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Biochemistry

α4, the major regulatory protein of herpes simplex virus type 1, is stably and specifically associated with promoter–regulatory domains of α genes and of selected other viral genes

(DNA–protein complexes/gel electrophoresis assay)

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ABSTRACT **Herpes simplex virus type 1 genes form at least five groups (α, β_1, β_2, γ_1, and γ_2) whose expression is coordinately regulated and sequentially ordered in a cascade fashion. Previous studies have shown that functional α4 gene product is essential for the transition from α to β protein synthesis and have suggested that α4 gene expression is autoregulatory. However, the mechanism by which α4 regulates gene expression remained unknown. We report that labeled DNA fragments containing promoter–regulatory domains of three α (α0, α4, and α27) and a γ_2 gene form stable complexes with proteins from infected-cell lysates as detected by a gel electrophoresis binding assay. The protein(s) exhibits sequence specificity since autologous DNA fragments but not heterologous DNA fragments, synthetic polydeoxynucleotide chains, or salmon sperm DNA competitively displace the DNA probe from the complexes. Murine monoclonal antibody to α4 protein added before or after DNA–protein complex formation further retarded the electrophoretic mobility of the complexes whereas monoclonal antibody to α0, α27, or to a viral glycoprotein had no effect. Complexes consisting of the promoter–regulatory domain of the β-class thymidine kinase gene and infected cell proteins were low in abundance and could be detected only in the presence of antibody to α4 protein. The α4 protein, therefore, forms stable complexes with promoter–regulatory domains of α genes and of selected other herpes simplex virus type 1 genes either alone or in combination with other proteins.**

We report that the major regulatory protein (α4) of herpes simplex virus type 1 (HSV-1) is present in complexes formed by infected cell proteins and small DNA fragments containing promoter and regulatory domains of several HSV-1 genes. Relevant to this report are the following considerations. (*i*) The HSV-1 genes form several groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion (1). The five α genes (α0, α4, α22, α27, and α47) are the first viral genes to be transcribed after infection. Functional α proteins, especially α4, are required for the expression of β and γ genes (ref. 2; reviewed in ref. 3); α22 and α27 appear to be required for optimal expression of the later groups of genes (4, 5). The two γ groups, γ_1 and γ_2, can be differentiated by the dependence of γ_2 genes upon DNA synthesis for their expression (3). (*ii*) α gene expression is enabled by at least two sets of factors. In the promoter–regulatory region of α genes, there are G+C-rich elements that respond to host factors (6, 7), and there are specific A+T-rich elements that respond to *trans*-acting factors packaged in the virion (6, 8–10). The gene specifying the α-*trans*-inducing factor (α-TIF) has been sequenced (11, 12), but its mechanism of action is not known. α gene expression appears to be turned off by at least three factors. First, the transcription of α genes appears to be autoregulated inasmuch as some temperature-sensitive (ts) mutants in the α4 protein reinitiate the transcription of α genes when shifted to the nonpermissive temperature (2). Second, in chemically or physically enucleated cells, α mRNA translation is inhibited coincident with the appearance of β or γ_1 proteins (13). Lastly, a virion component has been shown to destabilize α mRNA (14).

This report centers on the application of the DNA–protein gel electrophoresis technique (15–17) to identify the viral specific factors that play a role in α gene expression.

MATERIALS AND METHODS

Preparation of Cellular Extracts. HeLa cells were grown to confluency in 150-cm^2 flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were mock-infected or infected with 5 plaque-forming units (pfu) of HSV-1(F) (18), ts502Δ305 (8), or tsHA1tk$^-$ (19) for 12 hr. Whole-cell extracts were prepared as described (20) except that all buffers contained 0.5 mM phenylmethylsulfonyl fluoride (Sigma); 0.42 M nuclear extracts were prepared as described except that 2% polyvinylpyrrolidone was included prior to isolation of the nuclei (21, 22). Protein concentrations of whole-cell extracts ranged from 20 to 30 mg/ml as determined by Bio-Rad protein assays.

Cloning and Preparation of DNA Probes. The HSV-1 DNA fragments shown in Fig. 1 were cloned by standard techniques (30). DNA probes were prepared by extraction of cloned DNA fragments from polyacrylamide gels, dephosphorylating with calf intestinal alkaline phosphatase (Boehringer Mannheim), and end-labeling with T4 polynucleotide kinase (Boehringer Mannheim) and [γ-^{32}P]ATP (>7000 Ci/mm, New England Nuclear; 1 Ci = 37 GBq) as described (30, 31). The labeled fragments were extracted twice with phenol/chloroform, separated from free ATP (30), and precipitated with ethanol. The activity ranged from 10,000 to 15,000 cpm/ng of DNA fragment.

Competitor Nucleic Acids. Salmon sperm DNA (Sigma) was extracted twice with phenol/chloroform, precipitated with ethanol, and sonicated to an average chain length of 1500 base pairs (bp). Poly(dI)·poly(dC) and poly(dI-dC)·poly(dI-dC) were from Pharmacia P-L Biochemicals.

DNA Binding Assay. Protein–DNA binding assays were done as described in the legend to Fig. 2.

Monoclonal Antibodies. Monoclonal antibodies H640, H1113, H1083, and HC-1 were a gift of Lenore Pereira and have been described elsewhere (32, 33).

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Abbreviations: TK, thymidine kinase; bp, base pair; HSV-1, herpes simplex virus type 1; ts, temperature sensitive.

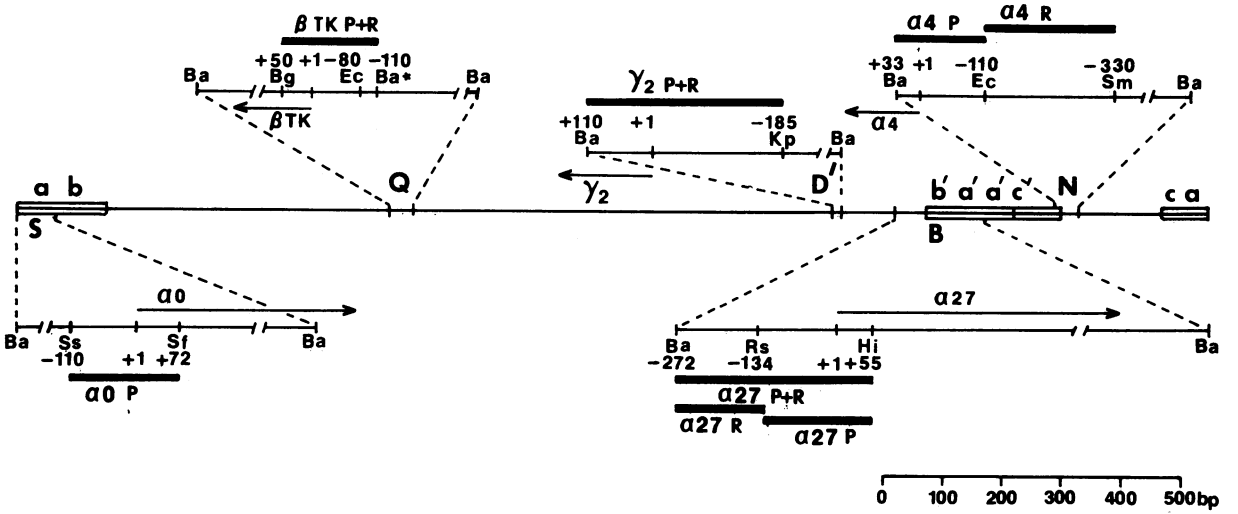

FIG. 1. Sequence arrangement and locations of HSV-1 DNA fragments used in these studies. Shown is a schematic illustration of the HSV-1 genome with the lined boxes representing the inverted repeat sequences *ab*, *b'a'a'c'*, and *ca* (23). Expanded scales show the restriction patterns and the locations of the promoter (P), regulatory (R), and coding sequences for the α genes *4*, *0*, and *27* (6, 24, 25), the β thymidine kinase gene (*βTK*) (26), and a γ_2 gene (19, 27). Arrows indicate the direction and approximate location of the mRNA for each gene. Filled bars represent the P, R, or P+R (promoter–regulatory) gene domains cloned and tested for DNA binding proteins: *α0* P, a 182-bp *Sst* II–*Sfa*NI fragment of *Bam*HI fragment S in the *Pst* I–*Sma* I site of pUC9 (28) (pRB3563); *βTK* P+R, a 160-bp *Bgl* II–*Bam*HI fragment derived from LS119/109 (29) (gift of S. L. McKnight) in the *Bam*HI site of pUC19 (pRB3597); γ_2 P+R, a 295-bp *Bam*HI–*Kpn* I fragment of *Bam*HI fragment D' in the *Bam*HI–*Kpn* I sites of pUC19 (pRB3628); *α27* P+R, a 325-bp *Bam*HI–*Hin*fI fragment of *Bam*HI fragment B in the *Sma* I site of pUC8 (pRB3052); *α27* P (138 bp) and *α27* R (187 bp), digestion products of pRB3052 with *Rsa* I/*Hin*dIII or with *Rsa* I/*Eco*RI, respectively; *α4* P, a 143-bp *Bam*HI–*Eco*RI fragment of *Bam*HI fragment N in the *Bam*HI–*Eco*RI sites of pUC9 (pRB3353); *α4* R, a 220-bp fragment of *Bam*HI fragment N in the *Hin*cII site of pUC9 (pRB3059). Restriction enzyme cleavage sites are abbreviated as follows: Ba, *Bam*HI; Ss, *Sst* II; Sf, *Sfa*NI; Bg, *Bgl* II; Ec, *Eco*RI; Kp, *Kpn* I; Rs, *Rsa* I; Hi, *Hin*fI; Sm, *Sma* I. *, Location of a synthetic *Bam*HI linker.

RESULTS

HSV-1-Infected Cell Lysates Contain Factors with High Affinity for the *α0* Promoter Domain. Fig. 2 shows the electrophoretic mobility of end-labeled *α0* promoter DNA fragment bound in mock-infected and infected whole-cell extracts in the presence of increasing amounts of unlabeled competitor nucleic acids. In the absence of competitor, all of the labeled DNA was bound by protein and remained at or near the origin of the gel. The addition of competitor removed proteins that were binding nonspecifically to the DNA, and stable DNA–protein bands became apparent. Bands 2 and 3 in Fig. 2 were formed in both mock-infected- and infected-cell lysates, whereas bands 1, 4, and 5 were formed only in the presence of infected-cell extracts. Bands 1 and 4 were stable even at a high ratio of competitor DNA to specific probe DNA and, therefore, were likely to represent complexes formed by sequence-specific, viral-encoded factors or cellular proteins whose relative abundance or activity was altered by the viral infection. Band 5 was very faint and was not investigated further in this study. Two observations should be noted. First, whole-cell extracts and nuclear extracts could not be differentiated with respect to the formation or stability of the *α0* promoter–protein complexes (data not shown). Second, the synthetic deoxypolynucleotide chain poly(dI)·poly(dC) did not significantly differ in competitive efficiency from salmon sperm DNA, even though they differed significantly in sequence complexity.

Specificity and Stability of *α0* Promoter–Protein Complexes.

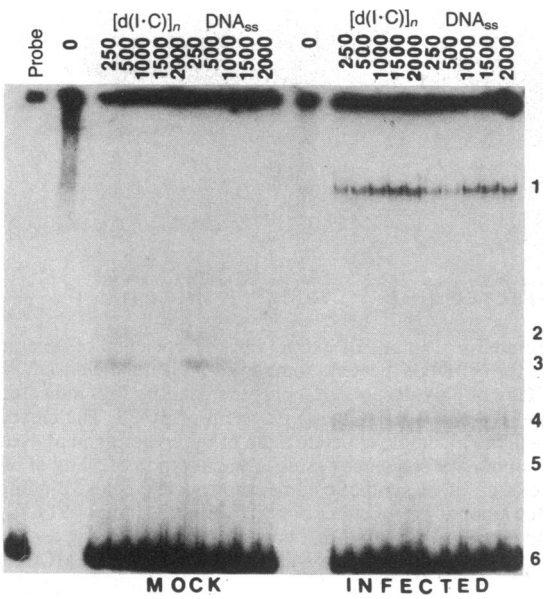

FIG. 2. Autoradiographic images of labeled *α0* promoter DNA complexed with protein in cell lysates in the presence of increasing amounts of competitor nucleic acids. All protein–DNA binding assays were done as follows unless otherwise stated. Labeled DNA probe (2.5 ng) was incubated with 1 μg of protein extract in the presence or absence of competitor DNA in 20 mM Tris, pH 7.6/50 mM KCl/0.05% Nonidet P-40/5% glycerol/50 μg of bovine serum albumin (Sigma) per ml/10 mM 2-mercaptoethanol/1 mM EDTA for 0.5 hr at 25°C. Protein extracts were diluted in the reaction buffer just prior to the assay and were added last to the reaction mixture by gentle pipetting. The quantities of DNA and protein were titrated to determine the optimal binding ratios. Reaction times between 0.5 and 2 hr did not affect the results. The reaction mixtures were loaded onto vertical 21-cm 4% polyacrylamide gels (acrylamide/bisacrylamide, 29:1; International Biotechnologies, New Haven, CT) and electrophoretically separated in 40 mM Tris borate/1 mM EDTA with buffer recirculation until the unbound DNA was near the bottom of the gel. The gels were dried and exposed to Kodak XS film with intensifying screens. For this experiment, the increasing quantities of competitors, poly(dI)·poly(dC) {lanes $[d(I{\cdot}C)]_n$} or salmon sperm DNA (lanes DNA_{ss}), were added prior to the addition of extracts of cells harvested 12 hr after mock-infection (MOCK) or HSV-1(F) infection (INFECTED). The quantity (ng) of competitor is shown at the top of each lane. The lane marked Probe contained the *α0* promoter probe DNA only. Lanes marked 0 contain the probe DNA and cell lysate in the absence of competitor nucleic acids. The DNA–protein complexes are numbered 1–5, whereas the unbound DNA migrates at position 6.

The stability and specificity of the infected-cell protein–$\alpha 0$ promoter DNA complexes were tested in several experiments. As shown in Fig. 3*A*, the unlabeled $\alpha 0$ promoter fragment effectively competed with the labeled $\alpha 0$ promoter fragment, whereas a similarly sized fragment (175-bp fragment derived from pUC9 and designated p175) did not significantly compete for the $\alpha 0$ promoter binding protein. Similarly, closed circular DNA of plasmid pRB3563 (contains $\alpha 0$ promoter) competed strongly with the formation of the $\alpha 0$ promoter probe–protein complex 1, whereas closed circular pUC9 plasmid DNA showed only a mild competitive effect, even at a base pair ratio of >2500:1. These experiments indicate that the formation of complex 1 is dependent on specific sequences within the $\alpha 0$ promoter DNA and not on the presence of high-affinity but sequence-independent DNA binding proteins. In contrast, the faster-migrating complexes evident in Fig. 3*A* were apparently unaffected by competition with either the probe-specific or nonspecific DNAs.

A comparison of the homopolymer poly(dI)·poly(dC) with the alternating copolymer poly(dI-dC)·poly(dI-dC) for the ability to compete with the labeled probe for DNA binding proteins is illustrated in Fig. 3*B*. In this instance, the pattern of $\alpha 0$ promoter DNA–protein complexes differed significantly depending on which competitor was used. The $\alpha 0$ promoter DNA–protein complex attributed to host proteins (band 2 in Fig. 3) was more stable in the presence of excess copolymer than in the presence of excess homopolymer. In contrast, the copolymer competed more efficiently than did the homopolymer for the infected-cell specific protein(s) present in band 1, although the band 1 complex was still detectable in the presence of >800-fold excess of the copolymer competitor. The reason for the competitive efficiency of the copolymer relative to the homopolymer for the proteins in this complex has not been determined. It is conceivable that the alternating purine-pyrimidine structure may be a determinant in the binding of the protein(s) in complex 1.

Fig. 3*C* illustrates the effect of NaCl concentration on the formation and stability of the $\alpha 0$ promoter–protein complex 1. The results indicate that the amounts of the $\alpha 0$ promoter–protein complex detected in gels were unaffected in the 25–75 mM range but were slightly decreased at 150 mM NaCl.

The Identification of the Protein in Infected Cell-Specific Complex 1 as an α Gene Product. The conclusion that an α gene product is present in the $\alpha 0$ promoter probe DNA–infected-cell lysate complex 1 rests on the following experiments. First, we tested lysates of cells infected with mutants ts502Δ305 and tsHA1tk$^-$ and maintained at the permissive (33°C) or the nonpermissive (39.5°C) temperatures for the ability to form stable $\alpha 0$ promoter–protein complexes. ts502Δ502 carries a ts lesion in the $\alpha 4$ gene and infected cells incubated at 39.5°C accumulate predominantly α proteins. tsHA1tk$^-$ carries a ts lesion in the major DNA binding protein (β_1 8), and the infected cells do not synthesize viral DNA or γ_2 proteins at 39.5°C. The lysates of cells infected with either mutant and maintained at 33°C or 39.5°C bound the $\alpha 0$ promoter probe DNA with equal efficiency in the presence of increasing concentrations of poly(dI)·poly(dC) (Fig. 4). This experiment suggested that the $\alpha 0$ promoter binding protein was either an α protein, a host protein that had been modified or made available during early phases of infection, or a virion component. The latter hypotheses were excluded, in part, by the observation that extracts of cells infected and maintained for 2 hr in the presence of actinomycin D (10 μg/ml) failed to form the complexes characteristic of the infected–cell lysates (data not shown). To test the hypothesis that the complexes contained α proteins, monoclonal antibody specific for $\alpha 4$ (H640), $\alpha 27$ (H1113), $\alpha 0$ (H1083), or control monoclonal antibody (HC-1) directed against glycoprotein C was added to the binding reaction either after or before complex formation. Monoclonal antibody H640 retarded the electrophoretic mobility of complex 1 when added either before or after the binding reaction (Fig. 4*B*). Similar results were obtained with four other independently derived anti-$\alpha 4$ monoclonal antibodies (data not shown). In contrast, none of the monoclonal antibodies used in these studies against $\alpha 0$, $\alpha 27$, or γ_2gC affected the mobility or inhibited the formation of $\alpha 0$ promoter–infected-cell protein complex 1.

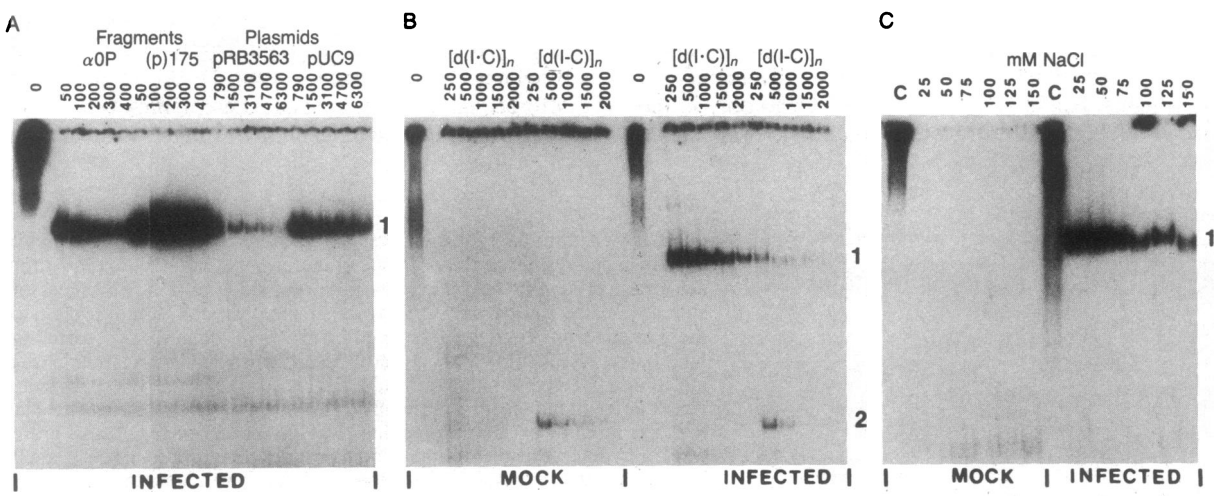

FIG. 3. Autoradiographic images of labeled $\alpha 0$ promoter DNA complexed with proteins in total cell extracts in the presence of diverse competitor nucleic acids and NaCl concentrations. (*A*) Competition of the $\alpha 0$ promoter (P) DNA–protein complexes with unlabeled DNA. Binding reactions were done in the absence (lane 0) or the presence (all others) of 2000 ng of poly(dI)·poly(dC) plus the quantity (ng) indicated on top of each lane of specific or nonspecific unlabeled competitor DNA. (p)175 is the 175-bp *Hin*dIII–*Pvu* II fragment of pUC9. The closed circular plasmid DNAs pRB3563 and pUC9 were added in the indicated quantity to equal the picomole concentration of the $\alpha 0$ P fragment used (i.e., the addition of 790 ng of pRB3563 results in the equivalent picomole concentration of $\alpha 0$ P sequences as does the addition of 50 ng of $\alpha 0$ P fragment). (*B*) Comparison of $\alpha 0$ P DNA–protein complexes formed in the presence of different synthetic competitor nucleic acids. Binding assays were done in the absence (lane 0) or in the presence (all other lanes) of the indicated amount (ng) of either poly(dI)·poly(dC) {lanes $[d(I\cdot C)]_n$} or poly(dI-dC)·poly(dI-dC) {lanes $[d(I\text{-}C)]_n$}. (*C*) Stability of the $\alpha 0$ P DNA–protein complexes at various NaCl concentrations. Binding assays were done as described except that the indicated concentration of NaCl was substituted for the KCl used in the standard reaction buffer. MOCK, uninfected cell extract; INFECTED, HSV-1(F)-infected cell extract.

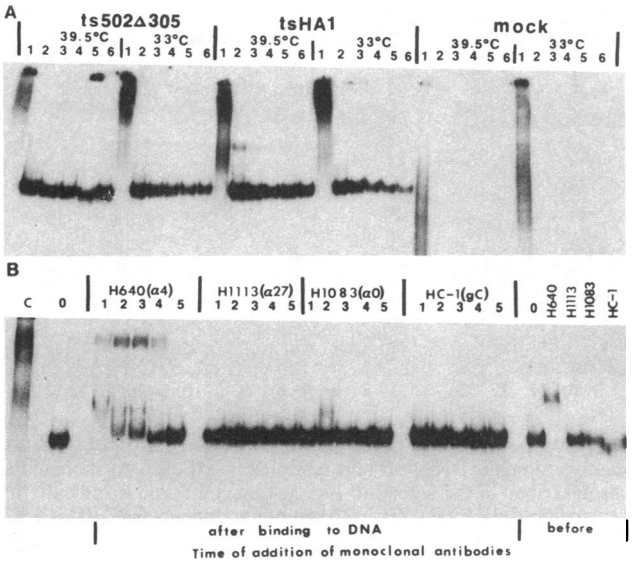

FIG. 4. Identification of an HSV-1 protein in the *α0* promoter DNA–protein complex 1 using ts mutant viruses and monoclonal antibodies to several HSV-1 proteins. (*A*) Binding of labeled *α0* promoter DNA to proteins of extracts prepared from cells mock-infected or infected with ts mutants and maintained at the permissive (33°C) or the nonpermissive (39.5°C) temperature. Lanes 1 through 6 differ in the amount of competitor poly(dI)·poly(dC) present during the binding reaction as follows: 1, 0 ng; 2, 250 ng; 3, 500 ng; 4, 1000 ng; 5, 1500 ng; 6, 2000 ng. (*B*) Electrophoretic mobility of the *α0* promoter DNA–infected-cell protein complex 1 in the presence of murine monoclonal antibodies against α4 (H640), α27 (H1113), α0 (H1083), and glycoprotein C (HC-1). The labeled probe was allowed to react in the presence of 1000 ng of poly(dI)·poly(dC) with an extract prepared from cells infected with ts502Δ305 and maintained at 33°C for 12 hr. The indicated monoclonal antibody was either added after protein–DNA binding or was preincubated with the protein extract for 0.5 hr prior to the addition of the extract to the reaction for an additional 0.5 hr. Lanes: C, DNA–protein complexes formed in the absence of poly(dI)·poly(dC); 0, no monoclonal antibody added; 1–5, amount of mouse ascites fluid protein, determined by Bio-Rad protein assays and added to the reaction as follows: 1, 500 ng; 2, 250 ng; 3, 100 ng; 4, 10 ng; 5, 1 ng. Preincubations were done with 500 ng of monoclonal antibody per 1000 ng of protein extract.

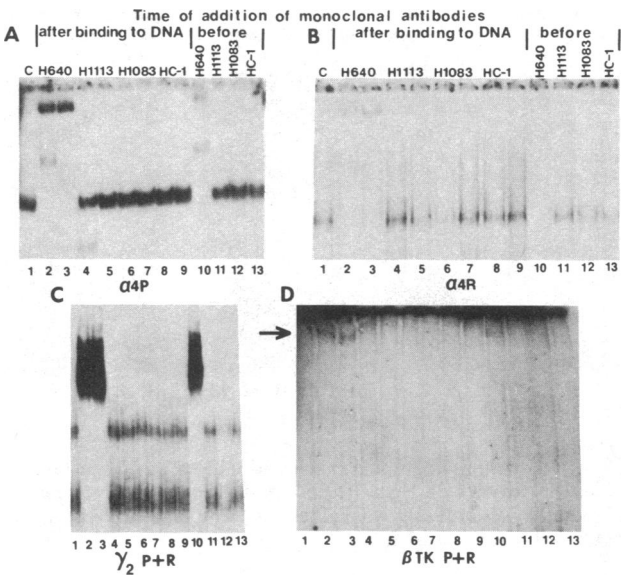

FIG. 5. Autoradiographic images of *α4*, *βTK*, and γ_2 DNA fragment probe–protein complexes exposed to monoclonal antibodies to selected viral proteins. Binding reactions in the absence and presence of the various monoclonal antibodies were done as described in the legend to Fig. 4*B*. Lanes: 1, no monoclonal antibody added; 2, 4, 6, and 8, 500 ng of the indicated monoclonal antibody; lanes 3, 5, 7, and 9, 250 ng of the indicated monoclonal antibody. Lanes 9–13 show complexes formed by DNA with infected-cell extracts (1000 ng) preincubated with the indicated monoclonal antibody (500 ng) as described in the legend to Fig. 4. (*A*) *α4* gene promoter domain (*α4* P). (*B*) *α4* regulatory domain (*α4* R). (*C*) γ_2 gene promoter–regulatory region (γ_2 P+R). (*D*) *βTK* gene promoter–regulatory region (*βTK* P+R). The arrow points to the location of the complex detected by the anti-α4 monoclonal antibody. The protein–DNA complex is not readily evident in the absence of monoclonal antibody.

Presence of α4 Protein in Complexes Formed by Promoter–Regulatory Domains of α, β, and γ_2 Genes with Infected-Cell Proteins. To determine whether α4 protein was present in complexes formed by other genes, the promoter and regulatory domains of other α genes and representative β and γ_2 promoter–regulatory domains, shown in Fig. 1, were allowed to react with infected-cell proteins. As shown by the slower migration of the DNA–infected-cell protein complexes in reactions containing monoclonal antibody H640, the α4 protein was present in complexes containing its own promoter (Fig. 5*A*) and regulatory domain (Fig. 5*B*), in two complexes formed by the representative γ_2 promoter–regulatory region (Fig. 5*C*), and in those formed by the promoter–regulatory domains of the *α27* gene (Fig. 6). In contrast, the presence of α4 protein in complexes formed by the *βTK* promoter–regulatory domain was only detected in the presence of monoclonal antibody and only after overexposure of the autoradiogram (Fig. 5*D*). In no instance were we able to demonstrate reactivity of the monoclonal antibodies to α27 or α0 used in these studies with any of the complexes tested.

DISCUSSION

The product of the *α4* gene, infected-cell polypeptide 4 (ICP4 in ref. 34) or α4 is a phosphoprotein (35). In its native state, the protein is a homodimer (36). The newly synthesized protein has an apparent molecular weight of ≈160,000, but the processed nuclear α4 protein forms at least three bands of higher apparent molecular weight in denaturing polyacrylamide gels (37). The protein has been reported to bind DNA (35, 38), but at least one report states that the binding is dependent on the presence of host proteins (38). We report that α4 protein is present in complexes formed by promoter and regulatory domains of selected α genes and a γ_2 gene with infected-cell proteins. The competition studies indicate that the binding of α4 protein to the promoter–regulatory domains of α and γ_2 genes is sequence specific, and preliminary identification of the binding site of α promoter–regulatory DNA supports this conclusion. Our results suggest that the *α4*, *α27*, and γ_2 promoter–regulatory regions contain multiple binding sites for α4 protein, whereas the *α0* promoter region contains at least one binding site. We could not demonstrate significant formation of stable complexes between a β gene promoter–regulatory domain and infected-cell proteins, although the trace amount of complex detected did contain α4 protein.

The significance of the results presented in this paper stems from two considerations. (*i*) The protein appears to induce the expression of a variety of genes other than HSV β or γ genes (39, 40) and, in this respect, is similar to the adenovirus E1A and pseudorabies virus immediate early proteins (41, 42). Moreover, the promoter sequence requirement of both HSV and non-HSV genes for induction by α4 protein has proved to be elusive. This apparent lack of sequence specificity has led to the suggestion that α4 protein acts by a general stimulation of transcription. (*ii*) As noted earlier in the text, current evidence supports the conclusion that α4 protein negatively regulates the transcription of α genes (2,

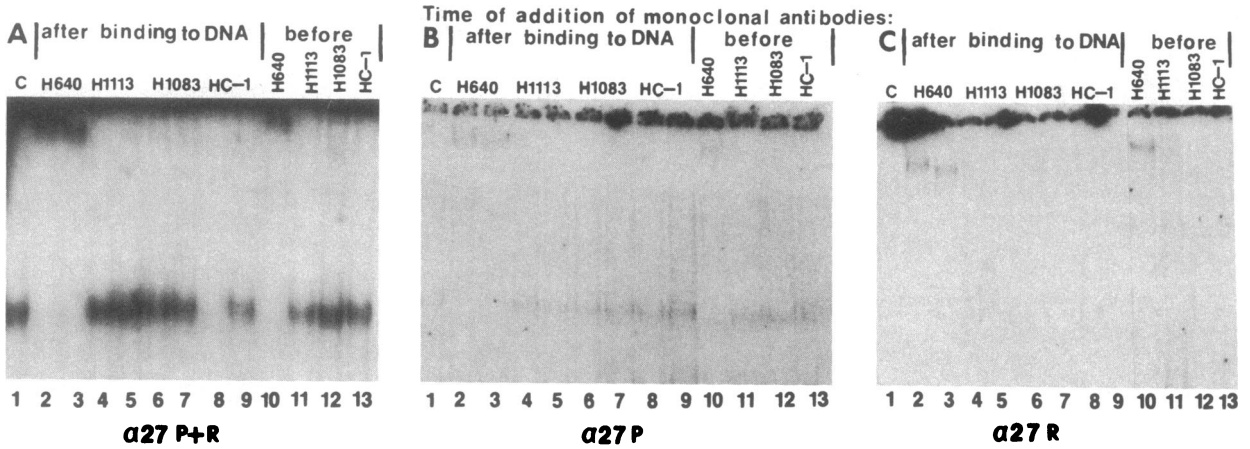

FIG. 6. Autoradiographic images of labeled $\alpha 27$ gene promoter or regulatory domain DNA–protein complexes exposed to monoclonal antibodies to selected HSV-1(F) proteins. The binding reactions were done as described in the legend to Fig. 5. Lanes marked C indicate that no monoclonal antibody was added. (*A*) The entire $\alpha 27$ promoter–regulatory region ($\alpha 27$ P+R). (*B*) $\alpha 27$ promoter domain ($\alpha 27$ P). (*C*) $\alpha 27$ regulatory domain ($\alpha 27$ R).

43). To stress the similarity in the gene products, the adenovirus E1A protein has also been implicated in the negative regulation of several viral enhancer elements (44).

The salient feature of the results presented in this report is that $\alpha 4$ protein binds to α promoter and regulatory domains on which it is expected to have a net negative effect, to a γ_2 promoter–regulatory domain on which it should have a positive effect, and minimally to a β promoter–regulatory domain of a gene known to be dependent on functional $\alpha 4$ protein for its expression. The possible explanations for these observations are as follows. (*i*) $\alpha 4$ protein has multiple DNA binding sites differing in specificity and affinity. It is conceivable that the $\alpha 4$ binding site for β promoter–regulatory domains is different from that of α or γ domains. Failure to detect significant amounts of $\alpha 4$ may reflect the conditions of the experiment, specifically the use of inappropriate competitor nucleic acids or the dependence upon specific ancillary factors for $\alpha 4$ protein binding. (*ii*) The effects of $\alpha 4$ protein hinge on the number and location of the $\alpha 4$ protein binding site(s) and their proximity to binding sites of transcriptional factors. In the case of the α genes, the $\alpha 4$ protein binds to both promoter and regulatory domains. It remains to be determined whether the binding of $\alpha 4$ interferes with the *α-trans*-inducing factor and host regulatory factors specific for sequences in the α regulatory domains. (*iii*) Consistent with the existence of multiple forms of the $\alpha 4$ protein, it is conceivable that the affinity of binding and the effect of the bound $\alpha 4$ protein are determined by its post-translational modification(s) (35, 37, 45). We cannot at this stage of the investigation differentiate between these possibilities nor are they mutually exclusive.

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