INTRODUCTION

Expression of herpes simplex virus type 1 (HSV-1) genes during the course of a lytic infection proceeds in a temporally ordered cascade (Hones and Roizman, 1974). The virus genes are classified in three major kinetic classes, immediate-early (IE), early (E), and late (L), based on their temporal pattern of expression during infection (Hones and Roizman, 1974, 1975). The IE genes, which are the first to be expressed, encode four regulators of gene expression [infected-cell proteins (ICPs) 4, 27, 0, and 22], and a protein, ICP47, that facilitates immune evasion by blocking the presentation of viral antigens (Hill et al., 1995; York et al., 1994). Regulation of virus gene expression by the IE proteins takes place at both the transcriptional and posttranscriptional levels. Transcriptional regulation is orchestrated by ICP4, the major transcriptional regulator of HSV-1. ICP4, which is essential for virus replication (DeLuca et al., 1985; Dixon and Schaffer, 1980; Knipe et al., 1978; Preston, 1979; Watson and Clements, 1978, 1980), is a sequence-specific DNA-binding protein (DiDonato et al., 1991; Faber and Wilcox, 1986; Michael and Roizman, 1989; Michael et al., 1988) that can act as both an activator and repressor of transcription. Although ICP4 appears to be the master switch in the coordinated temporal expression of HSV-1 genes (for a review, see Roizman and Sears, 1996), its exact molecular mechanism of action is largely unknown. Several lines of evidence suggest that it is involved in a large number of interactions with both viral and host proteins. ICP4 has been found to interact with the TATA binding protein (TBP), the basal transcription factor TFIIA, and the TBP-associated protein 250 (TAF250) (Carrazza and DeLuca, 1996; Gu and DeLuca, 1994; Gu et al., 1995; Smith et al., 1993). Therefore, it is possible that ICP4 regulates transcription by affecting transcription preini-

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tiation complex assembly. ICP4 also interacts with the HSV-1 regulatory proteins ICPO and ICP27 (Panagiotidis et al., 1997), and these interactions appear to affect the intracellular compartmentalization of these proteins (Mullen et al., 1995; Zhu et al., 1994; Zhu and Schaffer, 1995). This imposition of compartmental constraints by the virus regulatory proteins on each other is likely to have an impact on regulation of virus gene expression. Moreover, these interactions could alter the biochemical activities of the proteins involved. Indeed, the post-translational modification state of ICP4 is affected by ICP27 (McMahan and Schaffer, 1990; Rice and Knipe, 1988; Su and Knipe, 1989; Xia et al., 1996). Although the molecular basis for this phenomenon is not known, it is likely to involve physical interactions between ICP27 and hypomodified forms of ICP4 (Panagiotidis et al., 1997). This interaction may affect transcription because less modified forms of ICP4 have different DNA-binding specificities (Michael et al., 1988). In fact, the ability of ICP4 to bind to a regulatory region within the thymidine kinase (TK) gene depends on the presence of ICP27 (Panagiotidis et al., 1997).

The ability of ICP4 to interact with DNA, and thus regulate gene expression, can also be affected by components of the cell milieu that alter DNA structure. We have shown that polyamines (biological cations, which bind to and neutralize the anionic phosphate charges of DNA (Tabor and Tabor, 1984), enhance the DNA-binding activity of ICP4 at physiological concentrations (Panagiotidis et al., 1995). A recent report by Carrozza and DeLuca (1998) revealed that the high-mobility group (HMG) protein HMG I(Y) enhances ICP4 binding to the IE-3 gene cap site probe, and the complexes were analyzed by electrophoretic mobility shift assay (EMSA). The results of this analysis confirmed that rHMG I(Y) forms stable complexes with DNA containing the IE-3 cap site (Fig. 1A, lanes 3–6).

HMG I(Y) enhances ICP4 binding to the IE-3 gene cap site

The aim of the present study was to identify proteins that potentially alter the DNA-binding activity of ICP4. Therefore, we next asked whether HMG I(Y) affects the DNA-binding activity of ICP4 to the IE-3 gene cap site probe using recombinant HMG I(Y). The addition of increasing amounts of purified rHMG I to reactions containing 10 ng of a purified GST-fusion protein with the ICP4 DNA-binding domain (amino acids 245–523) revealed that HMG I(Y) enhances the DNA-binding activity of ICP4. In the absence of rHMG I, GST–ICP4–DNA complexes were barely detectable by EMSA (Fig. 1B, lanes 2–5). However, the abundance of these complexes was greatly enhanced with the addition of increasing amounts of rHMG I to the DNA-binding reaction mixture (Fig. 1B, lanes 2–5). Furthermore, the abundance of GST–ICP4–DNA complexes in the presence of increasing amounts of rHMG I was higher than that observed when
the DNA-binding reaction was performed in the absence of nonspecific competitor DNA (Fig. 1A, lane 2). This eliminates the possibility that the enhancement of the DNA-binding activity of ICP4 by rHMG I results from titration of the nonspecific sonicated salmon sperm DNA competitor by rHMG I.

The effect of HMG I on the DNA-binding activity of full-length ICP4 was also measured using nuclear extracts from HSV-1-infected HeLa cells as the source of ICP4. The addition of increasing amounts of rHMG I resulted in an increase in the accumulation of an ICP4–DNA complex (Fig. 2, lanes 3–6). Supershift analysis using 58S monoclonal antibody (MAb) confirmed that ICP4 was present in the HMG I-enhanced protein–DNA complex (Fig. 2, lanes 7–10). The results of this analysis demonstrate that the effect of HMG I is not limited to the bacterially expressed, purified DNA-binding domain of ICP4 but that it can be reproduced with the full-length ICP4. Furthermore, because rHMG I does not enhance the formation of other IE-3 cap site–protein complexes (Fig. 2), we believe that the HMG I effect is specific only for the binding of ICP4 to the IE-3 cap site and not for other IE-3 cap site-binding proteins. Further evidence for the specificity of the HMG I effect comes from our observation that HMG I does not enhance the binding of ICP4 to the TK B region of the HSV-1 TK gene that is bound by ICP4 (Papavassiliou and Silverstein, 1990b), which lacks an HMG I site and is not bound by the protein (data not shown).

HMG I(Y) binds the minor groove of DNA at AT-rich regions (Solomon et al., 1984; Thanos and Maniatis, 1992). To further demonstrate that the enhanced binding was specific, a poly(dA)/poly(dT) polynucleotide was used as a specific competitor and poly(dG)/poly(dC) was used as a nonspecific competitor in binding reactions. The resulting protein–DNA complexes were analyzed by EMSA. The addition of increasing amounts of either polynucleotide had little, if any, effect on the abundance ICP4–DNA complexes in the absence of exogenous rHMG I (Fig. 3, lanes 2–7). As expected, the addition of rHMG I to the binding reactions greatly increased the abundance of ICP4–DNA complexes (Fig. 3, compare lanes 2 and 8). However, only poly(dA)/poly(dT) competed for formation of both the rHMG I–DNA complexes and the HMG I-enhanced ICP4 complexes (Fig. 3, lanes 8–13). The decrease in the abundance of ICP4–DNA complexes paralleled that of HMG I–DNA complexes, as would be expected for an HMG I-specific effect (Fig. 3). Similar data are also obtained when distamycin, an oligopeptide antibiotic, is added to the binding reactions. Distamycin binds in the minor groove of DNA with similar specificity to HMG I(Y) (Bellorini et al., 1995; Mote et al., 1994). It also competes with HMG I(Y) for binding to DNA.
We verified this result, using the IE-3 cap site probe, and found that distamycin also competed with the HMG I-enhanced binding of ICP4 to DNA, whereas it did not affect the binding of ICP4 in the absence of exogenously added HMG I (data not shown).

Identification of the HMG I(Y) binding sites within the IE-3 promoter/regulatory region

DNase I protection footprint analysis was used to identify DNA sites within the IE-3 regulatory region that are bound by HMG I(Y). This analysis revealed that rHMG I protected, albeit weakly, two DNA sequences (from –2 to –4 and from +9 to +12, relative to the transcription start site) within the IE-3 cap site (Fig. 4). The weak protection by rHMG I is not unexpected because the IE-3 gene cap site bears little resemblance to sequences typically recognized by HMG I(Y). The addition of more rHMG I (at 120 and 200 ng) to the reactions resulted in DNase I hypersensitivity at two positions (+6 and –8) within the IE-3 cap site (Fig. 4). This may indicate that rHMG I binding to the IE-3 cap alters DNA structure. This putative alteration may facilitate the increased binding of ICP4 to this sequence.

The DNase I footprint analysis revealed that rHMG I binds to the IE-3 cap site and to two other sequences within the IE-3 promoter. One of these sites (from –26 to –21) corresponds to the TATA box. The strongest rHMG I-dependent protection within the IE-3 probe was observed further upstream (–106 to –98) at the DNA region overlapping the TAATGARAT motif. The binding of HMG I(Y) to several sites within the IE-3 gene promoter/regulatory region is consistent with the multivalent binding of this protein to several other promoters where it has

**FIG. 3.** Specificity of rHMG I enhancement of ICP4 binding. DNA-binding reactions with nuclear extracts from HSV-1-infected (7 h) HeLa cells and EMSAs were performed as described in Materials and Methods. Reactions were performed in the absence (–HMG I, lanes 1–7) or presence (+HMG I, lanes 8–13) of 100 ng of rHMG I. The amounts of poly(dA)/poly(dT) and poly(dG)/poly(dC) competitor polynucleotides are shown above the figure. No competitor was added in the reactions analyzed in lanes 2 and 8. The reaction in lane 1 (M) was performed using a nuclear extract from mock-infected HeLa cells. Arrows indicate the positions of the free IE-3 probe and its complexes with rHMG I or ICP4.

**FIG. 4.** DNase I footprinting analysis of HMG I binding sites within the IE-3 gene promoter/regulatory region. The reactions were performed as described in Materials and Methods with the indicated amounts of rHMG I added to each reaction. The black boxes at the left identify regions protected from DNase I action in the presence of rHMG I. The asterisks identify sites that become hypersensitive to DNase I action in the presence of rHMG I. The boxed sequences contain the TATA box and a high-affinity ICP4 recognition site. The arrow at +1 identifies the IE-3 gene transcription initiation site.
been found to alter DNA architecture (Carey, 1998; Maher and Nathans, 1996; Thanos and Maniatis, 1995).

**HMG I(Y) potentiates the repressive effect of ICP4 on the IE-3 promoter**

ICP4 binding to IE-3 promoter sequences results in repression of gene expression. Thus if *in vivo* HMG I(Y) enhances the binding of ICP4 to the IE-3 promoter, as it does *in vitro*, then it should enhance repression by ICP4. To test this hypothesis, the ability of ICP4 to repress the IE-3 promoter was measured in transfection experiments in the presence or absence of coexpressed HMG I. Vero cells were transfected with an IE-3 promoter-luciferase reporter construct and increasing amounts (10 ng to 1.5 μg) of a plasmid expressing ICP4 under the control of a cytomegalovirus (CMV) promoter, in the presence or absence of 1 μg of a CMV promoter-driven HMG I construct. As expected, ICP4 activated the IE-3 promoter at low ratios of ICP4 to IE-3 promoter-luciferase construct but repressed it when cotransfected at higher ratios (Fig. 5). However, the repressive effect of ICP4 on the IE-3 promoter was significantly enhanced when HMG I was coexpressed (Fig. 5). Specifically, the amount of input CMV-ICP4 DNA required to effect 50% repression was reduced from 750 ng, in the absence of coexpressed HMG I, to 40 ng in its presence. Similar results were also obtained with a construct expressing HMG Y (data not shown).

It was possible that the enhanced expression was caused by HMG I-mediated activation of the CMV promoter that was driving the expression of ICP4. If this were the case, then overproduction of ICP4 should generate similar results. Therefore, we determined the effect of cotransfecting a plasmid expressing HMG I with a CMV promoter-luciferase reporter construct on the activity of the CMV promoter. HMG I had only a very small effect (<5%) on the activity of the CMV promoter (data not shown). Thus the HMG I(Y)-mediated enhancement of the DNA-binding activity of ICP4 can take place not only *in vitro* but also within the intracellular milieu.

**Effect of HMG I(Y) on the activity of the IE-3 promoter**

HMG I(Y) is neither a transcriptional activator nor repressor (Carey, 1998; Thanos and Maniatis, 1992, and references therein). However, in its function as an architectural protein (Bustin and Reeves, 1996), it can alter the conformation of DNA-regulatory regions affecting the binding of transcriptional activators and repressors. The identification of three HMG I(Y) binding sites in the IE-3 gene promoter/regulatory region suggests that HMG I(Y) may alter the architecture of this promoter and affect its basal activity. To test this hypothesis, Vero cells were transfected with the IE-3 promoter-luciferase reporter constructs (1 μg) and increasing amounts (0.1 μg–2 μg) of plasmids expressing HMG I or antisense HMG I(Y) RNA, each of which was under the control of a CMV promoter. HMG I expression increased the activity of the IE-3 promoter by more than twofold, whereas expression of the antisense HMG I(Y) RNA resulted in a modest reduction (~30%) of the IE-3 promoter activity (Fig. 6). It is noteworthy that expression of either HMG I(Y), or the antisense HMG I(Y) RNA has no effect on the activity of the minimal IE-3 promoter (−108 to +32) reporter con-
Shepard plays an important role in the regulatory activities of ICP4 IE genes and an activator of E and L genes. DNA binding has been shown to act as a transcriptional repressor of mechanisms of the action of ICP4 are largely unknown, it

1996). Although the exact molecular mechanism or regulatory protein (for a review, see Roizman and Sears, 1989). Therefore, factors that affect the DNA-binding activity of ICP4 have the potential to modulate HSV-1 gene expression.

We previously demonstrated that a high-affinity binding site for ICP4, located at the transcriptional start site of the IE-3 gene, was bound by host proteins extracted from uninfected cells (Panagiotidis et al., 1991; 1995). This present study was initiated to identify host proteins that occupy sites in the HSV-1 chromosome that are subsequently bound by ICP4 after infection. The ICP4 DNA-binding site used in this analysis represents one of the most studied sites in the virus chromosome. Moreover, the biological consequences of ICP4 binding can be readily assessed, (i.e., ICP4 binding leads to IE-3 gene repression) (DeLuca et al., 1985; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985a; Roberts et al., 1988). Analysis of a HeLa cDNA expression library with a DNA probe containing the high-affinity binding site for ICP4 revealed that the chromatin architectural proteins HMG I and HMG Y bound to the probe. Binding of HMG I(Y) to the IE-3 cap site was further verified by in vitro experiments with purified recombinant HMG I (Fig. 1).

HMG proteins are a heterogeneous group of small, acid-soluble, non-histone chromatin proteins (Bustin and Reeves, 1996). They are classified in three general families: the HMG1/2, the HMG 14/17, and the HMG I(Y) families (Bustin and Reeves, 1996, and references therein). It was initially thought that HMGs represent structural components of the eucaryotic chromatin with limited gene-regulatory potential. They are now known to be involved in a wide variety of cellular functions involving DNA, such as transcription, replication, and recombination (Bustin and Reeves, 1996; Carey, 1998; Falvo et al., 1995; Fashena et al., 1992; Ogawa et al., 1995; Thanos and Maniatis, 1992, 1995). HMGs exert their effects by modifying chromatin architecture and/or by functionally interacting with regulatory and structural protein components of the chromatin (Bustin and Reeves, 1996; Carey, 1998; Falvo et al., 1995; Fashena et al., 1992; Ogawa et al., 1995; Thanos and Maniatis, 1992, 1995). HMG I(Y) binds to the minor groove of AT-rich DNA regions through three short basic amino acid repeats called the AT-hooks (Reeves and Nissen, 1990). Although not a transcriptional activator per se, HMG I(Y) influences the activity of several cellular and viral promoters (for a review, see Carey, 1998). It produces its effects in multiple ways by facilitating transcription factor binding to DNA, stabilizing higher-order multiprotein–DNA transcription-enhancing (enhanceosome) complexes, or competing with histone-mediated repression (Carey, 1998).

In this study, we demonstrate that HMG I(Y) induces the in vitro binding of both full-length ICP4 and a GST-fusion with the ICP4 DNA-binding domain to the IE-3 cap site DNA probe (Figs. 1 and 2). HMG I(Y) is known to exert its effects not only by altering the DNA structure but also by physically interacting with several transcription factors (Lehming et al., 1994; Thanos and Maniatis, 1992;
Wood et al., 1995; Zhou and Chada, 1998). Therefore, its effects on ICP4 could be a result of HMG I(Y)-induced DNA conformational change or changes and/or protein–protein interactions between HMG I(Y) and ICP4. However, despite repeated efforts using both immunoprecipitation and protein capture techniques, no evidence was found for protein–protein interactions between HMG I(Y) and ICP4. In contrast, binding of HMG I(Y) to the IE-3 cap DNA results in DNase I hypersensitivity of two basins in the vicinity of HMG I(Y) binding sites (Fig. 4). This hypersensitivity is indicative of HMG I(Y)-induced DNA conformational changes that could result in the enhancement of ICP4 binding in the presence of HMG I(Y).

ICP4 binding to the IE-3 gene cap site results in repression (Roberts et al., 1988). The enhanced binding of ICP4 in the presence of HMG I in vitro suggested to us that ICP4 binding in the presence of HMG I(Y) should potentiate the repressive effect of ICP4 in vivo. This hypothesis was tested in transfection experiments, and the HMG I and HMG Y isoforms were found to be equally effective in enhancing the repressing activity of ICP4 on the IE-3 promoter. These results indicate that HMG I(Y) has the potential to modulate the activity of ICP4 and, consequently, virus gene expression.

HMG I(Y), unlike other HMG-box containing proteins that induce sharp bends in the DNA structure, has only a relative modest effect on DNA conformation (Carey, 1998; Falvo et al., 1995). However, it frequently binds to multiple sites within target promoters, and this multivalent binding could produce extensive changes in chromatin architecture that might be essential for its effects on the binding of transcription factors. HMG I(Y) binds to at least three sites within the IE-3 gene promoter-regulatory sequences, as shown by DNase I footprint analysis (Fig. 4). Interestingly, these sites overlap with sequences that are important in the regulation of transcription from the IE-3 gene. These sequences include the TATA box, the TAATGARAT element, and the ICP4 binding site. Protection of these DNA elements suggests that the role of HMG I(Y) in the regulation of IE-3 gene expression is not limited to its effects on ICP4. Indeed, in transfection experiments, HMG I(Y) enhances the basal activity of the IE-3 promoter in the absence of viral factors (Fig. 6). A further role for HMG I(Y) in regulating transcription from this promoter is suggested by its failure to activate a minimal IE-3 promoter lacking the HMG I(Y) binding site that overlaps with the TAATGARAT sequence. This result suggests that multivalent HMG I(Y) binding to the IE-3 promoter/regulatory sequences is essential for its function. Alternatively, HMG I(Y) might recruit transcription factors that bind to upstream sequences that are missing from the minimal IE-3 promoter and/or it might facilitate the effects of upstream binding factors on the transcription preinitiation complex. To discriminate among these possibilities or to identify alternatives will require further experimentation.

The results presented here indicate that architectural protein components of the host cell chromatin may modulate HSV-1 gene expression by affecting the binding of host and virus-encoded transcription regulatory proteins. We do not at present know whether HMG I(Y) affects other classes of HSV-1 promoters. The recent observation (French et al., 1996) that HMG I(Y) binds to and modulates the activity of another HSV-1 promoter, the latency-active promoter 2 (LAP-2), points toward a more general role for HMG I(Y) in the regulation of virus gene expression. This study provides further evidence that architectural proteins can play a significant role in the regulation of HSV-1 gene expression.

MATERIALS AND METHODS

Cells and virus

Vero cells were grown and maintained in DMEM (GIBCO BRL, Grand Island, NY) containing 5% bovine calf serum (Hyclone Laboratories Inc., Logan, UT). The media were supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO BRL), unless otherwise noted. HeLa cells were propagated in monolayers as previously described (Papavassiliou and Silverstein, 1990a). HSV-1 strain 17 was used in this study.

Screening of the HeLa cDNA expression library with a DNA probe

The HeLa cDNA library, was constructed in the λ gt11 Sfi–Not vector (Promega, Madison, WI) by Jeh-Ping Liu and Scott Zeitlin (Columbia University). A 32P end-labeled 450-bp HindIII–EcoRI restriction fragment from plasmid pCPC-1B (see below) was used to screen ~750,000 plaques from the expression library for clones that produced protein that was bound by the probe. This DNA fragment contained six randomly concatamerized copies of the −17/+32 sequence from the HSV-1 IE-3 gene. The screening of the cDNA expression library was performed as previously described with some modifications (Singh et al., 1988). Filters were blocked with Blotto overnight at 4°C, with gentle agitation. The incubation was performed in 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 5% (v/v) glycerol, 1 mM EDTA, 4 mM dithiothreitol, and 0.2 mg/ml BSA, and 0.5 mM spermidine and salmon sperm DNA (100 µg/ml) was used as the nonspecific competitor. Positive plaques were isolated, verified by rescreening, and purified three times. The cDNA inserts were amplified by PCR using λ gt11 primers (Promega), and the amplimers were cloned into the TA cloning vector pCR1 (Invitrogen, San Diego, Ca.). The DNA sequence of each clone was determined using an ABI 377 automated sequencer (Perkin–Elmer, Foster City, CA) by the Columbia Cancer Center sequencing facility.

Plasmid constructions

Plasmid pCPC-1A, containing the −17/+32 region of the IE-3 gene, was constructed by subcloning an end-
filled 49 bp Avai–BamHI fragment from plasmid pIGA103 (Gelman and Silverstein, 1987) in the Hincll site of pUC19. Plasmid pCPC-1B, containing six tandem –17/ +32 IE-3 gene regions, was constructed by subcloning a self-ligated 68-bp Avai (filled) restriction fragment from pCPC-1A into the Hincll site of pUC19. IE-3 promoter-luciferase reporter constructs pCPC-4P–LUC (–323/+32) and pCPC-4P–LUC (–108/+32) were constructed as follows. Plasmid pCPC-4P–LUC was generated by cloning a BamHI fragment containing the luciferase coding sequences from plasmid p19LUC (van Zonneveld et al., 1988) into the BamHI site of pIGA103 (Gelman and Silverstein, 1987). Plasmid pCPC-4P–LUC was constructed in two steps. First, a BamHI and EcoRI fragment from plasmid pIGA103, containing the –108/+32 sequences from the IE-3 promoter, was cloned in vector pIC-20H (cut with BamHI–EcoRI I) to yield pCPC-4P1. Plasmid pCPC-4P–LUC was generated by cloning the BamHI fragment, containing the luciferase coding sequences, from plasmid p19LUC into the BamHI site of pCPC-4P1. Plasmids pCPC-I and pCPC-Y were constructed by ligating the PCR-amplified HMG I and HMG Y cDNAs with pCRII (InVitrogen). Plasmids pCPC-I– and pCPC-Y–, which express HMG I or HMG Y, respectively, under the control of the CMV promoter, were generated by subcloning the EcoRI (filled)–NotI fragments from plasmids pCPC-I and pCPC-Y in pcDNAI.neo (InVitrogen) that had been digested with EcoRV–NotI. Plasmid panti-HMG I(Y), expressing an antisense HMG I(Y) RNA (Thanos and Maniatis, 1992), was a gift from Dr. T. Maniatis, as was the bacterial expression construct pET15b-HMG I (Thanos and Maniatis, 1992). Recombinant HMG I (rHMG I) protein, carrying a hexahistidine tag fused at its amino terminus, was expressed in *Escherichia coli* BL21(DE3) transformed with plasmid pET15b-HMG I (Thanos and Maniatis, 1992). Purification of rHMG I was performed by affinity chromatography on a nickel-NTA agarose column (Qiagen), as previously described (Thanos and Maniatis, 1992). After purification, proteins were dialyzed overnight at 4°C against 200 volumes of 10 mM HEPES-KOH, pH 7.9, 50 mM NaCl, 1 mM EDTA, 4 mM dithiothreitol, and 20% (v/v) glycerol, with two changes.

Protein concentrations were determined by the Bradford method (Bradford, 1976), and protein purity was determined by SDS–PAGE (Laemmli, 1970). Proteins were stored in aliquots at −80°C.

**Transfections and luciferase assays**

Transfections were performed using the calcium phosphate precipitation method (Wigler et al., 1979). Briefly, Vero cells were seeded at a density of 5 × 10³ cells/60-mm plate the day before transfection. The culture media were replaced 5 h before transfection. Transfections were performed with calcium phosphate, using 15 µg of total plasmid DNA/plate, and the transfection mixtures were left on the cells overnight. The next day, the cells were refed with fresh media after three washes of the cell monolayers with PBS. The cells were harvested at 48 h posttransfection, and luciferase activities were determined as described previously (Brasier et al., 1989), using a Berthold Lumat LB9501 luminometer (Wallac Inc., Gaithersburg, MD). Luciferase activities were determined from triplicate transfections in at least two independent experiments.

**Nuclear extract preparation and EMSAs**

Nuclear extracts were prepared from uninfected and HSV-1 infected HeLa cells at 7 h postinfection (Dignam et al., 1983), with some modifications (Papavassiliou and Silverstein, 1990a). EMSAs were performed by incubating either 4 µg of nuclear or total cell extracts or the indicated amounts of the purified proteins with ³²P end-labeled restriction fragments from the IE-3 gene (–17/ +32), which contains a high-affinity ICP4 binding site, followed by native PAGE (Papavassiliou and Silverstein, 1990a). The DNA-binding reactions were incubated for 20 min at room temperature in 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 5% (v/v) glycerol, 1 mM EDTA, 4 mM dithiothreitol, and 0.2 mg/ml BSA in a final volume of 20 µl. Sonicated salmon sperm DNA or poly(dG)/poly(dC) (Pharmacia) were included as nonspecific DNA competitors. Supershift analyses were performed by adding 1 µg of purified anti-ICP4 mouse MAb 58S (Showalter, Zweig, and Hampar, 1981) to each binding reaction after 20 min of incubation, and the reaction mix was incubated for an additional 30 min. The 58S MAb was equilibrated in binding buffer by dialysis. After separation by native PAGE in 0.25× TBE, 4% gels, the DNA–protein complexes were visualized by autoradiography.

**DNase I footprint analysis**

The DNA probe used in the footprinting experiments was labeled on one strand by end-filling with the Klenow fragment of DNA polymerase I. The probe was a HindIII–NcoI fragment from plasmid pIGA103 (Gelman and Silverstein, 1987), and it was labeled at the HindIII site. DNase I protection experiments were performed as previously described (Panagiotidis et al., 1995), and the products of the digestion were analyzed on 6% sequencing gels.
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