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Repression of Host RNA Polymerase II Transcription by Herpes Simplex Virus Type 1

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Lytic infection of mammalian cells with herpes simplex virus type 1 (HSV-1) results in rapid repression of host gene expression and selective activation of the viral genome. This transformation in gene expression is thought to involve repression of host transcription and diversion of the host RNA polymerase (RNAP II) transcription machinery to the viral genome. However, the extent of virus-induced host transcription repression and the mechanisms responsible for these major shifts in transcription specificities have not been examined. To determine how HSV-1 accomplishes repression of host RNAP II transcription, we assayed transcription patterns on several cellular genes in cells infected with mutant and wild-type HSV-1. Our results suggest that HSV-1 represses RNAP II transcription on most cellular genes. However, each cellular gene we examined responds differently to the transcription repressive effects of virus infection, both quantitatively and with respect to the involvement of viral gene products. Virus-induced shutoff of host RNAP II transcription requires expression of multiple immediate-early genes. In contrast, expression of delayed-early and late genes and viral DNA replication appear to contribute little to repression of host cell RNAP II transcription. Modification of RNAP II to the intermediately phosphorylated (II_I) form appears unlinked to virus-induced repression of host cell transcription. However, full repression of host transcription is correlated with depletion of the hyperphosphorylated (IIO) form of RNAP II.

Infection with herpes simplex virus type 1 (HSV-1) profoundly alters the host cell synthetic machinery, diverting it from expression of the host genome and toward expression of the viral genome (reviewed in reference 46). This virus-induced transformation in gene expression is thought to involve the shutoff of host translation, destabilization of host mRNAs, and repression of host transcription. The virus then utilizes the translation and RNA polymerase II (RNAP II) transcription machinery of its host to synthesize its gene products in a defined temporal order. The question of how HSV-1 selectively represses transcription of most cellular genes while activating transcription of its own genes in a precise temporal order remains important to understanding both viral and cellular gene regulation.

The HSV-1 genes are classified into three temporal classes—immediate-early (IE), delayed-early (DE), and late (L)—based on their order of expression. HSV-1 genes are transcribed in the nucleus by host RNAP II, and their temporal regulation occurs at both transcriptional and posttranscriptional levels (7, 13, 27, 40, 55).

The five IE genes (encoding ICP0, ICP4, ICP22, ICP27 and ICP47) are expressed immediately following infection, and all IE gene products except ICP47 are regulatory proteins involved in controlling the expression of DE and L genes. Each of the IE gene promoters displays features of typical RNAP II TATA box promoters. In addition, each IE promoter contains a TAATGARAT element, which binds cellular factors including Oct1. On infection, the virion transcriptional activator protein VP16 enters the cell, associates with cellular factors including Oct1, and stimulates transcription of each IE gene (29). Another virion component, the vhs protein, triggers the shutoff of host cell translation and destabilization of host cell mRNAs (18). The IE gene product ICP4 is an essential viral transactivating protein which is required for transcriptional activation of the DE and L genes (46). ICP0 also acts as a transactivator in transfection assays; however, it is not required at high multiplicities of infection (MOI) for lytic growth in cultured cells (reviewed in reference 12). ICP27 is an essential viral protein that is required for DE and L gene expression and for normal levels of viral DNA replication (reviewed in reference 28). ICP27 is thought to act at posttranscriptional levels, perhaps controlling aspects of splicing, polyadenylation, and RNA stability. ICP22 is required, in some cultured cell types, for normal transcriptional activation of viral genes (35, 40, 43).

The DE genes encode proteins involved in viral DNA replication and are expressed only after IE genes are activated (starting at approximately 3 h postinfection). The mechanisms by which the DE genes are transcriptionally activated are not well understood. DE gene promoters are relatively simple, comprising TATA boxes, cap sites, and *cis*-acting sequences that bind basal cellular transcription factors such as Sp1 (reviewed in reference 46). The L genes encode structural components of the new virion, and their gene products begin to appear at approximately 6 h postinfection. L gene promoters are even simpler than those of the DE genes, often comprising only a TATA box and sequences surrounding the start site. Like DE genes, L genes contain no known promoter elements that bind viral IE gene products and could account for their temporal regulation (reviewed in reference 46). It is not clear why transcription occurs efficiently on viral DE and L gene promoters and not on cellular gene promoters which utilize the same RNAP II basal transcription factors and contain similar promoter elements.

Early studies documented a decrease in overall host RNA synthesis in HSV-infected cells (17, 54). Later studies of RNA

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synthesis in isolated nuclei verified that transcription by all three nuclear RNA polymerases declines to less than 50% of uninfected levels within 4 h postinfection (36). Transcription of adenovirus RNA in cells transformed by adenovirus type 5 is inhibited by more than 70% following infection with HSV. This inhibition of adenovirus transcription requires expression of HSV IE genes (52). Similarly, polyomavirus-specific RNA synthesis declines to less than 10% of uninfected levels when polyomavirus-transformed cells are infected with HSV (34).

A few studies have directly analysed transcription patterns on individual cellular genes by nuclear run-on transcription assays. These assays show that HSV infection brings about an approximately 40% reduction of histone H3 and actin gene transcription by 4 h postinfection (26) and a greater than 95% reduction of β-globin gene transcription in differentiated MEL cells by 5 h postinfection (44). Inhibition of β-globin gene transcription requires the expression of viral immediate-early genes (46). In addition, by 5 h postinfection, HSV infection results in an approximately 40% reduction in the transcription of genes encoding several unidentified cDNAs (20). In contrast, it has been reported that transcription of the cellular ubiquitin B gene, the interleukin-6 gene, and several unidentified genes increases following HSV infection (19–21, 37).

Some elegant studies examining the preferential activation of viral gene promoters involve the expression of cellular genes in the context of the viral genome. The rabbit β-globin gene (with its own promoter and 1,200 bp of 5′-flanking DNA), when stably integrated into the HSV-1 genome, is expressed to high levels as a viral DE gene after infection of the recombinant virus into differentiated MEL cells. While the viral copy of the β-globin gene is transcriptionally induced, transcription of the endogenous β-globin gene is efficiently repressed (44–46). Similarly, the human α-globin gene (under the control of its own promoter), when stably integrated into the HSV-1 genome, is expressed as a viral DE gene even in cells that do not express the endogenous α-globin gene (31). These experiments suggest that the differential effects of HSV infection on transcription of cellular and viral genes may not involve sequence differences between cellular and viral gene promoters. Instead, as suggested by Smiley et al. (46), the shift in transcription from cellular to viral genomes may involve sequence-independent mechanisms such as modifications to the RNAP II transcription machinery, alterations in DNA conformation, or localization of viral genes in specialized nuclear compartments.

We previously reported that infection of mammalian cells with HSV-1 results in rapid and aberrant phosphorylation of the large subunit of RNAP II and recruitment of RNAP II into viral replication compartments (39). Modification of RNAP II to the II_I form requires expression of the viral IE gene product ICP22, which is also necessary for appropriate transcription of DE and L genes in some cell lines (40).

To investigate the potential role of RNAP II modifications in the repression of host cell transcription and to determine how HSV-1 brings about selective repression of host RNAP II transcription, we have analyzed transcription of a number of cellular genes following infection with wild-type and mutant HSV-1. We conclude that multiple IE gene products contribute to host transcription repression and that virus-induced transcription repression occurs in gene-specific ways. Modification of RNAP II to the II_I form, although linked to efficient transcription of viral DE and L genes in some cell lines, does not appear to be necessary for repression of host gene transcription.

MATERIALS AND METHODS

Cells, viruses, and infections. HeLa S3 (human epithelioid cervical carcinoma), Vero (African green monkey kidney), and HEL (human embryonic lung) cells were used for infections. The cells were obtained from the American Type Culture Collection, Rockville, Md., and were grown as monolayer cultures in Dulbecco modified Eagle medium containing 10% heat-inactivated newborn calf serum (GIBCO). HSV-1 (KOS1.1) (wild type) was obtained from M. Levine (University of Michigan). The ICP4 null mutant virus *d*120 was obtained from Neal DeLuca (University of Pittsburgh School of Medicine). The ICP0 nonsense mutant virus *n*212 and the ICP22 nonsense mutant virus 22/*n*199 were obtained from Priscilla Schaffer (Dana-Farber Cancer Institute and Harvard Medical School). The ICP27 null mutant virus *d*27-1 has been described previously (38). The ICP6 mutant ICP6Δ was obtained from Sandy Weller (University of Connecticut School of Medicine). Virus strains were propagated and subjected to titer determination as described previously (40). A stock of wild-type HSV-1 (KOS1.1) was UV irradiated at 254 nm and inactivated as described previously (39). The UV irradiation reduced the viral titers by 4 to 5 orders of magnitude. The UV-inactivated virion stock contained functional VP16, as measured by the ability of the virions to transactivate an IE promoter fused to the chloramphenicol acetyltransferase (CAT) gene (39). In all experiments, cells were infected with HSV-1 strains at a MOI of 10 PFU per cell.

Western blots. Whole-cell extracts were prepared and Western blotting (immunoblotting) was performed as described previously (39, 40). The blots were probed with monoclonal antibody ARNA3 (Cymbus Bioscience Ltd.), which reacts with all phosphorylation variants of the large subunit of RNAP II.

Nuclear run-on transcription assays. HeLa S3 cells or HEL cells were mock infected or infected with wild-type or mutant viruses. Nuclei were isolated, and assays were performed on equal numbers of nuclei per sample, as described previously (40). In brief, transcription in isolated nuclei was allowed to proceed in the presence of [^{32}P]UTP and a buffer containing 150 mM KCl. RNA products were purified and hybridized to membranes (GeneScreen-Plus) bearing single-stranded bacteriophage M13 DNA probes specific for various cellular genes. The probes were designed to detect either sense or antisense transcription in the gene region of interest. The filters were washed and treated with RNase A to remove nonhybridizing portions of labeled RNAs.

The probes were constructed by cloning the following DNA fragments into either M13mp18 or M13mp19. The c-*myc* exon 1 probe was a 445-bp *Xho*I-*Pvu*II fragment from +66 to +511 of the human c-*myc* gene (49). The c-*myc* intron 1 probe was a 606-bp *Sst*I fragment from +936 to +1542 of the human c-*myc* gene (49). The c-*fos* 5′ probe was an 842-bp *Nar*I fragment from −82 to +760 (exon 1 and part of intron 1) of human c-*fos* genomic DNA. The c-*fos* 3′ probe was a 491-bp *Apa*I fragment from +1905 to +2396 (exon 4) of human c-*fos* genomic DNA. The γ-actin 5′ probe was a 583-bp *Bam*HI-*Bgl*I fragment from −100 to +483 (exons 1 to 4) of a human γ-actin cDNA. The γ-actin 3′ probe was a 600-bp *Bgl*I-*Sca*I fragment from +483 to +1083 (exons 4 to 6) of a human γ-actin cDNA (11). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene probe was a 979-bp fragment from +44 to +1023 of a human GAPDH cDNA (1). The histone H2b probe was a 302-bp *Bst*EII fragment from +110 to +412 of the chicken histone H2b gene (51).

Single-stranded M13 probes for the viral genes ICP4, ICP27, ICP8, gC, and UL36 have been described previously (40).

Radioactivity hybridizing to each probe was quantitated with a Fujix BAS100 bioimaging analyzer with MacBAS imaging software.

Photoaffinity labeling. Nuclei were isolated from mock-infected or HSV-1-infected HeLa S3 or Vero cells, and reactions were carried out as described previously (4). In brief, transcription was allowed to proceed in isolated nuclei in the presence of 100 mM ATP and GTP, 10 μCi of [α-^{32}P]CTP, and 0.1 mM 4-thio-UTP. In reactions involving α-amanitin, nuclei were preincubated with 2 μg of α-amanitin per ml for 10 min prior to addition of nucleotides. Nuclei were irradiated with UV light, as described previously (2), using a FisherBiotech 312-nm variable transilluminator set at maximum output for 5 min. The nuclei were then digested with RNase T_1 and DNase I. Some samples were also treated with calf intestinal alkaline phosphatase (Boehringer Mannheim). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (6% polyacrylamide). The gels were dried, and labeled proteins were visualized by autoradiography.

RESULTS

Transcription of host RNAP II genes is repressed following HSV-1 infection. Based upon pulse-labeling incorporation experiments and examination of transcription rates on transformed adenovirus and polyomavirus DNA and on several cellular genes, it has been assumed that the majority of host RNAP II genes are transcriptionally repressed following HSV-1 infection. However, few cellular RNAP II genes have been directly examined at the transcriptional level for effects of HSV-1 infection on initiation, elongation, or overall transcription rates. The nuclear run-on transcription assay is the only

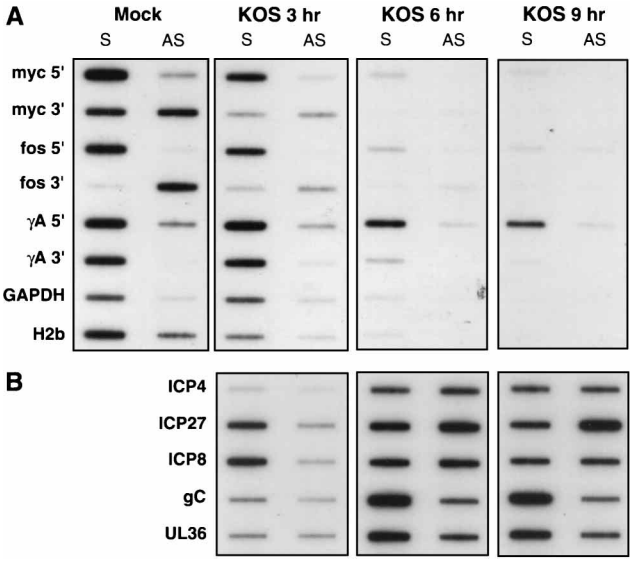

FIG. 1. Nuclear run-on transcription analysis of cellular and viral gene transcription in HeLa cells infected with wild-type virus, KOS1.1. Cells were infected, and nuclei were isolated at the times indicated. Assays were performed on equal numbers of nuclei per sample. RNA products of each sample were divided into two aliquots: 95% of the RNA was used to hybridize to the cellular gene filters (A), and the remaining 5% was used to hybridize to the viral gene filters (B). The filters contained single-stranded M13 DNA probes which detect sense (S) or antisense (AS) transcripts arising from the human c-*myc*, c-*fos*, γ-actin, GAPDH, and histone H2b genes or from the viral IE genes ICP4 and ICP27, the DE gene ICP8, and the L genes gC and UL36. Nuclear run-on transcription assays in mock-infected cells yielded no detectable hybridization to viral gene probes (data not shown).

method developed to date that directly measures RNA polymerase activity on specific gene regions, independent from the contributions of mRNA degradation and processing (25). Other in vivo assays of transcription, such as whole-cell pulse-labelling, measure the combined effects of RNA synthesis and rapid degradation.

To directly analyze repression of specific cellular RNAP II genes, we performed nuclear run-on transcription assays on nuclei isolated from HeLa S3 cells, mock infected or infected with the wild-type HSV-1 (KOS1.1). RNAs labeled during nuclear run-on assays were hybridized to filters containing single-stranded M13 DNA probes that detect either sense or antisense transcription from several endogenous cellular genes (Fig. 1A) or to filters containing probes that detect either sense or antisense transcription from several HSV-1 genes (Fig. 1B). Nuclei were counted in each sample and adjusted so that assays were performed on an equal number of nuclei per sample. As HSV-1 infection alters transcription by all three nuclear RNA polymerases and viral gene transcription is strongly induced during the first 9 h of infection, it is not possible to select a constant "internal" transcription control against which to measure transcription of cellular genes. Unfortunately, the use of DNA content or concentration of a viral or cellular gene (per microgram of DNA) would be useful as an internal control only in uninfected cells. As infection proceeds, viral DNA replication alters the total DNA content per cell, as well as the cellular-to-viral gene ratios. In addition, these parameters would be different after infection with viruses containing mutations in IE genes, which differ in their viral DNA replication efficiencies. Even protein levels per cell change during the infection period, as host translation is repressed and viral protein synthesis is strongly induced. Therefore, nuclear number was chosen as the best sample size control.

In mock-infected cells, sense transcription occurred within the c-*myc*, γ-actin, GAPDH, and histone H2b genes and at the 5′ end of the c-*fos* gene (Fig. 1A). The c-*myc* transcription pattern was predominantly a readthrough one, indicating that most of the transcription in exon 1 elongated into the intron 1 region. This pattern has been observed in other transformed human cell lines and is correlated with the presence of high levels of c-*myc* steady-state RNA (49). Transcription of the human c-*myc* gene is regulated, in part, by controlling the amount of promoter-paused RNAP II that is released into full-length transcription (23, 50). The c-*fos* transcription pattern was a typical transcription block pattern, indicating that most transcription at the 5′ end of the c-*fos* gene did not elongate into the exon 4 region. A block to transcription elongation in the murine and hamster c-*fos* genes has been documented, and release of this block may be one mechanism controlling the levels of steady-state c-*fos* mRNA (reviewed in reference 50). We observed increased readthrough transcription into the c-*fos* 3′ region when HeLa cells were stimulated to enter the cell cycle from quiescence (48). The HeLa cells used in these experiments were actively growing but were not serum stimulated prior to harvesting. The γ-actin gene showed a typical transcription readthrough pattern, indicating that most transcription complexes traversed the gene from the promoter region to beyond exon 4. The GAPDH and histone H2b probes were designed to detect transcription throughout the gene, and therefore transcription elongation patterns could not be determined. Antisense transcription was detected in the c-*myc* intron 1 and c-*fos* 3′ regions. The intensity of these antisense signals varied between experiments.

The buffer used in these transcription assays contained 150 mM KCl, which is adequate to release paused RNA polymerases (22, 41); therefore the run-on signals in these assays indicate the presence of both elongating and paused (but released during the assay) RNA polymerases. The inclusion of 2 μg of α-amanitin per ml in nuclear run-on transcription assays abolished transcription signals on all probes, indicating that transcription of these cellular genes was accomplished by RNAP II (data not shown). Although it has been reported that the c-*myc* gene can be transcribed by RNAP polymerase III in vitro and during transient-transfection assays (6, 53), RNA polymerase III transcription has not been detectable from the endogenous cellular c-*myc* gene in vivo or during nuclear run-on transcription assays (3, 3a).

In wild-type-HSV-1-infected cells, RNAP II transcription of all cellular genes declined over a 9-h infection period (Fig. 1A). Quantitation of nuclear run-on signals indicated that RNAP II transcription on most of these cellular genes declined to less than 11% of levels in mock-infected cells by 9 h postinfection with wild-type virus, KOS1.1 (Table 1). Exceptions were the γ-actin 5′ and 3′ regions, which retained higher levels of transcription by 9 h postinfection. All nuclear run-on transcription assays in this study were performed at least twice, and transcription levels on each probe region (expressed as a percentage of that obtained with mock-infected cells) varied as indicated in Table 1.

The transcription patterns on viral genes (Fig. 1B) were similar to those seen previously after infection of Vero and HEL cells with HSV-1 (13, 40, 55). At 3 h postinfection, sense transcription of ICP27 and ICP8 was abundant. By 6 and 9 h postinfection, high levels of sense and antisense transcription occurred in all regions of the HSV-1 genome.

RNAP II_I is the transcriptionally active form of RNAP II following HSV-1 infection. We previously hypothesized that

TABLE 1. Quantitative comparison of transcription on cellular genes following a 9-h infection with wild-type and mutant HSV-1[a]

Gene	Transcription intensity (% of mock-infected cells) (mean ± SD) for:				
	KOS 1.1	ICP22$^-$	ICP4$^-$	ICP0$^-$	ICP27$^-$
c-*myc* Ex1	4.8 ± 3.4	2.3 ± 1.3	10.7 ± 3.7	3.7 ± 5.3	17.4 ± 15.8
c-*myc* In1	4.3 ± 3.1	1.9 ± 2.6	4.4 ± 1.5	5.4 ± 1.5	14.6 ± 0.4
c-*fos* 5′	11.3 ± 5.1	15.3 ± 9.8	31.5 ± 4.0	30.5 ± 27.9	47.9 ± 19.6
γ-Actin 5′	40.8 ± 14.6	18.1 ± 13.7	40.4 ± 0.9	38.0 ± 15.1	54.8 ± 0.9
γ-Actin 3′	20.9 ± 19.6	8.2 ± 4.0	13.4 ± 1.6	20.4 ± 11.9	62.7 ± 41.7
GAPDH	8.7 ± 8.3	12.9 ± 5.7	14.4 ± 4.9	20.2 ± 9.5	80.1 ± 9.6
H2b	1.3 ± 1.7	5.6 ± 3.7	59.7 ± 4.3	17.2 ± 19.0	49.5 ± 10.7

[a] Intensities of nuclear run-on transcription signals were determined by PhosphorImager analysis. The levels of transcription are expressed as a percentage of mock infections after subtracting background hybridization to M13 single-stranded DNA. Data represent means of two independent assays (four independent assays for KOS infections) and standard deviations.

alterations in the phosphorylation of the large subunit of RNAP II may contribute to the transcription shift from cellular to viral genes that occurs following HSV-1 infection (39, 40). RNAP II occurs in two major forms in vivo, IIO and IIA, which differ in the extent of phosphorylation of the large subunit carboxy-terminal domain (CTD). (The large subunit of RNAP IIO is designated IIo, and the large subunit of RNAP IIA is designated IIa.) The IIA form binds to the promoter and enters into preinitiation complexes; the IIO form of the enzyme is involved in transcription elongation (for reviews, see references 8, 9, and 9a). Following infection of mammalian cells with HSV-1, the hyperphosphorylated (IIO) form of RNAP II is replaced by a polydisperse, intermediately migrating form (II_I) (Fig. 2A) (39, 40). It has been suggested that hyperphosphorylation of the RNAP II large subunit may be required for release of the enzyme complex from a promoter or for efficient elongation through nucleosomal DNA. Therefore, we have hypothesized that virus-induced modifications to the phosphorylation state of RNAP II may contribute to repression of transcription on cellular chromatin or to preferential transcription of viral nonnucleosomal DNA.

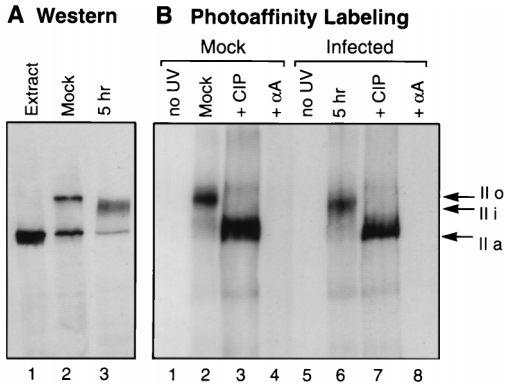

FIG. 2. Abundance and transcriptional activity of RNAP II large subunits in mock-infected and HSV-1-infected cells. (A) Vero cells were mock infected (lane 2) or infected with wild-type virus, KOS1.1, for 5 h (lane 3). Whole-cell extracts were prepared and analyzed by Western blotting. The blot was probed with monoclonal antibody ARNA3, which recognizes all forms of the large subunit of RNAP II. A nuclear extract from HeLa cells contains only the nonphosphorylated IIa form of the large subunit (lane 1). (B) Vero cells were mock infected (lane 1 to 4) or infected with wild-type virus, KOS1.1, for 5 h (lanes 5 to 8). Nuclei were isolated, and transcription was allowed to proceed in the presence of [α-^{32}P]CTP and 4-thio-UTP, as described in Materials and Methods. RNA polymerase subunits in contact with nascent RNA were UV cross-linked to 4-thio-UTP and thereby labeled with radioactive CTP. After RNase and DNase treatments, proteins were separated by SDS-PAGE and radioactive proteins were visualized by autoradiography. The positions of the hyperphosphorylated IIo (~240-kDa), the intermediately phosphorylated IIi (~220- to 230-kDa), and the nonphosphorylated IIa (~200-kDa) forms of the large subunit of RNAP II are indicated. Photoaffinity labeling performed in the presence of 2 μg of α-amanitin per ml (αA) (lanes 4 and 8) or in the absence of UV cross-linking (lanes 1 and 5) resulted in no labeling of RNAP II subunits. Treatment of photoaffinity assay products with calf intestinal alkaline phosphatase (CIP) reduced IIo and IIi to forms resembling IIa (lanes 3 and 7).

To verify that RNAP II_I was transcriptionally active in infected cells, we performed photoaffinity-labeling experiments. In this method, actively elongating RNAP II molecules are radioactively labeled by incorporating [^{32}P]CTP and 4-thio-UTP into nascent RNA and then cross-linking incorporated 4-thio-UTP to associated proteins with UV light (2, 4). Photoaffinity labeling has been used to show that RNAP IIO is the actively elongating form in vivo and in vitro (4, 24, 32).

Nuclei were isolated from Vero cells, either mock infected or infected with the wild-type virus, KOS1.1, for 5 h. Nascent RNA was labeled, and proteins were cross-linked to nascent RNA with UV light. After digestion with DNase I and RNases, the labeled proteins were visualized by SDS-PAGE (Fig. 2B). The identity of labeled bands was verified by transfer of proteins from gel to membrane and probing with monoclonal antibody ARNA3, which recognizes all phosphorylation variants of the large subunit of RNAP II (reference 39 and data not shown). The Western blot probing of this gel showed the positions of RNAP II large subunits IIo (~240 kDa), IIi (~220 to 230 kDa), and IIa (~200 kDa), as indicated. No labeling of the RNAP II large subunit occurred in the absence of UV cross-linking (Fig. 2B, lanes 1 and 5) or in the presence of α-amanitin, a specific inhibitor of RNAP II transcription elongation, at 2 μg/ml (lanes 4 and 8). In mock-infected cells, the hyperphosphorylated IIo form of the large subunit was photoaffinity labeled, consistent with previously published work (4) (lane 2). In HSV-1-infected cells, the intermediately phosphorylated IIi form was photoaffinity labeled (lane 6). Both the labeled IIo and IIi forms collapsed into forms comigrating with the nonphosphorylated IIa form after treatment of photoaffinity-labeled products with calf intestinal alkaline phosphatase (lanes 3 and 7). Although the experiment shown was performed with Vero cells, RNAP II_I was also the transcriptionally active form of RNAP II in HSV-1-infected HeLa cells (data not shown). These results indicate that RNAP II_I is the transcriptionally active form of RNAP II in HSV-1-infected cells.

Hyperphosphorylated forms of RNAP II are transcriptionally active in cells infected with the ICP22 mutant virus. We previously demonstrated that the IE protein ICP22 is required for efficient RNAP II_I induction in Vero cells, which are permissive for growth of ICP22 mutant viruses, and in HEL cells, which are restrictive. In addition, in HEL cells, ICP22 is re-

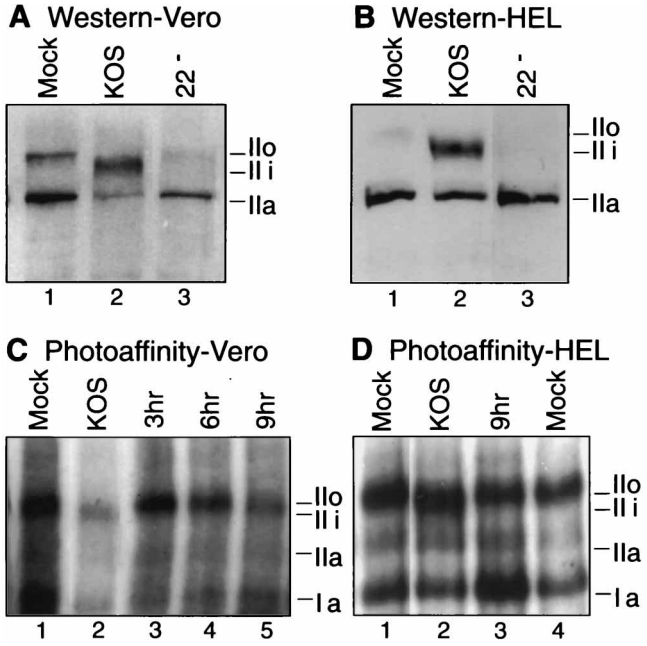

FIG. 3. Abundance and transcriptional activity of RNAP II large subunits in mock-infected cells or cells infected with wild-type or ICP22 mutant viruses. (A and B) Typical patterns of RNAP II large-subunit migration in Vero or HEL cells mock infected (lanes 1), infected with wild-type virus KOS1.1 (lanes 2), or infected with ICP22 nonsense mutant 22/*n*199 (lanes 3). At 10 h (A) or 8 h (B) postinfection, whole-cell extracts were prepared and analyzed by Western blotting. Blots were probed with monoclonal antibody ARNA3, which recognizes the large subunit of RNAP II. (C) Photoaffinity labeling of RNAP subunits in Vero cells that were mock infected (lane 1), infected with wild-type virus KOS1.1 for 9 h (lane 2), or infected with the ICP22 nonsense mutant 22/*n*199 for the times indicated. Nuclei were isolated, and photoaffinity labeling assays were performed as described in the legend to Fig. 2 and Materials and Methods. The positions of the three forms of RNAP II large subunit are indicated, as is the position of the largest subunit of RNAP I (Ia). (D) Photoaffinity labeling of RNAP subunits in HEL cells that were mock infected (lanes 1 and 4), infected with wild-type virus KOS1.1 for 9 h (lane 2), or infected with the ICP22 nonsense mutant 22/*n*199 (lane 3) for 9 h. Nuclei were isolated, and photoaffinity labeling assays were performed as described in the legend to Fig. 2 and Materials and Methods.

quired for efficient viral gene transcription (40). This raises the question of what form of RNAP II is transcriptionally active in ICP22 mutant-infected cells. To address this, we carried out photoaffinity-labeling experiments on nuclei from cells infected with the ICP22 mutant virus, 22/*n*199.

The Western blots shown in Fig. 3A and B are of whole-cell extracts from cells mock infected or infected with wild-type or ICP22 mutant virus and show the typical patterns of RNAP II large subunit migration before and after infection with HSV-1, as seen previously (39, 40). In mock-infected Vero and HEL cells, both IIa and IIo forms of the large subunit are visible, although the IIo form is less abundant in HEL cells than in HeLa cells (Fig. 3A and B, lanes 1). Infection with the wild-type virus, KOS1.1, results in the loss of IIo and its replacement with the intermediately migrating IIi form (Fig. 3A and B, lanes 2). When Vero cells are infected with an HSV-1 strain containing a nonsense mutation in the gene encoding ICP22, the IIo form is depleted; however, it is not replaced by a clearly visible IIi form. Despite this lack of a readily detectable IIi form, hyperphosphorylated forms of the large subunit that extend from the IIa to the IIo positions are sometimes detectable (Fig. 3A, lane 3) (40). Vero cells are permissive for growth of the ICP22 mutant virus, and viral gene transcription appears normal in Vero cells infected with this mutant (40). When HEL cells were infected with the ICP22 mutant virus, no hyperphosphorylated forms of the RNAP II large subunit were readily evident on Western blots (Fig. 3B, lane 3). HEL cells are restrictive for growth of the ICP22 mutant virus, and viral DE and L gene transcription is impaired in HEL cells infected with the ICP22 mutant (40).

Nuclei were isolated from Vero or HEL cells that were either mock infected or infected with the ICP22 mutant virus, 22/*n*199. Photoaffinity-labeling assays were performed as described above, and the labeled, elongating RNAP II subunits were visualized by SDS-PAGE and autoradiography. In both mock-infected Vero cells and mock-infected HEL cells, the IIo form of the large subunit of RNAP II was photoaffinity labeled (Fig. 3C, lane 1; Fig. 3D, lanes 1 and 4). The labeled species that migrated at approximately 180 kDa (Fig. 3C and D) has been previously identified as the largest subunit of RNA polymerase I, based on antibody affinity and labeling in the presence of α-amanitin (4).

In both Vero and HEL cells infected with the wild-type virus, KOS1.1, the IIi form of the large subunit was photoaffinity labeled (Fig. 3C and D, lanes 2). In Vero cells infected with the ICP22 mutant, transcriptionally active hyperphosphorylated forms of the large subunit of RNAP II were labeled at 3, 6, and 9 h postinfection (Fig. 3C, lanes 3 to 5), even though these were not readily visible in Western blot analyses of whole-cell extracts from Vero cells infected with the ICP22 mutant (Fig. 3A, lane 3). Similarly, hyperphosphorylated forms of the RNAP II large subunit were photoaffinity labeled in HEL cells infected with the ICP22 mutant, up to 9 h postinfection (Fig. 3D, lane 3), although no discrete hyperphosphorylated forms are visible on Western blots of whole-cell extracts from HEL cells infected with the ICP22 mutant (Fig. 3B, lane 3). As the recovery of labeled material differs between samples, it is not possible to make a quantitative comparison of RNAP II labeling in these assays.

These data show that hyperphosphorylated forms of the large subunit of RNAP II are reduced in abundance following ICP22 mutant infections; however, some fraction of the hyperphosphorylated forms that remain are involved in transcription elongation. We do not yet know what proportion of these elongating forms of RNAP II are involved in transcription of viral and cellular DNA or what proportion of the total amount of RNAP II in infected cells is involved in transcription elongation. The hyperphosphorylated forms of the large subunit that become labeled during photoaffinity-labeling experiments of ICP22 mutant-virus-infected cells must be minor components of the RNAP II pool, based on their abundance in Western blot analyses.

Host RNAP II transcription is repressed in cells infected with the ICP22 mutant virus. Earlier reports by Kemp and Latchman (20) indicated that transcription repression of several unidentified cellular cDNAs requires the expression of ICP22. In addition, we have found that induction of RNAP II_I requires the presence of ICP22, and that in HEL cells infected with an ICP22 mutant, the defect in RNAP II phosphorylation is accompanied by reduced viral gene transcription (40). To directly examine whether ICP22 affects the shutoff of transcription on known cellular RNAP II genes, HeLa or HEL cells were mock infected or infected with the ICP22 mutant virus, 22/*n*199 and nuclear run-on transcription assays were carried out with the same cellular gene probes as before (Fig. 4). The patterns of transcription in mock-infected cells were similar to those seen in Fig. 1. As infection with the ICP22 mutant proceeded, transcription signals on all probes declined similarly to that observed after infection with the wild-type virus (Fig. 4A and B; Table 1). Therefore, virus-induced shutoff of host cell

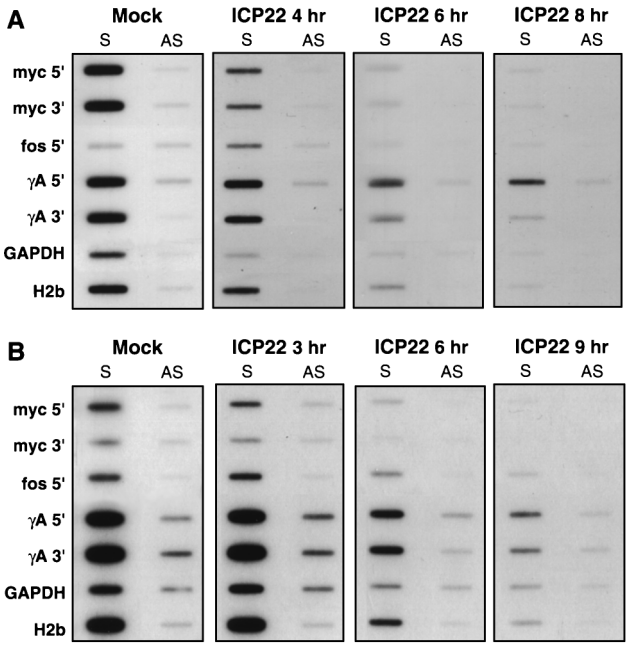

FIG. 4. Nuclear run-on transcription analysis of cellular gene transcription in cells infected with the ICP22 nonsense mutant 22/*n*199. HeLa cells (A) or HEL cells (B) were either mock infected or infected with the ICP22 mutant virus, and nuclei were isolated at the times indicated. Assays were performed on equal numbers of nuclei per sample. RNA products of each sample were hybridized to filters containing single-stranded M13 DNA probes which detect sense (S) or antisense (AS) transcripts arising from regions of the human c-*myc*, c-*fos*, γ-actin, GAPDH, and histone H2b genes.

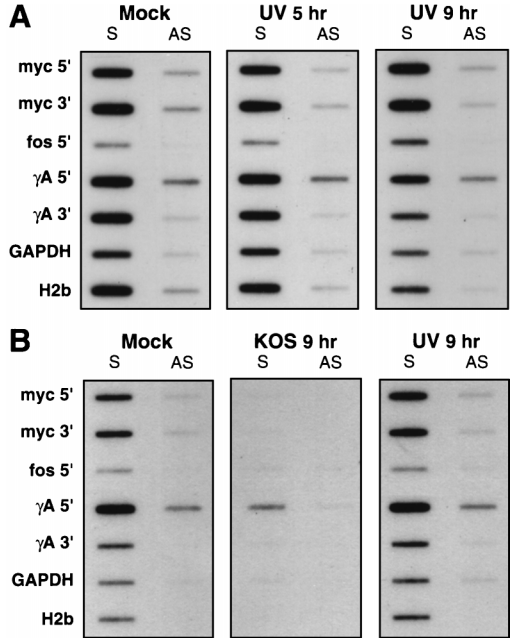

FIG. 5. Nuclear run-on transcription analysis of cellular gene transcription in cells infected with UV-inactivated virions or the wild-type virus, KOS1.1. (A) HeLa cells were either mock infected or infected with UV-inactivated virions for the times indicated. (B) In a separate experiment, cells were mock infected or infected with wild-type virus or UV-inactivated virions for 9 h. Assays were performed on equal numbers of nuclei per sample and analysed as described in the legend to Fig. 4.

genes occurs similarly after infection with both wild-type and ICP22 mutant viruses, even in cells restrictive for growth of the ICP22 mutant.

Efficient shutoff of host RNAP II transcription requires expression of the HSV-1 genome. The above data suggest that the presence of functional ICP22 and ICP22-dependent induction of RNAP II_I are not necessary for shutoff of host RNAP II transcription. To define which viral functions are necessary for host transcription repression, we performed nuclear run-on transcription assays on nuclei isolated from cells infected with UV-inactivated virus. Infection of cells with UV-inactivated virions allows one to define the relative contributions of virion components and viral gene expression to viral regulatory effects (39). Appropriate treatment of HSV-1 virions with short-wave (254-nm) UV light prevents transcription of viral genes by cross-linking viral DNA but does not damage virion proteins. Virion components that affect host functions include the Vhs protein, which triggers host translation shutoff, and the VP16 protein which activates transcription of the IE genes.

HeLa cells were mock infected or infected with UV-inactivated virions. The UV-inactivated virions were functional for VP16, as assayed by their ability to transactivate an IE promoter (39). The nuclei were isolated, and nuclear run-on transcription assays were performed on equal numbers of nuclei per sample. Transcription of viral genes, up to 9 h postinfection, was undetectable by nuclear run-on transcription assays (data not shown). Cellular gene transcription appeared unchanged by 5 h postinfection in cells infected with UV-inactivated virions but was marginally altered over some probes by 9 h postinfection (Fig. 5; Table 2). Transcription over the 5′ regions of the c-*myc* and c-*fos* genes was slightly elevated, compared with transcription in mock-infected cells. Transcription over the γ-actin, GAPDH, and histone H2b probes was variable between experiments, showing either modest transcription induction or repression following infection with UV-inactivated virions. These results suggest that virion components, in the absence of viral gene expression, contribute modestly, if at all, to repression of cellular gene transcription.

Repression of host RNAP II transcription requires IE gene expression. To define which aspects of viral gene expression are responsible for shutoff of host RNAP II transcription, we performed nuclear run-on transcription assays on nuclei isolated from cells infected with a virus bearing a null mutation in the IE gene encoding ICP4. Infection of cells with an ICP4 mutant virus allows one to define the relative effects of IE and DE/L gene expression. ICP4 mutant viruses express the IE genes (except for ICP4), as well as one DE gene, ICP6. How-

TABLE 2. Transcription of cellular genes following a 9-h infection with UV-inactivated virions[a]

Gene	Transcription intensity (% of mock-infected cells)		
	Expt A	Expt B	Mean ± SD
c-*myc* Ex1	142.6	131.3	136.9 ± 7.9
c-*myc* In1	106.6	86.8	96.7 ± 14.0
c-*fos* 5′	162.7	114.5	138.6 ± 17.0
γ-Actin 5′	46.8	148.6	97.7 ± 71.9
γ-Actin 3′	33.3	162.7	98.0 ± 91.5
GAPDH	74.4	154.5	114.5 ± 56.6
H2b	47.5	143.9	95.7 ± 68.2

[a] Intensities of nuclear run-on transcription signals were determined by PhosphorImager analysis of run-on transcription assays shown in Fig. 5. The levels of transcription are expressed as a percentage of the mock infections after subtracting background hybridization to M13 single-stranded DNA.

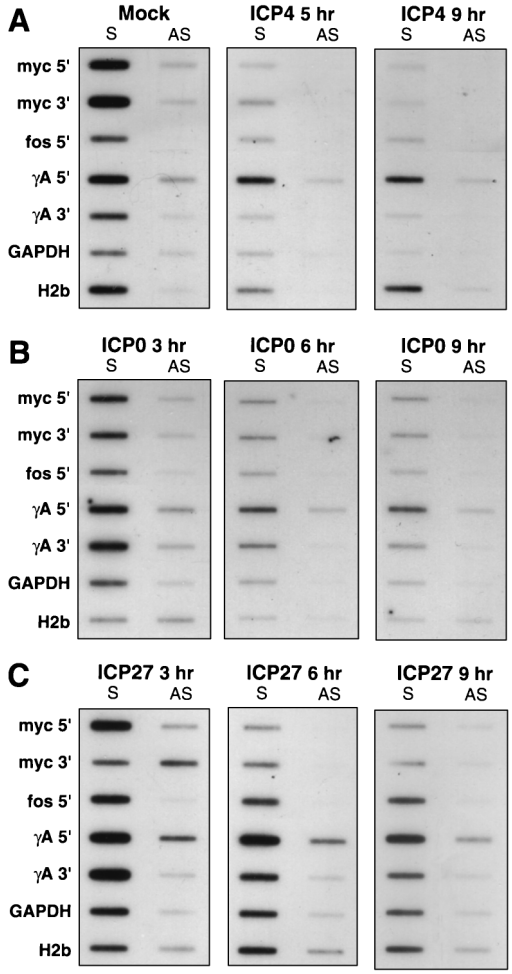

FIG. 6. Nuclear run-on transcription analysis of cellular gene transcription in cells infected with the ICP4, ICP0, or ICP27 mutant virus. HeLa cells were either mock infected or infected with the ICP4 null mutant, *d*120 (A), the ICP0 nonsense mutant, *n*212 (B), or the ICP27 null mutant, *d*27-1 (C). Assays were performed as described in the legend to Fig. 4.

ever, as ICP4 is required for all further viral gene expression, infections with ICP4 mutants are arrested at the IE stage.

HeLa cells were mock infected or infected with the ICP4 null mutant virus, *d*120. Nuclei were isolated, and run-on transcription assays were performed on equal numbers of nuclei per sample (Fig. 6A). Assays of viral gene transcription in ICP4 mutant-infected cells showed active transcription of the IE gene ICP27 but no detectable transcription in the DE gene ICP8 or in the L gene gC (data not shown). Cellular gene transcription was reduced by 5 and 9 h postinfection in a pattern similar to reductions seen after infection with wild-type virus (Fig. 6A; Table 1). Exceptions were the c-*fos* and histone H2b genes, which were transcriptionally more active after infection with the ICP4 mutant than after infection with the wild-type virus. These results show that the activities of one or more viral IE proteins are sufficient to obtain full transcription repression of most of the cellular genes that we examined. However, ICP4 itself, or a viral function activated by ICP4, may be necessary for full transcription shutoff of the histone H2b and c-*fos* genes.

Expression of the ICP0 and ICP27 genes contributes to repression of host RNAP II transcription. Having demonstrated that wild-type levels of transcription repression over most of the genes assayed requires the expression of viral IE genes, we examined the contributions of the IE gene products, ICP0 and ICP27. HeLa cells were mock infected or infected with viruses with mutations in genes encoding ICP0 and ICP27. Nuclear run-on transcription assays were performed on equal numbers of nuclei in each sample (Fig. 6B and C).

In cells infected with the ICP0 mutant, repression of cellular gene transcription appeared to be similar to that in cells infected with wild-type virus by 9 h postinfection (Fig. 6C; Table 1). One possible exception was the c-*fos* 5′ region, which was transcriptionally repressed to a lesser degree after infection with the ICP0 mutant virus. In cells infected with the ICP27 mutant, repression of cellular gene transcription was somewhat different from that in wild-type-infected cells (Fig. 6B; Table 1). At 9 h postinfection, levels of c-*myc* and γ-actin gene transcription in ICP27-mutant-infected cells were similar to those in wild-type-infected cells; however, transcription of the c-*fos* 5′, GAPDH, and histone H2b genes remained higher than in wild-type-infected cells. These results suggest that ICP27 gene expression may be required for full transcription repression of the c-*fos*, GAPDH, and histone H2b genes.

Finally, cells infected with viruses with mutations in the DE gene encoding ICP6 (which is expressed in ICP4 mutant infections) were transcriptionally repressed similarly to those infected with wild-type virus (data not shown).

DISCUSSION

HSV-1-induced repression of host RNAP II transcription occurs in gene-specific ways. The above data indicate that virus-induced repression of host RNAP II transcription occurs primarily through the effects of multiple IE proteins. They also show that cellular genes respond somewhat differently to these influences. To more easily compare transcription patterns on individual genes and gene regions, we present the data from Table 1 graphically in Fig. 7.

As seen in Fig. 7, the c-*myc*, c-*fos*, and γ-actin genes are repressed transcriptionally by both wild-type virus and viruses with mutations in each IE gene, although ICP4 and ICP27 (and possibly ICP0) mutant viruses are somewhat deficient in shutoff of c-*fos* transcription. As mutation in any individual IE gene does not fully restore transcription, it is possible that IE gene products are redundant in their effects on c-*myc*, c-*fos*, and γ-actin transcription repression. Alternatively, transcription repression of these genes may result directly or indirectly from the act of transcription of the viral genome independent of any specific function carried out by viral IE proteins. Infection with the ICP4 mutant virus results in efficient repression of host transcription; however, transcription of the viral genome is limited to that initiating on the five IE genes plus the IE-like gene encoding ICP6. As our infections are performed at an MOI of 10 with a particle-to-PFU ratio of approximately 10:1 to 100:1 anywhere from 600 to 6,000 IE promoters per cell could be engaged by RNAP II during an ICP4 mutant infection. It is possible that the establishment of RNAP II transcription initiation complexes on these promoters titrates limiting factors that are necessary for the transcription of many host genes. It is unlikely that transcription repression of c-*myc*, c-*fos*, and γ-actin genes is due merely to the presence of HSV-1 DNA in the nucleus. If this were the case, infection with UV-inactivated virions would be expected to result in transcription repression.

The observation that the c-myc exon 1 and intron 1 regions are transcriptionally repressed in parallel following HSV-1 infection suggests that repressive effects on c-*myc* transcription

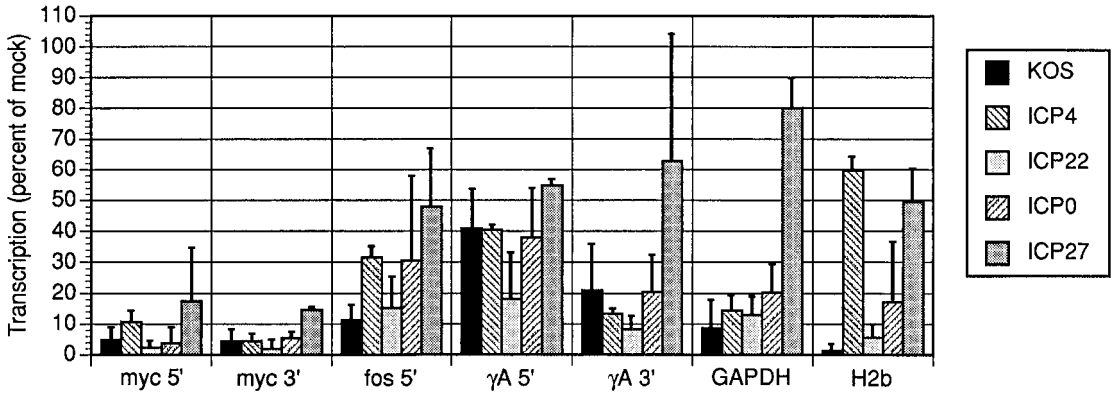

FIG. 7. Graphic representation of data from Table 1 showing transcription of cellular genes in cells infected with wild-type and mutant HSV-1. Transcription levels are expressed as a percentage of levels in mock-infected cells after subtraction of background hybridization to M13 single-stranded DNA. Standard deviations are indicated by vertical lines in each column.

are occurring via transcription initiation and not via transcription elongation alone.

Virus-induced repression of GAPDH transcription differs from that of c-*myc*, c-*fos*, or γ-actin in that the ICP27 mutant appears to be significantly defective in repression of GAPDH transcription. This suggests that ICP27 may play an important role in the transcriptional shutoff of this gene. Transcription of the histone H2b gene is repressed similarly following infection with wild-type viruses and the ICP0 and ICP22 mutant viruses. However, transcription shutoff is not as efficient after infection with the ICP4 and ICP27 mutant viruses, suggesting that full transcription repression of the histone H2b gene requires expression of the ICP4 and ICP27 genes.

Taken together, our results indicate that cellular genes respond somewhat differently to virus-induced repression of host RNAP II transcription. Each gene we have examined differs in its overall level of repression and in the relative contributions of IE genes to complete repression. Although we do not know the basis for these gene-specific differences, it is possible that the viral IE transactivator proteins ICP4 and ICP0 contribute both induction effects and repression effects on some genes in certain mutant backgrounds, altering their repression profiles.

The role of IE gene expression in the repression of host RNAP II transcription. The data in this paper indicate that repression of host RNAP II transcription by HSV-1 does not occur by a single, unified mechanism but is a complex, multifaceted process that is mediated by multiple IE proteins. Although the degree of repression or activation by UV-inactivated virus varies somewhat in our experiments, it appears that virion components alone are not sufficient to bring about significant transcription repression of cellular genes. However, they may act in concert with the IE genes to bring about full repression of host RNAP II transcription.

The viral IE protein which appears to have the most striking transcriptional effects is ICP27, since full transcription repression of the GAPDH and histone H2b genes is dependent upon ICP27 and since ICP27 may contribute to transcription repression of the c-*fos* 5′ region. These observations are somewhat surprising, as ICP27 is believed to be a posttranscriptional regulator of viral gene expression and to date has not been conclusively linked to transcriptional regulation. It has been noted previously that viral ICP27 mutants fail to fully repress cellular gene expression at the levels of protein synthesis and mRNA accumulation (15, 42). Since ICP27 inhibits pre-mRNA splicing in vitro (16) and alters the nuclear localization of splicing factors (33), it has been speculated that its effects on host shutoff result from inhibition of mRNA splicing. However, our results suggest that ICP27 may cause the transcriptional repression of certain cellular genes. This may be a direct effect of ICP27 on transcription, distinct from its effects on splicing. Another possibility is that repression of pre-mRNA processing by ICP27 feeds back to indirectly inhibit transcription. However, it is noteworthy that full repression of histone H2b gene transcription requires ICP27, yet this gene is devoid of introns and its mRNA is not polyadenylated. This would argue for a direct effect of ICP27 on transcription, at least for this cellular gene. Another possibility is that ICP27 acts indirectly by inducing DE or L viral genes, which are involved in transcription repression. However, our data from the ICP4 mutant infection indicate that IE gene expression alone is sufficient to repress host RNAP II transcription, indicating that DE and L genes are not required.

The IE protein ICP4 also appears to contribute to full transcription repression of some cellular genes, particularly c-*fos* 5′ and histone H2b. ICP4 is known to be a potent transcriptional activator of viral genes and transfected promoters and is absolutely required for viral gene expression at the transcriptional level (10, 13; reviewed in reference 46). It also represses the transcription of promoters that contain ICP4 binding sites near their transcription start sites (30). The ability of ICP4 to both activate and repress transcription may be related to its capacity to form complexes with the RNAP II basal transcription factors TFIIB, TATA-binding protein (TBP), and the TAF250 subunit of TFIID (5, 14, 47). Given the clear involvement of ICP4 in viral transcription regulation, it might also be expected to affect transcription of endogenous cellular genes. However, transcription repression of the host genes we examined is fairly efficient even in the absence of ICP4.

Our observation that repression of host gene transcription depends upon IE gene expression is in agreement with the results of an earlier study that examined the effects of HSV-1 infection on transcription of adenovirus-specific RNAs in a cell line transformed by adenovirus (52). Adenovirus-specific transcription is inhibited by 75% after infection of cells with either wild-type HSV-1 or an ICP4 mutant virus. Our results are also consistent with those of Smiley et al. (45). In their study, they observed strong transcription repression of the β-globin gene in differentiated MEL cells after infection with both wild-type HSV-1 and the ICP4 null mutant, *d*120. This also suggests that IE gene products, but not ICP4 alone, are required for host

transcription repression. Our observations, however, differ from those of Kemp and Latchman (20). In their study, transcription repression of a number of unidentified cellular cDNAs was dependent upon expression of ICP22. In our studies, ICP22 has little if any effect on the transcription repression of host genes. It is not clear why our results differ; however, it may be due to the use of different cell lines and virus strains.

Role of RNAP II modifications in repression of host RNAP II transcription. Previously, we showed that ICP22 expression is necessary for modification of RNAP II to the intermediately phosphorylated II_I form and in restrictive lines for efficient viral L gene transcription (39, 40). We have hypothesized that modifications to RNAP II_I may contribute to shifts in transcription after viral infection; however, in this study, we find that the presence of ICP22 and abundant RNAP II_I is unnecessary for efficient shutoff of host transcription, even in cell lines where ICP22 mutants replicate poorly. Our conclusions are based on Western blot analyses, which show that RNAP II_I is depleted or absent after ICP22 mutant infections but that host transcription is repressed similarly after infection with wild-type or ICP22 mutant viruses. Although ICP22 and RNAP II_I may enhance transcription of the viral genome, shutoff of host gene transcription must be triggered by other viral components.

It is interesting that RNAP IIO, which is the hyperphosphorylated elongating form of the enzyme in uninfected cells, is depleted in abundance after infection with all wild-type and mutant viruses, including ICP22 mutant viruses (40). However, it is not depleted after infection with UV-inactivated virions (39). Both depletion of RNAP IIO and repression of host transcription require expression of two or more IE genes, and IE genes are redundant in their effects on both transcription repression and RNAP IIO abundance. These data indicate that depletion of RNAP IIO is consistently correlated with efficient repression of host transcription, suggesting that the two events may be functionally linked.

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