JOURNAL OF VIROLOGY, Oct. 2001, p. 8909–8916 Vol. 75, No. 19
0022-538X/01/$04.00+0 DOI: 10.1128/JVI.75.19.8909–8916.2001

Activation of Interferon Response Factor-3 in Human Cells Infected with Herpes Simplex Virus Type 1 or Human Cytomegalovirus

CHRIS M. PRESTON,[1]* ANDREW N. HARMAN,[2] AND MARY JANE NICHOLL[1]

Medical Research Council Virology Unit, Glasgow G11 5JR, Scotland,[1] and Division of Virology, Department of Pathology, University of Cambridge, Cambridge CB2 1QP, England[2]

Received 27 April 2001/Accepted 11 June 2001

Activation of cellular interferon-stimulated genes (ISGs) after infection with herpes simplex virus type 1 (HSV-1) or human cytomegalovirus (HCMV) was investigated. The level of ISG54-specific RNA in human fetal lung (HFL) or human foreskin (BJ) fibroblasts increased substantially after infection with either virus in the presence of cycloheximide. HSV-1 particles lacking glycoprotein D or glycoprotein H failed to induce ISG54-specific RNA synthesis, demonstrating that entry of virus particles rather than binding of virions to the cell surface was required for the effect. A DNA-binding complex that recognized an interferon-responsive sequence motif was induced upon infection with HSV-1 or HCMV in the presence of cycloheximide, and the complex was shown to contain the cell proteins interferon response factor 3 (IRF-3) and CREB-binding protein. IRF-3 was modified after infection with HSV-1 or HCMV to a form of lower electrophoretic mobility, consistent with phosphorylation. De novo transcription of viral or cellular genes was not required for the activation of IRF-3, since the effect was not sensitive to inhibition by actinomycin D. Infection of HFL fibroblasts with HSV-1 under conditions in which viral replication proceeded normally resulted in severely reduced levels of the IRF-3-containing complex, defining the activation of IRF-3 as a target for viral interference with ISG induction. In BJ fibroblasts, however, significant activation of IRF-3 was detected even when the viral gene expression program progressed to later stages, demonstrating that the degree of inhibition of the response was dependent on host cell type. As a consequence of IRF-3 activation, endogenous interferon was released from BJ cells and was capable of triggering the appropriate signal transduction pathway in both infected and uninfected cells. Activation of ISG54-specific RNA synthesis was not detected after infection of human U-373MG glioblastoma cells, showing that the induction of the response by infection is cell type dependent.

Alpha/beta interferons (IFN-α/β) are important components of cellular antiviral responses. The effects of the interferons are mediated by interferon-stimulated gene (ISG) products, which include proteins with defined activities, such as 2′,5′-oligoadenylate synthetase as well as many others with unknown functions (reviewed in reference 39). Most studies on the antiviral activities of IFN-α/β have focused on the inhibition of RNA virus replication; the effects on DNA virus replication are less well understood. Herpes simplex virus type 1 (HSV-1) replication is inhibited by interferon pretreatment of cells due to a block at the level of immediate-early (IE) gene transcription and additional effects on protein synthesis that are manifested at later times in infected cells (2, 25, 30, 32). In mice, IFN-α/β are important host defenses, since attenuated HSV-1 mutants are much more virulent in animals lacking interferon receptors (21, 22).

The signaling pathway for IFN-α/β is well characterized (39) (Fig. 1). Binding of interferons to their cellular receptor results in the phosphorylation of Janus kinases (JAKs) JAK1 and Tyk2 and the consequent phosphorylation of signal transducers and activators of transcription (STATs) 1 and 2. The latter proteins are translocated to the nucleus, where they are recruited by interferon regulatory factor (IRF) 9 (IRF-9; also known as p48) to form a complex, named ISG factor 3 (ISGF3), on interferon-stimulated regulatory elements (ISREs) within ISG promoters. The acetylases p300 and CREB-binding protein (CBP) associate with STATs 1 and 2 and are probably important in mediating transcriptional activation.

Cells commonly produce interferon in response to virus infection, and significant progress has been made in understanding the cellular pathways activated upon infection with RNA viruses. Upon infection of human cells, the cellular factors NF-κB, ATF-2/c-Jun, IRF-3, and/or IRF-7 combine with high-mobility-group protein HMGI(Y) on the promoter controlling the IFN-β gene to form a stable transcription complex, the enhanceosome (41, 42). IRF-3 is a cytoplasmic protein that is phosphorylated in response to infection, resulting in translocation to the nucleus and binding to elements PRDI and PRDIII in the IFN-β promoter (4, 23, 42, 46). In addition, the activation of IRF-3 results in the induction of ISG-specific RNA synthesis through a mechanism that is independent of interferon or ISGF3, since ISREs have homology to the PRDI and PRDIII sequences found within ISG promoters. In this pathway, phosphorylated IRF-3 recruits CBP and binds to ISREs, thereby inducing ISG expression directly (Fig. 1). For RNA viruses, many lines of evidence suggest that double-stranded RNA is the trigger for the activation of IRF-3 (11, 38, 40, 44).

Typically, viruses encode products that antagonize various aspects of the interferon response in order to overcome host

* Corresponding author. Mailing address: Medical Research Council Virology Unit, Church St., Glasgow G11 5JR, Scotland. Phone: 44 141 330 3921. Fax: 44 141 337 2236. E-mail: c.preston@vir.gla.ac.uk.

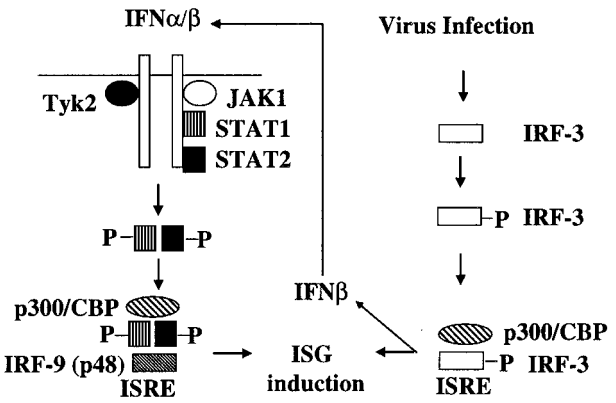

FIG. 1. Mechanisms of ISG induction. The pathway activated by IFN-α/β is depicted on the left side of the diagram, leading to formation of the complex known as ISGF3 (phosphorylated STATs 1 and 2 plus IRF-9). The route of virus activation of IRF-3 is shown on the right, leading to a complex containing phosphorylated IRF-3 plus CBP. The induction of ISGs is mediated by either complex; in addition, activated IRF-3 can induce the IFN-β gene, resulting in the production of IFN-β, which can engage the IFN-α/β receptor to activate the pathway leading to the formation of ISGF3.

defenses and facilitate virus replication (1, 13, 14, 39). In some situations, IRF-3 is the target for the virus. The influenza virus nonstructural protein NS1 blocks the activation of IRF-3 by binding and sequestering double-stranded RNA (40), whereas the human papillomavirus type 16 protein E6 abolishes IRF-3 activity by interacting with the protein (35). In a further strategy, human herpesvirus 8 encodes IRF homologs that act as decoys to compete with IRF-3 for binding to CBP (10).

Although most studies on direct induction of ISGs have focused on RNA viruses, it was shown that infection with human cytomegalovirus (HCMV) resulted in the stimulation of ISG synthesis and that an early event occurring prior to the translation of viral transcripts was responsible (47, 48). Treatment of cells with purified HCMV glycoprotein B (gB) also induced ISGs, suggesting that the binding of virus particles to the cellular receptor is sufficient to trigger the response (8). In common with the response to RNA virus infection, IRF-3 is activated following infection with HCMV, resulting in the formation of an ISRE-specific complex containing CBP (29). Similar investigations with HSV-1 demonstrated that infection resulted in the synthesis of ISG-specific RNAs, but only under conditions in which viral gene expression was blocked at an early stage (27, 31). Gene array analysis identified ISGs as the predominant set of cellular genes to be induced under these infection conditions (27). In contrast to the situation for HCMV, however, induction was not detected during normal infection, and this finding led to the conclusions that HSV-1 induces ISG synthesis shortly after the penetration of virus and that a viral gene product(s) disarms the response (27, 31). Viruses with mutations in individual IE genes all inhibited the production of ISG-specific RNAs, suggesting that the effect was not due solely to a single viral gene product (27).

The induction of ISG synthesis by HSV-1 may be relevant to the events that occur after infection of cells under conditions in which viral gene expression is severely limited. Upon infection with HSV-1 mutants containing multiple mutations in IE genes, especially that encoding ICP0, cells are not killed and the viral genome is retained in a nonreplicating "quiescent" state (26, 33, 36). During the early stages of such interactions, the viral genome is converted to an inactive state in which promoters, either homologous or heterologous, are repressed (33, 36). An apparently analogous repression occurs when IE gene transcription is inhibited by pretreatment of cells with IFN-α/β, suggesting that induced ISG products may play a role in attainment of the quiescent state (28, 30). This possibility is supported by other observations. An antiviral state is attained in cells infected with an IE gene-impaired mutant, since cultures treated in this way become resistant to superinfection with HSV-1 or RNA viruses (27). Furthermore, ISG-specific RNA is not induced in human osteosarcoma U2OS cells, a line in which repression of the viral genome does not occur, as judged by the fact that ICP0-deficient mutants replicate as efficiently as wild-type HSV-1 (27, 45).

We have investigated the signaling pathways activated by HSV-1 and HCMV during the early stages of infection and have investigated further the block to the response at later stages in the replication cycle. The results reveal novel interactions of these viruses with the host and demonstrate significant differences in responses depending on the nature of the host cells.

MATERIALS AND METHODS

Cells and viruses. Human fetal lung (HFL) fibroblasts were obtained from Flow Laboratories as Flow 2002 cells and propagated in Dulbecco's modified Eagle medium supplemented with 5% (vol/vol) fetal calf serum, 5% (vol/vol) newborn calf serum, 100 U of penicillin, and 100 μg of streptomycin per ml. Human foreskin fibroblasts transfected with the telomerase coding sequences (BJ cells) (6) were obtained from Geron Corporation and propagated in Dulbecco's modified Eagle medium containing 10% (vol/vol) medium 199 (Life Technologies),10% (vol/vol) fetal calf serum,1 mM sodium pyruvate, 100 U of penicillin, and 100 μg of streptomycin per ml. U-373MG cells were cultured in the same medium (and constituents) as HFL cells.

Wild-type HSV-1 was strain 17 or HFEM, and HCMV was strain AD169. HSV-1 mutant SC16gD.del.Z, lacking gD, was propagated in Vero gD+/19 cells, which express gD (5), and HFEM lacking gH was propagated in CR1 cells, which provide gH (7). To produce stocks lacking glycoproteins, Vero cells were infected with the mutants at a multiplicity of infection (MOI) of 10. After adsorption for 90 min, cells were washed with pH 3 buffer and incubated at 37°C for 24 h as described previously (34). Particles released into the growth medium were purified with Ficoll gradients as described by Rodger et al. (34), except that the gradients were 5 to 15% instead of 15 to 30% Ficoll. Virus particle numbers were estimated by comparison with latex particles of known concentrations using negatively stained preparations as described by Watson et al. (43).

Medium transfer. Culture medium from infected monolayers was removed and centrifuged at 25,000 × *g* for 1 h at 4°C. The medium was applied to fresh monolayers after the addition of cycloheximide to 100 μg/ml (except when cycloheximide was already present). Recombinant human IFN-α was obtained from Sigma, and anti-IFN-β antibody was obtained from CN Biosciences.

EMSA. For electrophoretic mobility shift assays (EMSA), whole-cell extracts were prepared as described by Navarro et al. (29), with the exception that Triton X-100 was added to the lysis buffer at 0.2% (vol/vol) instead of 1% (vol/vol) and a mammalian protease inhibitor cocktail (Sigma P-8340) was added at the concentration recommended by the manufacturer. Extracts were incubated at 15°C for 5 min in a buffer containing 20 mM HEPES (pH 7.0), 40 mM KCl, 20 mM NaCl, 10 mM NaF, 1 mM $MgCl_2$, 2 mM β-glycerophosphate, 0.5 mM dithiothreitol, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, 4% (vol/vol) Ficoll, 0.08% (vol/vol) Triton X-100, 2 μg of poly(dI)-poly(dC), and approximately 0.1 ng of ^{32}P-3′-end-labeled oligonucleotide, with the cell extract constituting 20% of the reaction volume. Mixtures were loaded onto a 6% acrylamide–0.2% *N*,*N*′-methylenebisacrylamide polyacrylamide gel. After electrophoresis at 7 V/cm for 4 h, the gel was dried and exposed for autoradiography. Double-stranded oligonucleotides representing the ISG15 ISRE were (top strand) 5′-GATCGGGAAAGGGAAACCGAAACTGAAG

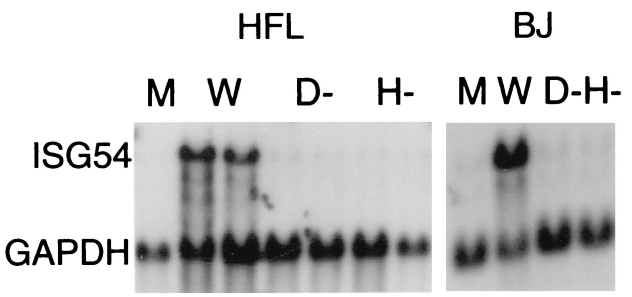

FIG. 2. Glycoprotein-deficient HSV-1 mutants do not induce ISG54-specific RNA. Monolayers of HFL cells (left panel) were mock infected (lane M) or infected with 200 particles (left lane of the pair) or 40 particles (right lane of the pair) of wild-type HSV-1 strain HFEM (W), gD-deficient virions (D−), or gH-deficient virions (H−) per cell in the presence of 100 μg of cycloheximide per ml. Monolayers of BJ cells (right panel) were mock infected or infected with 100 particles of wild-type HSV-1, gD-deficient virions, or gH-deficient virions per cell in the presence of 100 μg of cycloheximide per ml. At 5 h after infection, cytoplasmic RNA was extracted and hybridized to probes specific for ISG54 or GAPDH.

CCA and a mutated version that was identical except for the substitution of C for the underlined G (44). Extracts were preincubated at 4°C in a reaction mixture containing antibodies specific for IRF-3 (Santa Cruz sc-9082X) for 5 min or antibodies specific for CBP (Santa Cruz sc-369X) for 60 min prior to the addition of the radiolabeled oligonucleotide. Quantification was achieved by excising bands and measuring incorporated radioactivity.

Detection of IRF-3. Whole-cell extracts prepared as described above were analyzed by protein blotting with an anti-IRF-3 polyclonal antibody using methods described previously (18).

RNA analysis. Cytoplasmic RNA was extracted and analyzed by electrophoresis and hybridization with ISG54- or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific probes as described previously (31). Quantification was achieved by use of a Bio-Rad Molecular Imager and associated software.

RESULTS

Activation of ISG expression requires penetration of HSV-1. The response of cellular ISGs to infection with HSV-1 or HCMV was investigated with two human fibroblast lines, the HFL fibroblast line routinely used in our laboratory for investigation of repression of viral gene expression and BJ fibroblasts that had been transformed with the human telomerase coding sequences to confer an extended life span in cultures. HFL and BJ cells were treated with gradient-purified virions of HSV-1 or with virions lacking gD or gH and incubated at 37°C for 5 h in the presence of cycloheximide. As expected from previous studies (31), treatment of HFL cells with 200 or 40 particles of wild-type HSV-1 per cell or of BJ cells with 100 particles per cell resulted in the production of ISG54-specific RNA (Fig. 2). The addition of glycoprotein-deficient virions, however, failed to elicit this response in either cell type. Analysis of infected cells immediately after the adsorption period by Southern hybridization demonstrated that approximately equivalent amounts of viral DNAs were associated with cells treated with wild-type, gD-negative, or gH-negative preparations, confirming that the glycoprotein-deficient virions bound efficiently to the cell surface (results not shown). The experiment in Fig. 2 demonstrates that the entry of virus particles is required for the induction of ISG54-specific RNA, confirming and extending the observations of Mossman et al., who showed that virus particles lacking gD or gB did not induce ISG expression (27). The results also make an important distinction between the modes of action of HSV-1 and HCMV particles, since induction by the latter is thought to be a consequence of glycoprotein interaction with the cell membrane rather than entry of virions (8).

Activation of IRF-3 by HSV-1. In view of the differences in the modes of action of HSV-1 and HCMV and to characterize the mechanism of induction by HSV-1, the activation of IRF-3 was investigated. Lysates of HFL or BJ cells were analyzed on protein blots using an IRF-3-specific antibody (Fig. 3A). For both cell types, infection with HSV-1 or HCMV in the presence of cycloheximide resulted in a small but discernible decrease in the electrophoretic mobility of IRF-3, consistent with the change observed by others in response to phosphorylation (23, 46). Evidence for the activation of IRF-3 was also obtained by EMSA using extracts prepared after infection with HSV-1 or HCMV in the presence of cycloheximide (Fig. 3B). A novel complex was formed after incubation of infected cell extracts with a radiolabeled probe containing an ISRE. The complex was present in both cell types, but extracts of BJ cells routinely contained larger amounts of it and formed fewer "nonspecific" complexes in the upper part of the gel. In all other EMSA

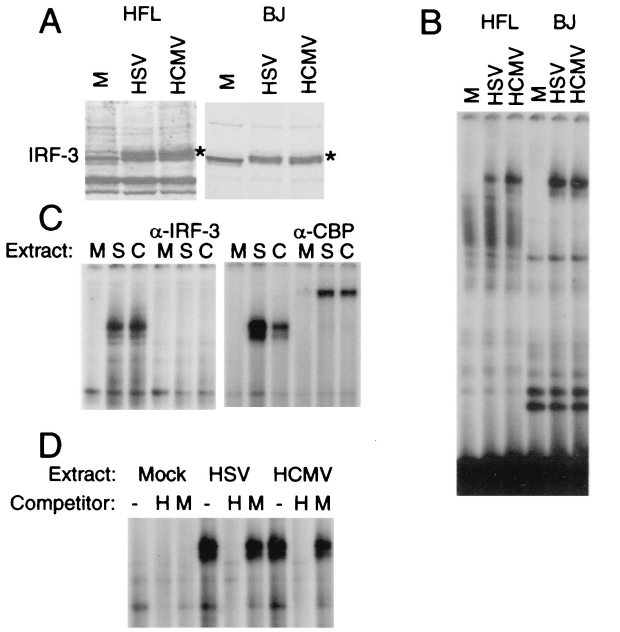

FIG. 3. Activation of IRF-3 in response to infection with HSV-1 or HCMV. Monolayers of HFL or BJ cells were mock infected (M) or infected with 5 PFU of HSV-1 (strain 17) or 1 PFU of HCMV per cell in the presence of 100 μg of cycloheximide per ml. At 5 h after infection, cells were harvested and extracts were prepared. (A) Extracts were analyzed on protein blots incubated with anti-IRF-3 serum. The position of IRF-3 is marked with an asterisk. (B) Extracts were analyzed by EMSA using an ISG15-specific oligonucleotide as a probe. (C) EMSA were carried out with extracts from BJ cells mock infected (lane M) or infected with HSV-1 (lane S) or HCMV (lane C). Anti-IRF-3 or anti-CBP antibody (α) was added as indicated. (D) EMSA were carried out with BJ cell extracts in the presence or absence (−) of a 100-fold excess of homologous oligonucleotide (lane H) or a mutant oligonucleotide with a single base pair difference from the probe (lane M).

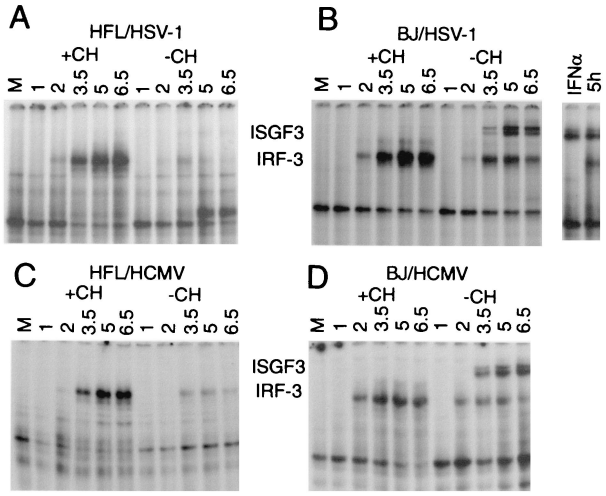

FIG. 4. Time course of IRF-3 and ISGF3 activation. Monolayers were mock infected (M) or infected with 5 PFU of HSV-1 (strain 17) or 1 PFU of HCMV per cell in the presence or absence of 100 μg of cycloheximide (CH) per ml. At various times (hours, shown above lanes) after infection, extracts were made. EMSA were carried out using an ISG15-specific probe. (A) HFL cells infected with HSV-1. (B) BJ cells infected with HSV-1. A comparison of an extract made 5 h after infection (without cycloheximide) with an extract made 1 h after treatment of BJ cells with 10^3 U of IFN-α and 100 μg of cycloheximide per ml is shown to the right. (C) HFL cells infected with HCMV. (D) BJ cells infected with HCMV.

analyses presented here, only the top portion of the autoradiograph is shown.

When a polyclonal antibody specific for IRF-3 was included in the binding reaction, formation of the complex induced by both HSV-1 and HCMV was specifically inhibited (Fig. 3C); when extracts were preincubated with an anti-CBP antibody, the complex exhibited a slower electrophoretic mobility (Fig. 3C). These experiments confirmed that the novel complex contained IRF-3 and CBP. The specificity of binding was demonstrated by competition with an excess of the probe oligonucleotide itself or with a derivative containing a single point mutation that is known to abrogate IRF-3 binding (44). The homologous oligonucleotide inhibited complex formation by extracts of HSV-1- or HCMV-infected BJ cells, but the mutant oligonucleotide had no detectable effect (Fig. 3D). This experiment confirms that the novel complex bound DNA with the specificity expected of IRF-3.

It appears, therefore, that despite differences in the viral signal for the induction of ISGs by HSV-1 and HCMV, at the cellular level the activation of IRF-3 is identical.

Effects of HSV-1 during normal infection. Previous studies showed that ISG-specific RNA synthesis was not induced during normal infection of HFL cells with wild-type HSV-1 or with many mutants that express viral gene products, suggesting that the potential induction by virion components was not realized due to the production of virus-specified products that blocked the response (27, 31). To investigate the stage at which inhibition of the response occurs and to examine the kinetics of IRF-3 activation, production of the complex was examined after infection of HFL or BJ cells in the presence or absence of cycloheximide (Fig. 4). In HFL cells with cycloheximide present, the novel complex was detectable by 2 h after infection, and the level rose during the next 3 h. Without cycloheximide present, the complex was detectable by 2 h after infection, but the level did not increase greatly during infection and declined after 3.5 h (Fig. 4A). Quantification revealed that the amount of radiolabeled probe in the complex at 3.5 h after infection was between 5 and 8% that present when extracts from cycloheximide-treated cells were analyzed (results not shown). In BJ cells, however, activation of IRF-3 was detectable during normal infection, reaching 30 to 50% the amount present in extracts made after infection in the presence of cycloheximide (Fig. 4B). Furthermore, two slower-migrating complexes were formed by extracts prepared at 3.5, 5, and 6.5 h after infection, and these species comigrated with the well-characterized ISGF3 produced in response to IFN-α/β. This experiment therefore suggests that interferon is produced in infected BJ cells.

The response to infection with HCMV followed a similar time course, again with poor induction of the IRF-3-containing complex in HFL cells infected without cycloheximide (Fig. 4C) but stronger activation of IRF-3 and subsequent formation of ISGF3 in BJ cells (Fig. 4D). The data in Fig. 4 further show that activated IRF-3 was first present at 2 h after infection and was not detectable in extracts prepared immediately after adsorption of HCMV to cells.

The presence of ISGF3 in infected BJ cells raised the possibility that endogenous IFN-β was synthesized, presumably following the pathway in which activated IRF-3, in conjunction with other cellular factors, forms the enhanceosome (41, 42). Previous studies (27; C. M. Preston, unpublished observations), however, concluded that interferon was not produced during infection of HFL cells with wild-type HSV-1 or with mutants blocked such that IE gene products were not expressed, based on the finding that medium from cells infected under these conditions did not inhibit virus replication and did not induce ISG-specific RNA synthesis when applied to fresh cells. To reconcile these observations with the data in Fig. 4, which implies the induction of IFN-β synthesis in BJ cells, ISG-specific RNA was investigated using cells infected with wild-type HSV-1; in addition, the medium from infected cells was centrifuged to remove virus and was applied to fresh monolayers to test for the induction of ISG-specific RNA. As was found previously, at an MOI of 5 no ISG54-specific RNA was detected in HFL cells infected without cycloheximide (Fig. 5A, lane 3), and the cell culture medium did not induce ISG54-specific RNA synthesis when applied to fresh cells (Fig. 5A). As expected, infection in the presence of cycloheximide induced ISG54-specific RNA synthesis (Fig. 5A, lane 2). In contrast, ISG54-specific RNA was detected after infection of BJ cells without cycloheximide (Fig. 5A, lane 5), and the medium from the cells contained interferon, since it caused the accumulation of ISG54-specific RNA when added to fresh monolayers (Fig. 5A). Equivalent results were obtained when the medium from HFL cells was applied to fresh BJ cell monolayers and vice versa (results not shown).

The identity of the inducing agent as IFN-β was confirmed by the observation that anti-IFN-β reduced the ISG54-specific RNA levels provoked by infected BJ cell medium but did not affect the potency of added IFN-α (Fig. 5B). Figure 5A also shows that infection of cells at an MOI of 5 resulted in a

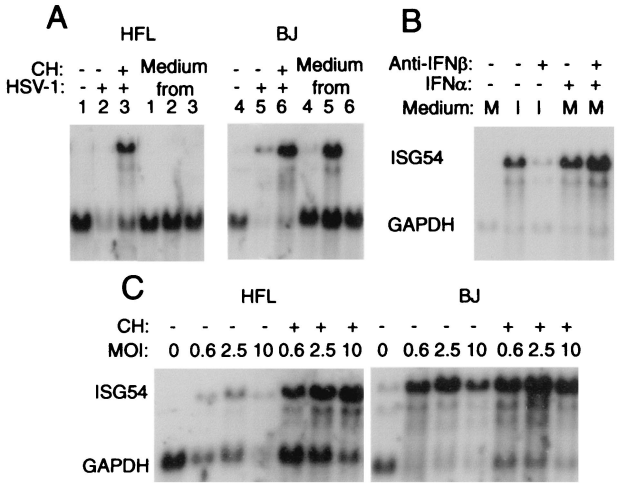

FIG. 5. Production of ISG54-specific RNA and IFN-β by infected BJ cells. (A) Monolayers of HFL (lanes 1 to 3) or BJ (lanes 4 to 6) cells were infected with 5 PFU of HSV-1 (strain 17) per cell in the presence or absence of 100 μg of cycloheximide (CH) per ml and incubated at 37°C for 5 h. RNA was extracted and analyzed by hybridization to ISG54- and GAPDH-specific probes, and the medium from the monolayers was centrifuged to remove virus. The medium samples were applied to fresh monolayers, which were incubated, with cycloheximide added, at 37°C for 5 h. RNA was extracted and analyzed as described above. (B) Medium from cells mock infected (lane M) or infected (lane I) with 5 PFU of HSV-1 per cell for 5 h at 37°C was centrifuged and incubated overnight at 4°C with or without 10^3 U of anti-IFN-β antibody. The medium samples were applied to fresh BJ cell monolayers with cycloheximide added and with or without 10^3 U of IFN-α per ml. After 5 h at 37°C, RNA was prepared and hybridized with ISG54- and GAPDH-specific probes. (C) HFL and BJ cell monolayers were infected with various MOIs of HSV-1 in the absence or presence of cycloheximide. MOIs of 0.6, 2.5, and 10 PFU per cell represent 12, 50, and 200 particles per cell, respectively. After incubation at 37°C for 5 h, RNA was prepared and analyzed by hybridization to ISG54- and GAPDH-specific probes.

reduction in the levels of cellular GAPDH RNAs in both cell types at 5 h after infection, presumably due to mRNA degradation through the virion host shutoff function and the later inhibition of protein synthesis that occurs upon infection with HSV-1 (12, 16, 19). When a lower MOI was used to reduce the degree of mRNA destruction, limited production of ISG54-specific RNA was detected in HFL cell monolayers, amounting to 8% (MOI, 0.6), 8% (MOI, 2.5), and less than 1% (MOI, 10) the levels in cycloheximide-treated cells (Fig. 5C). In BJ cells, the levels of ISG54-specific RNA were 67% (MOI, 0.6), 69% (MOI, 2.5), and 37% (MOI, 10) of those in cycloheximide-treated cells. Therefore, significant production of ISG54-specific RNA and endogenous IFN-β occurs during normal infection of BJ cells with HSV-1 and, presumably, HCMV, whereas in HFL cells the level of production of ISG54-specific RNA is lower and is observed reliably only after infection at a low MOI. The observations are in accord with the implications from the activation of IRF-3 and the formation of ISGF3 shown in Fig. 4.

RNA synthesis is not required for the activation of IRF-3. In many situations, the activation of IRF-3 is thought to be due to the intracellular production of viral double-stranded RNA; thus, it was possible that HSV-1-specific IE RNA was involved in ISG induction. The effects of adding actinomycin D, an inhibitor of RNA synthesis, on IRF-3 activation were therefore examined (Fig. 6). During infection in the presence of cycloheximide, actinomycin D had no effect on the amount of the IRF-3-specific complex formed (Fig. 6, lanes 3 and 4). Without cycloheximide, actinomycin D increased the amount of the complex to a level equivalent to that found in cycloheximide-treated cells and prevented the production of ISGF3 (lanes 1 and 2). Actinomycin D did not stimulate complex formation in uninfected cells (lanes 5 and 6). This experiment shows that newly synthesized RNA does not contribute to the activation of IRF-3. It also confirms that the reduction in activation during infection in the absence of cycloheximide, compared with infection in the presence of the inhibitor, is dependent upon transcription. Finally, the production of ISGF3 was inhibited by actinomycin D, suggesting that RNA synthesis is required for the induction of IFN-β production that occurs during infection of BJ cells with HSV-1.

Induction of ISG-specific RNA in U-373MG cells. Mossman et al. (27) demonstrated that the human osteosarcoma line U2OS failed to produce ISG-specific RNA after infection with UV-inactivated HSV-1, in contrast to human lung fibroblasts, which responded strongly, suggesting that the lack of a requirement for ICP0 correlates with insensitivity to the induction of ISGs by the virus. The generality of the response to HSV-1 was therefore investigated. The human cell line U-373MG is restricted for the replication of ICP0-deficient mutants, approximately as stringently as human fibroblasts (Preston, unpublished). Nonetheless, ISG54-specific RNA was not detected after infection in the presence of cycloheximide, even though IFN-α itself was active in this cell line (Fig. 7). Therefore, the induction of ISG54-specific RNA synthesis does not strictly correlate with permissiveness for ICP0-deficient mutants. Similarly, the induction of ISG54-specific RNA synthesis was not observed for HEC-1B (human) or Vero (monkey) cells, even though the replication of ICP0-deficient mutants was restricted (results not shown).

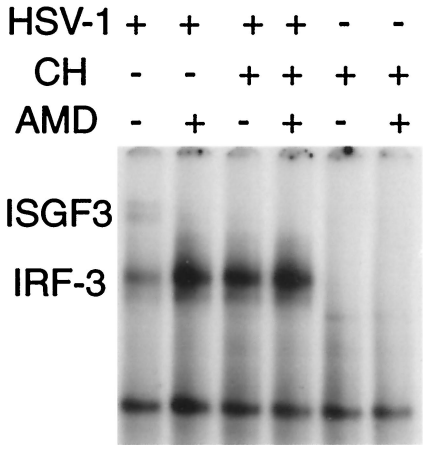

FIG. 6. Effects of actinomycin D on activation of IRF-3. BJ cell monolayers were infected with 5 PFU of HSV-1 (strain 17) per cell or mock infected in the presence of 1 μg of actinomycin D (AMD) or 100 μg of cycloheximide (CH) per ml. Extracts were made at 5 h after infection and analyzed by EMSA.

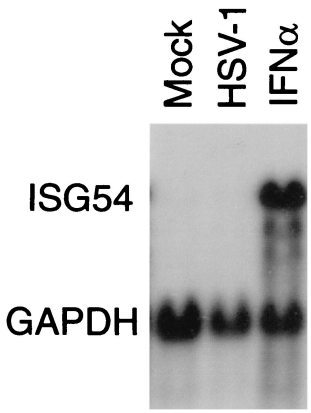

FIG. 7. Induction of ISG54-specific RNA in U-373MG cells. Monolayers of U-373MG cells were mock infected, infected with 5 PFU of HSV-1 (strain 17) per cell, or treated with 10^3 U of IFN-α per ml, treated with 100 μg of cycloheximide per ml, and incubated at 37°C for 5 h. RNA was extracted and hybridized to ISG54- and GAPDH-specific probes.

DISCUSSION

We investigated the cellular pathways involved in the induction and abrogation of ISG synthesis during HSV-1 and HCMV infections. In contrast to the situation with HCMV, entry of HSV-1 rather than binding was required for the cellular response, although the downstream cellular pathways appear to be equivalent for the two viruses. The induction and disarming of the response are each more complex than suggested from existing data, with the nature of the host cell being an important variable. BJ fibroblasts, which respond to infection with vigorous activation of IRF-3, are able to synthesize ISGs, including IFN-β, during normal infection with HSV-1 or HCMV. The degree of response shown by human fibroblasts is not exhibited by all human cell types.

The failure of virions lacking gD or gH to induce ISG54-specific RNA, together with the finding that gB-deficient virions are also inactive (27), suggests that virus entry, rather than signal transduction elicited by a glycoprotein interaction with the cell surface, is required for the cellular response. This conclusion is supported by the finding that IRF-3 activation was first detected at 2 h after infection rather than during the first hour, when adsorption takes place. The contrast between the mechanisms used by HSV-1 and HCMV is intriguing, but the differences in the approaches used to reach the conclusions should be noted. Application of purified glycoproteins, as was used for HCMV, may not equate to the events that occur during infection with virus, and it is unfortunate that glycoprotein-deficient mutants of HCMV are not available to allow experiments analogous to those carried out with mutant HSV-1 virions to be performed.

Activation of IRF-3 is a common response to infection with RNA viruses, and in most situations it is thought that double-stranded RNA is the trigger. Indeed, inhibition of Sendai virus replication by the addition of ribavirin or UV irradiation of virions prevented the activation of IRF-3 (38). The ability of UV-irradiated HSV-1 to induce ISG-specific RNA synthesis suggested that de novo synthesis of viral transcripts was not required for induction (27, 31), and the observation that stringent inhibition of RNA synthesis with actinomycin D did not affect the activation of IRF-3 confirmed this conclusion. It has recently been shown that HCMV particles contain viral and host cell RNAs (9, 15), possibly explaining the response to HCMV and to HSV-1, if analogous transcripts exist in its virions. Both viruses, however, contain many proteins in the tegument and capsid structures, including protein kinases, and any of these could have a role in triggering host defenses. If RNA is not required for the effects of HSV-1 and HCMV, IRF-3 activation must proceed through a novel pathway after infection with these viruses.

A major function of IRF-3 is the induction of IFN-β synthesis and hence protection of neighboring uninfected cells, rather than induction of ISG synthesis in initially infected cells. Our finding that endogenous IFN-β is produced in BJ cells confirms that the pathways downstream of IRF-3 are intact during the first 6.5 h after infection with HSV-1 and HCMV. Furthermore, since virtually every cell is infected at an MOI of 5, the presence of ISGF3 shows that the JAK/STAT pathway remains functional at least up to 5 h after infection with HSV-1 or HCMV. At later times after infection with HCMV, many components of the IFN-α signal transduction pathway are inhibited (24). The fact that HSV-1 can induce IFN-β synthesis through a signal that requires the entry of virions defines a new and unexpected way in which the virus can induce this cellular response, in contrast to previous reports. For peripheral blood mononuclear cells (PBMCs), the presentation of soluble gD or of cells expressing the protein was able to induce interferon synthesis (3, 20). In HFL or BJ cells, no induction of ISG54-specific RNA was detected even after the addition of 1 μM soluble gD, a concentration that gave maximum induction in PBMCs (3; Preston, unpublished). The production of IFN-β-specific RNA was detected in HSV-1-infected mouse embryo fibroblasts, but not until 12 h after infection, much later than what we observed for BJ cells (37).

Previous studies showed that the induction of ISG-specific RNA synthesis was not detectable at 6 h (31) or 24 h (27) after normal infection with HSV-1, although in both studies a general reduction in cellular transcript levels was also observed. Many HSV-1 mutants lacking intact IE genes also disarmed the response, although in these instances the effects on cellular RNAs were smaller than that seen with wild-type virus (27). By analogy to other viruses, it appeared that HSV-1 encoded one or more products dedicated to ablating the response and hence overcoming this aspect of host antiviral defense. The results presented here show that this aspect of the virus-host interaction is more complex and probably less specific than suggested by the initial observations. It is clear that IRF-3 is activated much less efficiently in HFL cells than in BJ cells after infection with HSV-1 without cycloheximide; therefore, IRF-3 is a possible target for viral inhibition of ISG synthesis. Even in BJ cells, the activation of IRF-3 is reduced in comparison with the levels that can be attained in the presence of actinomycin D or cycloheximide.

The way in which HSV-1 may interfere with IRF-3 or the pathways leading to its phosphorylation are unclear at present. The similarities in the responses to infection with HSV-1 and HCMV without cycloheximide are surprising, in view of the different time scales of the HSV-1 and HCMV replication cycles. As in HSV-1-infected cells, IRF-3 was activated poorly

in HCMV-infected HFL cells and more strongly, but not to the levels seen with cycloheximide present, in BJ cells. Two main possibilities exist to reconcile these observations. HSV-1 and HCMV may each inhibit the activation of IRF-3 in the same way, due to the production of specific viral proteins that have functional homologies, with the same timing after infection. Alternatively, the similarity in cell responses may signify the operation of normal cellular regulation of IRF-3 activity and may be unrelated to virus infection. If the latter possibility is valid, it follows that infection with HSV-1 does not specifically block ISG synthesis, but rather that the only negative effect attributable to the virus is the overall increase in the degradation of cellular mRNAs.

Even a relatively small change in host cell type, from human lung fibroblasts to human foreskin fibroblasts, results in significantly different responses to infection. This is a surprising finding, since both cell types are fibroblasts derived from human material. The life-extended status of the BJ cells used here does not appear to be an important factor, as a secondary human foreskin fibroblast line (HFFF2) responded in a manner similar to that of BJ cells, with IRF-3- and ISGF3-specific complexes being present in extracts made at 5 h after infection without cycloheximide (Preston, unpublished). Given the strong agreement between our findings with HFL cells and those of Mossman et al. (27) using human embryo lung fibroblasts from a different source, it appears that the tissue origin of the cells used may be the relevant variable. The basis for the cell type difference in response is largely attributable to the stronger activation of IRF-3 in BJ cells, but at present the kinases responsible for the activation of IRF-3 are unknown; thus, it is not possible to speculate on the identities of the cellular factors that respond to infection with HSV-1 (38).

Induction of ISG54-specific RNA synthesis does not correlate with the requirement for ICP0. The finding that ISG54-specific RNA is not produced in response to infection of U-373MG cells and other cells shows that this aspect of the virus-cell interaction is dependent upon the nature of the host cell. The correlation between the induction of ISGs and the dependence on ICP0 does not hold invariably, but it is possible that there are subtle variations in responses to infection and that ISGs other than ISG54 may indeed be switched on in a broader range of cell types. The production of IFN-β itself does not significantly influence the virus infection program in the infected cell, since the replication of HSV-1 is equivalent in HFL and BJ cells (Preston, unpublished). It would be expected that the antiviral effects of IFN-β rely on the activation of protein kinase R, and the work of He et al. (17) shows that viral protein ICP34.5 negates this event by activating a cellular phosphatase.

The results presented here extend current knowledge of the way in which HSV-1 and HCMV interact with host cells. Although the induction of ISG synthesis does not directly correlate with the lack of dependence on ICP0, the activation of IRF-3 can result in the secretion of interferons from human fibroblasts even in the context of a normal productive infection. The induction depends on viral entry rather than the action of viral glycoproteins binding to cells, in contrast to the proposed mechanisms for the responses of HCMV-infected fibroblasts or HSV-1-infected PBMCs. The inducing component is probably not RNA, suggesting a mechanism different from that which occurs in RNA virus-infected cells. Counteraction of the cellular antiviral response is partly due to a general degradation of cellular mRNAs, and although the reduction in the activation of IRF-3 during normal infection with HSV-1 accounts for the lower ISG-specific RNA levels, the effect may be due to normal cellular regulatory pathways rather than virus-specified products. Further investigation of the interactions of HSV-1 and HCMV with different cell types, particularly in vivo, may shed light on the biological significance of the cellular responses to infection that we report here.

ACKNOWLEDGMENTS

We thank Tony Minson for help in the preparation of virions and for interest in this work.

A. N. Harman was supported by the Wellcome Trust.

REFERENCES

1. **Alcami, A., and U. H. Koszinowski.** 2000. Viral mechanisms of immune evasion. Trends Microbiol. **8:**410–419.
2. **Altinkilik, B., and G. Brandner.** 1988. Interferon inhibits herpes simplex virus-specific translation: a reinvestigation. J. Gen. Virol. **69:**3107–3112.
3. **Ankel, H., D. F. Westra, S. Welling-Wester, and P. Lebon.** 1998. Induction of interferon-α by glycoprotein D of herpes simplex virus: a possible role of chemokine receptors. Virology **251:**317–326.
4. **Au, W.-C., P. A. Moore, W. Lowther, Y.-T. Juang, and P. M. Pitha.** 1995. Identification of a member of the interferon regulatory factor family that binds to the interferon-stimulated response element and activates expression of interferon-induced genes. Proc. Natl. Acad. Sci. USA **92:**11657–11661.
5. **Babic, N., G. Rodger, J. Arthur, and A. C. Minson.** 1999. A study of neuronal infection by mutants of herpes simplex virus type 1 lacking dispensable and non-dispensable glycoproteins. J. Gen. Virol. **80:**2403–2409.
6. **Bodnar, A. G., M. Ouellette, M. Frolkis, S. E. Holt, C.-P. Chiu, G. B. Morin, C. B. Harley, J. W. Shay, S. Lichtsteiner, and W. E. Wright.** 1996. Extension of life-span by introduction of telomerase into normal human cells. Science **279:**349–352.
7. **Boursnell, M. E. G., C. Entwhistle, C. Blakeley, D. Roberts, I. A. Duncan, S. E. Chisholm, S. E. Martin, R. Jennings, C. D. Ni, I. Sobek, S. C. Inglis, and C. S. Mclean.** 1997. A genetically inactivated herpes simplex virus type 2 (HSV-2) vaccine provides effective protection against primary and recurrent HSV-2 disease. J. Infect. Dis. **175:**16–25.
8. **Boyle, K. A., R. L. Pietropaolo, and T. Compton.** 1999. Engagement of the cellular receptor for glycoprotein B of human cytomegalovirus activates the interferon response pathway. Mol. Cell. Biol. **19:**3607–3613.
9. **Bresnahan, W. A., and T. T. Shenk.** 2000. A subset of viral transcripts packaged within human cytomegalovirus particles. Science **288:**2373–2376.
10. **Burysek, L., W. S. Yeow, B. Lubyova, M. Kellum, S. L. Schafer, Y. Q. Huang, and P. M. Pitha.** 1999. Functional analysis of human herpesvirus 8-encoded interferon regulatory factor 1 and its association with cellular interferon regulatory factors and p300. J. Virol. **73:**7334–7342.
11. **Daly, C., and N. C. Reich.** 1993. Double-stranded RNA activates novel factors that bind to the interferon-stimulated response element. Mol. Cell. Biol. **13:**3756–3764.
12. **Fenwick, M. L., and M. J. Walker.** 1978. Suppression of the synthesis of cellular macromolecules by herpes simplex virus. J. Gen. Virol. **41:**37–51.
13. **Garcia-Sastre, A.** 2001. Inhibition of interferon-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses. Virology **279:**375–384.
14. **Goodbourn, S., L. Didcock, and R. E. Randall.** 2000. Interferons: cell signalling, immune modulation, antiviral responses and virus countermeasures. J. Gen. Virol. **81:**2341–2364.
15. **Greijer, A. E., C. A. J. Dekkers, and J. M. Middledorp.** 2000. Human cytomegalovirus virions differentially incorporate viral and host cell RNA during the assembly process. J. Virol. **74:**9078–9082.
16. **Hardwicke. M. A., and R. M. Sandri-Goldin.** 1994. The herpes simplex virus regulatory protein ICP27 contributes to the decrease in cellular mRNA levels during infection. J. Virol. **68:**4797–4810.
17. **He, B., M. Gross, and B. Roizman.** 1997. The $\gamma_1$34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1a to dephosphorylate the α subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. Proc. Natl. Acad. Sci. USA **94:**843–848.
18. **Homer, E. G., A. Rinaldi, M. J. Nicholl, and C. M. Preston.** 1999. Activation of herpesvirus gene expression by the human cytomegalovirus protein pp71. J. Virol. **73:**8512–8518.
19. **Kwong, A. D., and N. Frenkel.** 1987. Herpes simplex virus-infected cells contain a function(s) that destabilizes host and viral mRNAs. Proc. Natl. Acad. Sci. USA **84:**1926–1930.

20. **Lebon, P.** 1985. Inhibition of herpes simplex virus type 1-induced interferon synthesis by monoclonal antibodies against viral glycoprotein D and by lysosomotropic drugs. J. Gen. Virol. **66:**2781–2786.
21. **Leib, D. A., T. E. Harrison, K. M. Laslo, M. A. Machalek, N. J. Moorman, and H. W. Virgin.** 1999. Interferons regulate the phenotype of wild-type and mutant herpes simplex viruses in vivo. J. Exp. Med. **189:**663–672.
22. **Leib, D. A., M. A. Machalek, B. R. G. Williams, R. H. Silverman, and H. W. Virgin.** 2000. Specific phenotypic restoration of an attenuated virus by knockout of a host resistance gene. Proc. Natl. Acad. Sci. USA **97:**6097–6101.
23. **Lin, R., C. Heylbroeck, P. M. Pitha, and J. Hiscott.** 1998. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. Mol. Cell. Biol. **18:**2986–2996.
24. **Miller, D. M., Y. Zhang, B. M. Rahill, W. J. Waldeman, and D. D. Sedmak.** 1999. Human cytomegalovirus inhibits IFN-α-stimulated antiviral and immunoregulatory responses by blocking multiple levels of IFN-α signal transduction. J. Immunol. **162:**6107–6113.
25. **Mittnach, S., P. Straub, H. Kirchner, and H. Jacobsen.** 1988. Interferon treatment inhibits onset of herpes simplex virus immediate-early transcription. Virology **164:**201–210.
26. **Mossman, K. L., and J. R. Smiley.** 1999. Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 renders expression of the immediate-early genes almost entirely dependent on ICP0. J. Virol. **73:**9726–9733.
27. **Mossman, K. L., P. F. MacGregor, J. J. Rozmus, A. B. Goryachev, A. M. Edwards, and J. R. Smiley.** 2001. Herpes simplex virus triggers and then disarms a host antiviral response. J. Virol. **75:**750–758.
28. **Mossman, K. L., H. A. Saffran, and J. R. Smiley.** 2000. Herpes simplex virus ICP0 mutants are hypersensitive to interferon. J. Virol. **74:**2052–2056.
29. **Navarro, L., K. Mowen, S. Rodems, B. Weaver, N. Reich, D. Spector, and M. David.** 1998. Cytomegalovirus activates interferon immediate-early response gene expression and an interferon regulatory factor 3-containing interferon-stimulated response element-binding complex. Mol. Cell. Biol. **18:**3796–3802.
30. **Nicholl, M. J., and C. M. Preston.** 1996. Inhibition of herpes simplex virus type 1 immediate-early gene expression by alpha interferon is not VP16 specific. J. Virol. **70:**6336–6339.
31. **Nicholl, M. J., L. H. Robinson, and C. M. Preston.** 2000. Activation of interferon-responsive genes after infection of human cells with herpes simplex virus type 1. J. Gen. Virol. **81:**2215–2218.
32. **Oberman, F., A. and Panet.** 1988. Inhibition of transcription of herpes simplex virus immediate early genes in interferon-treated human cells. J. Gen. Virol. **69:**1167–1177.
33. **Preston, C. M., and M. J. Nicholl.** 1997. Repression of gene expression upon infection of cells with herpes simplex virus type 1 mutants impaired for immediate-early protein synthesis. J. Virol. **71:**7807–7813.
34. **Rodger, G., J. Boname, S. Bell, and T. Minson.** 2001. Assembly and organization of glycoproteins B, C, D, and H in herpes simplex virus type 1 particles lacking individual glycoproteins: no evidence for the formation of a complex of these molecules. J. Virol. **75:**710–716.
35. **Ronco, L. V., A. Y. Karpova, M. Vidal, and P. M. Howley.** 1998. Human papilloma virus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. Genes Dev. **12:**2061–2072.
36. **Samaniego, L. A., L. Neiderhiser, and N. A. DeLuca.** 1998. Persistence and expression of the herpes simplex virus genome in the absence of immediate-early proteins. J. Virol. **72:**3307–3320.
37. **Sato, M., H. Suemori, N. Hata, M. Asagiri, K. Ogasawara, K. Nakao, T. Nakaya, M. Katsuki, S. Noguchi, N. Tanaka, and T. Taniguchi.** 2000. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-α/β induction. Immunity **13:**539–548.
38. **Servant, M. J., B. Ten Oever, C. Lepage, L. Conti, S. Gessani, L. Julkunen, R. Lin, and J. Hiscott.** 2001. Identification of distinct signaling pathways leading to the phosphorylation of interferon regulatory factor 3. J. Biol. Chem. **276:**355–363.
39. **Stark, G. R., I. M. Kerr, B. R. G. Williams, R. H. Silverman, and R. D. Schreiber.** 1998. How cells respond to interferons. Annu. Rev. Biochem. **67:**227–264.
40. **Talon, J., C. M. Horvath, R. Polley, C. F. Basler, T. Muster, P. Palese, and A. Garcia-Sastre.** 2000. Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. J. Virol. **74:**7989–7996.
41. **Thanos, D., and T. Maniatis.** 1995. Virus induction of human IFNβ expression requires the assembly of an enhancosome. Cell **83:**1091–1100.
42. **Wathelet, M. G., C. H. Lin, B. S. Parekh, L. V. Ronco, P. M. Howley, and T. Maniatis.** 1998. Virus infection induces the assembly of coordinately activated transcription factors on the IFN-β enhancer in vivo. Mol. Cell **1:**507–518.
43. **Watson, D. H., W. C. Russell, and P. Wildy.** 1963. Electron microscopy particle counts on herpes virus using the phosphotungstate negative staining technique. Virology **19:**250–260.
44. **Weaver, B. K., K. P. Kumar, and N. C. Reich.** 1998. Interferon regulatory factor 3 and CREB-binding protein/p300 are subunits of double-stranded RNA-activated transcription factor DRAF1. Mol. Cell. Biol. **18:**1359–1368.
45. **Yao, F., and P. A. Schaffer.** 1995. An activity specified by the osteosarcoma line U2OS can substitute functionally for ICP0, a major regulatory protein of herpes simplex virus type 1. J. Virol. **69:**6249–6258.
46. **Yoneyama, M., W. Suhara, Y. Fukuhara, M. Fukuda, E. Nishida, and T. Fujita.** 1998. Direct triggering of the type 1 interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. EMBO J. **17:**1087–1095.
47. **Zhu, H., J.-P. Cong, and T. Shenk.** 1997. Use of differential display analysis to assess the effect of human cytomegalovirus infection on the accumulation of cellular RNAs: induction of interferon-responsive RNAs. Proc. Natl. Acad. Sci. USA **94:**13985–13990.
48. **Zhu, H., J.-P. Cong, G. Mamtora, T. Gingeras, and T. Shenk.** 1998. Cellular gene expression altered by human cytomegalovirus: global monitoring with oligonucleotide arrays. Proc. Natl. Acad. Sci. USA **95:**14470–14475.