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Guanylylation and Adenylylation of the α Regulatory Proteins of Herpes Simplex Virus Require a Viral β or γ Function

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Herpes simplex virus genes form several groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion. Most of the products of the first group, the α genes, appear to have regulatory functions. We report that the α proteins, infected cell proteins 4, 0, 22, and 27 of herpes simplex virus 1 and 4, 0, and 27 of herpes simplex virus 2, were labeled in the isolated nuclei of infected HeLa cells with [α-^{32}P]GTP or [α-^{32}P]ATP late in infection and that these proteins represent the largest group of virus-specific proteins labeled in this fashion. Studies with [2-^{3}H]ATP, in which the label is in the purine ring, showed that a portion of the label in α proteins and in at least one other infected cell protein is due to nucleotidylylation. Analyses of the labeling reactions in nuclei of (i) cells infected with temperature-sensitive mutants at nonpermissive temperatures, (ii) cells infected with wild-type virus and harvested at different times postinfection, and (iii) cells treated with inhibitors of protein synthesis or of synthesis of viral DNA led to the conclusion that viral gene functions expressed after the synthesis of α proteins are required for the labeling of the α proteins with [α-^{32}P]GTP. We conclude that several of the α proteins are extensively posttranslationally modified and that these modifications include nucleotidylylation.

The genes of herpes simplex virus (HSV) form several groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion (21–23). The first group, the α genes, is transcribed by the host RNA polymerase II in the absence of de novo viral protein synthesis (9) and is induced by a structural protein of the virion, the α *trans*-inducing factor (αTIF or VP16) (3, 7, 32, 42). The expression of the later groups, β and γ, requires functional α proteins and especially infected cell polypeptide 4 (ICP4), the major viral regulatory protein. The β and γ groups comprise genes differing in timing and requirements of expression. Thus, β_1 genes and β_2 genes differ in the kinetics of synthesis of their proteins (41, 52), and the γ_1 and the γ_2 genes are differentiated on the basis of their dependence on viral DNA synthesis. Whereas γ_1 genes are expressed at a reduced level in the absence of viral DNA synthesis, the γ_2 genes are stringently dependent on viral DNA synthesis for their expression (reviewed in reference 52).

The α genes are of particular interest inasmuch as at least four of the α proteins, designated ICP4, ICP0, ICP22, and ICP27, have been shown to perform regulatory functions at either a transcriptional or posttranscriptional level. Several of these proteins, but most notably ICP4 and ICP27, have been shown to be multifunctional regulatory proteins (4, 10, 13–16, 18, 19, 29, 33, 34, 35, 37–39, 44, 45, 49–51, 54, 57, 59, 60, 62).

Previous studies from this laboratory have shown that the α proteins are extensively modified posttranslationally. Thus, ICP4, ICP0, ICP22, and ICP27 migrate as multiple species in denaturing one- and two-dimensional electrophoretic gels (1, 2, 36, 41, 52, 64). The α proteins are phosphoproteins inasmuch as they incorporate ^{32}P label during the course of a productive infection following the addition of $^{32}P_i$ to the medium (15, 64). ICP4 was reported to accept poly(ADP-ribosyl)ation in isolated nuclei (47), but recent studies indicate that ICP4 poly(ADP-ribosyl)ation in nuclei may differ from that occurring in the infected cell (5). Whereas the changes in electrophoretic mobility of ICP4 have been related to a function expressed by ICP27 (51, 62), the modification of ICP22 was shown to be influenced by a protein which has features in common with protein kinases and is encoded by the U_L13 open reading frame (48). In the latter case, changes in electrophoretic mobility correlate with changes in regulatory functions associated with this protein (48a).

Recently we reported that ICP4 encoded by HSV-2 is both adenylylated and guanylylated in cell-free nuclei of infected cells (6). Further studies described in this report showed that ICP4, ICP0, ICP22, ICP27, and several other HSV-1 proteins were labeled with [α-^{32}P]GTP or [α-^{32}P]ATP in isolated nuclei of infected cells. Also, exposure of isolated nuclei to [^{3}H]ATP, in which the tritium label is in the purine ring, demonstrated that a portion of the label is due to nucleotidylylation of the proteins. Finally, studies with temperature-sensitive viral mutants and metabolic inhibitors indicate that a viral gene product may be required for these modifications and that the function correlates with the expression of β or γ_1 genes.

MATERIALS AND METHODS

Cells and viruses. HeLa S3 cells obtained from the American Type Culture Collection were grown in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum. HSV-1(F) and HSV-2(G) are the prototype HSV-1 and HSV-2 strains, respectively, used in this laboratory (12, 27). As in the case of many HSV-1 isolates passaged a limited number of times in cells in culture, HSV-1(F) exhibits a temperature-sensitive lesion in the α4 gene (30), and at 39.5°C, the infected cells express only α proteins. R325 was derived from HSV-1(F) by deletion of 821 nucleotides from the coding domain of the α22 gene (43). Unless specifically stated in the text, subconfluent HeLa S3

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cultures containing approximately 4×10^6 cells were exposed to 5 PFU per cell for 1 h and then incubated at 37°C in medium containing 2% newborn calf serum.

Labeling of ICPs in nuclei. Unless specifically stated otherwise in Results, the procedures for labeling cells were the same as previously described (6). Briefly, mock-, HSV-2(G)-, or HSV-1(F)-infected HeLa cells were harvested at 20 h postinfection as follows. The medium was aspirated, the cells were rinsed once with phosphate-buffered saline (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1.5 mM K_2HPO_4 [pH 7.5]) containing 0.5 mM EDTA, reacted at 25°C for 5 min, and then suspended by tapping the culture flask in 1 ml of 50 mM Tris-HCl (pH 7.5)–5 mM $MgCl_2$. The membranes were solubilized by the addition of 4 μl of 10% Nonidet P-40 and incubation at 25°C for 5 min. Nuclei were separated from the cytoplasm by centrifugation for 1 s in a Brinkmann microcentrifuge, washed in 100 μl of 0.1% Nonidet P-40–50 mM Tris-HCl (pH 7.5)–5 mM $MgCl_2$, and centrifuged again for 1 s.

The nuclei were labeled in 50 μl of 50 mM Tris-HCl (pH 7.5)–5 mM $MgCl_2$–10 nM okadaic acid (Calbiochem, La Jolla, Calif.)–30 nM [α-^{32}P]GTP (3,000 Ci/mmol; Amersham, Arlington Heights, Ill.)–150 μM GTP (Sigma, St. Louis, Mo.)–150 μM GDP (Sigma) at 15°C for 30 min. Nuclear proteins (approximately 2 mg/ml) were extracted as described previously (11). Reactions with [2-^{3}H]ATP and [α-^{32}P]ATP (23 Ci/mmol and 3,000 Ci/mmol, respectively; Amersham) were done as described above in reaction mixtures containing ATP (Sigma) instead of GTP in the absence of nucleoside 5′-diphosphate and okadaic acid. In reaction mixtures containing [α-^{32}P]GTP but without GDP or okadaic acid, the concentration of the excess GTP or other single nucleotides was 150 μM. GMP and GTPγS were from Sigma; GTPβS was from Calbiochem.

Infection of cells. (i) Maintenance of infected cells at 39.5°C. Subconfluent HeLa S3 cells were placed at 4°C for approximately 30 min and then exposed to 5 PFU of HSV-1(F) or of HSV-2(G) per cell. After 2 h at the same temperature (4°C), the inoculum was replaced with medium containing 2% calf serum, and the cell culture flasks were immediately submerged into a circulating water bath stabilized at exactly 39.5°C. The cells were harvested 20 h postinfection, and the nuclei were separated from cytoplasm in wet ice. The nuclei were labeled as described above.

(ii) Maintenance of infected cells in the presence of cycloheximide and actinomycin D. Prior to infection, subconfluent HeLa S3 cells were incubated for at least 1 h at 37°C in medium containing 5% calf serum and 100 μg of cycloheximide (Sigma) per ml of medium. The cells were then exposed to 100 PFU of HSV-1(F) or HSV-2(G) per cell in the presence of 100 μg of cycloheximide per ml. After 1 h of exposure to virus, the inoculum was replaced with medium containing 5% calf serum and the same concentration of cycloheximide. After 5 h at 37°C, the cells were replenished with medium containing 5% calf serum, cycloheximide, and 50 μg of actinomycin D (Sigma) per ml and incubated at 37°C for 1 h. The cells were then washed seven times with phosphate-buffered saline and incubated for 14 h at 37°C in medium containing 5% calf serum and the same concentration of actinomycin D. Nuclei were prepared and labeled as above.

(iii) Maintenance of infected cells in the presence of PAA. Prior to infection, subconfluent HeLa S3 cells were incubated in medium plus 5% calf serum containing 300 μg of phosphonoacetic acid (PAA; gift of Abbott Laboratories, North Chicago, Ill.) per ml at 37°C for 1 h. The cells were then exposed to 5 PFU of HSV-1(F) or HSV-2(G) per cell in the presence of 300 μg of PAA per ml of medium containing 2% calf serum at 37°C. After 1 h of incubation, the inoculum was replaced with the same medium. At 20 h postinfection, nuclei were isolated and labeled as described above.

Polyacrylamide gel electrophoresis, transfer of electrophoretically separated polypeptides to nitrocellulose, and reaction with monoclonal antibodies. Electrophoretic separations of denatured proteins were done in 9.0, 9.5, or 11% polyacrylamide gels cross-linked with *N,N′*-diallyltartardiamide containing 0.1% sodium dodecyl sulfate (SDS). The separated polypeptides were electrically transferred to nitrocellulose sheets in a gel buffer containing 0.025% SDS at 120 V for 3 to 5 h at 4°C. ICPs were visualized after staining with specific antibodies in an immunoalkaline phosphatase-coupled reaction (1, 25). ICPs were visualized by using monoclonal antibody H640 to ICP4 (1, 25), monoclonal antibody H1113 to ICP27 (1), monoclonal antibody 1083 to ICP0 (1), polyclonal antibody R10 to ICP22 (2), and monoclonal antibody M28 to U_S11 (53). Fluorography was done by spraying the nitrocellulose sheets with En^{3}Hance as recommended by the vendor (New England Nuclear, Boston, Mass.). The labeled proteins were subjected to fluorography or autoradiography at −70°C on Kodak X-Omat film.

RESULTS

Comparison of HSV-1(F)- and HSV-2(G)-infected HeLa cell proteins labeled in nuclei with [α-^{32}P]GTP. An earlier report from this laboratory demonstrated that the ICP4 protein of HSV-2(G) is labeled by [α-^{32}P]GTP and that the labeling reaction is due to guanylylation (6). To determine whether this phenomenon was specific for HSV-2, these studies were repeated with HeLa cells infected with the HSV-1(F) strain. Mock-, HSV-1(F)-, and HSV-2(G)-infected HeLa cells were harvested 20 h postinfection, and nuclei were prepared as described in Materials and Methods. The cell-free nuclei were labeled in reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 nM okadaic acid, and 30 nM [α-^{32}P]GTP plus either 3, 30, 150, or 300 μM both unlabeled GTP and GDP at 15°C for 30 min. As described previously, the reaction conditions yield optimal labeling of HSV-2(G) ICP4 (6). The titration of unlabeled GTP and GDP was done to determine whether the labeling mixture most suitable for the HSV-2 ICP4 corresponded to that of its HSV-1(F) homolog. Following the labeling reaction, nuclear proteins were extracted, separated in a denaturing polyacrylamide gel, electrically transferred to nitrocellulose, probed for immune reactivity with monoclonal antibody H640, and subjected to autoradiography as described previously (11) and in Materials and Methods.

The results (Fig. 1) were as follows.

(i) The reactivity of HSV-1(F) and HSV-2(G) ICP4 with monoclonal antibody H640 to HSV-1 ICP4 was not affected by the incubation with labeling medium (Fig. 1A, lanes 1 to 7). Although equal amounts of protein were loaded in all lanes, the amount of HSV-2(G) ICP4 reactive with the HSV-1 monoclonal antibody (lanes 1 to 4) was greater than that reactive with the HSV-1(F) homolog (lanes 5 to 7). This was also observed in earlier studies (6) and suggests that the amounts of ICP4 produced in HSV-2(G)-infected cells may be higher than that produced in cells infected with HSV-1(F).

(ii) Consistent with the earlier report (6), the amount of [α-^{32}P]GTP label associated with the HSV-2(G) ICP4 was highest in reaction mixtures containing 150 μM GTP and

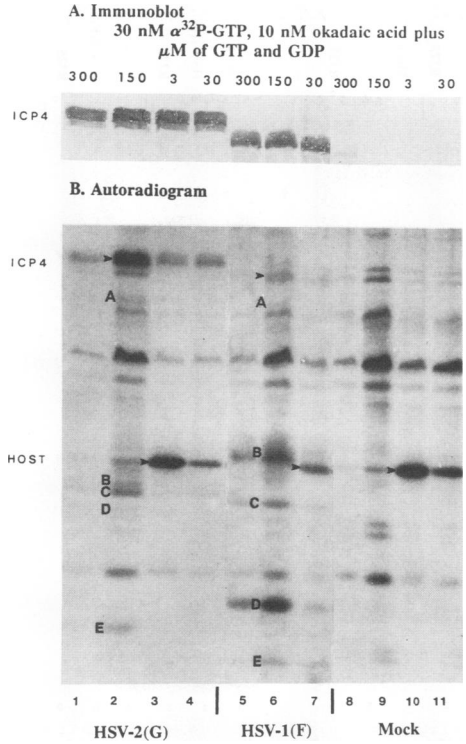

FIG. 1. Immune reactivities (A) and autoradiographic images (B) of nuclear proteins labeled with [α-^{32}P]GTP. Nuclear proteins labeled in the nuclei of mock-, HSV-(F)-, and HSV-2(G)-infected HeLa cells were separated in a 9.5% denaturing gel, transferred to nitrocellulose, and probed with antibody H640 as described in Materials and Methods. ICP4 and the major labeled host protein (HOST) are identified with arrowheads. Bands A to E identify additional ICPs labeled with [α-^{32}P]GTP.

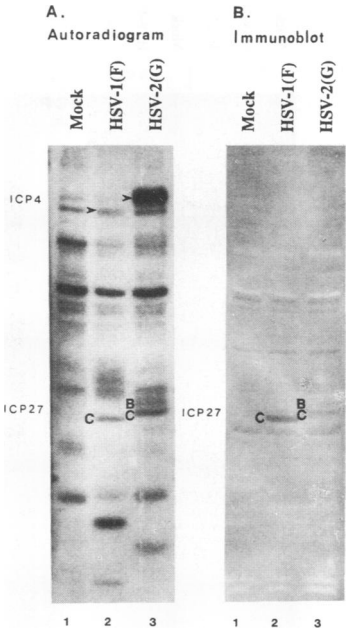

FIG. 2. Autoradiographic images (A) and immune reactivities (B) of nuclear proteins labeled with [α-^{32}P]GTP. Nuclear proteins labeled in the nuclei of mock-, HSV-1(F)-, and HSV-2(G)-infected HeLa cells were separated in a 9.5% denaturing gel, transferred to nitrocellulose, and probed with antibody H1113 as described in Materials and Methods. The ICP4 protein is identified with arrowheads. Bands labeled B and C are identical to those shown in Fig. 1. Note that the monoclonal antibody H1113 reacts more strongly with HSV-1 than with HSV-2 ICP27. Moreover, HSV-2 ICP27 forms two bands. The antibody reacts significantly better with the more rapidly migrating C band than with the slower B band.

GDP (Fig. 1B, lane 2). A protein, identified as HSV-1(F) ICP4 by its electrophoretic mobility and reactivity with the monoclonal antibody, was also labeled with [α-^{32}P]GTP (lanes 5 to 7). This protein was also optimally labeled in the presence of 150 μM GTP and GDP (lane 6), but the extent of labeling was considerably lower than that observed for the HSV-2(G) homolog (compare lanes 1 to 4 with lanes 5 to 7).

(iii) Also consistent with the earlier report (6), a host band was labeled with [α-^{32}P]GTP in the presence of the lowest GTP and GDP concentrations tested (3 μM; Fig. 1B, lanes 3 and 10). As the amounts of GTP and GDP increased to 300 μM, the extent of labeling of this band decreased (compare lane 3 with lanes 1, 2, and 4; compare lane 7 with lanes 5 and 6; compare lane 10 with lanes 8, 9, and 11).

(iv) The autoradiograms revealed several additional ICPs labeled with [α-^{32}P]GTP (Fig. 1B; compare lanes 8 to 11 with lanes 1 to 7). These proteins were also optimally labeled in reaction mixtures containing 150 μM GTP and GDP (lanes 2 and 6). To facilitate their description pending identification, they have been provisionally designated proteins A to E for both HSV-2(G) and HSV-1(F). Whereas ICP4 (lane 2) was the most highly labeled band from HSV-2(G), proteins B and D (lane 6) were the most highly labeled bands for HSV-1(F). Only protein A of HSV-2(G) and HSV-1(F) had a comparable electrophoretic mobility. The HSV-1(F) band designated B was broad and appeared to consist of two bands; the HSV-2(G) electrophoretic profile did not contain a homolog with a comparable electrophoretic mobility.

On the basis of these results, we conclude the following: (i) the HSV-1(F) ICP4 protein is labeled with [α-^{32}P]GTP in the nuclei of infected cells isolated late in infection, but the extent of this labeling is less than that observed for HSV-2(G) ICP4 even under optimal reaction conditions; (ii) the biochemical process which results in ICP4 labeling is not specific to ICP4, since additional ICPs are labeled with [α-^{32}P]GTP under these conditions; and (iii) although the numbers of bands labeled by [α-^{32}P]GTP were nearly identical, some of the HSV-1(F) and HSV-2(G) bands do not coincide with respect to electrophoretic mobility.

Band C labeled with [α-^{32}P]GTP is ICP27. The electrophoretic mobilities of bands C of HSV-1(F) and HSV-2(G) were similar to those of the corresponding ICP27 proteins of the two viruses (21–23, 36, 41), and as observed for ICP27 (36, 41), protein C from HSV-2(G) has a slower mobility than that from HSV-1(F). To determine whether ICP27 was labeled with [α-^{32}P]GTP, nuclei from mock-, HSV-1(F)-, and HSV-2(G)-infected HeLa cells were isolated, labeled, and subjected to electrophoresis in denaturing gels. The electrophoretically separated polypeptides were transferred to a nitrocellulose sheet and probed with monoclonal antibody H1113, specific for ICP27 (1).

The results (Fig. 2) show that monoclonal antibody H1113 to ICP27 reacted with two HSV-2(G) bands (Fig. 2B, lane 3) corresponding in electrophoretic mobility to bands B and C labeled with [α-^{32}P]GTP in Fig. 2A, lane 3. The same monoclonal antibody reacted with one band from HSV-1(F)-

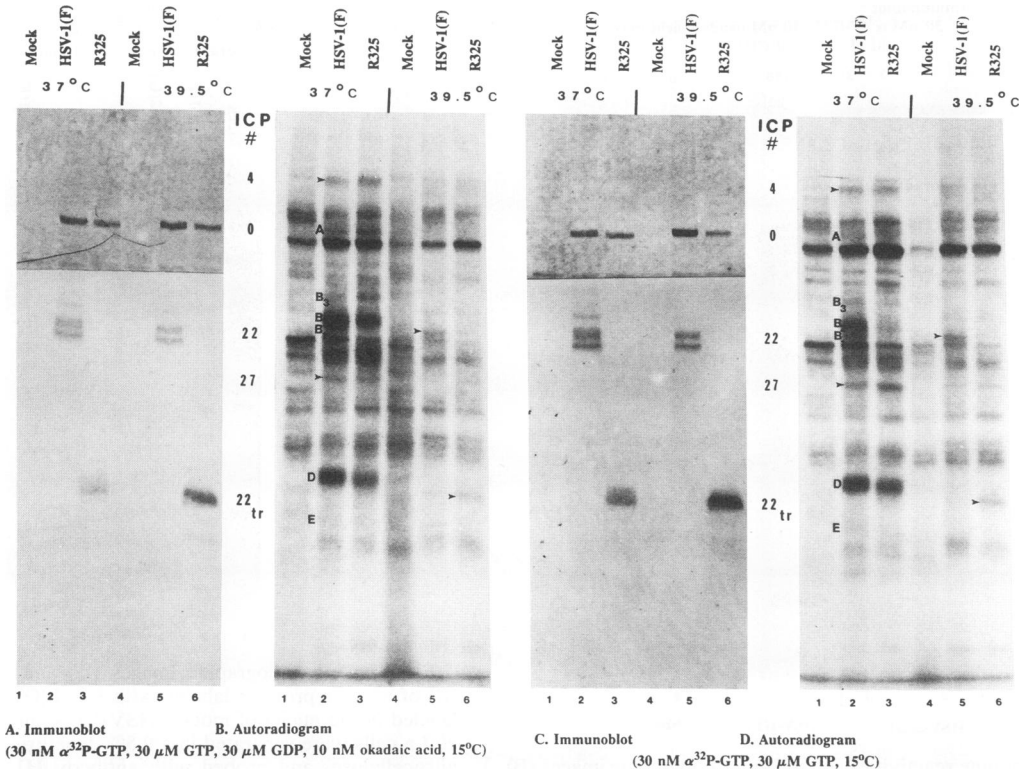

FIG. 3. Immune reactivities (A and C) and autoradiographic images (B and D) of nuclear proteins labeled with [α-^{32}P]GTP. Nuclear proteins labeled under two different sets of reaction conditions in the nuclei of mock-, HSV-(F)-, and R325-infected HeLa cells at two different infection temperatures (37 and 39.5°C) were separated in a 9.0% denaturing gel, transferred to nitrocellulose, and probed with antibodies H1083 and R10 as described in Materials and Methods. ICPs are indicated by numbers in the margins; 22_{tr} refers to the truncated form of the ICP22 protein and is marked with an arrowhead. ICP4, ICP22, and ICP27 are also marked with arrowheads. Bands labeled A to E are identical to those shown in Fig. 1, but band B is actually at least three bands labeled B_1, B_2, B_3.

infected cells (Fig. 2B, lane 2) which corresponded to the [α-^{32}P]GTP-labeled band C in Fig. 2A, lane 2. The monoclonal antibody identified the labeled B and C bands of HSV-2(G)-infected cells and band C of HSV-1(F)-infected cell as containing ICP27. We conclude that ICP27 was labeled by [α-^{32}P]GTP under the same reaction conditions as those which resulted in the labeling of ICP4.

ICP0 and ICP22 are labeled by [α-^{32}P]GTP in the presence and absence of GDP and okadaic acid. The observation that ICP27 is labeled by [α-^{32}P]GTP under the same conditions as those required to label ICP4 raised the question of whether the two remaining known α regulatory proteins, i.e., ICP0 and ICP22, were also labeled by [α-^{32}P]GTP under the same conditions. To determine whether ICP22 was one of the labeled proteins shown in Fig. 1, replicate cultures of HeLa cells were infected with HSV-1(F) or R325 and maintained at the permissive (37°C) or nonpermissive (39.5°C) temperature as described in Materials and Methods. The recombinant virus R325 was derived from HSV-1(F) and contains a genetically engineered 821-bp deletion in the coding domain of the α22 gene (43, 55, 56). As a consequence of the deletion, cells infected with this mutant express a truncated polypeptide which accumulates at the nonpermissive temperature.

Isolated nuclei were labeled in reaction mixtures containing [α-^{32}P]GTP plus either (i) okadaic acid and unlabeled 30 μM GTP and GDP or (ii) 30 μM unlabeled GTP only. Two sets of labeling conditions were used to determine whether GTP alone was sufficient for the labeling of the α proteins. The nuclear proteins were electrophoretically separated in a denaturing gel, transferred to nitrocellulose, probed with monoclonal antibody H1083, specific to ICP0, and polyclonal antibody R10, specific to ICP22, and subjected to autoradiography as described in Materials and Methods.

The results (Fig. 3) were as follows.

(i) The bands previously identified as ICP4, ICP27, and band A were labeled with [α-^{32}P]GTP with and without okadaic acid or GDP (Fig. 3B and D) but only at the permissive temperature (compare lanes 2 and 3 with lanes 5 and 6).

(ii) Bands corresponding in electrophoretic mobility to band A reacted with monoclonal antibody to H1083, specific to ICP0 (Fig. 3A and C).

(iii) The significant findings from Fig. 3A and C were that the polyclonal rabbit antibody R10 reacted with several bands from lysates of HSV-1(F)-infected cells. These bands comigrated with proteins labeled with [α-^{32}P]GTP, but this region of the gel also contained other protein bands similarly labeled with [α-^{32}P]GTP which are not ICP22 (Fig. 3B and D). The only labeled bands clearly identifiable as ICP22 were the ones marked with an arrow and which were distinctly labeled at 39.5°C. Concordance with the hypothesis that ICP22 was more highly labeled than either ICP4, ICP0, or ICP27 at 39.5°C emerged from the observation that cells infected with R325 exhibited a rapidly migrating band which reacted with the polyclonal antibody against ICP22. As expected, the band reactive with the serum was more prominent at 39.5°C than at 37°C. Surprisingly, this band was

also labeled with [α-^{32}P]GTP more extensively at 39.5°C than at the permissive temperature.

We conclude from these studies the following. (i) Both ICP22 and ICP0 are also labeled by [α-^{32}P]GTP with the same mixture and under the same conditions as those which resulted in labeling of ICP4 and ICP27. In the case of ICP22, only the fast-migrating truncated form of the protein could be clearly and unambiguously identified as labeled with [α-^{32}P]GTP. Since ICP0, ICP4, ICP22, and ICP27 are labeled in reactions in which only GTP is present, these conditions were used in all experiments described below. (ii) The labeling of ICP22 differs from that of ICP4, ICP27, and ICP0. Whereas ICP4, ICP27, and ICP0 accumulate at both permissive and nonpermissive temperatures, [α-^{32}P]GTP-labeled polypeptides were detected only, or in the case of ICP27, predominantly, at the temperature which led to a productive infection. In contrast, ICP22 was labeled with [α-^{32}P]GTP at both permissive and nonpermissive temperatures.

Labeling of HSV-1(F)-infected cell proteins in nuclei with [2-^{3}H]ATP. The results in Fig. 1 to 3 show that four of the HSV-1(F) α proteins are labeled with [α-^{32}P]GTP in the nuclei of infected cells. To determine whether the labeled proteins are nucleotidylylated, nuclei isolated from mock-, HSV-1(F)-, and HSV-2(G)-infected HeLa cells were labeled with either [α-^{32}P]ATP or [2-^{3}H]ATP, which contains the tritium label in the purine ring. The choice of the labeled compound used in this study was based on availability; [2-^{3}H]ATP was the only compound available at the required specific activity with the label at a specific site in the purine ring. The labeling conditions were done in the presence of excess ATP, and then nuclear proteins were extracted, electrophoretically separated in a denaturing gel, electrically transferred to nitrocellulose, and fluorographed for 4 weeks as described in Materials and Methods.

The results (Fig. 4) were as follows.

(i) The pattern of labeling obtained with [α-^{32}P]ATP was for the most part similar to that obtained with [α-^{32}P]GTP (Fig. 1). The increase in the number of bands shown in Fig. 4 may be due to differences in the amounts of protein loaded on the gels.

(ii) Autoradiographic images most prominent at the highest concentrations of electrophoretically separated proteins (lanes 7 to 9) did show [2-^{3}H]ATP-labeled bands corresponding to the positions of ICP4, ICP0, ICP22, and ICP27. In some instances, the [2-^{3}H]ATP bands were broader (e.g., ICP27) or migrated slightly more slowly than the [α-^{32}P]ATP-labeled band. Immunostaining of the blot with antibodies H640 and R10 confirmed the locations of ICP4 and ICP22, respectively (data not shown).

(iii) In contrast to the α proteins, the host protein band H1 labeled much more intensely with [2-^{3}H]ATP than with [α-^{32}P]ATP. We should note that this result was not unexpected, inasmuch as (a) the reaction conditions were optimized for labeling with [α-^{32}P]ATP or [α-^{32}P]GTP and not with [2-^{3}H]ATP and (b) under the optimal conditions for labeling α proteins with [α-^{32}P]GTP, the host protein labeled poorly (Fig. 1).

(iv) Of the two host bands, H2 and H3, labeled with [α-^{32}P]ATP, only the fastest-migrating band (H3) was labeled with [2-^{3}H]ATP. Some virus-specific bands, e.g., those marked by the vertical line, were strongly labeled by [α-^{32}P]ATP but not by [2-^{3}H]ATP. Other bands labeled intensively with [α-^{32}P]ATP (e.g., D and E) were poorly labeled with [2-^{3}H]ATP.

We conclude from this series of experiments the following.

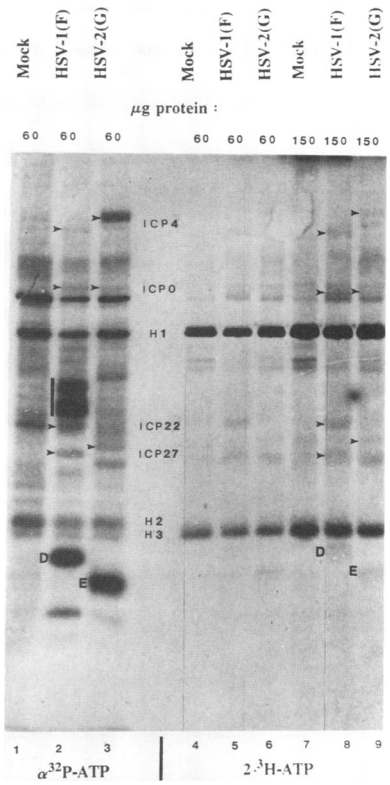

FIG. 4. Autoradiographic images of nuclear proteins labeled with [α-^{32}P]ATP and [2-^{3}H]ATP. Nuclear proteins labeled in the nuclei of mock-, HSV-1(F)-, and HSV-2(G)-infected HeLa cells were separated in a 9.5% denaturing gel, transferred to nitrocellulose, and fluorographed as described in Materials and Methods. The experiments in lanes 1 to 3 were identical to those in lanes 4 to 6 expect that two times the amount of protein was added onto the gel in the latter lanes. The ICP4, ICP0, ICP22, and ICP27 proteins are identified with arrowheads; protein D from HSV-1(F) and protein E from HSV-2(G) are identical to those shown in Fig. 1. H1, H2, and H3 refer to unknown host proteins. Proteins indicated by the bar were not observed in Fig. 1.

(i) In the instances in which ICPs were labeled with [α-^{32}P]ATP and not with [2-^{3}H]ATP, either the labeling conditions for [2-^{3}H]ATP labeling were not optimal or the labeling with [α-^{32}P]ATP resulted from a transfer of the α phosphate without the purine, and therefore the nature of the bond between these proteins and the phosphate-labeled moiety of [α-^{32}P]ATP remains unknown.

(ii) In the instances in which the proteins were labeled by both [2-^{3}H]ATP and [α-^{32}P]ATP, as was the case with ICP4, ICP0, ICP22, and ICP27, at least a portion of the [α-^{32}P]ATP label was due to adenylylation of the protein. The HSV-1(F) band D and the HSV-2(G) band E are of particular interest since they represent non-α proteins which are adenylylated. The origins of these viral specific bands are unknown, and studies designed to map their genes are currently under way.

Labeling of α proteins with [α-^{32}P]GTP differs from labeling with [γ-^{32}P]GTP. The results in Fig. 4 suggest that nucleotidylylation represents only a fraction of the label transferred to ICPs from α-^{32}P-labeled nucleoside 5′-triphosphates in isolated nuclei. The purpose of this series of experiments was to determine whether the labeling of infected cell proteins with [α-^{32}P]GTP differs from that observed with [γ-^{32}P]GTP since commonly, protein phospho-

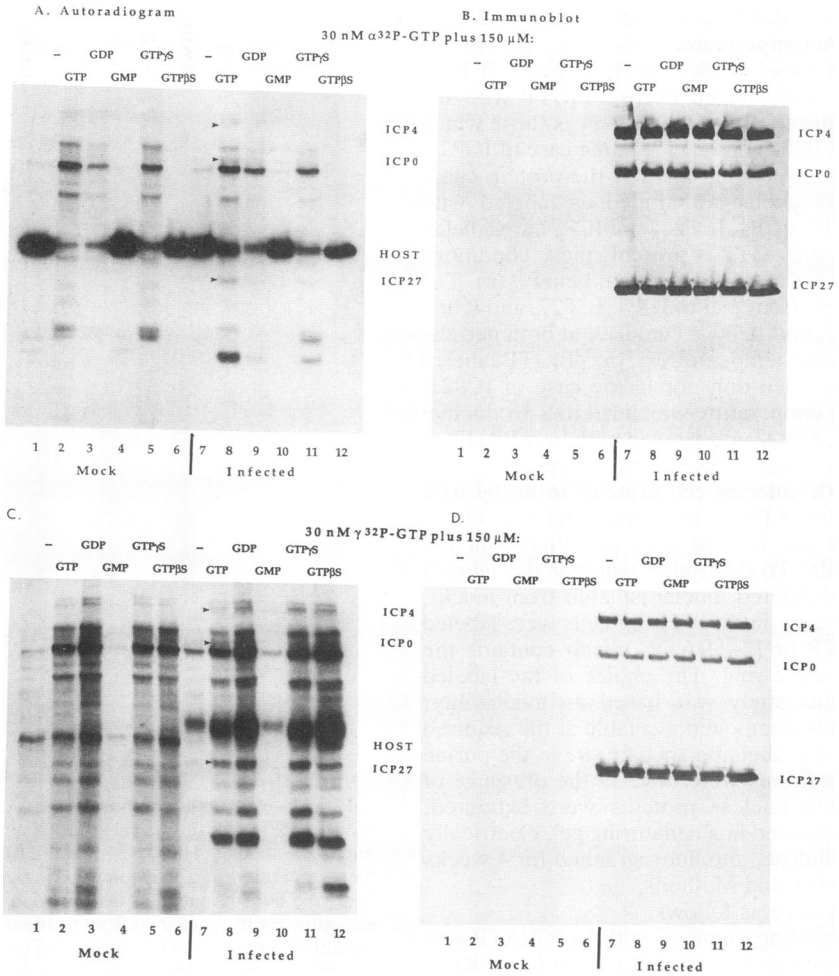

FIG. 5. Autoradiographic images (A and C) and immune reactivities (B and D) of nuclear proteins labeled with [α-^{32}P]GTP and [γ-^{32}P]GTP. Nuclear proteins from mock- and HSV-1(F)-infected HeLa cells were separated in a 9.5% denaturing gel, transferred to nitrocellulose, and probed with antibodies H640, H1083, and H1113 as described in Materials and Methods. ICP4, ICP0, and ICP27 are indicated by arrowheads. HOST refers to the major host protein labeled with [α-^{32}P]GTP in the absence of excess GTP, and the minus sign refers to only radiolabeled nucleotides present in the reactions.

rylation results from the transfer of a γ phosphate from a purine nucleotide, usually ATP, to an acceptor protein.

Nuclei from uninfected and HSV-1(F)-infected HeLa cells were isolated as described in Materials and Methods and reacted with either 30 nM [α-^{32}P]GTP or [γ-^{32}P]GTP alone or in the presence of 150 μM GTP, GDP, GMP, GTPγS, or GTPβS. Nuclear proteins were extracted, separated in a denaturing gel, electrically transferred to nitrocellulose, probed with antibodies H640, H1083, and H1113, and then subjected to autoradiography.

The results (Fig. 5) were the following.

(i) ICP4, ICP0, and ICP27 were labeled with both [α-^{32}P]GTP and [γ-^{32}P]GTP, and the labeling in both cases was stimulated by excess GTP (compare lanes 7 and 8 in Fig. 5A and C). However, labeling with [α-^{32}P]GTP was optimal in reactions containing excess GTP or GTPγS, while [γ-^{32}P]GTP labeling was best with excess GDP or GDPβS. For both sources of label, excess GMP inhibited labeling with both [α-^{32}P]GTP and [γ-^{32}P]GTP, whereas excess GTPβS inhibited [α-^{32}P]GTP labeling.

(ii) The nature of the labeled proteins differed depending on the source of the label. For example, in the absence of excess GTP, a host protein is the major labeled protein with use of [α-^{32}P]GTP (Fig. 5A, lane 7), while under similar conditions with [γ-^{32}P]GTP, a viral protein is the major labeled species (Fig. 5C, lane 7). Also, this viral protein, which is highly labeled with [γ-^{32}P]GTP, is not labeled with [α-^{32}P]GTP (lanes 7 to 12).

From these results, we conclude that the labeling of viral proteins with [α-^{32}P]GTP does not result from the presence of [γ-^{32}P]GTP in the reactions, which could arise from either de novo synthesis in nuclei or contamination of the commercial stock, inasmuch as the requirements for optimal labeling and the distributions of the labeled proteins with each source are not the same. That the α proteins are also labeled with [γ-^{32}P]GTP is not surprising, since it is known that these proteins are phosphoproteins (see the introduction).

The labeling of α proteins by [α-^{32}P]GTP requires a viral gene expressed later in infection. The results of the experiments done at the nonpermissive temperature and shown in Fig. 3 indicated that the labeling of ICP4, ICP0, and ICP27 with [α-^{32}P]GTP required a function expressed later in infection. To verify this conclusion, three series of experiments were done. In the first series, we examined the

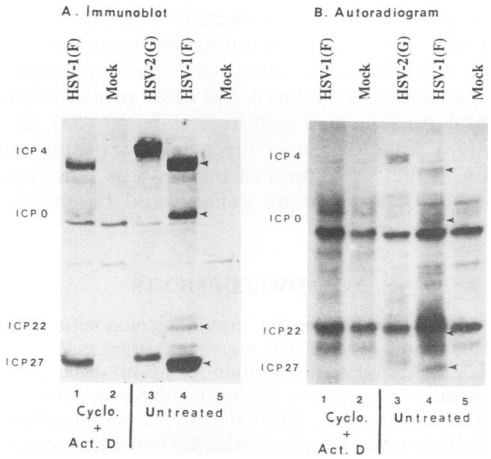

FIG. 6. Immune reactivities (A) and autoradiographic images (B) of nuclear proteins labeled with [α-^{32}P]GTP. Nuclear proteins labeled in the nuclei of mock-, HSV-1(F)-, and HSV-2(G)-infected HeLa cells were separated in a 9.5% denaturing gel, transferred to nitrocellulose, and probed with antibodies H640, H1083, R10, and H1113 as described in Materials and Methods. ICP4, ICP0, ICP22, and ICP27 proteins are indicated by arrowheads. Cyclo. + Act. D, cells were incubated and infected in the presence of cycloheximide and actinomycin D as described in Materials and Methods; Untreated, infections in the absence of drugs.

labeling of ICPs as a function of time after infection with HSV-1(F). Nuclei were labeled with [α-^{32}P]GTP, as described in Materials and Methods, at time points between 0 and 20 h postinfection to determine when the labeling reaction was first observed. The progress of the infection was monitored by immunostaining of the ICP4 protein with antibody H640 as described in Materials and Methods. The results (data not shown) were as follows. (i) As expected (21–23), the ICP4 protein was first observed at 2 h postinfection; its accumulation peaked between 2 to 5 h and remained constant until the end of the experiment at 20 h. (ii) The autoradiograms of the electrophoretically labeled proteins were consistent with previous results (Fig. 3), inasmuch as optimal labeling of α proteins, i.e., ICP4, ICP0, and ICP27, occurred between 5 and 8 h postinfection, while optimal labeling of proteins D and E (Fig. 1) with [α-^{32}P]GTP occurred between 8 and 10 h postinfection. We conclude that the function required for the labeling of the α proteins with [α-^{32}P]GTP is present at 5 h postinfection.

In the second series, HeLa cells were either mock or HSV-1(F) infected and maintained for 6 h in the presence of cycloheximide, an inhibitor of protein synthesis. At that time, medium containing the drug was replaced with fresh medium containing both cycloheximide and actinomycin D. After 1 h of incubation, the cycloheximide was removed and the cells were incubated for an additional 14 h in the presence of actinomycin D alone as described in Materials and Methods. Among the controls used were uninfected cells and infected untreated cells. Analyses of the proteins extracted from isolated nuclei and labeled with [α-^{32}P]GTP verified the absence of labeling of ICP4, ICP0, and ICP27 when the virus was precluded from expressing β and γ genes by actinomycin D (Fig. 6). Labeling of the α proteins was never observed under these conditions even after longer autoradiographic exposures of the gel or when larger amounts of protein were loaded on the gel (data not shown). On the basis of these results, we conclude that a viral gene product made later in infection is required for the labeling of the α proteins with [α-^{32}P]GTP.

To determine whether the modifying activity requires the expression of a viral γ_2 gene, in the third series of experiments, mock-infected HeLa cells or cells infected with either HSV-1(F) or HSV-2(G) were incubated in medium containing PAA, a specific inhibitor of viral DNA synthesis (24, 26). At the end of the incubation period, nuclei were isolated and labeled with [α-^{32}P]GTP plus excess GTP. Nuclear proteins were extracted, electrophoretically separated in an 11% denaturing gel, electrically transferred to nitrocellulose, and stained with antibody M28 as described in Materials and Methods. Antibody M28 is specific for the U_S11 protein, a product of a γ_2 gene (26, 53). The results (data not shown) were as follows: (i) infections in the presence of PAA precluded γ_2 gene expression, inasmuch as the U_S11 protein was not synthesized in the presence of PAA; and (ii) γ_2 gene expression was not required for the labeling of ICPs in nuclei with [α-^{32}P]GTP. Therefore, we conclude that the labeling of α proteins with [α-^{32}P]GTP requires either a β or γ_1 gene function.

DISCUSSION

The significant features of the results presented in this report are as follows.

(i) We have demonstrated the transfer of label from [α-^{32}P]GTP or [α-^{32}P]ATP to three sets of proteins in isolated nuclei of infected cells.

The first set consists of the HSV α proteins, i.e., ICP4, ICP0, ICP22, and ICP27 of HSV-1(F) and ICP4, ICP0, and ICP27 of HSV-2(G). ICP22 was identified on the basis of the observation that both the wild-type polypeptide and the product of the truncated gene specified by the recombinant R325 are labeled with [α-^{32}P]GTP but have vastly different electrophoretic mobilities. All other polypeptides were identified on the basis of their reactivity with monoclonal antibodies and the previously demonstrated type-specific differences in the electrophoretic mobilities of corresponding HSV-1 and HSV-2 proteins (1, 2, 25, 40). We should stress that the reason why we have not identified the ICP22 of HSV-2(G) among the labeled proteins is unclear. Although the HSV-1 and HSV-2 are closely related (27, 36, 46, 52, 59), previous studies have demonstrated differences in electrophoretic mobilities which in at least some instances have been shown to be related to differences in amino acid sequence. Whether ICP22 of HSV-2(G) does not accept either [α-^{32}P]GTP or [α-^{32}P]ATP or whether the protein does not accumulate to detectable levels late in infection is not clear at present time.

The second set of proteins [e.g., bands D and E of HSV-1(F) and HSV-1(G), respectively; Fig. 1] labeled with either [α-^{32}P]GTP or [α-^{32}P]ATP in nuclei of cells infected with HSV-1(F) or with HSV-2(G) also appear to be virus specific. Identification of the genes which encode these ICPs requires additional mapping studies currently under way. These proteins, however, are either β or γ proteins, inasmuch as they are made later in infection.

Lastly, the third set comprises host proteins. It is noteworthy, as stated in Results, that the requirements for maximal labeling of the host proteins appears to be different from that of the viral proteins.

(ii) A portion of the ^{32}P label transferred to the proteins from [α-^{32}P]ATP or [α-^{32}P]GTP appears to be due to nucleotidylylation. This conclusion is based on the observation that the α proteins and at least one other HSV-1 protein are

labeled in nuclei with [2-^{3}H]ATP, in which the tritium atom is in the purine ring.

The observation that several viral proteins were labeled with [α-^{32}P]ATP but no labeling of the same proteins occurred with [2-^{3}H]ATP raises several possibilities, i.e., (i) contamination of the [α-^{32}P]ATP with [γ-^{32}P]ATP, (ii) transfer of the α phosphate to a γ position, and (c) adenylylation followed by the removal of the purine, among other possibilities. The available data are not sufficient to identify the nature and origin of the phosphate transferred from [α-^{32}P]ATP or [α-^{32}P]GTP in the absence of the purine ring. It is noteworthy that Hilz et al. (20) recently described phosphoadenylylation of eukaryotic proteins. For reason described below, the contamination of the [α-^{32}P]ATP with [γ-^{32}P]ATP cannot account for the observations detailed in this report.

(iv) The labeling of the α proteins with [α-^{32}P]GTP and [γ-^{32}P]GTP differs, suggesting the mechanisms involved in the labeling reactions may be unique. The hypothesis that α-phosphate-dependent labeling is distinct from γ-phosphate-dependent labeling is supported by the observations that, when one compares [α-^{32}P]GTP and [γ-^{32}P]GTP, (i) the patterns of labeling of the α proteins differs and (ii) the requirements for optimal labeling of the α proteins differ according to the type of unlabeled nucleoside 5′-triphosphate present. The labeling of proteins with [γ-^{32}P]GTP is of interest since, with few exceptions, most protein kinases described to date prefer ATP over GTP and are not localized to the nucleus. The most notable exception is casein kinase II, which utilizes GTP and ATP equally well and was recently shown to be a nuclear kinase (28). In vitro, casein kinase II phosphorylates a variety of nuclear factors, the adenovirus E1A protein (8), and the human papillomavirus 16 E7 protein (17). One of its functions appears to be the attenuation of the AP-1 activity in vivo (31). Whether casein kinase II is involved in the transfer of phosphate from [γ-^{32}P]GTP to the α proteins remains to be determined.

(v) The guanylylation and adenylylation of the α proteins requires viral functions expressed by β or γ_1 gene products. The possibility that the labeling of α proteins with [α-^{32}P]ATP or [α-^{32}P]GTP is the sole function of a cellular protein is excluded by the observation that the α proteins were not labeled in cells in which viral gene expression was restricted to α genes only.

(vi) A most intriguing question is the role of the nucleotidylylation in the function of the α proteins. Recent studies from this laboratory (47a, 48) show that posttranslational modifications associated with phosphorylation of regulatory proteins play a significant role in infection. Thus, ICP22 was shown to form five bands differing in electrophoretic mobility (2). In cells infected with the U_L13 kinase-minus mutants, only the two most rapidly migrating forms appear to be phosphorylated and accumulate in significant amounts (48). The phenotype of the U_L13^- virus appears to be very similar to that of the R325 virus in which a large portion of the α22 gene had been deleted; both viruses replicate well in continuous cell lines derived from human and nonhuman primates but fail to replicate well or express late protein in rodent cell lines (47a). Preliminary studies indicate that the guanylylation of the ICP22 proteins is not dependent on the HSV-1 U_L13 kinase, inasmuch as α proteins are labeled with [α-^{32}P]GTP in cells infected with the U_L13 null mutant virus (6a).

That protein nucleotidylylation, especially adenylylation, can act as a regulatory function was shown for the glutamine synthase cascade of *Escherichia coli* in classic experiments by Stadtman and others (reviewed in reference 61). Several proteins of viral origin, including picornavirus, reovirus, rotavirus, hepadnavirus, adenovirus, and poxvirus, were shown to be nucleotidylylated, but these proteins function in either viral nucleic acid synthesis (55, 63, 65) or mRNA capping (58). Elucidation of the role of α protein nucleotidylylation in the replication of herpesvirus will require the development of appropriate genetic and biochemical systems.

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