Herpes Simplex Virus Immediate-Early Protein ICP22 Is Required for Viral Modification of Host RNA Polymerase II and Establishment of the Normal Viral Transcription Program

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Infection of cells with herpes simplex virus type 1 (HSV-1) results in a rapid alteration of phosphorylation on the large subunit of cellular RNA polymerase II (RNAP II), most likely on its C-terminal domain (S. A. Rice, M. C. Long, V. Lam, and C. A. Spencer, *J. Virol.* 68:988–1001, 1994). This phosphorylation modification generates a novel form of the large subunit which we have designated Ili. In this study, we examine the roles that HSV-1 gene products play in this process. An HSV-1 mutant defective in the immediate-early transcriptional activator protein ICP4 is able to efficiently induce Ili. Viruses having mutations in the genes for the ICP0, ICP6, or ICP27 proteins are also competent for Ili formation. In contrast, 22/n199, an HSV-1 mutant which contains a nonsense mutation in the gene encoding the immediate-early protein ICP22, is significantly deficient in Ili induction. This effect is seen in Vero cells, where 22/n199 grows relatively efficiently, and in human embryonic lung (HEL) cells, where 22/n199 growth is more restricted. RNAP II is recruited into viral replication compartments in 22/n199-infected cells, indicating that altered phosphorylation of RNAP II is not a prerequisite for nuclear relocation of RNAP II. In addition, we show by nuclear run-on transcription analysis that viral gene transcription is deficient in HEL cells infected with 22/n199. Viral late gene transcription does not occur efficiently, and antisense transcription throughout the genome is diminished compared with that of the wild-type HSV-1 infection. These transcriptional effects cannot be explained by differences in viral DNA replication, since 22/n199 replicates its DNA efficiently in HEL cells. Our results demonstrate that ICP22 is necessary for virus-induced aberrant phosphorylation of RNAP II and for normal patterns of viral gene transcription in certain cell lines.

Herpes simplex virus type 1 (HSV-1), a common human herpesvirus, provides a useful model to study various aspects of herpesvirus gene expression and regulation (for a review, see reference 47). Like all herpesviruses, HSV-1 replicates in the nucleus of its host cell, and to a large extent depends on the cell's synthetic machinery for the expression of its genes. Transcription of the viral genes is mediated by host RNA polymerase II (RNAP II). The genome of HSV-1 is a linear double-stranded DNA molecule, ~152 kb in size, encoding approximately 75 genes. For the most part, the HSV-1 genes are similar in structure to cellular genes, possessing typical eukaryotic promoter and polyadenylation sequences. During lytic infection, the viral genes are induced to high levels, while expression of cellular genes is greatly suppressed (for a review, see reference 56). Moreover, HSV-1 gene expression is tightly controlled. The viral genes have been grouped into three classes on the basis of the temporal order of, and requirements for, their expression (24). The first genes to be expressed are the immediate-early (IE; also called α) genes. Their expression does not require newly synthesized viral proteins but does require the action of the virion component VP16. The second class of genes are the delayed-early (DE; also called β) genes. The expression of DE (and later) genes is dependent upon newly synthesized IE gene products, in particular the ICP4

protein, which is an essential transcription activator. Many of the DE gene products encode enzymes involved in viral DNA replication, and DNA synthesis begins once these proteins accumulate. Viral DNA replication triggers the expression of the last set of viral genes, the late (L; also called γ) genes. Most of the L genes encode virion components. The L genes are subdivided into two classes, leaky-L (γ_1) and true-L (γ_2), depending on whether they are partially (leaky-L) or wholly (true-L) dependent on viral DNA replication for their expression.

Many of the changes in gene expression in HSV-1-infected cells are the consequence of transcriptional regulation. Direct evidence for transcriptional regulation comes from nuclear run-on transcription assays which demonstrate that the transcription rates of most cellular genes decline after infection (26, 54), while the transcription rates of most viral genes correlate temporally with viral protein synthesis levels (20, 64). Indirect evidence for transcriptional regulation comes from promoter-swapping experiments performed in the context of HSV-1 recombinant viruses (41, 53). These studies suggest that temporal control of many viral genes is conferred largely by their 5' promoter-regulatory regions. However, transcriptional control of HSV-1 genes must operate at levels beyond those dictated by 5' promoter regions, since viral genes or promoters outside the context of the viral genome are regulated quite differently from viral genes in their natural context (53). Furthermore, cellular genes embedded in the viral genome are not transcriptionally repressed in parallel with their cellular counterparts but instead are transcriptionally activated (35, 54, 57).

The *trans*-acting regulatory factors which determine tran-

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scriptional specificity in HSV-1-infected cells have only been partially elucidated. It is clear that the IE protein ICP4 is a critical component of HSV-1 transcriptional regulation, being a necessary factor for transcriptional induction of DE and L genes (14, 63). ICP4 has nonspecific DNA-binding activity (19) but exhibits a marked preference for particular DNA sequences (17). ICP4 interacts *in vitro* with the TATA-binding protein and the basal transcription factor TFIIB (58) and may assist in the formation of transcription preinitiation complexes on viral promoters. Although ICP4's DNA-binding activity appears necessary for its transcriptional activation function (36, 52), the mechanism by which ICP4 is specifically directed to viral DE and L genes is unclear, since many HSV-1 genes lack ICP4 binding sites in the vicinity of their promoters, and in one DE promoter which does have ICP4 binding sites, these sites can be removed without consequence (55).

A central question concerning transcriptional regulation in HSV-1-infected cells is how the viral DE and L promoters are activated in the apparent absence of specific binding sites for ICP4 or other viral *trans*-acting factors. A related issue concerns the mechanism by which cellular gene transcription is specifically repressed following HSV-1 infection. One possible mechanism to account for these global shifts in transcription is that HSV-1 infection modifies RNAP II, altering its function in a manner that allows genes residing in the viral genome to be preferentially transcribed (56). Consistent with this hypothesis, we recently discovered that HSV-1 infection results in two striking modifications to RNAP II (46). First, infection causes the recruitment of the enzyme from a relatively diffuse nuclear distribution into virus-induced subnuclear replication compartments. Second, infection induces the appearance of an abnormally phosphorylated form of RNAP II, as described below.

Eukaryotic RNAP II is a complex enzyme consisting of at least 10 subunits (for a review, see reference 66). The largest subunit is of particular interest because it contains sites for catalysis, RNA binding, and DNA binding. In addition, the large subunit possesses an unusual repeating structure at its C terminus known as the C-terminal domain (CTD) (for a review, see references 11–13). The CTD is composed of up to 52 repeats of the heptapeptide consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser, is conserved from yeasts to humans, and is required for cell viability. *In vivo*, RNAP II exists in two discrete forms, IIA and IIO, which differ only in the extent of CTD phosphorylation. The two forms of the large subunit found in IIA and IIO are designated IIA and IIO, respectively, and differ in their electrophoretic mobilities when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). IIA is an unphosphorylated form, while IIO is hyperphosphorylated on the CTD and on serine, threonine, and tyrosine residues (3, 4, 13). Substantial evidence indicates that the IIA and IIO forms of RNAP II are associated with different steps in the transcription cycle (13, 67). It is the unphosphorylated IIA form that is recruited to the preinitiation complex (30, 38), possibly via interactions with basal or specific transcription factors (1, 27, 62). In contrast, IIO is the form which is actively involved in RNA chain elongation (4, 30, 38). Biochemical evidence suggests that phosphorylation of IIA to IIO may be coincident with the act of transcriptional initiation (38) or with the conversion of an initiated but paused RNAP II into an elongation-competent form (34). After completion of a round of transcription, IIO must be dephosphorylated to IIA before RNAP II can reinitiate a second round (7). Although several mammalian kinases and phosphatases which can modify the CTD *in vitro* have been identified (3, 7, 9, 16, 18, 22, 31, 37, 48), the enzymes responsible for *in vivo* modification are as yet unknown.

As mentioned above, we found that HSV-1 infection results in the altered phosphorylation of RNAP II (46). Specifically, infection causes a general depletion of the IIA and IIO forms of the RNAP II large subunit and the appearance of a set of polydisperse forms which migrate on SDS-polyacrylamide gels at positions intermediate between IIA and IIO. For convenience, we refer to these new species as a single form, designated Iii (for intermediately migrating). *In vitro* phosphatase treatment of Iii converts it to IIA, indicating that it is a phosphorylation variant of the large subunit. Since phosphorylation of the large subunit appears to occur exclusively on the CTD (4), it is likely that Iii represents a form which contains intermediate levels of CTD phosphorylation. Our previous work also indicated that HSV-1 protein synthesis is required for the generation of Iii, since Iii is not induced when infection is carried out with UV-inactivated HSV-1 stocks or in the presence of protein synthesis inhibitors. This suggests that one or more newly synthesized viral gene products are specifically involved in Iii induction.

In this study, we demonstrate that efficient induction of the Iii form requires ICP22, an HSV-1 IE protein which has not been extensively characterized. Furthermore, in HEL cells infected with an HSV-1 ICP22 mutant, the defect in RNAP II phosphorylation is accompanied by a dramatic reduction in viral gene transcription. Our results suggest that ICP22 may alter transcriptional specificity in HSV-1-infected cells by modifying the function of RNAP II via a posttranslational modification.

MATERIALS AND METHODS

Cells, viruses, and infections. Vero (African green monkey kidney) and HEL 299 (human embryonic lung) cells were used for infections. Vero and HEL cells were obtained from the American Type Culture Collection, Rockville, Md., and were grown as monolayer cultures. The cells were propagated in Dulbecco modified Eagle's medium containing 10% heat-inactivated fetal calf serum. All tissue culture reagents were purchased from Gibco-BRL.

KOS1.1 (25) was the wild-type (WT) HSV-1 strain used in these experiments. The ICP4 mutant *d120* (14) was obtained from Neal DeLuca (University of Pittsburgh School of Medicine) and was grown and titered on E5 cells (14). The ICP6 mutant ICP6Δ (21) was obtained from Sandy Weller (University of Connecticut Health Center) and was grown and titered on Vero cells at 34°C. *n212* (5), an HSV-1 mutant containing a nonsense mutation in both copies of the ICP0 gene, was grown and titered in 0-28 cells (49). *d27-1* (45), an ICP27 deletion mutant, was grown and titered on V27 cells (45). Finally, 22/*n199* (2), a mutant containing a nonsense mutation in the ICP22 gene, and 22/*n199R*, a marker-rescued derivative of 22/*n199*, were grown and titered in Vero cells. The 22/*n199* mutant contains a 16-bp oligonucleotide linker sequence containing stop codons in all three reading frames inserted into the single *PvuII* site in the ICP22 coding region. The ICP22 gene in 22/*n199* encodes a truncated ICP22 protein consisting of the N-terminal 199 amino-terminal residues of the 420-residue ICP22 protein.

In all experiments, cells were infected with HSV-1 at a multiplicity of infection of 10 PFU per cell in phosphate-buffered saline (PBS) containing 0.1% glucose and 1% heat-inactivated newborn calf serum. The virus inoculum was allowed to adsorb to the cells for 1 h at 37°C. The inoculum was then replaced with medium 199 containing 1% newborn calf serum, and the cultures were incubated at 37°C.

Western blots and immunofluorescence. Western blotting (immunoblotting) was performed as described previously (46). Briefly, cells were mock infected or infected with WT or mutant viruses. At various times postinfection, cells were scraped in PBS containing protease inhibitors (50 µg of *N*-*p*-tosyl-L-lysine chloromethyl ketone and 25 µg of phenylmethylsulfonyl fluoride per ml), and lysed into an SDS-polyacrylamide gel sample buffer. In some experiments, the following phosphatase inhibitors were included in the scraping buffer: 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM tetrasodium phosphate, 20 mM EDTA, and 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid]. The presence of phosphatase inhibitors did not appear to affect the forms of the RNAP II large subunit detected by Western blotting. Proteins were separated by SDS-6% PAGE, transferred to Hybond ECL membranes, and probed with primary antibodies 8WG16 (61) and/or ARNA3 (28) and secondary antibody horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G. 8WG16 was obtained from Nancy Thompson (University of Wisconsin), ARNA3 was purchased from Cymbus Bioscience Ltd., Southampton, United Kingdom, and HRP-conjugated goat anti-mouse was purchased from Jackson ImmunoResearch Labs. The RNAP II large subunit was visualized by the ECL Western blot detection system from Amersham.

For immunofluorescence, Vero or HEL cells were grown to confluence on glass coverslips and infected. Immunofluorescence was performed as described previously (46) with the 8WG16 antibody. In some cases, replication compartments in infected cells were identified by costaining with 3-83, a polyclonal rabbit antiserum directed against ICP8. 3-83 was a gift of David Knipe, Harvard Medical School, Boston, Mass.

Nuclear run-on transcription assays. HEL or Vero cells were infected with either WT virus or 22/n199. Nuclei were isolated, and assays were performed as described previously (59). In brief, transcription was allowed to proceed in the presence of [³²P]UTP and RNA products were purified and hybridized to nitrocellulose filters (GeneScreen-Plus) bearing single-stranded bacteriophage M13 DNA probes specific for various HSV-1 genes. The probes were designed to detect RNAs corresponding to either sense or antisense transcription in the gene region of interest. The M13 probes for the ICP4, ICP8, ICP5, and gC genes have been described previously (20). The probes for the ICP27 gene were M13 mp18 and M13 mp19 clones containing a 1.2-kb *Bam*HI-*Sal*I insert taken from the 5' half of the HSV-1 ICP27 gene and were obtained from L. Su and D. Knipe. The probes for the VP16 gene were M13 mp18 clones containing a 1.2-kb *Sal*I insert from the HSV-1 VP16 (UL48) gene. The UL36 probes were M13 mp18 and M13 mp19 clones containing a 1.3-kb *Hind*III-*Bam*HI insert from the 5' end of the UL36 gene. Radioactivity hybridizing to each probe was quantitated with a Fujix BAS100 bio-imaging analyzer with MacBAS imaging software.

Analysis of viral DNA replication. HEL cells were infected with WT virus or 22/n199. After a 1-h adsorption at 37°C, the cells were washed extensively with medium to remove unadsorbed virus. Total DNA was prepared either immediately or at 3, 6, or 9 h postinfection (hpi) by the method described by Challberg (6). Five micrograms of each DNA preparation was then digested with *Bam*HI, electrophoresed on a 1% agarose gel, and transferred to a GeneScreen-Plus filter. Hybridization was carried out following standard procedures with the ³²P-labeled plasmid pBICP5, which contains a 1,544-bp HSV-1 *Bam*HI fragment inserted into pUC19. ³²P labeling of the probe was done by the random primer method. The relative radioactivity in each band of the Southern blot was quantitated with a Fujix BAS100 bio-imaging analyzer with MacBAS imaging software.

Artwork for Fig. 1 to 7. Autoradiographs were scanned on an Abaton 300 color scanner, with Adobe Photoshop software, and images were saved in TIFF. Images were assembled and labeled with QuarkXpress 3.0 software on a Macintosh IIx.

RESULTS

HSV-1-induced modification of RNAP II does not require ICP4. Previously, we found that a novel phosphorylated form of the RNAP II large subunit, termed Ili, appears within 3 to 4 h of HSV-1 infection (46). Experiments using metabolic inhibitors or UV-inactivated virus demonstrated that viral protein synthesis is required for the induction of Ili. In addition, Ili formation occurs in cells infected with a viral ICP8 gene deletion mutant, which is unable to replicate viral DNA or to efficiently express L genes. Together, our previous results suggest that expression of one or more HSV-1 IE or DE proteins (other than ICP8) is required for the induction of Ili.

To identify HSV-1 gene products involved in Ili induction, we first asked whether the IE protein ICP4, which is required for transcription of DE and L genes, is necessary for the formation of Ili. To test the effect of ICP4, we infected cells with the HSV-1 ICP4 deletion mutant *d120*, which encodes a severely truncated ICP4 molecule incapable of its transcriptional activation function (14). Total cell proteins from WT (strain KOS1.1)- or *d120*-infected Vero cells were prepared at 3, 6, and 9 hpi and subjected to SDS-PAGE and immunoblotting with a mixture of two monoclonal antibodies directed against the RNAP II large subunit. The monoclonal antibody 8WG16 (61) recognizes an epitope on the CTD; it reacts with the Ila and Ili forms of the large subunit but does not recognize Ilo. The monoclonal antibody ARNA3 (28) binds to the body of the large subunit and hence recognizes all forms. Ili was efficiently induced in *d120*-infected cells (Fig. 1, lanes 6 to 8) with kinetics that were comparable to those in the WT HSV-1 infection (Fig. 1, lanes 3 to 5). Therefore, expression of ICP4 is not required for induction of Ili. Furthermore, since DE and L gene products are not expressed in *d120* infections, this experiment excludes the possibility that newly expressed DE or

FIG. 1. Western blot analysis of the RNAP II large subunit in cells infected with an HSV-1 ICP4 deletion mutant. Vero cells were mock infected (lane 2) or infected with WT HSV-1, strain KOS1.1 (lanes 3 to 5), or the ICP4 deletion mutant *d120* (lanes 6 to 8). Cells were harvested at the times indicated and lysed into an SDS-polyacrylamide gel sample buffer. Lysates representing equal numbers of cells were run on an SDS-6% polyacrylamide gel and analyzed by Western blotting. The blot was probed with a mixture of 8WG16 and ARNA3, two monoclonal antibodies directed against the large subunit of RNAP II. 8WG16 binds to an epitope on the CTD and recognizes the Ila and Ili forms, while ARNA3 binds to the body of the large subunit and hence recognizes all forms. Lane 1 contains an aliquot of a nuclear in vitro transcription extract prepared from HeLa cells, in which only the Ila form of the large subunit is present. Ilo and Ili migrate at approximately 240 and 200 kDa, respectively.

L proteins are required for Ili induction. These results, together with the results of our previous UV-inactivation and protein synthesis inhibition experiments, suggest that the induction of Ili requires the expression of one or more IE proteins other than ICP4.

Ili induction requires the IE protein ICP22. In addition to ICP4, HSV-1 encodes four IE proteins: ICP0, ICP22, ICP27, and ICP47. HSV-1 encodes an additional protein, ICP6, which is not generally classified as an IE protein but which is expressed very early after infection and is not dependent on ICP4 for its expression (14, 15). These four IE proteins and ICP6 are thus potential factors involved in the induction of Ili. ICP0, ICP22, and ICP27 are nuclear proteins which play poorly understood regulatory roles in the expression of HSV-1 genes; ICP6 corresponds to the large subunit of the HSV-encoded ribonucleotide reductase and possesses an N-terminal region with autophosphorylating and possibly protein kinase activity (8, 10); and ICP47 is a cytosolic protein that inhibits antigen presentation to CD8⁺ T lymphocytes (65).

To determine whether ICP0, ICP6, ICP27, or ICP22 is required for the induction of Ili, we analyzed the RNAP II large subunit forms present in cells infected with HSV-1 mutants defective in each of these proteins. The mutants used were (i) *n212*, an ICP0 gene nonsense mutant which expresses only the N-terminal 212 residues of the 775-residue ICP0 protein and is deficient in ICP0 *trans*-regulatory functions (5); (ii) ICP6Δ, an ICP6 gene deletion mutant (21); (iii) *d27-1*, an ICP27 gene deletion mutant (45); and (iv) 22/n199, an ICP22 gene nonsense mutant which expresses only the N-terminal 199 residues of the 420-residue ICP22 (2). Proteins obtained from infected cells at 5 and 10 hpi were analyzed by SDS-PAGE and immunoblotting (Fig. 2). The Western blots were probed separately with ARNA3 (Fig. 2A) or 8WG16 (Fig. 2B). The ICP0, ICP6, and ICP27 mutants (Fig. 2, lanes 4 and 5, 8 and 9, and 10 and 11, respectively) were all similar to the WT virus (Fig. 2, lane 3) in their abilities to induce the Ili form of the RNAP II large

A. WESTERN - ARNA3 **B. WESTERN - 8WG16**

FIG. 2. Western blot analysis of the RNAP II large subunit in cells infected with HSV-1 IE mutants. Vero cells were mock infected (lane 2) or infected with WT HSV-1, strain KOS1.1 (lane 3), the ICP0 mutant *n212* (lanes 4 and 5), the ICP22 mutant *22/n199* (lanes 6 and 7), the ICP6 mutant *ICP6Δ* (lanes 8 and 9), or the ICP27 mutant, *d27-1* (lanes 10 and 11). Extracts were prepared, and blots were run as described in the legend to Fig. 1. Lane 1 contains an aliquot of an in vitro transcription extract prepared from HeLa cells. Blot A was probed with monoclonal antibody ARNA3, which reacts with the body of the large subunit of RNAP II, while blot B was probed with monoclonal antibody 8WG16, which reacts only with the Ila and Ili forms of the large subunit.

subunit. Furthermore, these mutant infections were similar to the WT infection in the degree to which both the Ila and Ilo forms were depleted as infection proceeded. In contrast, cells infected with the ICP22 mutant *22/n199* showed striking differences from the WT infection in the forms of the large subunit that were detectable at 5 and 10 hpi (Fig. 2A and B, lanes 6 and 7). First, the *22/n199* infection did not induce large amounts of Ili, although some comigrating immunoreactive material was observed. Second, although a significant depletion of the Ilo form occurred in the *22/n199* infection, the Ila form was not greatly depleted. The staining of a parallel Western blot with an ICP27-specific monoclonal antibody showed that *22/n199*-infected cells expressed high levels of ICP27, indicating that the cells had been successfully infected with HSV-1 (data not shown).

In addition to *22/n199*, at least two other HSV-1 ICP22 mutants have been isolated and phenotypically characterized (40, 51). A common feature of all three mutants is a cell-type-dependent replication defect, that is, they grow efficiently in some lines, such as Vero cells, but not in others, such as BHK (baby hamster kidney) or HEL (human embryonic lung) cells (2, 40, 51). It was therefore of interest to examine the effects of *22/n199* infection on RNAP II large subunit forms in a more restrictive infection. For these experiments we used HEL cells. Total proteins were prepared from infected HEL cells at 4 and 8 hpi and analyzed by SDS-PAGE and Western blotting (Fig. 3). Infection with WT virus (Fig. 3, lanes 3 and 7) resulted in RNAP II phosphorylation changes similar to those seen in Vero cells, that is, depletion of Ilo and Ila and the strong

WESTERN - ARNA3 + 8WG16

FIG. 3. Western blot analysis of the RNAP II large subunit in HEL cells infected with *22/n199* and a marker-rescued derivative. HEL cells were mock infected (lanes 2 and 6) or infected with WT HSV-1, strain KOS1.1 (lanes 3 and 7), *22/n199* (lanes 4 and 8), or a marker-rescued derivative, *22/n199R* (lanes 5 and 9). Extracts were prepared, and blots were run as described in the legend to Fig. 1. Lane 1 contains an aliquot of an in vitro transcription extract prepared from HeLa cells. The blot was probed with a mixture of monoclonal antibodies ARNA3 and 8WG16.

induction of Ili. As in Vero cells, *22/n199* infection resulted in an altered pattern of RNAP II large subunit forms (Fig. 3, lanes 4 and 8). At 4 hpi (Fig. 3, lane 4), *22/n199* appeared to induce some Ili, but the levels were quite reduced compared with those of the WT infection. The mutant pattern at 8 h (Fig. 3, lane 8) was dramatically different from the WT pattern. Although *22/n199* infection caused efficient depletion of Ilo, most of the Ila form was retained at this time, and there was little if any Ili observed. This latter result differed somewhat from those seen in Vero cells, in which *22/n199* appeared to induce low but detectable levels of Ili at late times (Fig. 2, lane 7). Thus, although the results are slightly different in the two cell lines, it appears that ICP22 is required in both Vero and HEL cells for the efficient induction of Ili as well as for the depletion of the Ila form.

To ensure that the effect on Ili induction is due to the engineered nonsense mutation in the ICP22 gene and not a result of an adventitiously acquired secondary mutation mapping elsewhere in the *22/n199* genome, we also analyzed Ili induction in cells infected with *22/n199R* (2), a revertant virus derived from *22/n199* by marker rescue with a plasmid DNA fragment spanning the ICP22 gene. The induction of Ili and the depletion of Ila and Ilo in *22/n199R*-infected HEL cells (Fig. 3, lanes 5 and 9) occurred similarly to that seen in the WT infection. Therefore, we can conclude that the observed effects of *22/n199* infection on RNAP II result from the engineered mutation in the ICP22 gene.

Redistribution of RNAP II into viral replication compartments in *22/n199*-infected cells. We showed previously that HSV-1 infection induces an additional alteration to RNAP II, that is, in its recruitment into subnuclear viral replication compartments (46). Furthermore, we found that the altered phosphorylation that leads to Ili does not require the relocation of the enzyme into viral replication compartments, since Ili induction occurs efficiently in cells infected with an HSV-1 ICP8 mutant, in which replication compartments do not form. However, it is possible that the converse is true, i.e., that the altered phosphorylation of RNAP II is a prerequisite for its recruitment into replication compartments. The identification of a mutant, *22/n199*, which fails to induce efficient induction of Ili allows us to test this hypothesis. To do this, the formation of replication compartments was examined in mock-, WT-, or

FIG. 4. Nuclear localization of RNAP II in infected Vero cells. Vero cells were mock infected (A to C) or infected with WT KOS1.1 (D to F) or 22/n199 (G to I). The cells were fixed at the times indicated and processed for immunofluorescence with the monoclonal antibody 8WG16. Panels A, D, and G are phase-contrast images corresponding to panels B, E, and H, respectively. Bar = 10 μ m.

22/n199-infected Vero cells. Infected cells were fixed at 4 and 6 hpi and processed for immunofluorescence with the 8WG16 antibody (Fig. 4). Consistent with our previous results, RNAP II in uninfected cells was localized in the nucleus in a mostly diffuse, nonnucleolar distribution (Fig. 4A to C), while RNAP II in HSV-1-infected cells is redistributed into viral replication compartments (Fig. 4D to F). A similar relocation of RNAP II occurs in the 22/n199-infected cells and with similar kinetics (Fig. 4G to I). The nuclear structures seen in WT- and 22/n199-infected cells were confirmed to be viral replication compartments, since they costained with a polyclonal antiserum directed against the HSV-1 ICP8 protein, which is a major marker for these structures (44) (data not shown). This experiment was repeated in HEL cells, with essentially identical results (not shown). We conclude that the recruitment of RNAP II into viral replication compartments does not require the efficient modification of the RNAP II large subunit into the III form.

Deficiencies in viral gene transcription in 22/n199-infected cells. Viral ICP22 mutants exhibit defects in viral gene expression at the level of protein synthesis (2, 40, 51). This has been observed primarily in restrictive infections, but some alterations in protein expression have also been reported in permissive infections of Vero cells. One of the common defects exhibited by ICP22 mutants is a delay in the synthesis of DE proteins as well as reductions in the synthesis of L proteins. One L gene, US11, has been examined in more detail; in this case the defect in protein synthesis appears to correlate with a reduction in the level of its mRNA (42). It is possible that the defects in DE and L gene expression exhibited by ICP22 mutants arise from an underlying defect in viral gene transcription. To date, however, transcription of viral genes in ICP22 mutant-infected cells has not been examined. Since our results demonstrate that ICP22 affects the phosphorylation of RNAP II, we wished to directly examine the transcription of viral genes in ICP22 mutant-infected cells.

To measure transcription rates, nuclear run-on transcription assays were performed. We first examined viral gene transcription in Vero cells, in which infection with 22/n199 is relatively efficient (2). Nuclei were prepared from WT- or 22/n199-infected cells at 3, 6, and 9 hpi, and RNA transcripts initiated in vivo were elongated in vitro in the presence of [32 P]UTP. The radiolabeled run-on transcripts were hybridized to single-stranded DNAs complementary to either specific viral mRNAs (sense probes) or to antisense RNAs from the same regions (antisense probes). The probes used detected two IE genes (ICP4 and ICP27), one DE gene (ICP8), and three L genes (VP16, gC, and UL36). The WT HSV-1 transcription pattern (Fig. 5, top panels) was largely consistent with the results of previous nuclear run-on analyses (20, 64). At 3 hpi, specific sense transcription of IE and DE genes occurred, and by 6 hpi the L genes also showed high levels of sense transcription. However, apparent antisense transcription occurred in all regions of the viral genome by 6 and 9 hpi. The phenomenon of antisense transcription of the HSV-1 genome has been observed in previous nuclear run-on studies and appears to be contingent upon active viral DNA replication (20, 64). The transcription pattern in 22/n199-infected Vero cells appeared generally similar to that of the WT virus. At 3 hpi, a WT pattern of sense transcription was observed, and significant antisense transcription was evident at 6 and 9 hpi.

Next, we examined transcription in HEL cells (Fig. 6A), in which 22/n199 replication is more restricted. The WT transcription pattern (Fig. 6A, top panels) was similar to that seen in Vero cells. However, the transcription pattern of 22/n199-infected HEL cells (Fig. 6A, bottom panels) was different from the WT pattern in two major respects. First, sense-strand transcription of all three L genes appeared significantly reduced compared with that of the WT infection at both 6 and 9 hpi. IE and DE gene transcription also appeared to be reduced but to a lesser extent. Second, the 22/n199 infection showed signifi-

FIG. 5. Nuclear run-on transcription analysis of viral gene transcription in infected Vero cells. Vero cells were infected with WT virus, strain KOS1.1 (top panels), or with the ICP22 mutant virus 22/*n*199 (bottom panels) for the times indicated. Nuclei were isolated, and transcription was allowed to proceed in the presence of [³²P]UTP as described in Materials and Methods. RNA products from equal numbers of nuclei per sample were hybridized to immobilized single-stranded DNA probes which detect sense (S) or antisense (AS) transcripts arising from the IE genes ICP4 and ICP27, the DE gene ICP8, and the L genes VP16 (ICP25), gC, and UL36 (ICP1-2). Single-stranded DNA of M13 mp19 was included as a background hybridization control. Nuclear run-on transcription assays of mock-infected cells yielded no hybridization to these probes (data not shown).

cantly less antisense transcription than the WT infection at both 6 and 9 hpi.

To quantitate the effects of the ICP22 mutation on transcription, the signals from the nuclear run-on experiment in Fig. 6A were analyzed by PhosphorImager, focusing on the ICP27, ICP8, and gC genes as representative members of the IE, DE, and L gene classes, respectively (Fig. 6B). The data from all genes indicate that the levels of antisense transcription at 6 and 9 hpi were significantly lower (by two- to fivefold) in the ICP22 mutant-infected HEL cells than in the WT-infected cells. In addition, sense-strand transcription was reduced for all three genes examined. However, the difference was most dramatic for the L gC gene (bottom panel). Sense transcription of the gC gene at 6 and 9 hpi in the 22/*n*199 infection was reduced more than fivefold compared with that of the WT infection. At these time points, the low level of gC gene sense-strand transcription in the mutant-infected cells was essentially equal to the low levels of gC antisense transcription. In contrast, gC gene sense transcription in WT-infected cells was two- to threefold higher than the antisense transcription levels.

Viral DNA replication is normal in 22/*n*199-infected HEL cells. The expression of HSV-1 L genes is stimulated by viral DNA replication. This effect is the most dramatic for true-L genes, such as gC and UL36, which are not expressed at readily

detectable levels in the absence of DNA synthesis (23, 32). Furthermore, the high levels of HSV-1 antisense transcription that are observed in nuclear run-on transcription assays are dependent upon viral DNA replication (20, 64). Therefore, it is possible that the altered transcription patterns seen in 22/*n*199-infected HEL cells are not due to a direct effect on transcription but rather to a lower rate of DNA replication. To test this possibility, we compared the rate at which 22/*n*199 and the WT virus replicate their DNA in infected HEL cells. Total DNA was isolated from infected cells at 1, 3, 6, and 9 hpi. Equal amounts of each DNA sample were restricted with *Bam*HI and subjected to Southern blot analysis with an HSV-1-specific probe (Fig. 7). The relative levels of HSV-1 DNA in the samples were quantitated by PhosphorImager analysis and are shown at the bottom of Fig. 7 (with the level of WT HSV DNA at 1 hpi being assigned the arbitrary value of 1.0). Although the cells were infected with equivalent PFUs, the WT infection showed approximately four times as much HSV-1 DNA at 1 hpi. This may indicate that the WT and 22/*n*199 virus stocks have different particle-to-PFU ratios, although we have not investigated this further. However, by 9 hpi, the amount of 22/*n*199 DNA was nearly equal to the amount of WT DNA (10.5 versus 14.2). This indicates that the ICP22 mutant replicates its DNA in HEL cells at a rate that is at least equal to that of the WT virus. Therefore, the alterations in transcription that are observed in 22/*n*199-infected HEL cells cannot be explained by differences in viral DNA replication but likely reflect direct effects of ICP22 on viral gene transcription.

DISCUSSION

HSV-1 ICP22 is required for modification of RNAP II. We previously demonstrated that HSV-1 infection leads to the altered phosphorylation of the RNAP II large subunit, most likely on its CTD, generating a polydisperse set of molecules which have electrophoretic mobilities intermediate between those of the two normal forms of the large subunit (46). These multiple novel species, designated II_i, predominate after several hours of infection. Thus, most of the RNAP II in HSV-1-infected cells at late times presumably exists in a modified form, which we term RNAP II_i. Recently, we have used a photoaffinity labeling technique to show that II_i can be efficiently cross-linked to nascent RNA in infected cells (60). Thus, RNAP II_i appears to be transcriptionally active. Although the functional characteristics of RNAP II_i have not yet been analyzed in detail, it is intriguing to speculate that it may have distinct properties which allow it to preferentially transcribe viral genes. If this hypothesis is correct, it might be expected that specific HSV-1 gene products would be involved in mediating the formation of II_i. In this study, we show that II_i induction does not occur efficiently in cells infected with 22/*n*199, an HSV-1 nonsense mutant encoding a truncated ICP22 polypeptide. Therefore, we have identified the IE protein ICP22 as a viral function which is critical for RNAP II modification to II_i.

The molecular functions of ICP22 (also called Vmw 68) during HSV-1 lytic infection have not been well characterized. The ICP22 polypeptide consists of 420 amino acid residues, localizes predominantly to the nucleus, and exists as several phosphorylated forms (42, 43). Much of the existing information concerning the function of ICP22 has come from the analysis of HSV-1 mutants possessing engineered mutations in the ICP22 gene (2, 40, 51). When inoculated into experimental animals, these mutants are highly attenuated for in vivo replication (2, 33, 39). However, in tissue culture cells, they exhibit cell-type-dependent replication defects, growing relatively ef-

FIG. 6. Nuclear run-on transcription analysis of viral gene transcription in infected HEL cells. (A) HEL cells were infected with WT virus, strain KOS1.1 (top panels), or with the ICP22 mutant virus 22/n199 (bottom panels) for the times indicated. Nuclear run-on assays were performed as described in the legend to Fig. 5. (B) Quantitation of the relative ³²P-hybridization signal to specific gene probes by PhosphorImager analysis: top, ICP27; middle, ICP8; bottom, gC. Nuclear run-on transcription assays of mock-infected cells yielded no hybridization to these probes.

ficiently in certain cells, such as Vero cells, but being highly restricted for growth in others, such as HEL cells. In restrictive host cells, ICP22 mutants show striking deficiencies in viral gene expression, including a prolonged expression of some DE proteins and a delay and reduction in the expression of L proteins (2, 40, 51).

The 22/n199 mutant does not efficiently induce the Ii form of the large subunit in Vero cells, in which it grows relatively efficiently, or in HEL cells, in which it grows poorly. Therefore, ICP22's effects on RNAP II do not appear to correlate with its cell-type-dependent growth phenotype. There does appear to be a subtle difference between the two cell types in that low levels of Ii are detectable at late times in Vero but not in HEL cells. It is important to remember that our Western blot analyses detect overall relative abundance of RNAP II phosphorylation variants and do not indicate the proportion of each form that is actively involved in transcription of viral or cellular genes. Preliminary data from photoaffinity labeling experiments suggest that in Vero cells infected with 22/n199 for 9 h, RNAP II_i is the transcriptionally elongating form, despite its minimal detection on Western blots (60). In contrast, RNAP II_o appears to be the transcriptionally elongating form in HEL cells infected with 22/n199 for 9 h, despite undetectable quantities of II_o (or Ii) at this time point. Further interpretation of these differences must await results of photoaffinity labeling

studies in conjunction with fractionation of viral and cellular transcription complexes.

ICP22 is required for normal viral gene transcription in HEL cells. In restrictive host cells, HSV-1 ICP22 mutants exhibit defects in viral L gene expression. This phenotype is consistent with, and could be explained by, an underlying defect in viral gene transcription. Since our results implicate ICP22 in a potentially important modification to RNAP II, we measured the rates of viral gene transcription in 22/n199-infected cells. In permissive Vero cells, the transcription pattern of the 22/n199 mutant appears generally similar to that of the WT virus. In HEL cells, however, the transcription pattern is significantly altered.

One major transcriptional difference exhibited by the ICP22 mutant has to do with the symmetrical or antisense transcription that is observed for WT HSV-1 when transcription is analyzed by nuclear run-on transcription assays (20, 64). We

FIG. 7. DNA replication in infected HEL cells. HEL cells were infected with 22/n199 (ICP22) or WT HSV-1, strain KOS1.1 (KOS), and total cellular DNA was prepared at the times indicated. Five micrograms of each DNA preparation was restricted with *Bam*HI and subjected to Southern blot analysis with a ³²P-labeled HSV-1 DNA probe which detects a 1.5-kb HSV-1 *Bam*HI fragment. An autoradiograph of the Southern blot is shown. Hybridization of the radioactive probe was quantitated by PhosphorImager analysis, and the relative HSV-1 DNA levels are indicated below each lane. The amount of DNA in the WT infection at 1 hpi was assigned the arbitrary value of 1.0.

find that this antisense transcription is significantly reduced in 22/n199-infected HEL cells. Previous studies have shown that antisense transcription of the WT genome is dependent on viral DNA replication, since it is not observed at early times after infection or when infection is carried out in the presence of viral DNA synthesis inhibitors (20, 64). The effect of the ICP22 mutation on antisense transcription, however, does not appear to be due to effects on DNA replication, as we find that viral DNA synthesis is efficient in ICP22 mutant-infected cells. In addition, viral replication compartments, believed to be sites of viral DNA synthesis, appear microscopically identical in Vero or HEL cells infected with WT or 22/n199 viruses (Fig. 4 and data not shown). Thus, in infected HEL cells, antisense transcription may be dependent not only on viral DNA replication, but also on a functional ICP22. The significance of this finding is unclear, since the biological relevance of antisense transcription is not understood. One interpretation of antisense transcription is that RNAP II molecules become relatively promiscuous in their transcriptional specificity after viral genome replication. An alternate interpretation of antisense transcription is that it is an artifact of the nuclear run-on assay and does not reflect real transcription *in vivo*. Support for this latter view comes from the work of Zhang and Wagner (68), who studied HSV-1 gene transcription by pulse-labeling infected cells with [³H]uridine and measuring incorporation of the radiolabel into nuclear RNA. Although these investigators did not specifically measure antisense strand transcription, they did observe that the transcription of several IE and DE genes declines to near background levels at late times, suggesting that nonspecific transcription is not occurring. However, this study does not definitively rule out antisense transcription *in vivo*, since significant RNA turnover can occur during pulse-labeling, but RNAs synthesized during nuclear run-on transcription assay are stable (29, 50). Therefore, if antisense transcripts are rapidly degraded in an intact cell, they might not be detected by a pulse-labeling protocol.

Whatever its origin, the phenomenon of antisense transcription complicates the interpretation of nuclear run-on experiments as applied to HSV-1 genes, since it is impossible to determine at late time points exactly how much of the sense-strand signal for any gene is due to bona fide, promoter-dependent transcription, as opposed to the unknown process which gives rise to the antisense signal. Despite this caveat, our

data strongly suggest that viral L gene transcription is deficient in 22/n199-infected HEL cells. The level of sense transcription for all three L genes we examined (VP16, gC, and UL36) is dramatically reduced when compared with that of the corresponding sense signals in the WT infection (Fig. 6A). In the case of the gC gene, the ICP22 mutant sense-strand signal at 6 and 9 hpi is not significantly different from the low levels of the antisense signals, whereas for the WT virus, sense-strand signals are two- to threefold higher than antisense signals at these times (Fig. 6B). Our data therefore indicate that bona fide L gene transcription is significantly reduced in 22/n199-infected HEL cells. These results suggest that a defect in viral gene transcription may underlie, at least in part, the cell-type-dependent growth defects of 22/n199 and other ICP22 mutants.

Role of ICP22 in the alteration of RNAP II phosphorylation. The mechanism by which ICP22 induces the Ili form of the RNAP II large subunit during infection is not yet known. One possible mechanism is that ICP22 alters the activity of an existing cellular CTD kinase or phosphatase or induces the activity of a novel enzymatic activity. In regard to the latter possibility, it is intriguing that studies from Roizman's laboratory suggest that ICP22 may interact with a virion-associated, virally encoded protein kinase, the product of the HSV-1 UL13 gene. It was found that the phosphorylation of ICP22 is dependent on the UL13 protein, suggesting that ICP22 itself is a substrate for UL13 protein kinase (43). In addition, an HSV-1 UL13 mutant has a cell-type-dependent growth phenotype similar to that of an ICP22 mutant (42). On the basis of these and our findings, we suggest the possibility that the UL13 protein is an ICP22-dependent CTD kinase. An obvious prediction of this hypothesis is that Ili formation would be deficient in cells infected with a UL13 mutant.

Any models describing the role of ICP22 in RNAP II phosphorylation must take into account our observation that 22/n199 infection still causes significant alterations in the phosphorylation of the RNAP II large subunit. Although 22/n199 infection does not induce Ili or cause depletion of Ila, it does result in the significant depletion of the hyperphosphorylated Ilo form. This suggests that another viral gene product may also significantly affect RNAP II metabolism. Another possibility that cannot be excluded at present is that the truncated ICP22 protein encoded by 22/n199 has some residual activity which results in Ilo depletion. This question can be resolved by examining RNAP II modifications in cells infected with a true ICP22 null mutant. It is relevant to point out that the phenotype of 22/n199 closely resembles that of an ICP22 null mutant in terms of cell-type-dependent growth and gene expression (2, 40). For this reason, we favor the idea that another viral gene product is involved in Ilo depletion. Ilo depletion occurs efficiently in cells infected with an ICP4 mutant but not in cells infected with UV-inactivated virus (46). These observations together suggest that an IE gene product is responsible for Ilo depletion. However, we find that infection with viruses mutant in each of the IE genes ICP22, ICP4, ICP27, ICP0, and ICP6 all result in depletion of Ilo. This may seem inconsistent with the hypothesis that an IE function causes the depletion of Ilo. However, it is possible that IE genes are redundant in their ability to cause Ilo depletion. It will be interesting to investigate RNAP II phosphorylation in cells infected with viruses possessing multiple IE gene mutations.

In summary, we find that the HSV IE protein ICP22 is required for the efficient formation of RNAP II_l, a novel form of the enzyme which appears to have intermediate levels of CTD phosphorylation. In addition, we find that ICP22 is required in HEL cells for normal viral gene transcription. We hypothesize that these two phenomena are functionally linked,

that is, that the alteration of RNAP II phosphorylation by ICP22 is critical for the enzyme's ability to efficiently transcribe viral genes. One observation inconsistent with this hypothesis in its simple form is that the 22/1199 mutant does not induce modification of RNAP II to the II₁ form in Vero cells, yet HSV-1 transcription is apparently normal. Host cell factors may therefore be critical in determining the requirements for efficient viral gene transcription. At present, we are carrying out experiments designed to delineate the biochemical pathway by which ICP22 alters RNAP II phosphorylation. We are also interested in determining if and how this modification alters the transcription specificity of the enzyme. It will be particularly interesting to examine the effects of ICP22 on the inhibition of cellular gene transcription after infection. We believe that the HSV-1 system will prove to be a valuable one in which to investigate the role of CTD phosphorylation in transcriptional regulation.

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