ICP4, the Major Regulatory Protein of Herpes Simplex Virus, Shares Features Common to GTP-Binding Proteins and Is Adenylated and Guanylated

JOHN A. BLAHO AND BERNARD ROIZMAN*

*The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago,
910 East 58th Street, Chicago, Illinois 60637*

Infected cell protein 4 (ICP4), the product of the $\alpha 4$ gene, regulates herpes simplex virus 1 and herpes simplex virus 2 gene expression at the transcriptional level both positively and negatively. Previous studies have shown that ICP4 is extensively modified posttranslationally. We report that ICP4 was labeled in isolated nuclei of infected cells by $[\alpha\text{-}^{32}P]GTP$ or $[\alpha\text{-}^{32}P]ATP$. The labeling of ICP4 by $[\alpha\text{-}^{32}P]GTP$ or $[\alpha\text{-}^{32}P]ATP$ required excess GTP, ATP, GDP, and ADP and occurred also in the presence of excess $GTP(\gamma)S$. While GDP and ADP activated the labeling process, only GTP and ATP labeled ICP4. Accumulation of labeled ICP4 was favored at temperatures from 15 to 27°C and in the presence of okadaic acid. The conditions for labeling ICP4 with $[\alpha\text{-}^{32}P]GTP$ or $[\alpha\text{-}^{32}P]ATP$ and the stability of the labeled protein were different from those of ICP4 labeled with $[\gamma\text{-}^{32}P]ATP$. Labeling studies with tritiated ATP and GTP showed that ICP4 is nucleotidylated, and chemical degradation of ICP4 labeled with $[\alpha\text{-}^{32}P]GTP$ yielded ribose-5-phosphate. Pulse-chase experiments indicated that the adenylation and guanylation are independent processes. These results, and the observation that ICP4 contains four regions which possess consensus GTP-binding elements, suggest that ICP4 may belong to a class of GTP-binding proteins which function in transcriptional transactivation.

The herpes simplex virus 1 (HSV-1) genome contains at least 76 open reading frames encoding at least 73 diverse polypeptides (3, 18, 25, 26). Three of the open reading frames, i.e., those encoding the infected cell polypeptides (ICPs) 0, 4, and 34.5, are contained in the inverted repeats flanking the long (U_L) and short (U_S) unique sequences and are therefore present in two copies per genome. The genes containing these 76 open reading frames 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 (19, 20). A key regulator of this cascade is ICP4, a product of the $\alpha 4$ gene (2, 5, 6, 11, 16, 17, 32, 33, 38, 39, 43, 44, 48–49, 50). Several features of ICP4 are of considerable interest in that they set this regulator apart from other viral transactivators reported to date (45). These features are as follows.

(i) ICP4 is predicted to have a translated molecular weight of 132,835 corresponding to 1,298 amino acids (26). The apparent molecular weight of the newly translated protein (ICP4a) is 160,000 in denaturing polyacrylamide gels cross-linked with N,N'-diallyltartardiamide (DATD) (30, 41). In solution, ICP4 is present as a homodimer, although it has been reported that higher-order oligomers are also present (12, 22, 27). ICP4 is posttranslationally modified; separation of ICP4 by electrophoresis in one-dimensional denaturing gels yields at least three bands, ICP4a, 4b, and 4c, with apparent molecular weights of 160,000, 163,000, and 170,000, respectively (1, 30, 41). In two-dimensional gels, ICP4 forms numerous bands differing in apparent molecular weight and charge (1).

(ii) ICP4 has been shown to incorporate ^{32}P in the course of the reproductive cycle (12, 51). Pulse-chase experiments (51) have shown that the phosphate cycles on and off forms 4a and 4c. ICP4 has also been reported to accept poly(ADP) ribosylation in vitro (42). Recently, the extent of phosphorylation of ICP4 was shown to contribute to ICP4's ability to form complexes with DNA (37). In these studies, phosphorylation of ICP4 was required for ICP4 interactions with binding sites in β and γ genes, whereas dephosphorylated ICP4 was able to bind its high-affinity sites present in α genes.

(iii) ICP4 binds directly to DNA (2, 8, 13–15, 21, 22, 24, 28, 29, 31, 35, 36, 39, 40, 47, 50). The locations of the binding sites vary: in some genes, the binding site is in the 5' untranscribed domain (2, 13, 24); in others, it is present both in the 5' untranscribed and in transcribed noncoding (5' leader) domains (8, 14, 15, 24, 28, 29, 31, 35, 36, 50). A consensus sequence (13, 14) derived from examination of a small number of binding sites does not appear to account for or describe all of the binding sites reported to date (2, 8, 13–15, 24, 28, 29, 31, 35, 36, 50). Although there exist no data that exclude the participation of other proteins in ICP4-DNA complexes (35, 36), ICP4 electrophoretically separated in denaturing gels, electrically transferred to nitrocellulose, and renatured specifically bound DNA fragments containing both the reported consensus and nonconsensus binding sites, indicating that ICP4 alone is sufficient for binding (29). Inasmuch as both consensus and nonconsensus sites bound two molecules of ICP4 (28), the differences in binding sites could not be attributed to the number of ICP4 molecules in the complex.

(iv) ICP4 regulates genes both positively and negatively (2, 5, 6, 11, 15–17, 32–34, 38, 40, 43, 44, 47–50). Analyses of deletion mutants attributed to specific domains of the molecule the positive and negative regulation as well as the binding of ICP4 to DNA (6, 38, 39). The most compelling evidence for negative regulation is that which concerns the $\alpha 4$ gene (5, 6, 33, 40, 44, 47). It has been suggested (8, 14, 24, 31, 40, 44, 47) that negative regulation results from binding of

* Corresponding author.

ICP4 to a site straddling the transcription initiation site of the α4 gene.

The studies described in this report stemmed from the observation that the three electrophoretic forms of ICP4, i.e., 4a, 4b, and 4c, did not have equal affinity for the various DNA-binding sites (29). To relate function to a specific posttranslationally modified form, it became important to elucidate the nature of these modifications. We report that the amino acid sequence of ICP4 contains sequences characteristic of GTP-binding proteins. Analyses of ICP4 for its capacity to bind GTP led to development of a cell-free system for the incorporation of both GTP and ATP into ICP4. In this report, we describe the system and the nature of the interaction of ATP and GTP with ICP4.

MATERIALS AND METHODS

Cells and virus. HSV-2(G) is the prototype HSV-2 strain used in this laboratory (10, 23). HeLa S3 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium with 5% newborn calf serum. Cultures containing 4×10^6 cells were exposed to 5 PFU per cell for 1 h and then incubated at 37°C in medium containing 2% calf serum.

Labeling of ICP4 in isolated nuclei. Except where modified as described in the text, the procedure for labeling the α4 protein was as follows. Infected cells in monolayer culture were exposed to phosphate-buffered saline (PBS-A; 140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1.5 mM K_2HPO_4, pH 7.5) containing 0.5% of the nonionic detergent Nonidet P-40 (NP-40). The suspended nuclei were washed once in PBS-A containing 0.1% NP-40 and incubated with 30 nM radiolabeled purine 5'-triphosphates (3,000 Ci/mmol; New England Nuclear, Boston, Mass.) in the presence of excess unlabeled purine 5'-triphosphates in 50 mM Tris-HCl (pH 7.5)–5 mM $MgCl_2$ for 30 min at 25°C. Labeling reactions in the presence of okadaic acid (Moana Bioproducts, Inc., Honolulu, Hawaii) were done as described above except that okadaic acid was dissolved in dimethylformamide (Baker) before addition to the reaction mixtures. Reactions with [2-^{3}H]ATP (23 Ci/mmol; Amersham, Arlington Heights, Ill.) and [8-^{3}H]GTP (7.8 Ci/mmol; Amersham) were performed as described above after adjustment of the tritiated nucleoside 5'-triphosphate concentrations to 300 μM by partial lyophilization. [2-^{3}H]ATP was brought to 7.8 Ci/mmol by dilution with unlabeled ATP. At the end of the reaction, nuclear proteins were extracted as previously described (9).

Polyacrylamide gel electrophoresis, transfer of separated polypeptides to nitrocellulose, and reaction with monoclonal antibodies. Electrophoretic separations of denatured proteins were done in 9.3% polyacrylamide gels cross-linked with DATD and containing 0.1% sodium dodecyl sulfate (SDS). The separated polypeptides were electrically transferred to nitrocellulose sheets in gel buffer containing 0.025% SDS at 120 V for 3 h at 4°C. ICP4 was visualized after staining with monoclonal antibody H640 (gift from Lenore Pereira, University of California at San Francisco) in an immunoperoxidase-coupled reaction (1, 21, 30, 41). Fluorography was done by soaking the SDS-DATD-polyacrylamide gel in dimethyl sulfoxide (DMSO), impregnating the dehydrated gel with 20% 2,5-diphenyloxazole (PPO) in DMSO, and drying. The labeled proteins were autoradiographed or fluorographed at −70°C on Kodak X-Omat film.

Chemical treatment of radiolabeled polypeptides. The labeled ICP4 bands were identified by autoradiography of wet gels. ICP4, as well as the region of identical electrophoretic

mobility in the mock lanes, was excised, electrically eluted into 50 mM NH_4HCO_3–0.025% SDS by using a Bio-Rad (Richmond, Calif.) model 422 Electro-Eluter, and lyophilized. The amounts of radioactivity recovered ranged from 60 to 80% of that initially measured. Polypeptides were resuspended in either distilled water or 6 M HCl, heated at 110°C for 5 h, lyophilized, resuspended in distilled water, and spotted onto polyethyleneimine-cellulose thin-layer chromatography plates containing a fluorescent indicator (EM Science, Gibbstown, N.J.). The plates were washed once in methanol, developed with 1 M acetic acid–1 M lithium chloride, and autoradiographed. GTP, GDP, GMP, and cyclic GMP markers (Sigma) were spotted on appropriate lanes and visualized by UV shadowing. Ribose-5-phosphate (Sigma) was visualized by staining with 5% anisaldehyde (Sigma) in 90% ethanol-5% sulfuric acid-1% acetic acid (vol/vol/vol).

RESULTS

The predicted amino acid sequence of ICP4 contains sequences homologous to the consensus domains of GTP-binding proteins. GTP-binding proteins have been reported to share three amino acid sequences (7). These elements have the consensus sequences (i) GXXXXGK, (ii) DXXG, and (iii) NKXD, where X is any amino acid and the spacing between each element is about 40 to 80 amino acids. The first two elements are required for phosphate binding, and the third is required for guanine specificity binding. Analyses of the predicted amino acid sequence of ICP4 (26) indicated that it contains four regions which are homologs of the first two elements of the GTP-binding domains. The four regions in ICP4 are as follows (the amino acids are represented by single-letter code and the numbers in parentheses are the amino acid numbers): 1, GGGGPRPPKTKKSG (739 to 752), DPLG (809 to 812); 2, ASAAPREGRKRKSPG (719 to 733), DAPG (754 to 757); 3, LSEAAPK (244 to 250), DATG (278 to 281); and 4, RYDRAQKG (457 to 464), DDEG (497 to 500). Region 1 in ICP4 contains only one amino acid mismatch from the consensus; however, none of the homologs are a perfect copy of the consensus. Although the third consensus element was never observed in the ICP4 sequence, the spacings between the first and second elements in all four ICP4 regions were conserved and are consistent with the spacings required for the GTP-binding consensus. Since the lysine in the first consensus element is conserved, it is important to note that seven of eight of the lysines present in ICP4 are contained in these regions. These regions are present in a domain of ICP4 which has been reported to be important for DNA binding, transactivation, and autoregulation (5, 39). Lastly, two of these regions contain the conserved amino acids which ICP4 shares with its varicella-zoster virus (VZV) homolog (26). VZV differs from HSV in numerous genes which are present in HSV and absent in VZV. The VZV homolog differs from ICP4, and the homology may be restricted to a subset of the GTP consensus sites.

For convenience, the following studies were done on HSV-2 ICP4. It should be noted that HSV-1 and HSV-2 are colinear genomes inasmuch as a vast variety of intertypic recombinants have been constructed and are viable (30, 43, 49). HSV-1 and HSV-2 ICP4 are functionally homologous (48, 49). Essentially identical results were obtained with ICP4 of HSV-1(F) (data not shown).

Labeling of ICP4 protein with [α-^{32}P]GTP. In preliminary studies designed to test the hypothesis that ICP4 is a GTP-binding protein, lysates of infected cells were separated
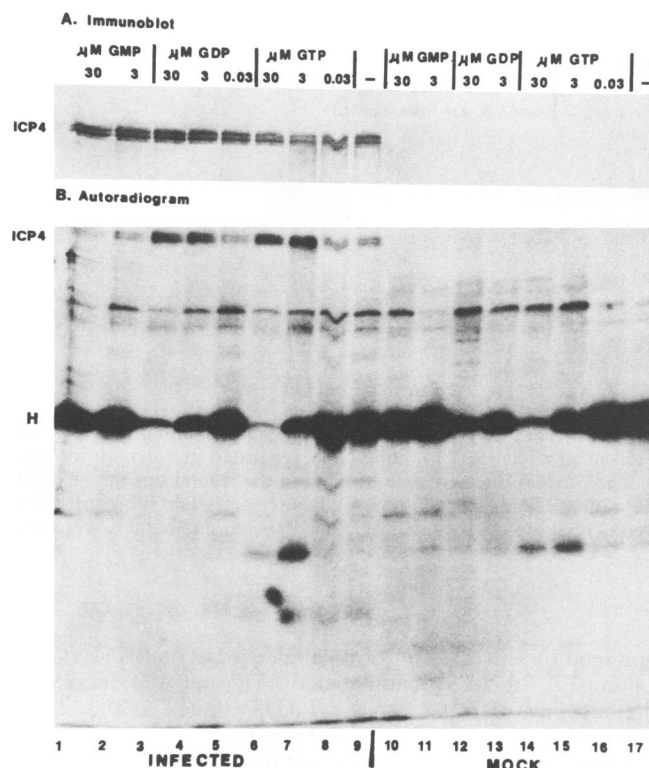
A. Immunoblot

| μM GMP | μM GDP | μM GTP | | μM GMP | μM GDP | μM GTP | |
| 30 3 | 30 3 0.03 | 30 3 0.03 | — | 30 3 | 30 3 | 30 3 0.03 | — |

ICP4

B. Autoradiogram

ICP4

H

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1   2   3   4   5   6   7   8   9   |  10  11  12  13  14  15  16  17
          INFECTED                  |              MOCK
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FIG. 1. Immunologic and autoradiographic images of ICP4 labeled by [α-^{32}P]GTP in the presence of unlabeled GTP, GDP, and GMP. Immunologic (A) and autoradiographic (B) visualization of electrophoretically separated polypeptides from extracts of mock- or HSV-2(G)-infected HeLa cell nuclei incubated with [α-^{32}P]GTP and excess unlabeled purine nucleotides. At 16 h after infection with 5 PFU per cell and incubation at 37°C, the nuclei of HeLa cells were isolated by lysis with 0.5% NP-40 in PBS-A, washed with 0.1% NP-40 in PBS-A, and incubated at 25°C for 30 min in 50 mM Tris-HCl (pH 7.5)–5 mM MgCl$_2$–30 nM [α-^{32}P]GTP (3,000 Ci/mmol; New England Nuclear) containing 0, 0.03, 3, or 30 μM GTP, GDP, and GMP. Nuclear proteins were extracted, electrophoretically separated on a preparative SDS–9.3% polyacrylamide gel, and electrically transferred to nitrocellulose, which was then dried, stained with antibody H640 (A) in an immunoperoxidase-coupled reaction, and exposed to Kodak X-OMAT film (B). Lanes marked by minus signs indicate no unlabeled nucleotides added; H indicates the major labeled host protein band.

in denaturing polyacrylamide gels, electrically transferred to a nitrocellulose sheet, renatured, and reacted with α-^{32}P-labeled GTP. The results (not shown) were unimpressive. In the course of attempts to label the nondenatured protein, we demonstrated that ICP4 does become labeled by [α-^{32}P] GTP. In these experiments, HeLa cell nuclei isolated 16 h after HSV-2(G) or mock infection, as described in Materials and Methods, were reacted at 25°C for 30 min in 50 mM Tris-HCl (pH 7.5)–30 nM [α-^{32}P]GTP containing 0, 0.03, 3, or 30 μM GMP, GDP, GTP, ATP, ADP, or AMP. Nuclear proteins were extracted, electrophoretically separated on a preparative SDS-polyacrylamide-DATD gel, and electrically transferred to nitrocellulose. The band containing ICP4 was identified by its immune reactivity with monoclonal antibody H640 (1, 21). The results (Fig. 1 and 2) were as follows.

(i) ICP4 was labeled by [α-^{32}P]GTP in the presence of excess amounts of unlabeled GTP, GDP, GMP (Fig. 1, lanes 1 to 8), ATP, ADP, and AMP (Fig. 2, lanes 11 to 18). Similar
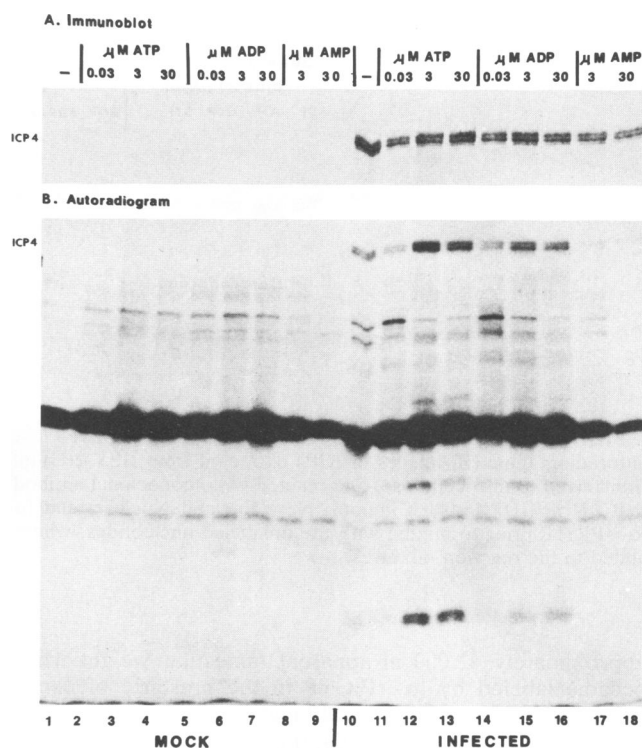
A. Immunoblot

| | μM ATP | μM ADP | μM AMP | | μM ATP | μM ADP | μM AMP |
| — | 0.03 3 30 | 0.03 3 30 | 3 30 | — | 0.03 3 30 | 0.03 3 30 | 3 30 |

ICP4

B. Autoradiogram

ICP4

H

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1   2   3   4   5   6   7   8   9   |  10  11  12  13  14  15  16  17  18
          MOCK                      |              INFECTED
```

FIG. 2. Immunologic and autoradiographic images of ICP4 labeled by [α-^{32}P]GTP in the presence of unlabeled ATP, ADP, and AMP. Immunologic (A) and autoradiographic (B) visualization of electrophoretically separated polypeptides from extracts of nuclei incubated with [α-^{32}P]GTP plus unlabeled purine nucleotides. Experiments were done exactly as for Fig. 1 except that the reactions contained 0, 0.03, 3, or 30 μM unlabeled ATP, ADP, and AMP. Lanes marked by minus signs indicate no unlabeled nucleotides added; H indicates the major labeled host protein band.

ICP4 labeling by [α-^{32}P]GTP was also observed in the presence of excess unlabeled GTP(γ)S, a nonhydrolyzable analog of GTP (data not shown). In all lanes containing electrophoretically separated infected cell polypeptides (Fig. 1, lanes 1 to 9; Fig. 2, lanes 10 to 18), the monoclonal antibody staining resolved two ICP4 bands, and both bands appeared to be labeled by GTP.

(ii) The labeling of ICP4 by [α-^{32}P]GTP was dependent on the total nucleotide concentration of unlabeled GTP which could be interchanged with GDP, ATP, ADP, or even with GTP(γ)S, but not with GMP or AMP. Thus, the labeling of ICP4 by labeled GTP in the presence of unlabeled GTP, GDP, ATP, or ADP was most efficient at concentrations of unlabeled diphosphates or triphosphates 1,000-fold higher than that of the labeled compound. In contrast, the labeling either decreased (Fig. 1, lanes 1 and 2 versus lane 9) or was inapparent (Fig. 2, lanes 17 and 18 versus lane 10) in the presence of unlabeled GMP and AMP, respectively. It is significant that the labeling of ICP4 is specific and selective and does not reflect a cellular event which affects the labeling of all proteins. Thus, the labeling of a prominent host band (H in Fig. 1 and 2) by [α-^{32}P]GTP showed reverse characteristics in that it was labeled more efficiently in the presence of low concentrations of unlabeled GDP and GTP (Fig. 1, lanes 3 to 8) or with equal efficiency in the presence of excess ATP and ADP (Fig. 2, lanes 11 to 16).

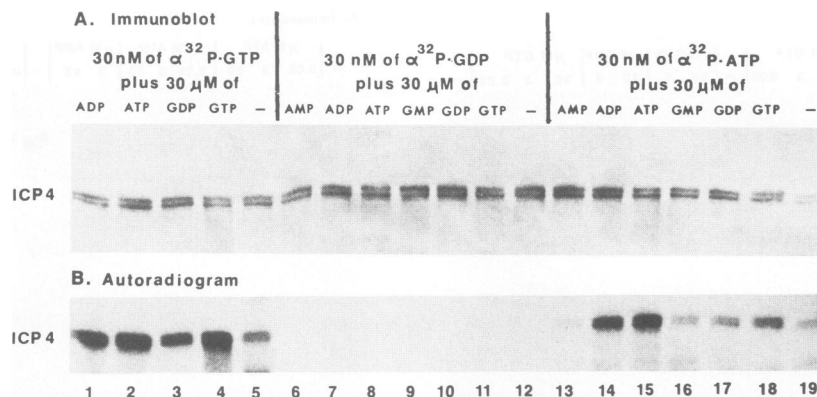(iii) The lysates of infected cells contained a protein of

FIG. 3. Immunologic and autoradiographic images of ICP4 labeled with [α-^{32}P]GTP, [α-^{32}P]ATP, and [α-^{32}P]GDP. Immunologic (A) and autoradiographic (B) images of ICP4 extracted from HSV-2(G)-infected HeLa cell nuclei electrophoretically separated in a denaturing gel, transferred to nitrocellulose, and reacted with monoclonal antibody H640. The procedures were as described in the legend to Fig. 1 except that [α-^{32}P]GDP (5,000 Ci/mmol; New England Nuclear) and [α-^{32}P]ATP (3,000 Ci/mmol; New England Nuclear) were substituted for [α-^{32}P]GTP prior to mixing with the unlabeled nucleotides, where indicated. The minus signs indicate that unlabeled nucleotides were not added to the reaction mixtures.

approximately 42,000 in apparent molecular weight which became labeled by [α-^{32}P]GTP in the presence of excess unlabeled ATP (Fig. 2, lanes 12 and 13) but not under other labeling conditions. Like ICP4, this infected cell protein was labeled more efficiently at higher ATP concentrations.

We conclude from these experiments the following: (i) only a small set of proteins in isolated nuclei from infected and mock-infected HeLa cells is labeled by [α-^{32}P]GTP; (ii) the labeling of ICP4 appears to be stable inasmuch as it is retained by the protein during denaturation in SDS, electrophoresis in SDS-polyacrylamide gels, and electrical transfer to a nitrocellulose sheet; and (iii) the activation of labeling requires a high concentration of GTP, GDP, ATP, or ADP, inasmuch as labeling was most efficient when the concentrations of unlabeled diphosphates and triphosphates were approximately 100- to 1,000-fold higher than that of the [α-^{32}P]GTP. The small protein labeled by [α-^{32}P]GTP was not identified and is not considered further.

Comparison of the efficiency of labeling of ICP4 with [α-^{32}P]GTP, [α-^{32}P]ATP, and [α-^{32}P]GDP. The capacity of the four nucleotides GDP, GTP, ADP, and ATP to stimulate the labeling of ICP4 with [α-^{32}P]GTP prompted us to determine whether ICP4 could also be labeled with ATP and GDP. In this series of experiments, nuclei obtained from HSV-2(G)-infected HeLa cells were reacted with [α-^{32}P]GDP, [α-^{32}P]GTP, or [α-^{32}P]ATP in the presence of 1,000-fold excesses of unlabeled AMP, ADP, ATP, GMP, GDP, and GTP for 30 min at 25°C and processed as above. The results (Fig. 3) were as follows.

(i) ICP4 was labeled by [α-^{32}P]GTP (lanes 1 to 5) or [α-^{32}P]ATP (lanes 13 to 19) but not by [α-^{32}P]GDP (lanes 6 to 12). As in the experiments illustrated in Fig. 1 and 2, monoclonal antibody staining resolved two ICP4 bands, and both bands were labeled.

(ii) ICP4 labeling by either [α-^{32}P]ATP or [α-^{32}P]GTP was enhanced by the addition of a 1,000-fold excess of unlabeled GTP, GDP, ATP, and ADP but not by AMP and GMP. The addition of unlabeled ATP and ADP enhanced labeling of ICP4 by [α-^{32}P]ATP (lanes 14 and 15), whereas unlabeled AMP had an adverse effect (lane 13). Although unlabeled GDP and GTP increased the labeling of ICP4 by [α-^{32}P]ATP (lanes 16 to 18), the increase was not as pronounced as that

obtained by the addition of unlabeled ADP and ATP (lanes 14 and 15). In contrast, unlabeled ADP and ATP increased the labeling of ICP4 by [α-^{32}P]GTP (lanes 1 and 2) as efficiently as did unlabeled GDP and GTP (lanes 3 and 4).

From these results we conclude that the process of ICP4 labeling was activated by either a purine triphosphate or diphosphate, but only a purine triphosphate acted as a substrate. In principle, the effect of the diphosphates and triphosphates could be on the labeling enzyme or on ATPases and GTPases, which would affect the general phosphate distribution in a cell-free system. We discount the latter possibility because the host protein (H in Fig. 1 and 2) became labeled at low purine diphosphate or triphosphate concentrations and its labeling was actually diminished under conditions that yielded maximal labeling of ICP4. The basis for the inhibitory effect of AMP is not known.

Optimization of reaction conditions for ICP4 labeling by [α-^{32}P]GTP. The purpose of this series of experiments was to optimize the labeling of ICP4 with [α-^{32}P]GTP. In the first series of experiments (Fig. 4A and B), we tested different procedures for the preparation of nuclei of infected cells and different conditions for the labeling of ICP4 in harvested nuclei. In the first part of this series (Fig. 4A and B), HeLa cells nuclei were harvested 16 h after infection with HSV-2(G) in either Tris-MgCl$_2$, Tris-EDTA, or PBS-A in the presence of various concentrations of NP-40. The harvested nuclei were labeled in 50 mM Tris-HCl (pH 7.5)–5 mM MgCl$_2$–30 nM [α-^{32}P]GTP–30 μM unlabeled GTP. In the second part of this series of experiments (Fig. 4C and D), nuclei were harvested from 16-h-infected HeLa cells in PBS-A containing 0.5% NP-40 and were reacted at 25°C for 30 min in buffers described in the legend to Fig. 4 and containing 30 nM [α-^{32}P]GTP and 30 μM unlabeled GTP. In all experiments, the reaction mixtures were denatured and electrophoretically separated in denaturing gels. The ICP4 bands were identified by monoclonal antibody staining, and the labeling was measured by autoradiography. The results (Fig. 4) were as follows.

(i) The nonionic detergent NP-40 had an effect on the mobility and labeling of ICP4 in nuclei harvested in either Tris-MgCl$_2$ or PBS-A buffer. In both buffer systems, the electrophoretic mobility increased and the labeling of ICP4

A. Immunoblot

B. Autoradiogram

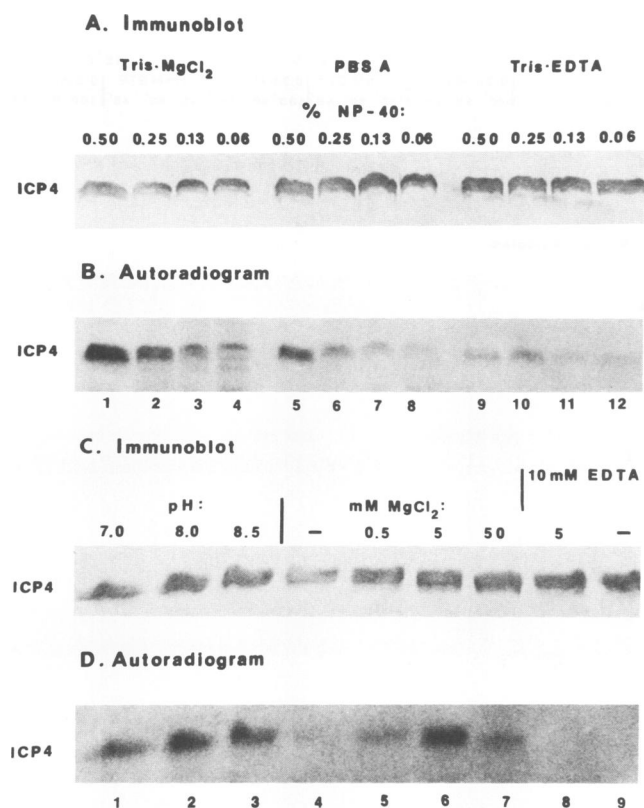## C. Immunoblot

D. Autoradiogram

FIG. 4. Autoradiographic images (B and D) and immune reactivities (A and C) of polypeptides from nuclei of infected HeLa cells prepared and labeled with [α-³²P]GTP under various conditions. Infected nuclei were isolated as described in Materials and Methods except that in panels A and B the cells were lysed with various amounts of NP-40 in either (i) 50 mM Tris-HCl (pH 7.5)–5 mM MgCl₂, (ii) PBS-A, or (iii) 10 mM Tris-HCl (pH 8.0)–0.1 mM EDTA prior to labeling with [α-³²P]GTP. Labeling reactions were carried out as described in Materials and Methods except that in panels C and D either the pH of the Tris-HCl buffer was changed or the amount of MgCl₂ was varied. Minus signs refer to no MgCl₂ added. EDTA to 10 mM was added to the reactions in lanes 8 and 9 of panels C and D.

A. Immunoblot

B. Autoradiogram

C. Immunoblot

D. Autoradiogram

FIG. 5. Labeling of ICP4 by [α-³²P]GTP and [α-³²P]ATP at different temperatures and reaction times. Autoradiographic images (B and D) and immune reactivities (A and C) of polypeptides extracted from HSV-2(G)-infected HeLa cell nuclei, electrophoretically separated in a denaturing gel, transferred to nitrocellulose, and reacted with monoclonal antibody H640. Infected cell nuclei were isolated following exposure of the cells to 10 mM Tris-HCl (pH 8.0)–0.1 M EDTA containing 0.5% NP-40. Labeling reactions were carried out as described in Materials and Methods except that in panels A and B, only 0.3 or 0.03 mM of unlabeled GTP was added to the reaction mixture for the given times (in minutes) and temperatures, whereas in panels C and D, [α-³²P]ATP was mixed with 0.3 mM unlabeled ATP and [α-³²P]GTP was mixed with 0.3 mM unlabeled GTP prior to incubation for 60 min at the temperatures shown. All other procedures were as described in Materials and Methods.

increased at the higher NP-40 concentrations tested (Fig. 4A, lanes 1 to 8). In contrast, NP-40 had no effect on electrophoretic mobility and only a marginal effect on the labeling of ICP4 in nuclei harvested in Tris-EDTA buffer (lanes 9 to 12).

(iii) Labeling of ICP4 did not vary significantly in the pH range from 7.0 to 8.5 (Fig. 4D, lanes 1 to 3 and 6). The difference in the electrophoretic mobility apparent in Fig. 4C and D is a gel artifact and not reproducible.

(iv) Optimum labeling of ICP4 required the presence of MgCl₂ at a concentration of 5 mM (Fig. 4D, lanes 4 to 7). Addition of EDTA to 10 mM, either alone or with 5 mM MgCl₂, to the reaction mixture completely inhibited labeling of ICP4 by [α-³²P]GTP (lanes 8 and 9). Varying the conditions of the labeling reaction did not affect the reactivity of ICP4 with the monoclonal antibody H640 (Fig. 4C).

We conclude the following from these experiments: (i) ICP4 labeling by [α-³²P]GTP was independent of the pH within the range tested and required Mg²⁺ ion; (ii) the buffer system in which the infected cell nuclei were harvested affected both the electrophoretic mobility and the labeling of
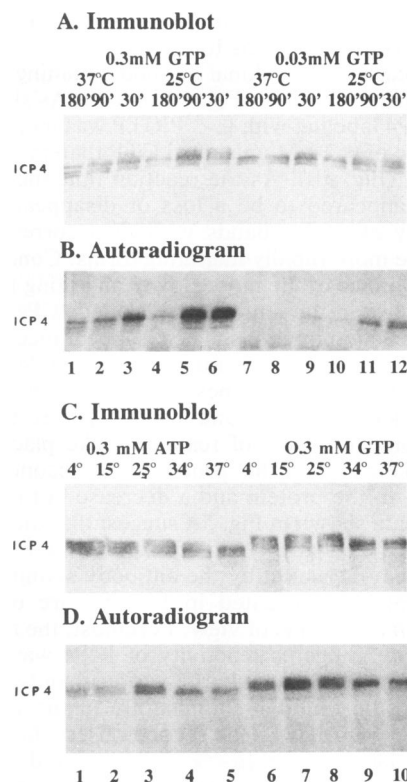
ICP4 by [α-³²P]GTP; and (iii) there was a concurrent increase in electrophoretic mobility and labeling of ICP4 in nuclei extracted with higher concentrations of NP-40 which may be attributed to a partial denaturation of ICP4 or greater availability of the substrate.

The purpose of the second series of experiments (Fig. 5) was to determine the effects of temperature and duration of the reaction on the efficiency of ICP4 labeling by [α-³²P]GTP and [α-³²P]ATP. In these studies, nuclei from HSV-2(G)-infected HeLa cells were isolated 16 h after infection by exposure to 10 mM Tris-HCl (pH 8.0)–0.1 mM EDTA containing 0.5% NP-40. Nuclei were reacted with 30 nM [α-³²P]GTP in the presence of 1,000- or 10,000-fold excesses of GTP for different time intervals. Similar experiments were done with [α-³²P]ATP (30 nM) in the presence of a 10,000-fold excess of ATP. Nuclear proteins were extracted, electrophoretically separated in a denaturing gel, and electrically transferred to nitrocellulose. The ICP4 band was identified

by its immune reactivity with monoclonal antibody H640. The results (Fig. 5) were as follows.

(i) In all cases, monoclonal antibody staining resolved at least three bands of ICP4 (Fig. 5A and C). As shown earlier (Fig. 1), ICP4 labeling with [α-^{32}P]GTP was more efficient in the presence of 0.3 mM unlabeled GTP than in the presence of 0.03 mM (Fig. 5B). As the reaction time increased (Fig. 5A), there appeared to be a loss or disappearance of the more slowly migrating bands without a corresponding increase in the more rapidly migrating forms. Concurrent with the disappearance of the more slowly migrating forms, there was a decrease in the amounts of labeled ICP4 detected at the longer reaction intervals. The disappearance of the more slowly migrating forms was more efficient at 34°C (Fig. 5C, lanes 4 and 9) and 37°C (lanes 5 and 10) than at the lower temperatures (lanes 1 to 3 and 6 to 8). The results indicate that two competing sets of reactions take place. The first results in labeling of the protein; the second results in degradation of the protein and a decrease in the amount of label. The data shown in Fig. 5A suggest that the decrease in the amount of label at 180 min of reaction is greater than the loss of protein measured by the antibody staining.

(ii) The results presented in Fig. 5C are of particular interest from two points of view. Foremost, the results show that while the antigenic reactivity of ICP4 was distributed approximately evenly among the various bands, the labeled ICP4 was to be found predominantly in the more slowly migrating forms of ICP4. The second observation is that the ICP4 bands labeled with [α-^{32}P]GTP migrated more slowly or exhibited additional, more slowly migrating bands than those labeled with [α-^{32}P]ATP (compare lanes 1 to 3 with lanes 6 to 9).

We conclude from these results the following. (i) The conditions for recovering maximally labeled ICP4 required reaction times less than 90 min at 4 to 25°C. (ii) The ICP4 populations recovered from nuclei after the reaction were not homogeneously labeled; the electrophoretic mobility of the ICP4 forms labeled with GTP or ATP was slower than that of the unlabeled or less labeled forms. (iii) The ICP4 modified in the presence of GTP formed additional bands which migrated more slowly than the most slowly migrating bands of ICP4 modified in the presence of ATP (compare Fig. 5C and D, lanes 1 to 5 with lanes 6 to 10).

Differentiation of labeling of ICP4 with ATP and GTP. The purpose of this series of experiments was to determine whether the optimal conditions for labeling ICP4 with [α-^{32}P]ATP and [α-^{32}P]GTP were similar. In these experiments, nuclei of mock-infected and 16-h-infected HeLa nuclei were harvested in a buffer containing 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, and 0.5% NP-40 and were reacted with 30 nM [α-^{32}P]ATP or [α-^{32}P]GTP in the presence of a 10,000-fold excess of unlabeled ATP or GTP, respectively, for various time intervals at 15 or 25°C. Nuclear lysates were then electrophoretically separated in a denaturing polyacrylamide gel and electrically transferred to a nitrocellulose sheet. ICP4 was identified by its immune reactivity with monoclonal antibody H640. The results (Fig. 6) were as follows.

(i) Consistent with previous results (Fig. 3 and 5), under identical reaction conditions, ICP4 was labeled more efficiently by [α-^{32}P]GTP than by [α-^{32}P]ATP (Fig. 6B, lanes 7 to 18). ICP4 labeling with either [α-^{32}P]ATP (lanes 10 to 12 and 16 to 18) or [α-^{32}P]GTP (lanes 7 to 9 and 13 to 15) was more efficient at 15 than at 25°C.

(ii) At 15°C, the extent of ICP4 labeling by [α-^{32}P]GTP decreased with prolonged reaction time (Fig. 6B, lanes 13 to
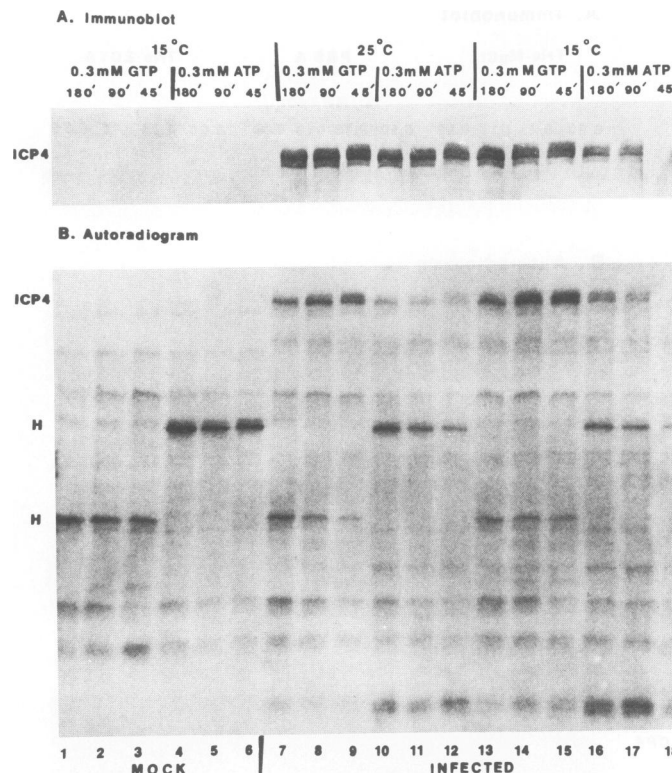

FIG. 6. Immune reactivities (A) and autoradiograhic images (B) of polypeptides extracted from mock- and HSV-2(G)-infected HeLa cell nuclei reacted with ATP or GTP for different intervals and at different temperatures. The procedures for labeling the nuclei for the times (in minutes) and temperatures shown and the protocol for electrophoresis, immunoblotting with monoclonal antibody H640, and autoradiography were as described in Materials and Methods. H identifies host protein bands.

15), whereas ICP4 labeling by [α-^{32}P]ATP remained constant (lanes 16 and 17). At 25°C, however, ICP4 labeling by [α-^{32}P]ATP (lanes 10 to 12) decreased with increasing reaction time more rapidly than that observed with [α-^{32}P]GTP (lanes 7 to 9).

(iii) For both [α-^{32}P]GTP and [α-^{32}P]ATP, labeling of the respective major host bands (H) increased with time at both temperatures (Fig. 6B, lanes 1 to 6).

We conclude from these results that the metabolic processes which affect ICP4 labeling by [α-^{32}P]ATP differ from those which determine the labeling of ICP4 by [α-^{32}P]GTP, inasmuch as the patterns of labeling were not identical. The processes that affect the amount of label observed at the end of the reaction reflect both the labeling and the removal of the label from ICP4, the hydrolysis of labeled triphosphates by cellular enzymes, and degradation of the ICP4. Earlier in the text we noted that the pattern of labeling of the major host protein was different from that of ICP4 and therefore the labeling of proteins was not a reflection of the availability or distribution of the labeled substrate in the nucleus. These results suggest that the labeling of ICP4 by [α-^{32}P]GTP is not merely the result of transfer of the labeled phosphate from one of the substrates to another.

Okadaic acid enhances ICP4 labeling by [α-^{32}P]GTP and [α-^{32}P]ATP. The purpose of these experiments was to differentiate between the labeling of proteins with [α-^{32}P]ATP,
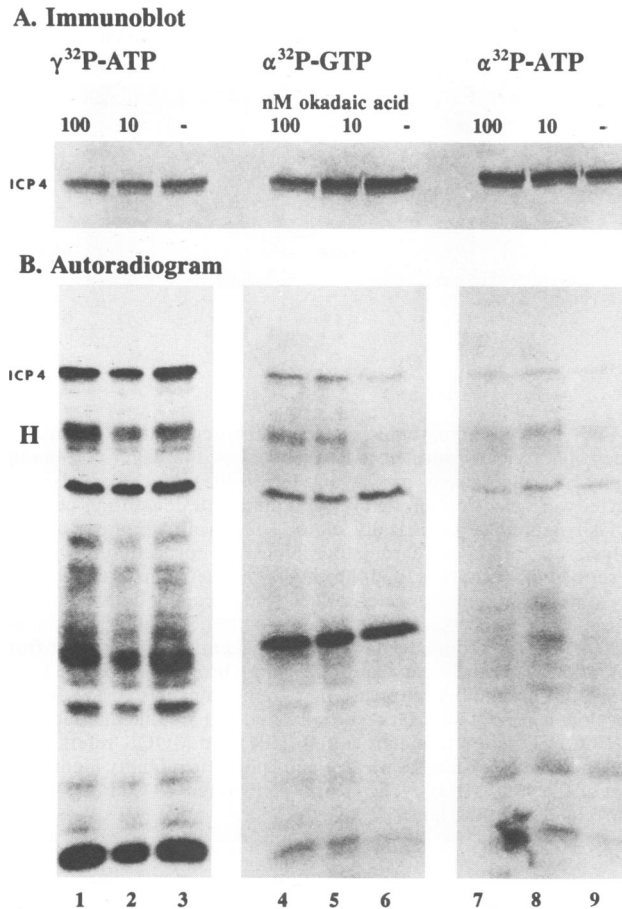
A. Immunoblot

FIG. 7. Immune reactivities (A) and autoradiographic images (B) of polypeptides extracted from HSV-2(G)-infected HeLa cell nuclei reacted with [α-^{32}P]GTP, [α-^{32}P]ATP, and [γ-^{32}P]ATP in the presence of okadaic acid. The procedures were as described in Materials and Methods except that the nuclei were also labeled with [γ-^{32}P]ATP and the reactions were done at 25°C for 30 min in the presence of okadaic acid dissolved in dimethylformamide. The minus signs indicate that dimethylformamide but not okadaic acid was present in the reaction mixture. H identifies host protein bands.

[α-^{32}P]GTP, and [γ-^{32}P]ATP. Specifically, it has been reported that okadaic acid blocks the dephosphorylation of serine and threonine amino acids in proteins (4). The usual source of this phosphate is [γ-^{32}P]ATP. The question was whether okadaic acid had a similar effect with γ-^{32}P- and α-^{32}P-labeled ICP4. In this series of experiments, nuclei isolated as described in Materials and Methods from HSV-2(G)-infected HeLa cells were reacted with 30 nM [α-^{32}P]GTP, [α-^{32}P]ATP, or [γ-^{32}P]ATP in the presence of a 10,000-fold excess of the corresponding unlabeled triphosphate in reactions containing 0, 10, and 100 nM okadaic acid. After incubation at 25°C for 30 min, nuclear proteins were extracted, electrophoretically separated in a denaturing polyacrylamide gel, and transferred to nitrocellulose. ICP4 was identified by its immune reactivity with monoclonal antibody H640. The results (Fig. 7) were as follows.

(i) In all labeling reactions, the presence of okadaic acid did not alter the electrophoretic mobilities of ICP4 and did not curtail ICP4 recognition by monoclonal antibody H640 (Fig. 7A).

(ii) The accumulation of ICP4 labeled by [γ-^{32}P]ATP was not affected by the presence of okadaic acid in the concentration range tested (Fig. 7B, lanes 1 to 3).

(iii) Within the same concentration range of okadaic acid, the accumulation of ICP4 labeled in isolated nuclei with [α-^{32}P]GTP (Fig. 7B, lanes 4 to 6) and [α-^{32}P]ATP (lanes 7 to 9) increased at higher concentrations of okadaic acid, although the increase in ICP4 labeling with [α-^{32}P]GTP was more pronounced than that with [α-^{32}P]ATP. The drug had a similar effect on the major labeled host protein band (H; lanes 4 to 9).

We conclude from these experiments that (i) the labeled adduct in ICP4 is subject to enzymatic degradation which is blocked by okadaic acid; (ii) the degradation of the substrate, ATP or GTP, if it occurs, is not as significant as that which affects the labeled adduct on ICP4, inasmuch as the effect of okadaic acid on the accumulation of labeled ICP4 by [γ-^{32}P]ATP labeling was insignificant; and (iii) the labeled adducts on ICP4 derived from [γ-^{32}P]ATP and [α-^{32}P]GTP or [α-^{32}P]ATP must be different.

The reaction of ICP4 with ATP or GTP results in adenylation or guanylation of the protein. One hypothesis that could explain the pattern of labeling observed in the studies described above is that ICP4 is both phosphorylated and nucleotidylated. If this were the case, the label for phosphorylation would be derived from [γ-^{32}P]ATP whereas the label for guanylation or adenylation would be derived from [α-^{32}P]GTP or [α-^{32}P]ATP, respectively. To test this hypothesis, HeLa cell nuclei isolated 16 h after HSV-2(G) infection were reacted with 300 μM [2-^{3}H]ATP or [8-^{3}H]GTP for 30 min at 25°C. All of the procedures were as described in Materials and Methods. Control experiments were done with [α-^{32}P]ATP and [α-^{32}P]GTP in the absence or presence of excess unlabeled ATP and GTP, respectively. Nuclear proteins were extracted and electrophoretically separated in a denaturing gel. The gel was impregnated with a fluor, dried, and fluorographed. The results (Fig. 8) were that ICP4 was labeled by both [2-^{3}H]ATP (lane 3) and [8-^{3}H]GTP (lane 4), although the labeling of ICP4 with tritiated GTP was less intense and less readily demonstrable in photographs than that obtained with ATP. In the control lanes, ICP4 was more efficiently labeled by [α-^{32}P]GTP in the presence of excess unlabeled GTP than in its absence (compare lanes 5 and 6). The major host band (H) was labeled (lane 4) after incubation with 300 μM [8-^{3}H]GTP almost as efficiently as with [α-^{32}P]GTP in the absence of unlabeled GTP (lane 6). However, the efficiency of labeling of this host protein by [α-^{32}P]GTP was greatly reduced when excess unlabeled GTP (300 μM) was added (lane 5).

Chemical analysis of the phosphate label in ICP4 labeled with [α-^{32}P]GTP. The objective of this experiment was to determine the molecular nature of the label in ICP4 extracted from the nuclei of cells reacted with [α-^{32}P]GTP. Nuclei from mock-infected and HSV-2(G)-infected HeLa cells were isolated 16 h after infection and labeled with 30 nM [α-^{32}P]GTP in the presence of a 1,000-fold excess of unlabeled GTP. Nuclear proteins were extracted and separated on a denaturing gel. The ICP4 band and the region in the mock lane with identical electrophoretic mobility were electrically eluted and lyophilized. Following addition of either distilled water or 6 M HCl and heating of the protein at 110°C for 5 h, the degradation products were resolved by thin-layer chromatography and visualized by autoradiography. The identities of the labeled degradation products were determined by comparing their chromatographic mobilities with
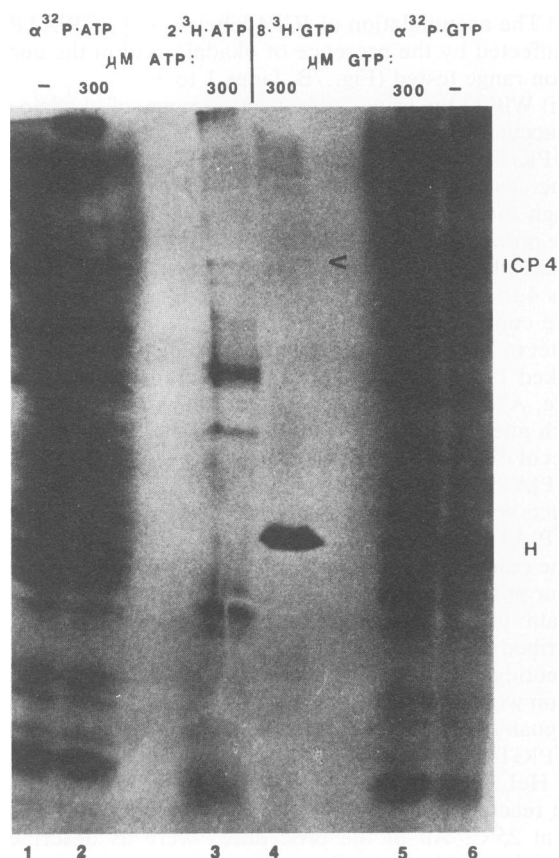
FIG. 8. Fluorographic image of polypeptides extracted from HSV-2(G)-infected HeLa cell nuclei labeled with [2-^{3}H]ATP and [8-^{3}H]GTP. The nuclear lysate was electrophoretically separated in a denaturing polyacrylamide gel. The gel was impregnated with PPO-DMSO, dried, and subjected to fluorography. The procedures for labeling the nuclei were as described in Materials and Methods except that the nuclei were labeled with 300 μM [2-^{3}H]ATP and [8-^{3}H]GTP. Control reactions were done with [α-^{32}P]ATP and [α-^{32}P]GTP containing excess unlabeled ATP and GTP, respectively. Minus signs indicate that no unlabeled ATP or GTP was added. H identifies a host protein band.
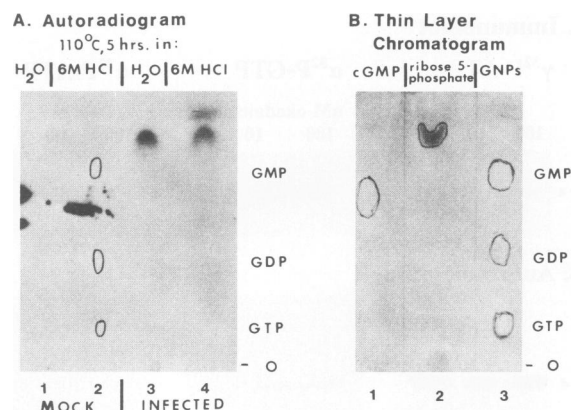

FIG. 9. Autoradiographic images of the degradation products of electrophoretically purified ICP4 resolved by thin-layer chromatography. Extracts from mock- and HSV-2(G)-infected HeLa cell nuclei were electrophoretically separated in a denaturing gel. The ICP4 bands were electrically eluted, chemically treated, and resolved by thin-layer chromatography. The procedures were as described in Materials and Methods. Nuclei were labeled with [α-^{32}P]GTP in the presence of a 1,000-fold excess of unlabeled GTP. O indicates the origin of the chromatogram; the direction of the buffer front was from bottom to top. The location of the buffer front migration was at the extreme top of the chromatogram and is not shown in the photograph. Chromatograms in panels A and B were developed separately. Markers are circled and identified at the right. INFECTED refers to electroeluted ICP4, and MOCK refers to the region in the mock lanes with an electrophoretic mobility identical to that of ICP4. cGMP is cyclic GMP; GNPs refers to GTP, GDP, and GMP loaded on the lanes.

those of authentic GTP, GDP, GMP, cyclic GMP, and ribose-5-phosphate. The results (Fig. 9) were as follows.

(i) Heating ICP4 labeled with [α-^{32}P]GTP in water at 110°C for 5 h yielded a labeled compound which migrated faster than GMP (Fig. 9A, lane 3). Similar treatment of [α-^{32}P]GTP-labeled ICP4 with 6 M HCl generated two labeled species; of one was identical in mobility to that released by water heating (lane 3), whereas the other had a higher mobility (lane 4). Under these chromatographic conditions, neither of the labeled species comigrated with the buffer front, which was at the top of the chromatogram and not shown in the photograph.

(ii) Only authentic ribose-5-phosphate migrated faster than GMP in the chromatogram (Fig. 9B, lanes 1 to 3). The position of ribose-5-phosphate on the chromatogram (lane 2) was essentially identical to that of the slowest-migrating labeled species liberated from ICP4 (Fig. 9A, lanes 3 and 4). Although the chromatograms shown in Fig. 9 were developed separately, the mobilities of the slowest-migrating species liberated from ICP4 and that of authentic ribose-5-

phosphate relative to their appropriate buffer fronts were identical and are therefore comparable.

From these results, we conclude that the form of the ICP4 adduct derived from [α-^{32}P]GTP is GMP rather than phosphate, since its degradation product comigrated with ribose-5-phosphate and 6 M HCl treatment further degraded this compound to another, faster-migrating labeled substance, which we presume to be P$_i$.

Relationship of the guanylation and adenylation events. The experiments described in this section were designed to determine whether the adenylation and guanylation of the ICP4 molecule involve the same or different events. In all of the experiments in this series, infected cell nuclei were reacted with purine triphosphates twice, initially with labeled or unlabeled GTP or ATP (the pulse), followed by a second reaction in the presence of labeled or unlabeled purine triphosphate (the chase). Thus, for the pulse, the nuclei of 16-h HSV-2(G)-infected cells were first reacted for 90 min at 15°C with 30 nM [α-^{32}P]GTP or [α-^{32}P]ATP in the presence of a 1,000-fold excess of unlabeled GTP or ATP, respectively, or with the equivalent amount of unlabeled purine triphosphate. For the chase, the nuclei were washed three times in buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl$_2$, and 0.1% NP-40 and reincubated for 90 min in a mixture containing either the same or the alternate purine triphosphate, either labeled or unlabeled. In other experiments, the chase was done without washing the nuclei after the first reaction. The latter experiments (not shown) were not readily interpretable because labeling initiated during the pulse continued in the presence of unlabeled added triphosphates during the chase interval. The results of an experiment in which the nuclei were washed between the pulse and

A. Immunoblot

Pulse: GTP* GTP* GTP* GTP* GTP ATP GTP ATP ATP*ATP* ATP* ATP*
Chase: — GTP*GTP ATP GTP*GTP*ATP* ATP*GTP ATP ATP* —

ICP4

B. Autoradiogram

ICP4

H

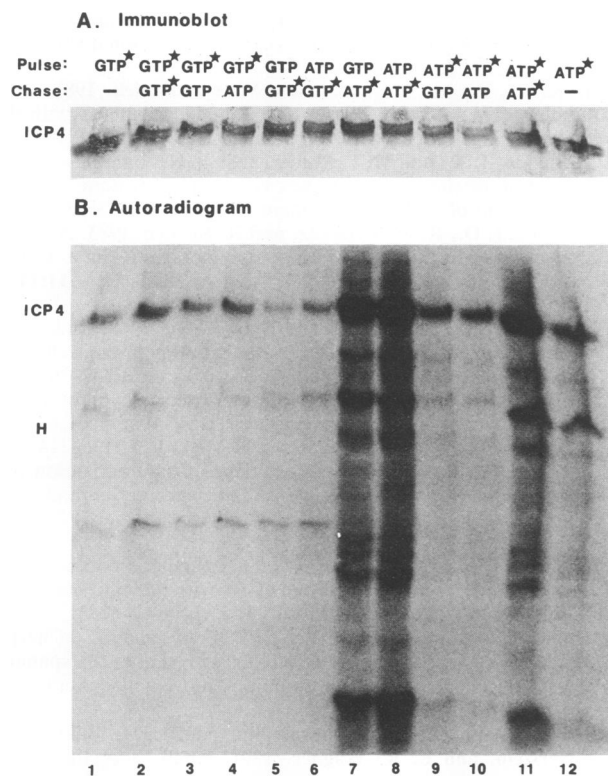1 2 3 4 5 6 7 8 9 10 11 12

FIG. 10. Immune reactivities (A) and autoradiographic images (B) of polypeptides extracted from HSV-2(G)-infected HeLa cell nuclei labeled with ATP or GTP during a pulse or a chase. Pulse refers to the initial interval of incubation of the nuclei for 90 min at 15°C; chase refers to the second, 90-min reaction which followed the pulse and washing of the cells after the pulse. The procedures were as described in Materials and Methods. The stars indicate the presence of labeled purine triphosphate (either $[\alpha\text{-}^{32}P]GTP$ or $[\alpha\text{-}^{32}P]ATP$) in the reactions. H identifies the major labeled host protein band, and the minus signs indicate that no chase reaction occurred.

the chase are illustrated in Fig. 10. The salient features of the results were as follows.

(i) The amounts of ICP4 recovered from each of the reaction mixtures were approximately similar (Fig. 10A).

(ii) There were no significant differences between the amounts of ICP4 labeled with $[\alpha\text{-}^{32}P]GTP$ during the pulse only (Fig. 10B, lane 1) and ICP4 labeled during the pulse and then chased either with GTP (lane 3) or with ATP (lane 4). Furthermore, there are no significant differences between ICP4 labeled during the pulse only and during both pulse and chase (lanes 1 and 2). The results are not consistent with the notion that the label transferred to ICP4 by $[\alpha\text{-}^{32}P]GTP$ was chased by unlabeled GTP or ATP.

(iii) ICP4 was labeled less intensely when $[\alpha\text{-}^{32}P]GTP$ was present during the chase after the incubation of the nuclei in the presence of unlabeled ATP or GTP (Fig. 10B, lanes 5 and 6). The results are consistent with the hypothesis that the conditions favorable for labeling with GTP occurred during the pulse interval.

(iv) The labeling of ICP4 with $[\alpha\text{-}^{32}P]ATP$ was far more effective during the chase (Fig. 10B, lanes 7 and 8) than during a pulse (lane 12). Furthermore, the incubation of the nuclei with unlabeled ATP or GTP did not affect the amount of label incorporated during the chase (lanes 7 and 8).

Labeling during both pulse and chase intervals (lane 11) was not demonstrably more effective than during a chase (lanes 7 and 8).

(v) Preincubation of ICP4 (pulse) with unlabeled ATP or GTP did not preclude labeling of the protein during the chase interval with $[\alpha\text{-}^{32}P]GTP$ (Fig. 10B; compare lane 1 with lanes 5 and 6) or $[\alpha\text{-}^{32}P]ATP$ (lanes 7 and 8). However, in chase reactions containing $[\alpha\text{-}^{32}P]GTP$, the extent of ICP4 labeling was slightly diminished when the nuclei were preincubated with unlabeled GTP (lane 5) as opposed to unlabeled ATP (lane 6).

(vi) The major labeled cellular protein (H) incorporated label during the pulse (Fig. 10B, lane 12), and this label was chased during the second reaction containing either ATP or GTP (lanes 9 and 10). Under the same test conditions, both the $[\alpha\text{-}^{32}P]ATP$ and $[\alpha\text{-}^{32}P]GTP$ labels on ICP4 were stable.

We conclude from these studies that (i) the reactions involving the transfer of label by $[\alpha\text{-}^{32}P]GTP$ and $[\alpha\text{-}^{32}P]ATP$ are distinct enzymatic reactions which require different physiologic conditions met during the pulse and chase, respectively, and (ii) under the conditions tested, the $[\alpha\text{-}^{32}P]ATP$ and $[\alpha\text{-}^{32}P]GTP$ did not compete for common sites.

DISCUSSION

The salient conclusions presented in this report are as follows.

(i) ICP4 is nucleotidylated; i.e., it is both guanylated and adenylated. This conclusion is based on three observations. First, the protein is labeled by $[\alpha\text{-}^{32}P]GTP$ or $[\alpha\text{-}^{32}P]ATP$. Second, ICP4 was labeled, albeit weakly, with $[8\text{-}^{3}H]GTP$ and $[2\text{-}^{3}H]ATP$. Lastly, chemical degradation of ICP4, labeled with $[\alpha\text{-}^{32}P]GTP$, yielded a labeled compound that comigrated in thin-layer chromatography with ribose-5-phosphate. Our experiments indicate that the conditions of labeling with $[\alpha\text{-}^{32}P]GTP$ or $[\alpha\text{-}^{32}P]ATP$ and the stability of ICP4 labeled with these compounds are different from those observed with $[\gamma\text{-}^{32}P]ATP$. For example, ICP4 was more highly labeled with $[\gamma\text{-}^{32}P]ATP$ than with $[\alpha\text{-}^{32}P]GTP$ or $[\alpha\text{-}^{32}P]ATP$. Moreover, okadaic acid increased the accumulation of $[\alpha\text{-}^{32}P]GTP$- or $[\alpha\text{-}^{32}P]ATP$-labeled ICP4 but not that of $[\gamma\text{-}^{32}P]ATP$-labeled protein.

(ii) The enzymes involved in the nucleotidylation of ICP4 have not been identified, but they appear to be different from those present and active in mock-infected cells. Thus, in both infected and mock-infected cells within the molecular weight range tested (>25,000), there were few bands of proteins labeled with $[\alpha\text{-}^{32}P]GTP$ or $[\alpha\text{-}^{32}P]ATP$. Moreover, the conditions for labeling of the host proteins and of ICP4 by $[\alpha\text{-}^{32}P]GTP$ or $[\alpha\text{-}^{32}P]ATP$ were different. Thus, the labeling of ICP4 by either $[\alpha\text{-}^{32}P]GTP$ or $[\alpha\text{-}^{32}P]ATP$ required relatively high concentrations of triphosphates or diphosphates, whereas the labeling of host proteins required lower concentrations of GTP or ATP; in fact, the conditions which were optimal for labeling of ICP4 were suboptimal for labeling of the host proteins.

It should be stressed that the guanine and adenine nucleotides appeared to have two functions. Only GTP or ATP nucleotidylated ICP4, inasmuch as GDP did not label ICP4 efficiently. However, excesses of either the purine diphosphates or triphosphates and even the nonhydrolyzable $GTP(\gamma)S$ were capable of activating the labeling enzyme pathway. The absence of an enzymatic pathway with properties similar to that operative in nucleotidylation of ICP4 from mock-infected cells requires confirmation; if indeed

such a pathway is absent, it would suggest that it is induced by or is a function of viral proteins.

(iii) As noted earlier in the text, the adduct, in ICP4 labeled with [α-^{32}P]GTP or [α-^{32}P]ATP, and ICP4 itself appeared to be unstable in isolated nuclei. We have also observed that ICP4 was not labeled uniformly and that the more rapidly migrating ICP4 bands were less labeled than the more slowly migrating bands. The significance of this observation is uncertain. We do not know whether our observations reflect the physiologic conditions of isolated nuclei or whether these processes also occur in intact infected cells. Previous studies have shown that phosphate label, whose nature was not known, appeared to cycle on and off at least two of the three resolved forms of ICP4.

(iv) The results from the pulse-chase experiments indicate that the optimal physiologic conditions for the labeling of ICP4 with [α-^{32}P]ATP and [α-^{32}P]GTP differ and that [α-^{32}P]ATP and [α-^{32}P]GTP do not compete for sites on the ICP4 molecule. However, the latter conclusion must be tempered by the fact that the labeling reaction does not appear to reach saturation by 90 min. Conceivably, the lack of competition reflects the possibility that only a small number of sites are actually labeled during the 90-min interval.

The most intriguing questions to emerge from these studies are the role of nucleotidylation in the function of ICP4 and the relationship of the consensus sequences for GTP-binding proteins to the nucleotidylation reaction. Recently, another transcriptional regulator, the simian virus 40 large T antigen, was shown to possess some of the properties of GTP-binding proteins (46). The central question is whether this and other regulatory proteins share with ICP4 similar properties, and specifically whether all transactivating GTP-binding proteins become nucleotidylated or whether ICP4 is unique in that respect. The hypotheses that guanylation and adenylation of ICP4 reflect the presence of sequences common to GTP-binding proteins and that in fact ICP4 is a G protein whose function is both positive and negative regulation of transcription of the viral genome remain to be tested further. Studies designed to ablate the consensus sequences as well as studies designed to map and modify the sites of nucleotidylation of ICP4 should resolve these questions.

ACKNOWLEDGMENTS

REFERENCES

1. **Ackermann, M., D. K. Braun, L. Pereira, and B. Roizman.** 1984. Characterization of herpes simplex virus 1 α proteins 0, 4, and 27 with monoclonal antibodies. J. Virol. **52:**108–118.
2. **Beard, P., S. Faber, K. W. Wilcox, and L. I. Pizer.** 1986. Herpes simplex virus immediate early infected-cell polypeptide 4 binds to DNA and promotes transcription. Proc. Natl. Acad. Sci. USA **83:**4016–4020.
3. **Chou, J., and B. Roizman.** 1990. The herpes simplex virus 1 gene for ICP34.5, which maps in inverted repeats, is conserved in several limited-passage isolates but not in strain 17syn+. J. Virol. **64:**1014–1020.
4. **Cohen, P.** 1989. The structure and regulation of protein phosphatases. Annu. Rev. Biochem. **58:**453–508.
5. **DeLuca, N. A., and P. A. Schaffer.** 1985. Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. Mol. Cell. Biol. **5:**1997–2008.
6. **DeLuca, N. A., and P. A. Schaffer.** 1988. Physical and functional domains of the herpes simplex virus transcriptional regulatory protein ICP4. J. Virol. **62:**732–743.
7. **Dever, T. E., M. J. Glynias, and W. C. Merrick.** 1987. GTP-binding domain: three consensus sequence elements with distinct spacing. Proc. Natl. Acad. Sci. USA **84:**1814–1818.
8. **DiDonato, J. A., and M. T. Muller.** 1989. DNA binding and gene regulation by the herpes simplex virus type 1 protein ICP4 and involvement of the TATA element. J. Virol. **63:**3737–3747.
9. **Dignam, J. D., R. M. Lebovitz, and R. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
10. **Ejercito, P. M., E. D. Kieff, and B. Roizman.** 1968. Characterization of herpes simplex virus strains differing in their effect on social behavior of infected cells. J. Gen. Virol. **2:**357–364.
11. **Everett, R. D.** 1984. Trans activation of transcription by herpes virus products: requirement for two HSV-1 immediate-early polypeptides for maximum activity. EMBO J. **3:**3135–3141.
12. **Faber, S. W., and K. W. Wilcox.** 1986. Characterization of a herpes simplex virus regulatory protein: aggregation and phosphorylation of a temperature-sensitive variant of ICP4. Arch. Virol. **91:**297–312.
13. **Faber, S. W., and K. W. Wilcox.** 1986. Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA. Nucleic Acids Res. **14:**6067–6083.
14. **Faber, S. W., and K. W. Wilcox.** 1988. Association of herpes simplex virus regulatory protein ICP4 with sequences spanning the ICP4 gene transcription initiation site. Nucleic Acids Res. **16:**555–570.
15. **Flanagan, W. M., A. G. Papavassiliuo, M. Rice, L. B. Hecht, S. Silverstein, and E. K. Wagner.** 1991. Analysis of the herpes simplex virus type 1 promoter controlling the expression of U_L38, a true late gene involved in capsid assembly. J. Virol. **65:**769–786.
16. **Gelman, I. H., and S. Silverstein.** 1985. Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proc. Natl. Acad. Sci. USA **82:**5265–5269.
17. **Gelman, I. H., and S. Silverstein.** 1986. Co-ordinate regulation of herpes simplex virus gene expression is mediated by the functional interaction of two immediate early gene products. J. Mol. Biol. **191:**395–409.
18. **Honess, R. W., and B. Roizman.** 1973. Proteins specified by herpes simplex virus. XI. Identification and relative molar rates of synthesis of structural and nonstructural herpes virus polypeptides in the infected cell. J. Virol. **12:**1347–1365.
19. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14:**8–19.
20. **Honess, R. W., and B. Roizman.** 1975. Regulation of herpesvirus macromolecular synthesis requires functional viral polypeptides. Proc. Natl. Acad. Sci. USA **72:**1276–1280.
21. **Hubenthal-Voss, J., R. A. Houghten, L. Pereira, and B. Roizman.** 1988. Mapping of functional and antigenic domains of the α4 protein of herpes simplex virus 1. J. Virol. **62:**454–462.
22. **Kattar-Cooley, P., and K. W. Wilcox.** 1989. Characterization of the DNA-binding properties of herpes simplex virus regulatory protein ICP4. J. Virol. **63:**696–704.
23. **Kieff, E. D., S. L. Bachenheimer, and B. Roizman.** 1971. Size, composition, and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. J. Virol. **8:**125–132.
24. **Kristie, T. M., and B. Roizman.** 1986. α4, the major regulatory protein of herpes simplex 1 is stably and specifically associated with the promoter-regulatory domains of α genes and of selected other viral genes. Proc. Natl. Acad. Sci. USA **83:**4700–4704.
25. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69:**1531–1574.
26. **McGeoch, D., A. Dolan, S. Donald, and D. H. K. Bauer.** 1986. Complete DNA sequence of the short region in the genome of

herpes simplex virus type 1. Nucleic Acids Res. **14**:1727–1745.
27. **Metzler, D. W., and K. W. Wilcox.** 1985. Isolation of herpes simplex virus regulatory protein ICP4 as a homodimeric complex. J. Virol. **55**:329–337.
28. **Michael, N., and B. Roizman.** 1989. Binding of the herpes simplex major regulatory protein to viral DNA. Proc. Natl. Acad. Sci. USA **86**:9808–9812.
29. **Michael, N., D. Spector, P. Mavromara-Nazos, T. Kristie, and B. Roizman.** 1988. The DNA binding properties of the major regulatory protein of herpes simplex virus. Science **239**:1531–1534.
30. **Morse, L. S., L. Pereira, B. Roizman, and P. A. Schaffer.** 1978. Anatomy of herpes simplex virus (HSV) DNA. X. Mapping of viral genes by analysis of polypeptides and functions specified by HSV-1 X HSV-2 recombinants. J. Virol. **26**:289–410.
31. **Muller, M. T.** 1987. Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site. J. Virol. **61**:858–865.
32. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53**:751–760.
33. **O'Hare, P., and G. S. Hayward.** 1985. Three *trans*-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. J. Virol. **56**:723–733.
34. **O'Hare, P., and G. S. Hayward.** 1987. Comparison of upstream sequence requirements for positive and negative regulation of a herpes simplex virus immediate-early gene by three virus-encoded *trans*-acting factors. J. Virol. **61**:190–199.
35. **Papavassiliou, A. G., and S. J. Silverstein.** 1990. Characterization of DNA-protein complex formation in nuclear extracts with a sequence from the herpes simplex virus thymidine kinase gene. J. Biol. Chem. **265**:1648–1657.
36. **Papavassiliou, A. G., and S. J. Silverstein.** 1990. Interaction of cell and virus proteins with DNA sequences encompassing the promoter/regulatory and leader regions of the herpes simplex virus thymidine kinase gene. J. Biol. Chem. **265**:9402–9412.
37. **Papsvassiliou, A. G., K. W. Wilcox, and S. J. Silverstein.** 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. EMBO J. **10**:397–406.
38. **Paterson, T., and R. D. Everett.** 1988. Mutational dissection of the HSV-1 immediate early protein Vmw175 involved in transcriptional transactivation and repression. Virology **166**:186–196.
39. **Paterson, T., and R. D. Everett.** 1988. The regions of the herpes simplex virus type 1 immediate early protein Vmw175 required

for site specific DNA binding closely correspond to those involved in transcriptional regulation. Nucleic Acids Res. **16**:11005–11025.
40. **Paterson, T., V. G. Preston, and R. D. Everett.** 1990. A mutant of herpes simplex virus type 1 immediate early polypeptide Vmw175 binds to the cap site of its own promoter *in vitro* but fails to autoregulate *in vivo*. J. Gen. Virol. **71**:851–861.
41. **Pereira, L., M. H. Wolff, M. Fenwick, and B. Roizman.** 1977. Regulation of herpesvirus macromolecular synthesis. V. Properties of α polypeptides made in HSV-1 and HSV-2 infected cells. Virology **77**:733–749.
42. **Preston, C. M., and E. L. Notarianni.** 1983. Poly ADP-ribosylation of a herpes simplex virus immediate early polypeptide. Virology **131**:492–501.
43. **Preston, V. G., A. J. Davison, H. S. Marsden, M. C. Timbury, J. H. Subak-Sharpe, and N. M. Wilkie.** 1978. Recombinants between herpes simplex virus types 1 and 2: analyses of genome structures and expression of immediate-early polypeptides. J. Virol. **28**:499–517.
44. **Roberts, M. S., A. Boundy, P. O'Hare, M. C. Pizzorno, D. M. Ciufo, and G. S. Hayward.** 1988. Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (α4) promoter and a specific binding site for the IE175 (ICP4) protein. J. Virol. **62**:4307–4320.
45. **Roizman, B., and A. M. Sears.** 1990. Herpes simplex viruses and their replication, p. 1795–1841. *In* B. Fields and D. Knipe (ed.), Virology, 2nd ed. (Raven Press, New York).
46. **Scheffner, M., R. Knippers, and H. Stahl.** 1989. RNA unwinding activity of SV40 large T-antigen. Cell **57**:955–963.
47. **Shepard, A. A., and N. A. DeLuca.** 1991. A second-site revertant of a defective herpes simplex virus ICP4 protein with restored regulatory activities and impaired DNA-binding properties. J. Virol. **65**:787–795.
48. **Smith, C. A., and P. A. Schaffer.** 1987. Mutants defective in herpes simplex type 2 ICP4: isolation and preliminary characterization. J. Virol. **61**:1092–1097.
49. **Smith, C. A., and P. A. Schaffer.** 1987. Intertypic recombinants of herpes simplex virus types 1 and 2 infected-cell polypeptide 4. Virology **160**:17–182.
50. **Tedder, D. G., R. D. Everett, K. W. Wilcox, P. Beard, and L. I. Pizer.** 1989. ICP4-binding sites in the promoter and coding regions of the herpes simplex virus gD gene contribute to activation of in vitro transcription by ICP4. J. Virol. **63**:2510–2520.
51. **Wilcox, K. W., A. Kohn, E. Sklyanskaya, and B. Roizman.** 1980. Herpes simplex virus phosphoproteins. I. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA. J. Virol. **33**:167–182.