol in the presence of glycogen

(10 μg/sample; BMB). After reprecipitation, the products were washed twice with 80% and 90% ethanol, respectively, dried, and resuspended in 5 μl of formamide buffer. Samples were then heated at 95°C for 5 min, quenched in ice water, and equal Cerenkov c.p.m. from the bound and free fractions were analyzed by separation in a 12% 1 × TBE polyacrylamide gel (19:1) in the presence of 8.3 M urea. Following electrophoresis, the gel was fixed, transferred to Whatman 3MM paper, vacuum dried, and exposed to Kodak XAR-5 film at −70°C with an intensifying screen.

Off-rate analysis of the DNA−protein complexes

The relative dissociation rate analyses of the various complexes formed with each probe in untreated and acid phosphatase-treated extracts were performed exactly as previously described (Papavassiliou and Silverstein, 1990a). The unlabeled α-leader probe served as the challenging DNA sequence. Two- to three-fold alterations in the amounts of salmon-sperm DNA, ^{32}P-labeled probe DNA, unlabeled α-leader probe, and nuclear extract did not affect the results, ensuring that the $K_{d(obs)}$ followed first-order kinetics (data not shown).

Western immunoblot analysis

HeLa cells were infected at a m.o.i of 5 p.f.u. of HSV-1 (strain F) per cell, and at 2 and 5 h post infection, cells were harvested and nuclear extracts were prepared as described above. Samples of nuclear extracts (28 μg) were then treated as indicated in the appropriate figure legends, heated at 95°C for 3 min in the presence of 2% SDS, and proteins were separated by SDS−PAGE in linear gradient gels of 2−10% polyacrylamide (Lambin, 1978) (acrylamide:bis-acrylamide weight ratio of 49:1) and then electrophoretically transferred to a nitrocellulose filter. The filter was blocked by incubation overnight at 4°C in BLOTTO buffer (5% w/v nonfat dry milk in PBS) with gentle agitation. It was subsequently washed in PBS−Tween buffer (0.05% Tween-20 in PBS) and incubated with a 1:1000 dilution of H640 in washing buffer containing 1% nonfat dry milk for 2 h at RT. This was followed by four washes in PBS−Tween buffer containing 1% nonfat dry milk and 0.1% NP40, and the filter was then reacted with a 1:1000 dilution of goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma A-4656) in washing buffer for 2 h at RT. The filter was washed four times as described above and two additional times with TNM buffer (100 mM Tris−HCl (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$). Finally, the filter was developed in 0.01% (w/v) Nitroblue Tetrazolium (Grade III, Sigma N-6876) and 3-indoxyl phosphate (Sigma I-5505) both dissolved in TNM buffer.

Acknowledgements

These studies were supported by grants from the USPHS to S.J.S. and K.W.W.

References

Beard,P., Faber,S., Wilcox,K.W. and Pizer,L.I. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 4016−4020.

Blair,E.D., Blair,C.C. and Wagner,E.K. (1987) *J. Virol.*, **61**, 2499−2508.

Bradford,M. (1976) *Anal. Biochem.*, **72**, 248−254.

Cherry,J.R., Johnson,T.R., Dollard,C., Shuster,J.R. and Denis,C.L. (1989) *Cell*, **56**, 409−419.

Coen,D.M., Weinheimer,S.P. and McKnight,S.L. (1986) *Science*, **234**, 53−59.

Courtney,R.J. and Benyesh-Melnick,M. (1974) *Virology*, **62**, 539−551.

Davison,M.J., Preston,V.G. and McGeoch,D.J. (1984) *J. Gen. Virol.*, **65**, 859−863.

DeLuca,N.A. and Schaffer,P.A. (1987) *Nucleic Acids Res.*, **15**, 4491−4511.

DeLuca,N.A. and Schafer,P.A. (1988) *J. Virol.*, **62**, 732−743.

DeLuca,N.A., McCarthy,A.M. and Schaffer,P.A. (1985) *J. Virol.*, **56**, 558−570.

Dignam,J.D., Lebowitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475−1489.

Dixon,R.F. and Schaffer,P.A. (1980) *J. Virol.*, **36**, 189−203.

Eisenberg,S.P., Coen,D.M. and McKnight,S.L. (1985) *Mol. Cell. Biol.*, **5**, 1940−1947.

Faber,S.W. and Wilcox,K.W. (1986a) *Nucleic Acids Res.*,**14**, 6067−6083.

Faber,S.W. and Wilcox,K.W. (1986b) *Arch. Virol.*, **91**, 297−312.

Flanagan,W.M., Papavassiliou,A.G., Rice,M., Hecht,L.B., Silverstein,S. and Wagner,E.K. (1991) *J. Virol.*, in press.

Gelman,I.H. and Silverstein,S. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5365−5269.

Ghosh,S. and Baltimore,D. (1990) *Nature*, **344**, 678−682.

Halpern,M.E. and Smiley,J.R. (1984) *J. Virol.*, **50**, 733−738.

Harris-Hamilton,E. and Bachenheimer,S.L. (1985) *J. Virol.*, **53**, 144−151.

Hollander,V.P. (1971) In Boyer,P.D.E. (ed.), *The Enzymes*. Academic Press, New York and London, Vol. IV, pp. 450−498.

Homa,F.L., Otal,T.M., Glorioso,J.C. and Levine,M. (1986) *Mol. Cell. Biol.*, **6**, 3652−3666.

Homa,F.L., Glorioso,J.C. and Levine,M. (1988) *Genes Dev.*, **2**, 40−53.

Honess,R.W. and Roizman,B. (1974) *J. Virol.*, **14**, 8−19.

Honess,R.W. and Roizman,B. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 1276−1280.

Johnson,P.A. and Everett,R.D. (1986) *Nucleic Acids Res.*, **14**, 8247−8264.

Kattar-Cooley,P. and Wilcox,K.W. (1989) *J. Virol.*, **63**, 696−704.

Knipe,D.M., Senechek,D., Rice,S.A. and Smith,J.L. (1987) *J. Virol.*, **61**, 276−284.

Kristie,T.M. and Roizman,B. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 4700−4704.

Lambin,P. (1978) *Anal. Biochem.*, **85**, 114−125.

Luscher,B., Christenson,E., Litchfield,D.W., Krebs,E.G. and Eisenman,R.N. (1990) *Nature*, **344**, 517−522.

Marsden,H.S., Crombie,I. and Subak-Sharpe,J.H. (1976) *J. Gen. Virol.*, **31**, 347−372.

Mavromara-Nazos,P. and Roizman,B. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 4071−4075.

Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499−560.

McCarthy,A.M., McMahan,L. and Schaffer,P.A. (1989) *J. Virol.*, **63**, 18−27.

McGeoch,D.J., Dolan,A., Donald,S. and Brauer,D.M.K. (1986) *Nucleic Acids Res.*, **14**, 1727−1745.

McGeoch,D.J., Dalrymple,M.A. Davison,A.J., Dolan,A., Frame,M.C., McNab,D., Perry,L.J., Scott,J.E. and Taylor,P. (1988) *J. Gen. Virol*, **69**, 1531−1574.

Metzler,D.W. and Wilcox,K.W. (1985) *J Virol*,**55**, 329−337.

Michael,N., Spector,D., Mavromara-Nazos,P., Kristie,T.M. and Roizman,B. (1988) *Science*, **239**, 1531−534.

Muller,M.T. (1987) *J. Virol.*, **61**, 858−865.

Nigg,E.A., Hilz,H., Eppenberger,H.M. and Dutly,F. (1985) *EMBO J.*, **4**, 2801−2806.

Papavassiliou,A.G. and Silverstein,S.J. (1990a) *J. Biol. Chem.*, **265**, 1648−1657.

Papavassiliou,A.G. and Silverstein,S.J. (1990b) *J. Biol. Chem.*, **265**, 9402−9412.

Paterson,T. and Everett,R.D. (1988) *Nucleic Acids Res.*, **16**, 11005−11025.

Pereira,L., Wolff,M.H., Fenwick,M. and Roizman,B. (1977) *Virology*, **77**, 733−749.

Poteat,H.T., Chen,F.Y., Kadison,P., Sodroski,J.G. and Haseltine,W.A. (1990) *J. Virol.*, **64**, 1264−1270.

Preston,C.M. (1979a) *J. Virol.*, **32**, 357−369.

Preston,C.M. (1979b) *J. Virol.*, **29**, 275−284.

Preston,C.M. and Notarianni,E.L. (1983) *Virology*, **131**, 492−501.

Randall,R.E. and Dinwoodie,N. (1986) *J. Gen. Virol.*, **67**, 2163−2177.

Raychaudhuri,P., Bagchi,S. and Nevins,J.R. (1989) *Genes Dev.*, **3**, 620−627.

Raychaudhuri,P., Bagchi,S., Neill,S. and Nevins,J.R. (1990) *J. Virol.*, **64**, 2702−2710.

Resnick,J., Boyd,B.A. and Haffey,M.L. (1989) *J. Virol.*, **63**, 2497−2503.

Raibowol,K.T., Fink,S., Gilman,M.Z., Walsh,D.A., Goodman,R.H. and Feramisco,J.R. (1988) *Nature*, **336**, 83−86.

Rice,S.A. and Knipe,D.M. (1990) *J. Virol.*, **64**, 1704−1715.

Roberts,M.S., Boundy,A., O'Hare,P., Pizzorno,M.C., Ciufo,D.M. and Hayward,G.S. (1988) *J. Virol.*, **62**, 4307−4320.

Sacks,W.R., Greene,C., Aschman,D. and Schaffer,P.A. (1985) *J. Virol.*, **55**, 796−805.

Sekyulovich,R.E., Leary,K. and Sandri-Goldin,R.M. (1988) *J. Virol.*, **62** 4510−4522.

Shepard,A.A. and DeLuca,N.A. (1989) *J. Virol.*, **63**, 1203−1211.

Shepard,A.A., Imbalzano,A.N. and DeLuca,N.A. (1989) *J. Virol.*, **63**, 3714−3728.

Shepard,A.A., Tolentino,P. and DeLuca,N.A. (1990) *J. Virol.*, **64**, 3916−3926.

Sikorska,M., Whitfield,J.F. and Walker,P.R. (1988) *J. Biol. Chem.*, **263**, 3005−3011.

Strauss,F. and Varshavsky,A. (1984) *Cell*, **37**, 889−901.

Su,L. and Knipe,D.M. (1989) *Virology*, **170**, 496−504.

Tedder,D.G. and Pizer,L.I. (1988) *J. Virol*, **62**, 4661−4672.

Tedder,D.G., Everett,R.D., Wilcox,K.W., Beard,P. and Pizer,L.I. (1989) *J. Virol.*, **63**, 2510−2520.

Watson,R.J. and Clements,J.B. (1980) *Nature*, **285**, 329−330.

Wilcox,K.W., Kohn,A., Skylanskaya,E. and Roizman,B. (1980) *J. Virol.*, **33**, 167–182.
Wu,L., Rosser,D.S.E., Schmidt,M.C. and Berk,A.J. (1987) *Nature*, **326**, 512–515.
Wu,C.-L. and Wilcox,K.W. (1990) *Nucleic Acid Res.*, **18**, 531–538.
Yamamoto,K.K., Gonzalez,G.,A., Biggs III,W.H. and Montminy,M.R. (1988) *Nature*, **334**, 494–498.
Yoshinaga,A., Dean,N., Han,M. and Berk,A.J. (1986) *EMBO J.*, **5**, 343–354.
Zipser,D.L., Lipsich,L. and Kwoh,J. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 6276–6280.

Received on October 19, 1990; revised on November 26, 1990