Characterization of a Nerve Growth Factor-Inducible Cellular Activity That Enhances Herpes Simplex Virus Type 1 Gene Expression and Replication of an ICP0 Null Mutant in Cells of Neural Lineage

ROBERT JORDAN, JOSH PEPE, AND PRISCILLA A. SCHAFFER*

Division of Molecular Genetics, Dana-Farber Cancer Institute, and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

Received 23 December 1997/Accepted 20 March 1998

Herpes simplex virus type 1 (HSV-1) ICP0 is required for efficient viral gene expression during lytic infection, especially at low multiplicities. A series of cellular activities that can substitute for ICP0 has been identified, suggesting that when the activity of ICP0 is limiting, these activities can substitute for ICP0 to activate viral gene expression. The cellular activities may be especially important during reactivation of HSV from neuronal latency when viral gene expression is initiated in the absence of prior viral protein synthesis. Consistent with this hypothesis, we have identified an inducible activity in cells of neural lineage (PC12) that can complement the low-multiplicity growth phenotype of an ICP0 null mutant, n212. Pretreatment of PC12 cells with nerve growth factor (NGF) or fibroblast growth factor (FGF) prior to infection produced a 10- to 20-fold increase in the 24-h yield of n212 but only a 2- to 4-fold increase in the yield of wild-type virus relative to mock treatment. Slot blot analysis of nuclear DNA isolated from infected cells treated or mock treated with NGF indicated that NGF treatment does not significantly affect viral entry. The NGF-induced activity in PC12 cells was expressed transiently, with peak complementing activity observed when cells were treated with NGF 12 h prior to infection. Addition of NGF 3 h after infection had little effect on virus yield. The NGF-induced cellular activity was inhibited by pretreatment of PC12 cells with kinase inhibitors that have high specificity for kinases involved in NGF/FGF-dependent signal transduction. RNase protection assays demonstrated that the NGF-inducible PC12 cell activity, like that of ICP0, functions to increase the level of viral mRNA during low-multiplicity infection. These results suggest that activation of viral transcription by ICP0 and transcriptional activation of cellular genes by NGF and FGF utilize common signal transduction pathways in PC12 cells.
10 (ND10). Colocalization of ICP0 with ND10 antigens correlates with their redistribution in the infected cell (48, 49). In addition, ICP0 coimmunoprecipitates with a 135-kDa protein homologous to members of the ubiquitin-specific protease family of proteins (22, 52). Redistribution of ND10 antigens and coimmunoprecipitation with the 135-kDa protein requires intact transcriptional activating domains of ICP0, suggesting that these phenomena are functionally related (48, 51). ICP0 also destabilizes the catalytic subunit of a DNA-dependent protein kinase (DNA-PK) following viral infection (41). Thus, ICP0's broad transactivating activity likely involves interactions with cellular proteins.

In studies of ICP0 null mutant viruses, several cell cycle-regulated and cell-type-specific cellular activities that are able to substitute for the transactivating activity of ICP0 have been identified (8, 84). Vero cells growth arrested in G0/G1 by isolation deprivation express an activity after release from growth arrest that enhances the plating efficiency of an ICP0 null mutant virus (8). Similarly, Vero cells and cells of neural origin express an activity after release from growth arrest that activates HSV-1 gene expression in transient expression assays (57). Finally, U2OS cells, an osteosarcoma cell line, constitutively express high levels of an activity that stimulates the growth of an ICP0 null mutant virus (84). These observations demonstrate that cellular functions can substitute for the transactivating activity of ICP0.

Initiation of viral gene expression at the onset of reactivation occurs in the absence of ICP0 and other viral transcriptional activators. Since ICP0 is an important activator of viral gene expression, the existence of cellular activities able to substitute for ICP0 suggests a possible mechanism by which viral gene expression is activated during reactivation. In this study, we describe an activity in cells of neural lineage (PC12) induced by treatment with two physiologically relevant growth factors, nerve growth factor (NGF) and fibroblast growth factor (FGF), that enhances gene expression and replication of an ICP0 null mutant, n212. The ICP0-like, n212-complementing activity is expressed transiently and can be blocked by inhibitors of NGF-dependent signal transduction. Moreover, like ICP0, the cellular complementing activity functions at the level of mRNA accumulation. We suggest that the NGF/FGF-induced n212-complementing activity may be responsible for the initiation of viral gene expression during reactivation from neuronal latency when no ICP0 is present.

MATERIALS AND METHODS

Cells and viruses. PC12 cells were the generous gift of John Wagner (Cornell University Medical College, New York, N.Y.) and were cultured in Dulbecco's modification of Eagle's minimal essential medium supplemented with 10% fetal bovine serum and 5% horse serum as described previously (26). MM17-26 cells were kindly provided by Geoffrey Cooper (Dana-Farber Cancer Institute, Boston, Mass.) and were cultured as described for PC12 cells. Vero cells, L7 cells, which contain a stably integrated copy of ICP0, and the osteosarcoma line U2OS were cultured as previously described (63, 65, 84).

Wild-type HSV-1, strain KOS, and the ICP0 nonsense mutant, n212, derived from KOS were propagated as described previously (7, 67). Viral titers were measured by standard plaque assay on Vero cell monolayers for strain KOS and from KOS were propagated as described previously (53, 56). The numbers of viral genomes and the number of ICP4-expressing cells active genomes was determined by quantitating the number of ICP4-expressing virus stocks by slot blot analysis of viral DNA, and the number of biologically active genomes was determined by standard plaque assays on Vero (KOS) or L7 or U2OS (n212) cell monolayers as described previously (37).

RESULTS

NGF and FGF stimulate replication of the ICP0 null mutant, n212, in PC12 cells. Reactivation of latent HSV-1 correlates with changes in levels of stress-induced growth factors and neurotrophins (32, 79). NGF, FGF, and epidermal growth factor (EGF) are among the many growth factors induced by stress that have a wide range of biological effects in vivo and in cell culture. In PC12 cells, physiological concentrations of NGF and FGF activate cellular signaling cascades leading to morphological and biochemical differentiation (28). Differentiated PC12 cells resemble sympathetic neurons in that they decrease electrically excitable activity, and express a number of neuron-specific genes (27, 28). In contrast, physiological concentrations of EGF stimulate mitogenesis in PC12 cells, even though many of the same signaling molecules are activated by all three factors (47). To determine the effects of growth factor-induced cellular activities on viral replication,
PC12 cells were treated with NGF, FGF, or EGF or were mock treated for 3 h prior to infection with 0.01 PFU of wild-type HSV-1 strain KOS or the ICP0 null mutant n212 per cell. At 3 and 24 hpi, virus yields were measured by plaque assay. Treatment of PC12 cells with NGF increased the 24-h yields of n212 and KOS; 14- and 3-fold, respectively (Fig. 1). Similarly, FGF stimulated n212 and KOS replication nine- and fourfold, respectively. These treatments had little effect on the levels of infectious virus at 3 hpi (data not shown). EGF had only minor but reproducible effects on 24-h yields of n212 (1.5-fold) and KOS (2.3-fold). The results of these tests indicate that NGF and FGF, but not EGF, induce activities in PC12 cells that complement n212 and, to a lesser extent, enhance replication of KOS.

One of the most dramatic morphological changes associated with differentiation of PC12 cells is the formation of neurite-like processes. Not only is neurite formation induced by NGF and FGF, but neurite formation can also be induced by combined treatment with EGF and KCl (31, 46). EGF activates cellular signaling pathways, while KCl activates voltage-sensitive calcium channels, leading to elevated intracellular calcium levels (3). The combined effects of stimulating EGF-dependent signal transduction, while increasing intracellular calcium levels leads to neurite formation (31, 46). Notably, the extent of neurite formation following EGF and KCl treatment is significantly less than that of NGF or FGF treatment (31, 46). To test whether neurite formation correlates with induction of the n212-complementing activity, PC12 cells were treated with EGF or EGF in combination with 50 mM KCl for 3 h prior to infection with 0.01 PFU of KOS or n212 per ml. Again, virus yields were measured at 24 hpi. In these tests, KCl alone and the combination of EGF and KCl (which produced moderate neurite outgrowth) had only modest effects (<2-fold) on replication of n212 or KOS (Fig. 1). Taken together, these results suggest that the n212-complementing activity does not correlate with neurite formation.

To examine more carefully the effects of NGF on growth of KOS and n212, PC12 cells were treated with NGF or mock treated 3 h prior to infection with 0.02 or 5.0 PFU of either virus per cell. Infected cultures were harvested at 3, 6, 9, 12, 18, and 24 hpi, and virus titers were measured by plaque assay. As shown in Fig. 2, at a low multiplicity of infection (0.02 PFU/cell), NGF treatment caused an increase in n212 replication from 6 to 24 hpi compared to mock treatment. In contrast, KOS replication increased only slightly in response to NGF relative to mock-treated samples at all times postinfection. At a high multiplicity of infection (5.0 PFU/cell), NGF treatment did not affect replication of n212 or KOS. These results suggest that NGF treatment of PC12 cells stimulates replication of n212 after low-multiplicity infection and that ICP0 and high-multiplicity infection are dominant with regard to the NGF-dependent, n212-complementing activity.

**NGF treatment does not affect viral entry.** It is conceivable that NGF treatment of PC12 cells may affect viral entry, even though the dramatic morphological changes associated with
NGF-dependent differentiation require 24 h or more to develop. To test whether NGF treatment affects viral infectivity, PC12 cells were treated with NGF 3 h prior to infection with KOS or n212. At 3 hpi, nuclear DNA was isolated and immobilized on a nylon membrane by slot blot hybridization. The membrane was probed with radiolabeled KOS DNA. The blot was then stripped and reprobed with a radiolabeled RNA probe specific for the cellular gene, GAPDH. The results of these tests show that NGF treatment had no significant effect on the amount of nuclear KOS or n212 DNA recovered from PC12 cell nuclei (Fig. 3). These results indicate that ICP0 and NGF treatment do not affect viral entry or transport of viral DNA to the nucleus. Similar results were obtained when entry was measured by indirect immunofluorescence (i.e., by counting the number of ICP4-expressing cells in NGF-treated or mock-treated cells infected with KOS or n212 [data not shown]).

The NGF-induced n212-complementing activity requires activation of multiple NGF-dependent signal transduction pathways. (i) Inhibitor studies. NGF activates multiple signal transduction pathways, including ras-dependent signaling pathways, whose downstream endpoint is the expression of differentiation-specific genes (16). The PC12-derived cell line MM17-26 constitutively expresses a dominant negative ras allele that blocks downstream functions of ras (69). Consequently, MM17-26 cells fail to differentiate in response to NGF or FGF treatment. To test whether ras is required for the NGF- or FGF-dependent stimulation of n212 replication, MM17-26 cells or PC12 cells were pretreated with NGF or FGF 3 h prior to infection with 0.01 PFU of n212 per cell. At 24 hpi, n212 yields were measured and compared to those on NGF-treated PC12 cells. As shown in Fig. 4, the replication efficiency of n212 in NGF- and FGF-treated MM17-26 cells was only 18% of that observed in NGF- and FGF-treated PC12 cells. These results indicate that the NGF/ras-dependent stimulation of n212 replication is partially ras dependent.

NGF-dependent differentiation requires the activities of multiple serine/threonine kinases which function sequentially to regulate downstream differentiation-specific activities (16, 69). Serine/threonine kinase inhibitors that block NGF-dependent differentiation have been used to identify specific kinases involved in the differentiation process (56, 76). To test whether selected kinase inhibitors block the NGF-dependent complementation of n212 replication, PC12 cells were treated for 30 min with K252a (a broad-spectrum serine/threonine kinase inhibitor), K5720 (a protein kinase A [PKA]-specific inhibitor), calphostin C (a PKC-specific inhibitor), or PD98059 (an inhibitor specific for mitogen-activated protein kinase). After the 30-min incubation, NGF was added to cultures containing inhibitors for 3 h prior to infection. The treated cultures were infected with 0.01 PFU of n212 per cell in the presence and absence of NGF and inhibitors. Virus yields were measured at 24 hpi. In control experiments, treatment of PC12 cells with each inhibitor blocked NGF-dependent differentiation (data not shown). The results of these tests show that the broad-spectrum kinase inhibitor K252a, which blocks multiple NGF-dependent signaling pathways, had the greatest inhibitory effect on virus yield, almost completely blocking the ability of NGF to stimulate replication of n212 (Fig. 4). Calphostin C blocked NGF-dependent replication of n212 by 44%, whereas PD98059 reduced the NGF-dependent stimulation of n212 replication by ~50%. Treatment with K5720 had very little effect on the ability of NGF to stimulate replication of n212 (Fig. 4). Taken together, these results show that kinase inhibitors able to block multiple NGF-dependent signaling pathways were able to inhibit NGF-dependent replication of n212 more effectively than inhibitors that blocked fewer NGF-dependent signaling pathways, indicating that multiple NGF-dependent signaling pathways must be activated to complement n212 replication. Neither the inhibitors used in this study nor the presence of the dominant negative ras allele in MM17-26 cells had a significant effect (−2-fold) on replication of KOS or n212 in the absence of NGF (data not shown). These observations suggest either that HSV-1 does not require these enzymes for productive infection or that multiple redundant signaling pathways are used by the virus.

(ii) Activator studies. Many of the intracellular signaling pathways activated by NGF can be stimulated indirectly by activators of cellular protein kinases or second messenger analogs. To test whether direct stimulation of protein kinases or

![Fig. 3. NGF does not affect viral entry. PC12 cells were mock treated or treated with NGF (100 ng/ml) for 3 h prior to infection with KOS or n212. Nuclear DNA was harvested prior to the onset of viral DNA replication at 3 hpi and applied to a nylon membrane by slot blot hybridization (inf.). Cesium chloride-purified KOS DNA was applied to the membrane as indicated (std.). The blot was probed for viral DNA by using 32P-labeled nick-translated KOS DNA. The blot was stripped and reprobed for cellular DNA by using a 32P-labeled antisense RNA probe specific for the cellular gene, GAPDH. The image was visualized by PhosphorImager analysis. The range values for the image display were set at 0 to 791 counts for the KOS probe and 0 to 258 counts for the GAPDH probe.

![Fig. 4. Serine/threonine kinase inhibitors block NGF-dependent replication of n212. PC12 cells (106/35-mm-diameter dish) were incubated for 30 min prior to NGF addition with serine/threonine kinase inhibitors at the following concentrations: K252a, 0.25 μM; K5720, 0.5 μM; PD98059, 20 μM; and calphostin C, 0.5 μM. At 3 h posttreatment, the cultures were infected with n212 (0.01 PFU/cell) in the presence and absence of inhibitors and NGF. At 24 hpi, the cultures were harvested and virus yields were measured. The data are expressed as percentage of NGF-induced n212 replication, which was set at 100% to show all of the data in a single figure. MAPKinase, mitogen-activated kinase.
TABLE 1. Effects of PMA and second messenger analogs on replication of n212 and KOS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n212 Fold (SD)</th>
<th>KOS Fold (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>1.0 (1.3)</td>
<td>1.0 (1.2)</td>
</tr>
<tr>
<td>NGF</td>
<td>10.0 (3.1)</td>
<td>1.7 (1.2)</td>
</tr>
<tr>
<td>PMA</td>
<td>1.9 (1.2)</td>
<td>1.1 (0.8)</td>
</tr>
<tr>
<td>PMA + NGF</td>
<td>10.5 (2.1)</td>
<td>1.6 (1.4)</td>
</tr>
<tr>
<td>PMA + dbcAMP</td>
<td>1.8 (0.1)</td>
<td>0.6 (0.4)</td>
</tr>
<tr>
<td>PMA + dbcAMP + NGF</td>
<td>10.8 (7.8)</td>
<td>1.0 (0.1)</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>2.6 (2.0)</td>
<td>0.4 (0.2)</td>
</tr>
<tr>
<td>dbcAMP + NGF</td>
<td>9.4 (4.0)</td>
<td>1.1 (0.5)</td>
</tr>
<tr>
<td>Forskolin</td>
<td>1.6 (0.4)</td>
<td>1.4 (0.7)</td>
</tr>
<tr>
<td>Forskolin + NGF</td>
<td>4.3 (0.6)</td>
<td>3.3 (0.4)</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>1.0 (0.8)</td>
<td>0.4 (0.1)</td>
</tr>
<tr>
<td>Ionomycin + NGF</td>
<td>9.0 (0.1)</td>
<td>1.6 (0.3)</td>
</tr>
<tr>
<td>Ionomycin + dbcAMP</td>
<td>0.7 (0.6)</td>
<td>1.0 (0.9)</td>
</tr>
<tr>
<td>Ionomycin + dbcAMP + NGF</td>
<td>10.4 (3.9)</td>
<td>2.1 (0.2)</td>
</tr>
</tbody>
</table>

*PC12 cells were treated with compounds as indicated for 3 h prior to infection with 0.01 PFU of n212 or KOS per cell. At 24 hpi, the cultures were harvested and viral yields were measured. The data are expressed as fold increase in virus titer at 24 hpi relative to no treatment. Each value represents the average of at least three independent experiments.

second messenger pathways result in n212-complementing activity, and to test whether NGF can synergize with these activities, PC12 cells were treated alone or in combination with PMA, forskolin, dbcAMP, and ionomycin in the presence and absence of NGF for 3 h prior to infection with 0.01 PFU of KOS or n212 per cell. At 24 hpi, virus yields were measured (Table 1). PMA activates PKC and stimulates mitogenesis in many cell types, including PC12 cells (75). PMA had only minor effects on replication of both KOS and n212. PMA did not affect NGF-induced replication of n212 since PC12 cells treated with both PMA and NGF produced a 10.5-fold increase in n212 yield relative to mock-treated cultures. This increase in replication efficiency was consistent with the 12-fold increase in n212 replication observed in NGF-treated cultures in the absence of PMA (Fig. 1). The combination of PMA and dbcAMP treatment in the presence and absence of NGF also had little effect on virus replication (Table 1).

Forskolin increases intracellular cAMP levels, which in turn activate cAMP-dependent enzymes (43). In a similar manner, dbcAMP, a membrane-permeable analog of cAMP, directly activates cAMP-dependent enzymes (43). Treatment of PC12 cells with forskolin or dbcAMP stimulated n212 replication (1.6- or 2.6-fold, respectively) and KOS replication (1.4- or 0.4-fold, respectively) only slightly (Table 1). Treatment of PC12 cells with forskolin and NGF increased replication of n212 4.8-fold and that of KOS 3.3-fold. While we do not have a satisfactory explanation for the increased replication efficiency of KOS in the presence of forskolin and NGF, it is a real and reproducible effect. The results of these tests indicate that activation of cAMP-dependent kinases did not induce the n212-complementing activity.

Ionomycin, a calcium-specific ionophore, increases intracellular calcium levels, thereby activating calcium-dependent enzymes (44). Treatment of PC12 cells with ionomycin alone or in combination with dbcAMP in the presence and absence of NGF had little effect on replication of both KOS or n212 (Table 1). Dimethyl sulfoxide, which was used as the vehicle for ionomycin delivery, had little effect on replication of KOS and n212 (data not shown). Taken together, the results of these tests indicate that activators of cellular protein kinases and second messenger analogs cannot substitute for NGF- or FGF-dependent stimulation of n212 replication in PC12 cells.

The NGF-induced ICP0-complementing activity is expressed transiently. To determine the optimal time of addition and length of NGF treatment relative to the time of infection required to produce maximal expression of the NGF-induced n212-complementing activity, PC12 cells were treated with NGF 3 h prior to infection and at the time of infection, or 15 min, 1 h, or 3 h after infection with 0.01 PFU of KOS or n212 per cell. Twenty-four hours after infection, virus yields were measured by plaque assay. Addition of NGF to cultures 3 h prior to infection and at the time of infection produced six- and ninefold increases, respectively, in the 24-h yield of n212 (Fig. 5A). However, addition of NGF 15 min, 1 h, or 3 h after infection produced a progressive decrease in the 24-h yield of n212 (Fig. 5A). These treatments had only minor effects (ap-
proximately twofold) on the 24-h yield of KOS (Fig. 5A). The results of these tests suggest either that (i) the NGF-induced activity which is able to substitute for ICP0 is required within the first 3 h prior to infection and the 30 min immediately after infection or (ii) by 3 hpi, infected PC12 cells no longer respond to stimulation by NGF.

To measure the duration of the NGF-induced complementing activity, PC12 cells were treated with NGF for 144, 72, 48, 24, 12, 6, and 3 h prior to infection, at the time of infection, or 3 h after infection with 0.01 PFU of KOS or n212 per cell. Virus yields were measured 24 h later. Pretreatment of PC12 cells with NGF from 0 to 12 h prior to infection produced a 10- to nearly 20-fold increase in the yield of n212, while only minor (<2-fold) effects on the yield of KOS were observed (Fig. 5B). The NGF-induced stimulation of n212 replication was less apparent in PC12 cells treated with NGF for 48 h or longer prior to infection. Thus, treatment of PC12 cells from 48 to 144 h prior to infection had only modest (≤5-fold) effects on the replication of n212 and very little effect on the replication of KOS (Fig. 5B). Consistent with the findings presented in Fig. 5B, by 3 hpi NGF had little effect on replication of either n212 or KOS (Fig. 5B). The results of these tests indicate that the NGF-induced stimulation of n212 replication is transient and is maximal if initiated 12 h prior to infection.

NGF treatment of PC12 cells increases the steady-state levels of viral mRNA. ICP0 increases the steady-state levels of viral E and L mRNAs during low-multiplicity infection by increasing the rate of initiation of mRNA synthesis (37). To test whether the NGF-induced n212-complementing activity functions at the same level as ICP0 to increase the steady-state level of viral mRNA, PC12 cells were treated with NGF or mock treated for 12 h prior to infection with 0.1 PFU of KOS or n212 per cell. At 0, 4, 7, and 10 hpi, cytoplasmic RNA was isolated and levels of ICP4, TK, and gC mRNAs were measured by quantitative RNase protection assay. The range values for the image display were set at 0 to 2,000 (ICP4), 0 to 1,000 (TK), and 0 to 200 (gC).

FIG. 6. NGF induces viral mRNA accumulation in infected PC12 cells. PC12 cells (5 × 10⁶/60-mm-diameter dish) were mock treated or treated with NGF (100 ng/ml) for 12 h prior to infection with 0.1 PFU of KOS or n212 per cell. At 0, 4, 7, and 10 hpi, cytoplasmic RNA was isolated and levels of ICP4, TK, and gC mRNAs were measured by quantitative RNase protection assay. The range values for the image display were set at 0 to 2,000 (ICP4), 0 to 1,000 (TK), and 0 to 200 (gC).
biological consequences of this induction are not well understood. To determine whether ICP0, like the NGF-induced n212-complementing activity, is involved in the herpesvirus infection-specific induction of cellular primary response genes, PC12 cells were infected with KOS or n212 for 60, 90, or 120 min or treated with NGF for 0, 30, 60, 90 or 120 min. At the times indicated (Fig. 7) cytoplasmic RNA was isolated. The RNA was separated according to molecular weight by denaturing gel electrophoresis and transferred to a nylon membrane by Northern blotting. The membrane was probed with radiolabeled RNA probes specific for the human c-fos message and the rat GAPDH mRNA. As shown in Fig. 7, the kinetics and extent of activation of c-fos mRNA were similar in cells treated with NGF or infected with KOS or n212, suggesting that ICP0 is not involved in the herpesvirus-induced activation of cellular primary response genes. Similar results were observed when northern blots were probed with radiolabeled c-jun, junD, and krox24 probes (data not shown). Moreover, as a control, addition of media in the absence of NGF did not induce cellular primary response gene expression. The results of these tests indicate that ICP0 is not involved in the herpesvirus infection-specific induction of cellular primary response gene expression.

**DISCUSSION**

The balance between productive and latent infection is ultimately determined by the transcriptional permissivity of the infected cell. Growth factors and extracellular signals influence the transcriptional permissivity of the cell by activating intracellular signaling cascades. Viral IE proteins circumvent the need for extracellular signaling by increasing the transcriptional permissivity of the infected cell, thereby promoting viral gene expression. During reactivation, in the absence of viral regulatory proteins, activation of cellular signaling cascades likely changes the transcriptional permissivity of the infected cell and stimulates viral gene expression. In support of this hypothesis, Tal-Singer et al. have reported that viral E and L genes may be induced prior to IE gene expression during reactivation in mice, suggesting that cellular functions can substitute for viral IE genes during reactivation (70). In addition, the levels of cyclic nucleotides, which function as second messengers during signal transduction, influence the maintenance and reactivation of latent virus in mice (23, 64). Moreover, activators of PKA and PKC as well as second messenger analogs stimulate reactivation of latent HSV-1 in primary rat neurons latently infected in vitro (68). While activators of PKA and PKC induce reactivation in this system, they fail to induce the ICP0-like activity, suggesting that activation of these enzymes alone is not sufficient to complement replication of n212. Taken together, these observations suggest that intracellular signaling plays a major role in regulating viral gene expression during reactivation.

NGF and FGF are two of the many neurotropic factors whose concentrations change during the host stress response and may contribute to the signals that induce HSV reactivation (45). NGF induces expression of the LAT promoter in a rasedependent manner in PC12 cells, suggesting that during latency, LAT activity may be regulated by NGF or NGF-like extracellular signals (26). Changes in the levels of stress-induced growth factors like NGF may well lead to activation of cellular signaling pathways which in turn induce activities that stimulate viral gene expression.

We have identified and characterized an NGF/FGF-inducible cellular activity that stimulates replication of the ICP0 null mutant, n212. The NGF-induced cellular activity is partially ras-dependent and requires activation of multiple NGF-dependent signaling pathways. This activity is transiently expressed, with peak complementing activity observed within the first 12 h of NGF treatment. Like ICP0, the NGF-dependent n212-complementing activity functions to stimulate viral E and L mRNA accumulation.

The NGF-induced complementing activity is cell type specific. The NGF/FGF-induced n212-complementing activity is specific to PC12 cells. NGF treatment of a human neuroblastoma cell line, SY5Y, had little effect on viral growth (data not shown). Notably, even clonal isolates of SY5Y constitutively expressing the trkA gene, which encodes the NGF receptor, failed to induce the n212-complementing activity in response to NGF. Furthermore, treatment of Vero cells with NGF, FGF, or EGF produced little difference in virus yields at 3 and 24 hpi relative to mock treatment. Although Vero cells do not express NGF receptors, they do express FGF and EGF receptors (12, 19). Notably, FGF and EGF treatment of Vero cells stimulates mitogenesis, indicating that these receptors are biologically active (12). These data imply that NGF/FGF signaling in conjunction with some other factor(s) in PC12 cells is required for complementation of n212 and that NGF/FGF signaling alone is not sufficient for induction of the n212-complementing activity.

**Cellular signaling, phosphorylation, and transcription factor activation.** How do NGF and FGF complement n212? A well-recognized endpoint of NGF/FGF-induced signal transduction is the phosphorylation of nuclear transcription factors (58, 66, 82). Activation of transcription factors by phosphorylation is a common mechanism by which growth factors initiate changes in cellular transcription (35). NGF and FGF activate multiple serine/threonine kinase cascades including the ras/raf-1/MEK/ERK pathway and cyclin-dependent kinase activities (16, 69). Once activated, these kinases phosphorylate transcription factors such as cAMP response element binding protein and serum response factor, thereby stimulating their transcriptional activating activities (43, 58, 66). NGF also induces phosphorylation of SP1 and c-Fos and stimulates NF-kB DNA binding activity (72, 81, 83). The NGF/FGF-induced n212-complementing activity may require several of these activated kinase signaling pathways to phosphorylate and activate...
transcription factors which are then used by the virus in the absence of ICP0 to stimulate viral gene expression. The observation that serine/threonine kinase inhibitors partially block absence of ICP0 to stimulate viral gene expression. The observations that the ubiquitin-dependent stimulation of n212 replication (Fig. 4) is consistent with this hypothesis.

Like the cellular ICP0-like activity, ICP0 may indirectly regulate the phosphorylation state of viral and cellular proteins during the course of infection. ICP0 physically interacts with HuAUSP, a component of the ubiquitin-dependent proteolysis system (22). Ubiquitin-dependent proteolysis of phosphorylated transcription factors is a mechanism by which cells down regulate cellular transcription induced by extracellular signaling (11, 39, 53, 77). The interaction of ICP0 with HuAUSP may serve to modify cellular enzymes that directly phosphorylate transcription factors. Indeed, ICP0 is required to destabilize the catalytic subunit of a host DNA-PK (41). In the absence of DNA-PK, the intranuclear phosphorylation state of numerous proteins may be altered, potentially affecting the ability of these proteins to interact with DNA. Thus, ICP0 and the NGF-induced n212-complementing activity may function to stimulate transcription indirectly by altering the levels of phosphorylation of transcription factors which activate viral transcription. Consistent with this hypothesis, two-dimensional gel electrophoresis of infected-cell nuclear proteins from KOS- and n212-infected PC12 cells shows significant differences in the pattern of phosphorylation of multiple nuclear phosphoproteins (37a).

A comparison of ICP0-like cellular activities. A comparison of the known ICP0-like cellular activities is shown in Table 2. Like ICP0, both the NGF-induced activity in PC12 cells and the cell cycle-regulated activity in Vero cells stimulate viral E and L but not IE gene expression. In contrast, the activity expressed constitutively in U2OS cells and the cell cycle-regulated activity in Nb41A3 cells preferentially stimulate basal IE gene expression in transient expression assays. These differences may be due to the differential effects of the cellular activity on DNA delivered by transfection versus infection. In addition, it is unknown whether increased basal IE gene expression is sufficient to complement ICP0 null mutant replication. The U2OS cell activity also stimulates IE gene expression during infection; however, it is unknown whether this effect occurs throughout infection or only at later times postinfection.

It is conceivable that all of the activities that complement ICP0 null mutants require activation of cellular kinases and phosphorylation of transcription factors. Quiescent cells entering the cell cycle induce cell cycle-regulated kinases that phosphorylate cellular transcription factors (2, 15, 59, 62, 74). Likewise, NGF induces many of these same activities in PC12 cells (15, 81, 83). Although it is unknown whether U2OS cells express elevated levels of kinase activity, transcription factors that regulate viral IE gene expression are activated (84). Thus, in at least two instances (Vero cells entering the cell cycle from G0 and NGF treatment of PC12 cells), induction of the ICP0-like cellular activity correlates with activation of cellular kinases.

Cellular kinase activation may be only one part of the ICP0-like activity. Peak expression of the NGF-induced n212-complementing activity in PC12 cells and the cell cycle-regulated ICP0-like activity in Vero cells occurs after the initial activation of cellular primary response genes (8) (Fig. 5B). Moreover, ICP0 does not affect the kinetics or level of expression of c-fos mRNA following infection of PC12 cells (Fig. 7). These observations suggest that the cellular functions that complement ICP0 null mutants require activities expressed downstream of initial signaling events.

ICP0 activates viral gene expression during productive infection and promotes efficient reactivation from latency both in vitro and in vivo. Thus, ICP0 regulates viral gene expression during all phases of the HSV-1 life cycle. Cellular activities that functionally substitute for ICP0 and complement replication of ICP0 null viruses have been described (8, 84). The existence of these activities may provide insight into the mechanisms that regulate viral gene expression during productive infection and reactivation from latency. We have shown that cellular activities induced by NGF and FGF in a neurally derived cell line can substitute for ICP0 and stimulate viral gene expression. We suggest that these activities may be similar to the activities that regulate viral gene expression during reactivation from latency.

ACKNOWLEDGMENTS

We thank Anh Nguyen-Huynh, Lily Yeh, David Fraser, and Luis Schang for helpful discussions of this work. This research was supported by Public Health Service grants R37CA20260 and PO1NS55138-10 (P.A.S.) and F32 AI09127 (R.J.).

REFERENCES