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Activation of Gene Expression by Herpes Simplex Virus Type 1 ICP0 Occurs at the Level of mRNA Synthesis

ROBERT JORDAN AND PRISCILLA A. SCHAFFER*

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

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ICP0 is a nuclear phosphoprotein involved in the activation of herpes simplex virus type 1 (HSV-1) gene expression during lytic infection and reactivation from viral latency. Although available evidence suggests that ICP0 acts at the level of transcription, definitive studies specifically addressing this issue have not been reported. In the present study we measured the ability of ICP0 to activate gene expression (i) from promoters representing the major kinetic classes of viral genes in transient expression assays and (ii) from the same promoters during viral infection at multiplicities of infection ranging from 0.1 to 5.0 PFU/cell. The levels of synthesis and steady-state accumulation of mRNA, mRNA stability, and levels of protein synthesis were compared in cells transfected with a reporter plasmid in the presence and absence of ICP0 and in cells infected with wild-type HSV-1 or an ICP0 null mutant, *n*212. In transient expression assays and during viral infection at all multiplicities tested, the levels of steady-state mRNA and protein were significantly lower in the absence of ICP0, indicating that ICP0 activates gene expression at the level of mRNA accumulation. In transient expression assays and during infection at low multiplicities (<1 PFU/cell) in the presence or absence of ICP0, marked increases in the levels of viral mRNAs accompanied by proportional increases in the levels of protein synthesis were observed with increasing multiplicity. At a high multiplicity (5 PFU/cell) in the presence or absence of ICP0, mRNA levels did not increase as a function of multiplicity and changes in the levels of protein were no longer related to changes in the levels of mRNA. Collectively, these tests indicate that transcription of viral genes is rate limiting at low multiplicities and that translation is rate limiting at high multiplicities, independent of ICP0. Consistent with the lower levels of mRNA detected in the absence of ICP0, the rates of transcription initiation measured by nuclear run-on assays were uniformly lower in cells infected with the ICP0 null mutant at all multiplicities tested, implying that ICP0 enhances transcription at or before initiation or both. No evidence was found of posttranscriptional effects of ICP0 (i.e., effects on the stability of mRNA, nuclear-cytoplasmic distribution, polyribosomal mRNA distribution, or rates of protein synthesis). Taken together, these results suggest that ICP0 activates gene expression prior to or at the level of initiation of mRNA synthesis in transient expression assays and during viral infection. Based on these findings, we hypothesize that the exaggerated multiplicity-dependent growth phenotype characteristic of ICP0 null mutants reflects the requirement for ICP0 under conditions where the steady-state level of mRNA is rate limiting, such as during low-multiplicity infection and reactivation from latency.

The temporal regulation of herpes simplex type 1 (HSV-1) gene expression is coordinated primarily by the activities of viral immediate-early (IE) proteins and cellular factors (7, 10, 12, 17, 28, 40, 46, 51, 62). Of the more than 75 viral genes expressed during lytic infection, only 5 encode IE proteins. These proteins are designated infected cell polypeptides (ICP) 0, 4, 22, 27, and 47. The functions of the IE proteins were initially inferred from the phenotypes of viruses with mutations in IE genes (11, 35, 38, 55, 56, 59, 66). Transient gene expression assays and analysis of the properties of IE proteins and peptides have corroborated many of the functions defined by genetic studies (12, 16, 43, 44, 61). Two of the five IE proteins, ICP4 and ICP27, are essential for viral replication (10, 55). ICP4 is the major transcriptional regulator of viral gene expression, repressing synthesis of its own mRNA and that of other IE genes and activating transcription of early (E) and late (L) viral genes (10–12, 24, 34, 44, 53). ICP27 controls 3′ mRNA processing and may also regulate gene expression at the transcriptional level (25, 38, 39, 48, 51, 57, 61). ICP22, which is dispensable for viral replication, alters the phosphorylation state of the host cell RNA polymerase II, which in certain cell types is correlated with efficient viral gene expression during lytic infection (52, 59). ICP47, although not directly involved in the regulation of HSV-1 gene expression, has been implicated in blocking viral antigen presentation on the surface of the infected cell, thus facilitating immune evasion (71).

ICP0 is a nuclear phosphoprotein required for efficient viral gene expression during lytic infection (7, 8, 18, 27, 66). In transient expression assays, ICP0 activates expression of any gene, viral or cellular, whose promoter exhibits basal activity and functions cooperatively with ICP4 to activate viral gene expression (16, 43, 44). Because ICP0 activates expression of all three major classes of viral genes, ICP0 null mutants exhibit significantly reduced levels of gene expression and virus yields, especially at low multiplicities of infection (7, 8, 18, 66). ICP0 is also required for efficient reactivation from latency in mouse and rabbit models (4, 9, 21, 54). The reduced reactivation frequency of ICP0 null mutants appears to be due in part to the reduced efficiency with which latency is established in these mutants (4). In an in vitro model of HSV-1 latency, ICP0 is both necessary and sufficient for reactivation (26, 54, 72). ICP0 is also required for efficient de novo viral gene expression following transfection of cultured cells with infectious HSV

* Corresponding author. Present address: Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104. Phone: (215) 573-9863. Fax: (215) 573-5344. E-mail: pschfr@mail.med.upenn.edu.

DNA (5). Initiation of gene expression from transfected DNA resembles reactivation from latency in that it occurs in both instances in the absence of preexisting viral proteins.

The growth of ICP0 null mutants is highly multiplicity dependent (7, 8, 18, 66). Thus, following low multiplicity infection, gene expression and replication are severely impaired in ICP0 null mutant-infected cells relative to wild-type virus-infected cells, whereas at high multiplicities, replication and gene expression are similar in mutant- and wild-type virus-infected cells (7, 8, 18, 66). The dependence of ICP0 mutant replication efficiency on multiplicity is not simply a function of genome copy number, since ICP0 null mutants replicate significantly less efficiently than wild-type virus at multiplicities of 0.1 PFU/cell and lower, well below the multiplicity required for more than one genome to enter the same cell (7, 18). Furthermore, it is unlikely that virion-associated ICP0 contributes to the multiplicity dependence of ICP0 null mutants, since these mutants, which contain no virion-associated ICP0, exhibit marked multiplicity-dependent replication (7, 8, 18, 66). The near wild-type replication efficiencies of ICP0 null mutants at high multiplicities implies that some factor other than ICP0, but associated with the process of infection, activates gene expression. Consistent with this hypothesis, following low multiplicity infection with an ICP0 deletion mutant, replication was enhanced by superinfection with varicella-zoster virus and human cytomegalovirus (HCMV) but not adenovirus (65). Although varicella-zoster virus contains a gene homologous to ICP0 (gene 61), an HCMV homolog of ICP0 has not been identified. Although multiplicity is a determinant of the efficiency of gene activation in the absence of ICP0, the factor(s) that compensates for the absence of ICP0 at high multiplicities of infection has not been identified.

Some insight into the mechanism of gene activation by ICP0 has been provided by the observation that cellular activities can complement the growth of ICP0 null mutants. In Vero cells, a cell-cycle-dependent activity expressed 6 to 8 h after release from growth arrest in G_0/G_1 enhances the plating efficiency of the ICP0 null mutant 7134 (6). A similar activity expressed with similar kinetics in both Vero cells and cells of neural lineage (NB41A3) can activate HSV-1 IE promoters in transient gene expression assays (50). An activity constitutively expressed by the osteosarcoma cell line U2OS enhances the plating efficiency of the ICP0 null mutant *n*212 200-fold relative to the plating efficiency of *n*212 on Vero cell monolayers (70). These observations indicate that cell-cycle-regulated or cell-type-dependent cellular activities can substitute for the gene-activating activity of ICP0.

Since both ICP0 and the ICP0-complementing cellular activities promote gene expression, it is possible that the function of ICP0 is to "turn on" these cellular activities. If this were the case, ICP0 and the cellular activities would activate gene expression by the same mechanism. Because the levels at which ICP0 and the cellular activities activate gene expression (pre- or posttranscriptionally or translationally) have not been determined definitively, it is not known whether they function by the same or different mechanisms. In the present study, we attempted to identify the level at which ICP0 activates gene expression. Specifically, the ability of ICP0 to activate gene expression was measured (i) from promoters representing the major kinetic classes of viral genes in transient expression assays and (ii) from the same promoters during viral infection at various multiplicities of infection. The results of these tests indicate that in both transient expression assays and during viral infection at all multiplicities tested, the level of steady-state mRNA was higher in the presence than in the absence of ICP0 and that the reduction in steady-state levels of mRNA in the absence of ICP0 was due to lower rates of initiation of mRNA synthesis and not to posttranscriptional effects. Notably, the reduced levels of E and L gene expression seen in ICP0 mutant-infected cells relative to wild-type-infected cells became progressively less marked with increasing multiplicities of infection, such that the levels of E and L gene expression correlated directly with the multiplicity-dependent growth of the mutant. Moreover, changes in the levels of IE, E, and L mRNA correlated directly with changes in the levels of reporter enzyme activity or protein accumulation in transient expression assays and during infection at low multiplicities, suggesting that the efficiency of mRNA translation was not affected by ICP0. Taken together, these findings demonstrate that ICP0 activates gene expression at the level of mRNA synthesis.

MATERIALS AND METHODS

Cells and viruses. Vero, 0-28, and U2OS cells were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and 5% newborn bovine serum as previously described (55, 56, 70). The 0-28 cell line was constructed by stable transfection of the KOS ICP0 gene in combination with the gene encoding G418 resistance into Vero cells as previously described (56). The U2OS cell line was derived from a human osteosarcoma and constitutively expresses an activity that complements ICP0 mutant viruses as efficiently as 0-28 cells (70).

Wild-type HSV-1 strain KOS and the ICP0 nonsense mutant *n*212, derived from KOS, were propagated as previously described (5, 58). Virus P32, containing the gene encoding chloramphenicol acetyltransferase (CAT) under the control of the HSV-1 ICP8 promoter inserted into the ICP0 locus, was constructed and propagated as described below. Viral titers were measured on Vero cell monolayers for strain KOS and on 0-28 or U2OS cells for ICP0 mutant viruses *n*212 and P32. Genome numbers were determined for all virus stocks by slot blot analysis and by quantitating the number of ICP4-expressing cells after low-multiplicity infection in Vero cells by indirect immunofluorescence. The genome numbers and the numbers of ICP4-expressing cells correlated with the titers of KOS measured by plaque assay on Vero cell monolayers and *n*212 measured on U2OS cell monolayers.

Plasmids. Plasmid pWRICP0, which expresses CAT under control of the wild-type ICP0 promoter-regulatory region, was constructed as previously described (50). Plasmid pWRICP8, which expresses CAT under control of the KOS ICP8 promoter-regulatory region, was constructed by removing the ICP8 promoter-regulatory sequences (−335 to +91) within a 435-bp *Bst*YI fragment of the ICP8 promoter-CAT expression vector p8CAT and inserting it into the unique *Bam*HI site of pGem7Zf+ (Promega Inc., Madison, Wis.) (67). The ICP8 promoter, flanked by *Sac*I and *Hin*dIII restriction sites, was removed as a *Sac*I-*Hin*dIII fragment and cloned into the *Sac*I-*Hin*dIII site of pWR-CAT. Plasmid pWR-CAT was derived from pGemCAT (Promega Inc.) and contains three tandem copies of the simian virus 40 polyadenylation signal in an 810-bp fragment inserted immediately 5′ of the ICP8 promoter (50). The polyadenylation signal reduces read-through transcription originating from cryptic promoters contained within vector sequences (50).

Plasmids prp4, prpTK, and prpgC, used to generate probes for RNase protection assays, were constructed as follows. Plasmid prp4 was derived from p4Sma which contains a *Sma*I fragment of the HSV-1 strain KOS ICP4 gene cloned into the *Sma*I site of pGEM3Zf+. Plasmid p4Sma was cleaved with *Hin*dIII immediately downstream of the SP6 promoter and the ends made blunt by treatment with T4 DNA polymerase and deoxynucleoside triphosphates. In a separate reaction, plasmid pGEM7Zf+ was cleaved with *Hae*III, and the 142-bp *Hae*III fragment was isolated, treated with T4 DNA polymerase and deoxynucleotides, and cloned into the *Hin*dIII site of p4Sma to generate prp4. Plasmid prp4 was linearized with *Xcm*I. Runoff RNA transcripts generated by transcription with SP6 RNA polymerase produced a 441-nucleotide probe and, after RNase protection, a 299-nucleotide protected product.

Plasmid prpTK was derived from pTK. Plasmid pTK contains a *Bam*HI-*Hin*dIII fragment of the HSV-1 strain KOS TK gene cloned into the *Bam*HI-*Hin*dIII site of pGEM3Zf+. Plasmid pTK was cleaved with *Sac*I, and a 142-bp blunt-ended *Hae*III fragment from pGEM7Zf+ was cloned into the *Sac*I site of pTK. RNase protection probes were generated by cleaving plasmid prpTK with *Hin*dIII, and runoff transcripts, synthesized by SP6 RNA polymerase, produced a 673-nucleotide probe and, after RNase protection, a 531-nucleotide protected product.

Plasmid prpgC was constructed as follows. Plasmid prp4 was cut with *Sma*I, and vector DNA was separated from ICP4 DNA sequences by gel electrophoresis and religated to generate pRNAP. Plasmid pRNAP was cut with *Bam*HI and *Xba*I, and a 901-bp *Eco*RI-*Xba*I fragment from pgCEX-pUC19 was inserted into the *Bam*HI-*Xba*I site of pRNAP. Plasmid pgCEX-pUC19 contains an *Eco*RI-*Xba*I fragment of the gC gene from HSV-1 strain KOS inserted into the *Eco*RI-

*Xba*I site of pUC19 (20). Plasmid prpgC was linearized with *Bsg*I to give rise to a 383-nucleotide probe and, after RNase protection, a 241-nucleotide protected product.

As an intermediate in the construction of virus P32 (described below), a plasmid containing the CAT gene under control of the ICP8 promoter-regulatory region (pWRICP8-CAT) flanked by HSV-1 DNA sequences immediately 5′ and 3′ of the ICP0 gene was constructed by W. Ralph (Dana-Farber Cancer Institute) as follows: the 1,087-bp *Aat*II-*Pst*I fragment from pSH, which contains sequences 3′ of the ICP0 gene (positions 120,466 to 121,553) (47), was inserted into the *Aat*II-*Pst*I site of plasmid pMRICP8 to generate plasmid pMRICP8-3′. In a separate reaction, plasmid pSG28, which contains the entire *Eco*RI E-K fragment, containing sequences 5′ of the ICP0 gene (positions 124,814 to 128,624) (47), was inserted into the *Eco*RV site of pGEM 5Zf(−), resulting in plasmid p10, which contains the *Stu*I insert flanked by a *Bsp*HI site on its 5′ side and a *Pst*I site on its 3′ side, with respect to its natural orientation. The 4.6-kbp *Bsp*HI-*Pst*I fragment in p10 was isolated and inserted at the *Nsi*I-*Afl*III site of plasmid pMRICP8-3′, placing the original *Stu*I fragment immediately 5′ of the promoter-regulatory region of the ICP8 gene. The resulting plasmid was designated pGRICP8.

Plasmid pSH containing the ICP0 gene under control of the ICP0 promoter-regulatory region was constructed as previously described (5).

Mutant virus construction. The ICP8-promoter CAT gene was transferred into the HSV-1 genome by W. Ralph in the following manner. Vero cells (5×10^5/60-mm-diameter dish) were transfected with 2 μg of infectious 7134 DNA, 4.5 μg of linearized pGRICP8, and 0.5 μg of pSH. The HSV-1 mutant virus 7134, an ICP0 null virus that contains the bacterial *lacZ* gene in place of both copies of the ICP0 gene, replicates poorly in the absence of ICP0 (5). In order to enhance viral DNA replication and recombination between linearized plasmid DNA and infectious 7134 DNA, 0.5 μg of pSH was added to the transfection mixture to supply ICP0 exogenously. Four days later, when cytopathic effects were apparent, cultures were harvested, frozen at −20°C, thawed, sonicated (Heat Systems-Ultrasonics Inc.) for 1 min at 60% efficiency, and used to infect Vero cell monolayers. Infected monolayers were overlaid with medium containing methylcellulose. After incubation at 37°C for 4 days, 2 ml of neutral red containing 300 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) (GIBCO/BRL, Gaithersburg, Md.)/ml was added to each 35-mm-diameter petri dish. Plaques produced by 7134 were blue. White plaques initiated by recombinant viral genomes were picked and plaque purified. Two plaque-purified isolates resulting from the cotransfection of infectious 7134 DNA and linearized pGRICP8 plasmid DNA were designated P32. P32 viral DNA was digested with restriction endonucleases and analyzed by Southern blot analysis (63). In this way the ICP8-CAT sequences were shown to be inserted in the correct position and orientation within the genome.

Transfections. Vero cells were seeded at 2.5×10^6 cells per 100-mm-diameter dish, and medium was changed 2 h prior to transfection. DNA was added as a calcium phosphate precipitate according to the method of Graham and van der Eb (22). At 18 h posttransfection, monolayers were washed twice with 5 ml of trypsin diluent (0.4 mM sodium phosphate, 5.6 mM dextrose, 0.04 mM potassium phosphate, 140 mM sodium chloride, 2% phenol red) and 10 ml of fresh medium was added. The cells were harvested at the indicated times posttransfection by two washes with 5 ml of 1× Tris-buffered saline (TBS) (25 mM Tris-HCl [pH 7.4], 140 mM sodium chloride, 5 mM potassium chloride) at 0°C and scraping into 1 ml of 1× TBS at 0°C. The cells were collected by centrifugation at $800 \times g$ for 4 min at 4°C. The cell pellet was resuspended in 0.5 ml of 1× TBS at 0°C, and 100 μl was removed and stored at −70°C for measurement of CAT activity. The remaining cells were used to isolate cytoplasmic RNA and nuclear DNA as described below.

Cytoplasmic RNA and nuclear DNA isolation. Vero cell pellets containing 3×10^6 to 6×10^6 transfected or infected cells were resuspended in 0.375 ml of RNA lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM potassium acetate, 5 mM magnesium acetate, 0.5% Nonidet P-40, 1 mM dithiothreitol) and incubated at 0°C for 5 min. Under these conditions more than 95% of the cells were lysed as measured by microscopic examination and trypan blue exclusion. The nuclei and insoluble debris were isolated by centrifugation at $14,000 \times g$ for 2 min at 4°C. The supernatant fluids were transferred to a new microcentrifuge tube and sodium dodecyl sulfate (SDS) and proteinase K were added to final concentrations of 0.1% and 100 μg/ml, respectively. The mixture was incubated at 55°C for 15 min, extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol) and once with an equal volume of chloroform-isoamyl alcohol (24:1, vol/vol). Sodium acetate was added to the aqueous phase to a final concentration of 0.3 M, and the RNA was precipitated at −20°C for 12 to 18 h after the addition of 1 ml of 95% ethanol.

Nuclei from the cytoplasmic RNA isolation procedure were resuspended in 400 μl of TES (10 mM Tris-HCl [pH 7.6], 0.5 mM EDTA, 0.1% SDS, 100 μg of proteinase K/ml) and incubated at 37°C for 4 to 8 h. The mixture was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol and once with an equal volume of chloroform-isoamyl alcohol and precipitated in ethanol as described above. The DNA precipitate was collected by centrifugation, dried under vacuum, and resuspended in 400 μl of TE (10 mM Tris-HCl [pH 7.6], 0.1 mM EDTA) containing 100 μg of RNase A/ml. The DNA solution was incubated at 37°C for 30 min. SDS and proteinase K were added to final concentrations of 0.1% and 100 μg/ml, respectively. The mixture was incubated at 55°C for 15 min and phenol-chloroform-isoamyl alcohol extracted and ethanol precipitated as described above. Slot blot hybridizations were carried out as previously described (70).

RNase protection. Cytoplasmic RNA (5 to 20 μg), stored as a precipitate in ethanol at −20°C, was concentrated by centrifugation at 4°C for 15 min at $14,000 \times g$, washed once with 0.5 ml of a solution containing 75% ethanol and 25% 0.1 M sodium acetate and dried under vacuum. The RNA pellet was resuspended in 100 μl of a solution containing 50 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 1 mM dithiothreitol, and 5 U of RNase-free DNase I (Promega Inc.), and the mixture was incubated at 37°C for 15 min. The RNA was extracted once with an equal volume of phenol-chloroform-isoamyl alcohol and ethanol precipitated. The RNA precipitate was concentrated by centrifugation, resuspended in 100 μl of a solution containing 0.3 M sodium acetate and 4 ng of [α-^{32}P]GTP-labeled RNA probe (2.5×10^5 cpm/ng), and reprecipitated in ethanol. After collecting the precipitate by centrifugation, the RNA pellet was resuspended in 30 μl of 1× hybridization buffer {80% formamide, 20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] [pH 6.8], 200 mM sodium chloride, 0.5 mM EDTA}, heated at 85°C for 10 min, and then incubated at 56°C for 12 to 18 h. The hybridization mixture was cooled to room temperature (22°C), 0.35 ml of RNA digestion buffer (10 mM Tris-HCl [pH 7.6], 300 mM sodium chloride, 5 mM EDTA, 0.2 μg of RNase A/ml, 0.5 μg of RNase T_1/ml) was added, and the mixture was incubated at 30°C for 30 min. The reaction was terminated by addition of SDS and proteinase K to 0.1% and 100-μg/ml final concentrations, respectively. The reaction mixture was incubated for 15 min at 30°C and extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The RNA was precipitated in ethanol after the addition of sodium acetate to 0.3 M and the addition of 20 μg of *Escherichia coli* tRNA. The precipitate was collected by centrifugation, and the RNA pellet was resuspended in 10 μl of RNA loading buffer (95% formamide, 10 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol). The RNA sample was heated for 5 min at 90°C prior to size separation by denaturing polyacrylamide gel electrophoresis (PAGE).

A technical problem routinely encountered in quantitative measurement of viral mRNA levels in HSV-1-infected cells is the lack of suitable cellular mRNA controls to normalize for recovery. Both cellular actin and rRNA probes, when present in excess, detected decreased signal intensity as a function of increasing multiplicity of infection or time postinfection. Consequently, these probes were not used to normalize for viral mRNA recovery. With this in mind, all RNA and protein isolations from KOS- and *n*212-infected cells were performed at the same time and processed together to minimize variability in recovery and analysis. Comparisons were made from samples collected from cells infected at the same multiplicity and harvested at the same times postinfection in multiple tests. Because the differences in RNA recovered were similar, we can assume that the differences detected between KOS- and *n*212-infected cells are due to the absence of ICP0 and not to differences in recovery of RNA and protein from KOS- and *n*212-infected cells.

CAT assay. Cell suspensions from transfection or infection experiments were thawed at room temperature and sonicated for 20 s at 40% power in a W-380 sonicator. The cell lysate was incubated at 60°C for 10 min to inactivate deacetylases, and the insoluble cell debris was collected by centrifugation at $14,000 \times g$ for 10 min at 4°C. The supernatant fluid was assayed for CAT activity by the method of Seed and Sheen (60). CAT activity did not change with increasing concentrations of chloramphenicol or butyryl-coenzyme A as substrates. In addition, the change in CAT activity was linear over the time of the assay and exhibited first-order dependence with respect to the concentration of enzyme extract. These control experiments indicate that CAT activity was measured under steady-state conditions and that the level of enzyme activity was proportional to the level of CAT enzyme in crude cell extracts.

TK assay. Thymidine kinase (TK) activity was measured in crude soluble enzyme extracts as previously described (62). Briefly, 3×10^6 infected cells were washed twice with 5 ml of 1× TBS at 0°C. The cells were scraped into 1 ml of 1× TBS and collected by centrifugation at $800 \times g$ for 4 min at 4°C. The cell pellet was resuspended in 0.5 ml of 1× TBS, 0.25 ml of the cell suspension was transferred to a separate tube for cytoplasmic RNA isolation, and the remaining cells were concentrated by centrifugation at $800 \times g$ for 4 min at 4°C. The cell pellet was resuspended in 40 μl of 1× TBS containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μg of TPCK (tolylsulfonyl phenyalanyl chloromethyl ketone)/ml, quick frozen in liquid nitrogen, and stored at −70°C. The cells were thawed on ice and sonicated at 0°C for 20 s as described above. The insoluble cell debris was collected by centrifugation at $14,000 \times g$ for 10 min at 4°C.

A reaction mixture containing 150 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 3 mM ATP, 10 mM creatine phosphate, 0.6 U of creatine kinase, 1 μg of TPCK/ml, 0.2 mM PMSF, 100 μM thymidine, 5 μCi of [^{3}H]thymidine (50 Ci/mmol), and 150 to 200 μg of crude cell lysate (40 μl) in a 100-μl total volume was incubated at 37°C for 20 min. At 4-min intervals, 10 μl of the reaction mixture was removed and spotted onto a DEAE filter disk and air dried. The filter disks were washed twice with 5 ml of 30 mM ammonium formate and once with 5 ml of H_2O and were rinsed in 95% ethanol. Radiolabeled material bound to the filters was counted by liquid scintillation counting. The rate of product formation over time was determined by linear regression of the data by using the program Enzfitter (Cambridge Biosoft, Cambridge, Mass.).

TK activity, expressed as a specific activity (rate/microgram), was linear over

the course of the assay. In control experiments, TK activity exhibited a first-order dependence on extract concentration and did not change with increasing concentrations of ATP or thymidine substrates. These experiments demonstrate that TK activity was measured under steady-state conditions and that changes in TK activity were proportional to changes in TK levels.

Quantitative immunoprecipitation. Vero cells (10^6 cells/60-mm-diameter dish) were infected with KOS or *n*212 at the indicated multiplicities. At 1 h postinfection the virus inoculum was removed and replaced with methionine-free Dulbecco's modified Eagle's medium supplemented with 20 μCi of [^{35}S]methionine/ml and 2% fetal calf serum. The cells were incubated at 37°C in 5% CO_2 for the indicated times. At the time of harvest, monolayers were washed twice with 2 ml of 1× TBS containing 1 μg of TPCK/ml and 0.2 mM PMSF at 0°C. The cells were scraped into 1 ml of 1× TBS and collected by centrifugation at 800 × *g* for 4 min at 4°C. The cell pellet was resuspended in 0.4 ml of radioimmunoprecipitation assay (RIPA) buffer (1× TBS, 1 μg of TPCK/ml, 0.2 mM PMSF, 0.1% SDS, 1% deoxycholate) and incubated on ice for 15 min, and the insoluble material was collected by centrifugation at 14,000 × *g* for 15 min at 4°C. The supernatant fluid (350 μl) was transferred to a new tube and 0.4 μl of monoclonal antibody H112 against ICP4 was added (Goodwin Institute, Plantation, Fla.). The mixture was incubated, with constant mixing, for 2 h at 4°C. A 22.5-μl slurry of rabbit anti-mouse immunoglobulin G antibody bound to protein A-Sepharose (Gibco/BRL, Grand Island, N.Y.) was added to the mixture and incubated, with constant mixing, for 1 h at 4°C. The immunoprecipitate was collected by centrifugation at 800 × *g* for 4 min at 4°C. The pellet was washed twice in 500 μl of RIPA buffer and once in 500 μl of 1× TBS. The final pellet was resuspended in 40 μl of SDS-PAGE sample buffer and boiled for 5 min, and the insoluble material was collected by centrifugation at 10,000 × *g* for 2 min at 22°C. The proteins in the supernatant were separated by SDS-PAGE.

The changes in the amount of radiolabeled ICP4 in the immunoprecipitate were linear with increasing concentrations of cell lysate. In addition, the amount of ICP4 recovered in the immunoprecipitate did not change with increasing concentrations of H112 antibody. Furthermore, we could not immunoprecipitate additional ICP4 protein from the supernatant fluids of the initial immunoprecipitation reaction. These control experiments indicate that the concentration of antibody was saturating for immunoprecipitation of ICP4.

mRNA stability. Vero cells (3×10^6 cells/100-mm-diameter dish) were infected with 5 PFU/cell of KOS or *n*212. At 6 or 9 h postinfection (hpi), medium containing actinomycin D at a concentration of 5 μg/ml was added, and at 0, 0.5, 1, 2, 3, and 5 h after addition, infected cells were harvested and cytoplasmic mRNA was isolated. The mRNA was separated by gel electrophoresis and transferred to nylon membranes by Northern blotting (1). The membranes were probed for TK mRNA as described below. In experiments measuring CAT mRNA stability, cells transfected with the CAT expression vectors were treated with actinomycin D at 48 h posttransfection. Cytoplasmic mRNA was isolated at 0, 0.5, 1, 2, 3, 5, and 8 h posttreatment, and CAT message was measured by quantitative RNase protection assay.

Nuclear-cytoplasmic mRNA distribution. Vero cells (3×10^6 cells/100-mm-diameter dish) were infected with 0.5, 1.0, and 5.0 PFU/cell of KOS or *n*212. At the times indicated, infected cells were lysed in RNA lysis buffer and fractionated by differential centrifugation. Nuclei were resuspended in RNA lysis buffer and pelleted through a 20% sucrose cushion to remove contaminating membrane components. The RNA from cytoplasmic and nuclear fractions was isolated, separated by gel electrophoresis, and transferred to nylon membranes by Northern blotting (1). Northern blots were probed with ^{32}P-labeled antisense mRNA specific for TK message. The amount of radioactivity in the band corresponding to TK mRNA was quantitated by phosphorimager analysis. The range value for the image display was the same for *n*212 and KOS at each multiplicity.

Polyribosome distribution. Vero cells (4×10^7 per roller bottle) were infected with 0.1, 1.0 or 10.0 PFU/cell of KOS or *n*212, and at 6 hpi cells were harvested and cytoplasmic mRNA was isolated. Heparin sulfate was added to a final concentration of 1 mg/ml. The RNA was layered on a 0.5 to 1.1 M sucrose gradient (12 ml) and centrifuged at 180,000 × *g* for 110 min at 4°C. Fractions (0.5 ml) were collected from the top of the gradient, and absorbance at 260 nm was monitored. The RNA in each fraction was quantified by RNase protection assay. Fractions containing 40S, 60S, and 80S ribosomal subunits (subpolysomal pool) were determined by visual inspection of the ethidium bromide-stained agarose gels prior to RNase protection assay and from the absorbance measurements at 260 nm.

Protein synthesis rates. Protein synthesis rates in Vero cells infected with KOS or *n*212 were determined as previously described (41).

RESULTS

ICP0 activates gene expression at the level of mRNA accumulation. (i) Cotransfection experiments. As a first step towards elucidating the level of gene activation by ICP0, we performed transient expression assays and measured the levels of mRNA and protein accumulation in the presence and absence of ICP0 (Fig. 1). Vero cells were cotransfected with a constant amount of a reporter plasmid containing the CAT gene under the control of the promoter of the ICP8 gene (an E gene) and increasing concentrations of plasmid pSH, an ICP0-expressing plasmid. Aliquots of the transfected cell suspension were used to measure the levels of transfected DNA

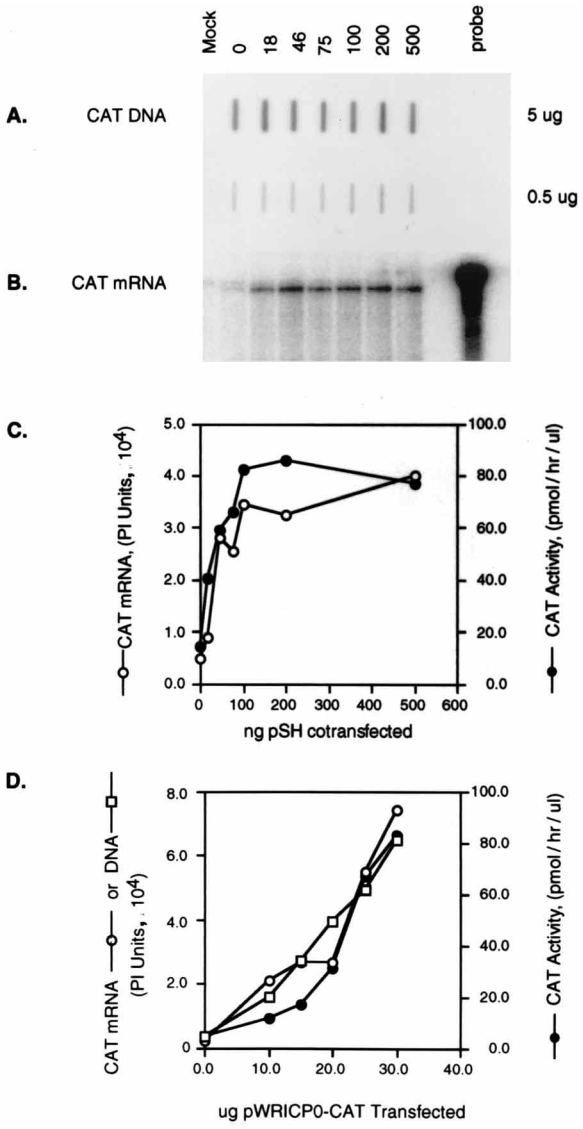

FIG. 1. Activation of CAT mRNA and CAT activity by ICP0 in transient gene expression assays. Vero cells (3×10^6/100-mm-diameter dish) were cotransfected with 15 μg of pMRICP8-CAT and various amounts of plasmid pSH as indicated. (A) At 48 hours posttransfection the nuclei from 20% of the cells were used to prepare nuclear DNA to measure transfection efficiencies by slot blot hybridization probing for CAT DNA sequences. Nuclear DNA was applied to the slot blot apparatus in 5- and 0.5-μg amounts. (B) Cytoplasmic mRNA was purified from 80% of the cells, and the concentration of CAT mRNA was measured by quantitative RNase protection assay. (C) Lysates were prepared from the remaining cells, and CAT activity and CAT mRNA levels were measured. CAT activity is expressed as picomoles/hour/microliter of extract and was measured in the linear range of the assay (less than 40% substrate utilized). Also shown are CAT mRNA levels quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). CAT mRNA values are expressed in PhosphorImager units (PI units). The maximum range value for the image display was set so that the band having the highest range value was just within the visual linear range. The lower range value for the image display was set at the lowest setting (0.012 PI units). (D) Control experiment showing the linearity of the CAT assay. Vero cells were transfected with 0 to 30 μg of pWRICP0-CAT. Plasmid pWRICP0-CAT contains the promoter-regulatory region of ICP0 from plasmid pSH driving expression of CAT. At 48 h posttransfection the amounts of CAT mRNA, transfected DNA, and CAT activity were measured.

(Fig. 1A), CAT mRNA (Fig. 1B and C), and CAT activity (Fig. 1C).

Transfection efficiencies of pMRICP8-CAT measured by slot blot hybridization of nuclear DNA were similar in the presence and absence of pSH (Fig. 1A). The amount of CAT mRNA and CAT activity in crude enzyme extracts of cells transfected with pMRICP8-CAT increased with increasing amounts of pSH added to the transfection mixture, saturating at 100 ng of pSH (Fig. 1B and C). Amounts of pSH greater than 1,000 ng were inhibitory to the accumulation of both CAT mRNA and CAT activity (data not shown). Similar effects were observed when pWRICP0-CAT (a plasmid in which the CAT gene is regulated by the ICP0 promoter) was transfected with increasing amounts of pSH (data not shown). The saturation of CAT activity at 100 ng of pSH was shown not to be due to squelching of the ICP0 promoter or to the inability of transfected cells to take up additional DNA. Thus, in control experiments (Fig. 1D), the amounts of transfected DNA, and the resulting levels of CAT mRNA and CAT activity expressed from pWRICP0-CAT, were linear when 10 to 30 μg of reporter plasmid was added to the transfection mixture. Finally, no major differences were observed in the half-life of CAT mRNA in cells transfected with pMRICP8-CAT in the presence ($t_{1/2} = 2.9 \pm 0.5$ h) or absence ($t_{1/2} = 3.9 \pm 2.1$ h) of pSH. Taken together, these data suggest that ICP0 activates gene expression at the level of mRNA accumulation in transient-expression assays and that the changes in steady-state amounts of mRNA observed in these experiments occur at the level of mRNA synthesis. Since these experiments measured cytoplasmic CAT mRNA, however, we cannot rule out the possibility that mRNA transport is affected by ICP0.

(ii) Transfection and superinfection experiments. Although ICP0 alone is sufficient to activate gene expression in transient expression assays, ICP0 is known to cooperate with other viral factors (e.g., ICP4) during infection to regulate HSV-1 gene expression (7, 15, 16, 43, 44). The ability of ICP0 to activate gene expression in the presence of other viral proteins was therefore tested by transfecting Vero cells with the reporter plasmid pMRICP8-CAT and infecting these cells with 5 PFU/cell of either wild-type virus or the ICP0 null mutant *n*212. Levels of cytoplasmic CAT mRNA and CAT activity were measured at selected times postinfection.

As shown in Fig. 2, the levels of CAT activity and CAT mRNA increased through 10 hpi in cells infected with wild-type virus, whereas CAT activity and CAT mRNA levels decreased progressively over time in cells infected with *n*212, suggesting that ICP0, when expressed together with other viral proteins, activates expression of transfected genes at the level of mRNA accumulation. Notably, changes in the levels of CAT activity were roughly proportional to changes in the levels of CAT mRNA in cells infected with either wild-type virus or *n*212, indicating that ICP0 had no major effect on the efficiency of translation during viral infection.

(iii) Viral infection experiments. Transient gene expression assays have proven useful in identifying many of the *cis*-acting elements and *trans*-acting factors involved in the regulation of HSV-1 gene expression (2, 16, 43, 44, 51, 61). Transient expression assays can be problematic, however, in that the regulatory activities defined in these assays are not always observed during viral infection (11, 19, 32). To determine whether ICP0 activates expression of viral genes in their natural context at the level of mRNA accumulation, as observed in Fig. 1 and 2, the levels of ICP4, TK, and gC mRNA were measured in Vero cells infected with wild-type virus and *n*212 at multiplicities of infection ranging from 0.1 to 5.0 PFU/cell. At times when the rates of RNA and protein synthesis from the test genes were maximal (ICP4, 3 hpi; TK, 6 hpi; gC, 9 hpi) infected cells were harvested, and cell extracts were prepared for isolation and measurement of cytoplasmic ICP4, TK, and gC mRNA by quantitative RNase protection assay (Fig. 3).

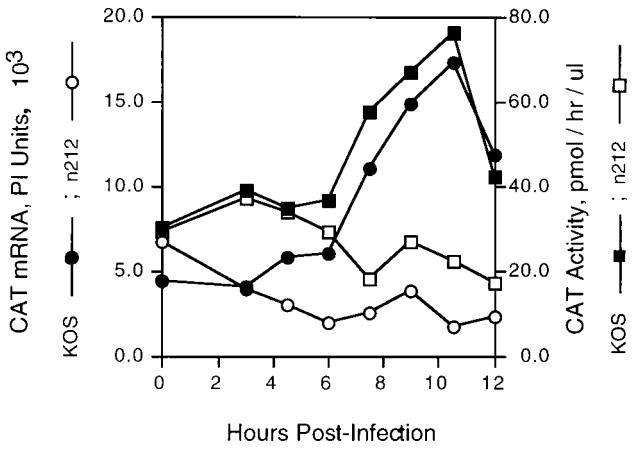

FIG. 2. Effect of ICP0 on CAT mRNA and CAT activity following viral superinfection. Vero cells (3×10^6/100-mm-diameter dish) were transfected with 15 μg of pMRICP8-CAT, and 24 h later cells were infected with 5.0 PFU/cell of either KOS or *n*212. At the indicated times, cells were harvested and soluble enzyme extracts were prepared from approximately 10% of the cells to measure CAT activity. The remaining cells were used to prepare cytoplasmic RNA, and CAT mRNA was measured by quantitative RNase protection assay.

The results of these tests demonstrate that for selected transcripts representing the major classes of viral genes, the levels of cytoplasmic E and L (but not IE) gene-specific mRNAs in *n*212-infected cells were markedly lower than in wild-type virus-infected cells, especially at low multiplicities of infection. The most dramatic differences in TK and gC mRNA levels in KOS- and *n*212-infected cells were observed at multiplicities lower than 1 PFU/cell. Similar results were obtained when Northern blotting was used to measure levels of TK mRNA (data not shown). In contrast to TK and gC mRNAs, the level of ICP4 mRNA was only slightly reduced in *n*212-infected cells relative to KOS-infected cells, suggesting that ICP0 had only a minor effect on the level of accumulation of at least one IE mRNA (ICP4). Collectively, these results are consistent with the observations of Cai and Schaffer which showed that the absence of ICP0 has a greater effect on E and L gene expression than on IE gene expression (7).

In order to study the effects of ICP0 on the kinetics of expression of individual viral genes at low and high multiplicities of infection, ICP4 and TK mRNA and protein accumulation were measured over time in Vero cells infected with KOS or *n*212 at multiplicities of 0.2 and 5.0 PFU/cell. As shown in Fig. 4, the times of peak synthesis of both ICP4 and TK mRNA in KOS- and *n*212-infected cells (4 hpi) occurred before the times of peak protein synthesis at the higher multiplicity, suggesting that at high multiplicities translation is rate limiting for gene expression early in infection with or without ICP0. In contrast, at the lower multiplicity (0.2 PFU/cell) KOS ICP4 (Fig. 4A), KOS TK (Fig. 4C), and *n*212 TK (Fig. 4D) mRNA accumulation peaked at later times than at the higher multiplicity and the kinetics of protein accumulation correlated closely with the kinetics of mRNA accumulation, suggesting that transcription is rate limiting for gene expression at low multiplicities. Only in the case of *n*212 ICP4 did the kinetics of mRNA and protein accumulation at the lower multiplicity resemble those seen at the higher multiplicity. The reduced

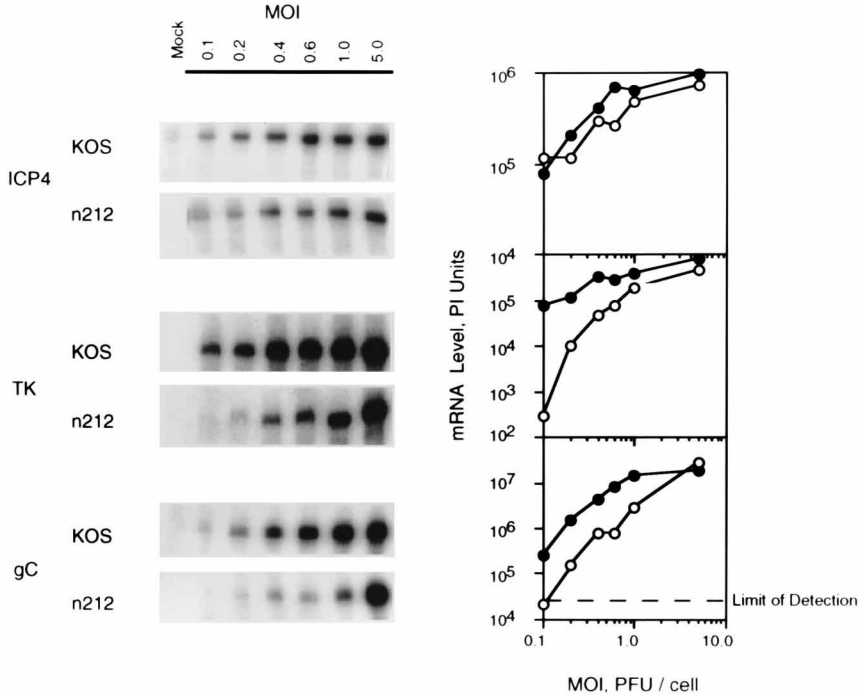

FIG. 3. ICP0 effects the steady-state level of mRNA accumulation during lytic infection at several multiplicities. Vero cells (10^6/100-mm-diameter dish) were infected with KOS (●) or *n*212 (○) at the indicated multiplicities (MOI, multiplicities of infection). Cells were harvested as described below, and cytoplasmic RNA was prepared. Twenty micrograms of infected-cell RNA was used to measure the amounts of ICP4, TK, and gC transcripts by quantitative RNase protection assay. The data are plotted to show the amount of each transcript as a function of multiplicity. The data shown are from infections harvested at 3 (ICP4), 6 (TK), and 9 (gC) hpi. The maximum range value for the image display was set so that the band having the highest range value was just within the visual linear range. The lower range value for the image display was set at the lowest setting (0.012 phosphorimager [PI] units).

levels of *n*212 ICP4 (Fig. 4B) and *n*212 TK (Fig. 4D) mRNA accumulation observed in cells infected with 0.2 PFU/cell further confirm the requirement for ICP0 for wild-type levels of transcription at low multiplicities of infection.

Effect of multiplicity of infection on the accumulation of viral mRNA and protein in the presence and absence of ICP0. To examine more closely the effect of multiplicity of infection on gene expression in the presence and absence of ICP0, Vero cells were infected at multiplicities ranging from 0.1 to 5.0 PFU/cell with KOS, *n*212, or P32, and the expression of ICP4, TK, and CAT was measured. P32 is an ICP0 null mutant in which the ICP0 locus has been replaced by the CAT gene under control of the ICP8 promoter. The growth kinetics of P32 and *n*212 are similar and both viruses grow more slowly than KOS at low multiplicities of infection (49a). At 3 (ICP4) and 6 (TK and CAT) hpi infected cells were harvested and ICP4, TK, and CAT mRNA and protein levels or enzyme activities were measured (Fig. 5).

Several points can be made from these experiments. (i) The levels of ICP4 and TK mRNA and ICP4 protein and TK activity in KOS-infected cells were higher than the corresponding levels of ICP4 and TK mRNA and protein and TK activity in *n*212-infected cells, consistent with the data shown in Fig. 3 and 4. Similar results were observed for ICP8 and gC mRNA (data not shown). (ii) The levels of ICP4 and TK mRNA and protein in KOS- and *n*212-infected cells, and of CAT mRNA and CAT activity in P32-infected cells, increased roughly in proportion to the amount of input virus at multiplicities below 1.0 PFU/cell. However, at a multiplicity greater than 1.0 PFU/cell, the increased levels of mRNA and protein were no longer proportional to the change in multiplicity. For example, in KOS-infected cells the level of TK activity at 5.0 PFU/cell is approximately 1.2-fold greater than the level of TK activity at 1.0 PFU/cell. If the level of protein correlated with the multiplicity of infection one would expect five times more TK activity at 5.0 PFU/cell than at 1.0 PFU/cell. These data suggest that independent of the presence or absence of ICP0, gene expression is saturable at high multiplicities of infection.

Notably, the saturation of gene expression shown in Fig. 5A at 5.0 PFU/cell was not due to saturation of the components of the assays used to measure mRNA levels or protein expression but rather is a characteristic of the infection process. Thus, in control experiments changes in TK activity, and changes in ICP4, ICP8, and gC mRNA levels, were proportional to the amount of cell lysate or infected-cell mRNA added to the assay (data not shown).

Replotting the data in Fig. 5A to compare the amount of protein detected per unit of mRNA revealed that the levels of protein increased proportionally with changes in the levels of mRNA for KOS and *n*212 at low concentrations of mRNA for ICP4, TK, and CAT (Fig. 5B). At high concentrations of mRNA, however, a saturation point was reached and changes in the levels of protein were no longer proportional to the changes in concentrations of mRNA. This observation was most apparent for CAT gene expression, where at the highest concentration of mRNA there was actually slightly less CAT activity in three independent experiments. A similar pattern was observed for ICP4 and TK gene expression (Fig. 5B). The lines of best fit achieved by logarithmic regression of the data, especially for TK and CAT, are consistent with processes exhibiting saturable kinetic behavior. These observations imply that at low mRNA concentrations, the level of mRNA is rate limiting for gene expression and that at high, saturating mRNA concentrations, the translational capacity of the infected cell is

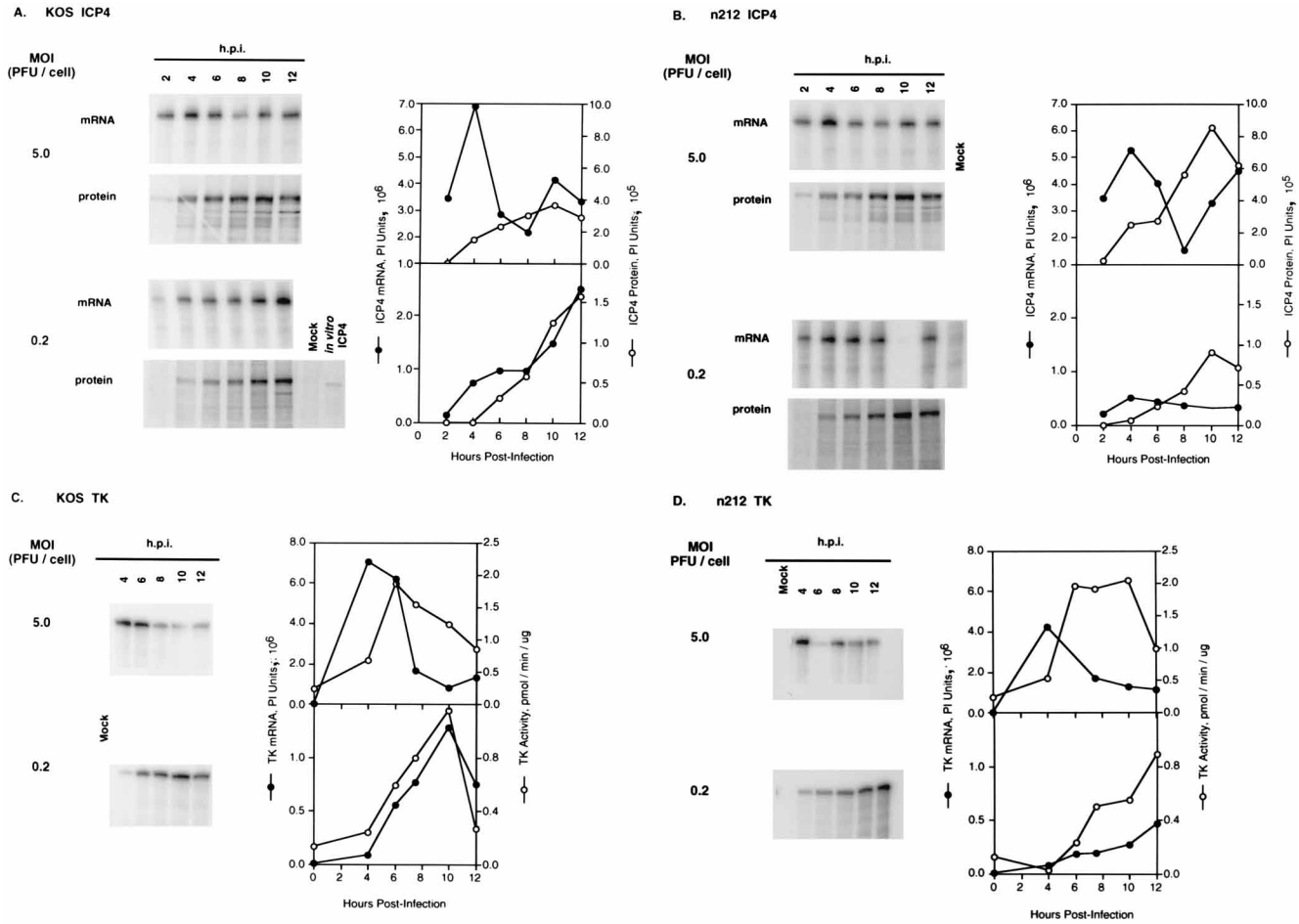

FIG. 4. Kinetics of ICP4 and TK mRNA and protein accumulation in KOS- and *n*212-infected cells. Vero cells (3×10^6/100-mm-diameter dish) were infected with either 0.2 or 5.0 PFU/cell of KOS (A and C) or *n*212 (B and D). (A and B) At 1 hpi the medium was removed and replaced with medium containing either [^{35}S]methionine or unlabeled methionine. At 2-h intervals, infected radiolabeled cells were harvested, and ICP4 was immunoprecipitated with monoclonal antibody H112. The radiolabeled immunoprecipitate was separated by SDS-PAGE. At the same time intervals, cytoplasmic RNA was isolated from the remaining unlabeled cultures, and the level of ICP4 mRNA was measured by quantitative RNase protection assay. ICP4 mRNA and protein were quantitated with a phosphorimager. (C and D) Vero cells were infected as described above; however, at 2-h intervals, cells were harvested and approximately 50% of cells were used to prepare crude soluble enzyme extracts and TK activity was measured. Cytoplasmic RNA was isolated from the remaining cells, and the level of TK mRNA was measured by quantitative RNase protection assay. The data are plotted to show the amounts of mRNA and protein detected as a function of time. Note the difference in scales used to measure mRNA and protein for infections performed at 0.2 and 5.0 PFU/cell. In vitro ICP4 (panel A), in vitro-transcribed and -translated ICP4 used as a marker. The 10-h ICP4 mRNA sample (panel B) and the 6-h TK sample (panel D) were lost during isolation. The data points in the graphs representing these samples have been omitted.

rate limiting. Therefore, gene expression can be saturated at high multiplicities of infection (Fig. 5A) and at high concentrations of mRNA (Fig. 5B). Notably, the effects of multiplicity were independent of ICP0.

ICP0 does not affect mRNA stability but activates gene expression at the level of or prior to initiation of mRNA synthesis. Steady-state accumulation of mRNA is dependent on the rate of mRNA degradation as well as on the rates of initiation and elongation of mRNA synthesis. To measure the stability of TK mRNA in KOS- and *n*212-infected cells, Vero cells infected with 5.0 PFU/cell of KOS and *n*212 were treated with actinomycin D at 6 and 9 hpi, and mRNA was isolated at 0, 0.5, 1, 2, 3, and 5 h post-actinomycin D treatment. The levels of mRNA were measured by Northern blotting using ^{32}P-radiolabeled RNA antisense to TK mRNA as a probe. The reduction in TK mRNA signal as a function of time post-actinomycin D treatment was used to calculate the half-life of TK mRNA. As shown in Table 1, no major differences in the half-life of TK mRNA in the presence or absence of ICP0 were observed at 6 or 9 hpi. The half-lives of these mRNAs are consistent with previously published results (45). These findings indicate that the changes in steady-state mRNA accumulation shown in Fig. 3 to 5 were not due to differences in the stability of mRNA in KOS- and *n*212-infected cells but to changes in the rate of initiation or elongation of mRNA synthesis.

To measure mRNA initiation rates, nuclear run-on experiments were performed. Vero cells were infected with either KOS or *n*212 at multiplicities of 0.4, 1.0, or 5.0 PFU/cell. (At multiplicities lower than 0.4 PFU/cell, hybridization signals became difficult to quantify.) At 5 hpi, nuclei were isolated and newly initiated mRNA was labeled to high specific activity in vitro. The radiolabeled mRNA was hybridized to immobilized single-stranded DNA specific for either sense or antisense transcripts of ICP8, gC, and VP5.

The results shown in Fig. 6 demonstrate that the amount of radioactivity in newly initiated mRNA that hybridized to immobilized DNA was higher in KOS-infected cells than in *n*212-

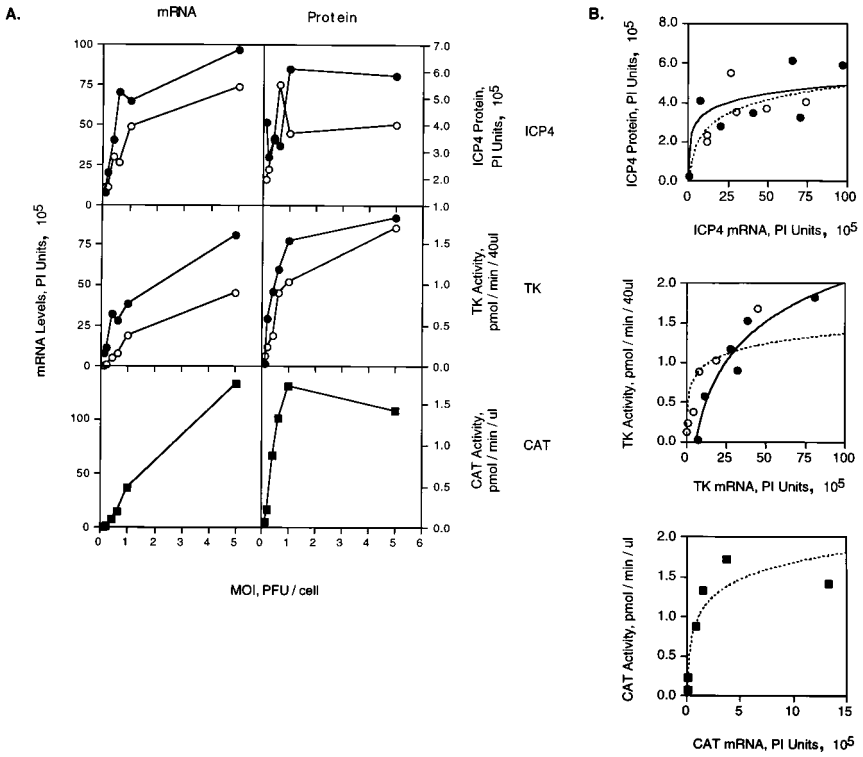

FIG. 5. Multiplicity-dependent synthesis of mRNA and protein in KOS- and *n*212-infected cells. Vero cells (3×10^6/100-mm-diameter dish) were infected at the indicated multiplicities with either KOS (●), *n*212 (○), or P32 (■). Virus P32 contains the ICP8-CAT construct from pMRICP8-CAT inserted in place of the *lacZ* gene in the ICP0 locus. At 3 hpi for ICP4 and 6 hpi for TK and CAT, the levels of mRNA and protein or enzyme activity were measured as described in Materials and Methods. (A) The levels of mRNA and protein or enzyme activity were plotted as a function of multiplicity. The actual ICP4 and TK mRNAs for these tests are shown in Fig. 3. (B) The data shown in panel A were replotted to show the amount of ICP4 protein or TK and CAT activity as a function of mRNA level. A logarithmic regression of the data points is shown (KOS, solid line; *n*212, dotted line). The *r* values for the regression are as follows: 0.444 and 0.601 for ICP4 in KOS- and *n*212-infected cells, respectively; 0.959 and 0.826 for TK in KOS- and *n*212-infected cells, respectively; and 0.929 for CAT in P32-infected cells. PI, phosphorimager.

infected cells at all three multiplicities tested. These data indicate that differences in the levels of mRNA accumulation in *n*212- and KOS-infected cells occur at or before initiation of mRNA synthesis. They do not rule out the involvement of ICP0 in mRNA elongation, however.

ICP0 does not activate gene expression by posttranscriptional mechanisms. Although the results of nuclear run-on assays indicated that ICP0 activates gene expression at or before initiation of mRNA synthesis, it is possible that ICP0 may also act at a posttranscriptional level. To test this possibility the nuclear-cytoplasmic mRNA distribution, polyribosomal distribution, and protein synthesis rates were measured in KOS- and *n*212-infected cells. Vero cells were infected with KOS or *n*212 at multiplicities of 0.5, 1.0, or 5.0 PFU/cell, harvested at 1 to 2 hpi, and fractionated by differential centrifugation into nuclear and cytoplasmic fractions. Total mRNA from each pool was isolated, and the level of TK mRNA in each pool was measured by Northern blotting (Fig. 7). The effectiveness of the fractionation procedure and the gel loading efficiencies were monitored by measuring the amount of preribosomal and ribosomal mRNA in ethidium bromide-stained agarose gels prior to Northern blotting, and they were found to be equivalent in all cases (data not shown). Although the overall levels of nuclear and cytoplasmic TK mRNA were slightly lower in *n*212- than in KOS-infected cells at the lowest multiplicity, and were slightly higher in *n*212- than in KOS-infected cells at the highest multiplicity (Fig. 7, blots), no major differences were observed in the levels of TK mRNA in the nucleus of KOS- or *n*212-infected Vero cells (Fig. 7, graphs).

TABLE 1. Half-life of TK mRNA in KOS- and *n*212-infected cells[a]

Time (hpi)	Virus	TK mRNA half-life (h)			
		Expt 1	Expt 2	Expt 3	Mean (SD)
6	KOS	0.5	1.0	1.8	1.1 (0.7)
	*n*212	1.9	0.9	1.2	1.3 (0.6)
9	KOS	1.1	3.8	ND	2.5 (1.9)
	*n*212	2.2	1.4	ND	1.8 (0.6)

[a] Vero cells were infected with KOS or *n*212 at a multiplicity of infection of 5.0 PFU/cell. At 6 or 9 hpi actinomycin D was added to a concentration of 5 μg/ml. At time zero, mRNA synthesis was inhibited by addition of actinomycin D at a concentration of 5 μg/ml. At 0, 0.5, 1, 2, 3, 5, and 8 h postaddition of actinomycin D, cells were harvested and cytoplasmic mRNA was isolated. The level of TK mRNA was determined by Northern blotting analysis. The decrease in TK signal intensity as a function of time post-actinomycin D treatment was fit to a single exponential decay equation by using the program Enzfitter to calculate the rate of mRNA decay.

The distribution of polyribosomes on mRNA encoding ICP4 or ICP8 was measured in KOS- and *n*212-infected cells. Cytoplasmic RNA from Vero cells infected with 0.1, 1.0, or 10.0 PFU/cell of either *n*212 or KOS was fractionated by velocity sedimentation on sucrose density gradients. The RNA from each fraction was purified, and the amount of ICP4 or ICP8 mRNA was measured by RNase protection assay (Fig. 8). Although most of the infected-cell mRNA copurified with monosome or low-number polysome fractions, no major differences were observed in the polyribosomal distribution for either ICP4 (Fig. 8A) or ICP8 (Fig. 8B) in KOS- and *n*212-

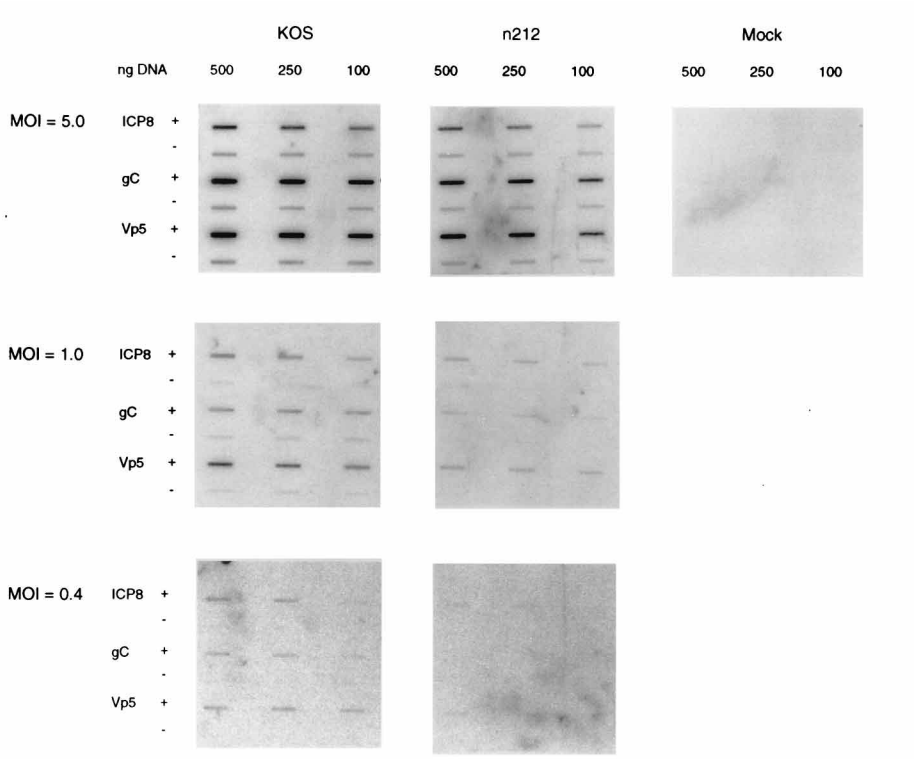

FIG. 6. Nuclear run-on assays. Vero cells (3×10^6 cells/100-mm-diameter dish) were infected with 0.4, 1.0, or 5.0 PFU/cell of either KOS or *n*212. At 5 hpi nuclei were isolated and newly initiated mRNA was labeled to high specific activity in vitro with [^{32}P]GTP. The radiolabeled mRNA was hybridized to single-stranded DNA specific for either the sense (+) or antisense (−) strand of the indicated genes immobilized on nylon membranes.

infected cells. These data indicate that ICP0 does not affect the distribution of polyribosomes on ICP4 or ICP8 mRNA.

ICP0 may activate gene expression by multiple mechanisms which may lead to broad effects on cellular metabolism. Although our findings strongly suggest that ICP0 activates gene expression at a transcriptional or pretranscriptional level, it is also possible that ICP0 has an effect on translation. Indeed, ICP0 was recently reported to interact with a component of the translational apparatus in vitro and in cells in culture (30). No evidence of translational effects of ICP0 during viral infection were reported, however. To determine whether ICP0 exerts a global effect on the regulation of protein synthesis in virus-infected cells, the relative rates of protein synthesis were measured in Vero cells infected with 0.1, 1.0, and 10.0 PFU/cell of either KOS or *n*212. At 3 hpi the rate of incorporation of [^{35}S]methionine into trichloroacetic acid (TCA)-precipitable material was measured. To account for differences in amino acid pool sizes, infected Vero cells were treated with medium containing [^{35}S]methionine or [^{35}S]methionine supplemented with a known concentration of unlabeled methionine. The rates of [^{35}S]methionine incorporation into TCA-precipitable material following each treatment were used to calculate the relative rates of protein synthesis in KOS- and *n*212-infected cells (Table 2). A value of 1.0 corresponds to an equal rate of protein synthesis. As shown in Table 2, the rate of ^{35}S incorporation into TCA-precipitable material in both *n*212- and KOS-infected cells approached 1.0, indicating that no major differences in the relative rates of protein synthesis occurred in KOS- or *n*212-infected Vero cells. Taken together, these results suggest that the contribution of ICP0 to posttranscriptional events is not great.

DISCUSSION

ICP0 activates a wide variety of viral and cellular promoters without apparent DNA sequence specificity. Although the broad transactivating activity of ICP0 has been well-documented, the mechanism by which ICP0 activates gene expression is poorly understood. Because the levels at which proteins function to regulate gene expression reflect their mechanisms of action, we attempted to determine the level at which ICP0 functions. For this purpose, we measured the accumulation of the mRNA and protein products of selected viral genes in transient expression and infection assays in the presence and absence of ICP0. In this paper we report that ICP0 activates gene expression at the level of mRNA accumulation, and more specifically, at or before initiation of transcription. Notably, ICP0 had no major effects on the stability, nuclear cytoplasmic distribution, or polyribosomal distribution of mRNA, or on the rates of protein synthesis, indicating that ICP0 does not affect viral gene expression at a posttranscriptional level. In both transfections and infections, ICP0 had a greater effect on E and L transcript accumulation than on IE transcript accumulation, consistent with previous reports.

ICP0 functions to enhance mRNA synthesis at low multiplicities of infection. A unique characteristic of ICP0 null mutants is the exaggerated multiplicity dependence of their growth relative to wild-type virus (7, 8, 18, 56, 66). At low multiplicities viral gene expression at the mRNA and protein levels, and consequently virus yields, are severely reduced (7, 8, 18, 56). At high multiplicities of infection, however, gene expression and virus yields approach wild-type levels (7, 8, 18, 56). Interestingly, the low-multiplicity growth defect of ICP0

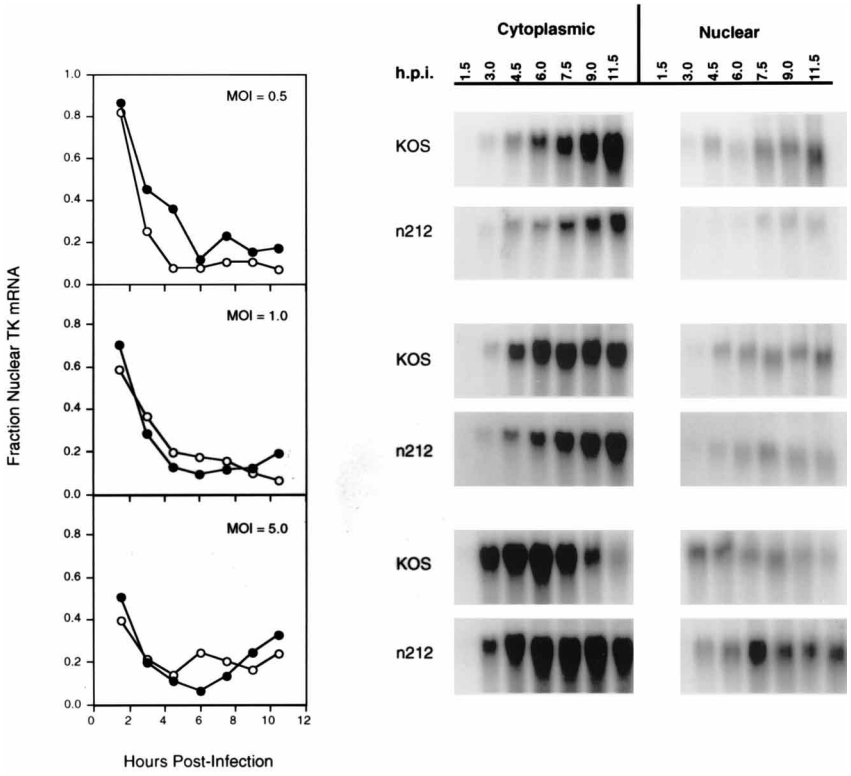

FIG. 7. Nuclear-cytoplasmic distribution of TK mRNA during infection with KOS or *n*212. Vero cells (3×10^6 cells/100-mm-diameter dish) were infected with 0.5, 1.0, or 5.0 PFU/cell of KOS or *n*212. At the times indicated, infected cells were fractionated and RNA was isolated from cytoplasmic and nuclear fractions. Cytoplasmic and nuclear RNAs were separated by gel electrophoresis and transferred to nylon membranes by Northern blotting. Northern blots were probed with ^{32}P-labeled antisense mRNA specific for TK message. The amount of radioactivity in the band corresponding to TK message was quantitated by phosphorimager analysis. The range value for the image display is the same for *n*212 and KOS at each multiplicity. Loading efficiency was monitored by the intensity of ethidium bromide staining of ribosomal or preribosomal mRNA (data not shown). The graphs show the fraction of mRNA relative to the total amount of TK mRNA for KOS (●) and *n*212 (○) at each time point.

null mutants can be complemented by superinfection with HCMV, for which no homolog of ICP0 has been identified (65). These observations suggest that the exaggerated multiplicity dependence of ICP0 null mutants may reflect an activity associated with the herpesvirus infection process that activates viral gene expression and viral replication in the absence of ICP0.

In order to determine whether the level (transcriptional, translational, etc.) at which multiplicity-dependent gene expression occurs is the same or different in the presence and absence of ICP0, the levels of mRNA and protein synthesis from genes representing each of the major kinetic classes of viral genes were measured as a function of multiplicity in KOS- and *n*212-infected cells. The results of these tests demonstrate that at low multiplicities of infection mRNA accumulation is rate limiting and that at high multiplicities translation is rate limiting for gene expression both in the presence and absence of ICP0, although the levels of mRNA and protein were uniformly lower in *n*212- than in KOS-infected cells.

The observation that ICP0 null mutants exhibit markedly reduced levels of mRNA at low multiplicities relative to wild-type virus, and that translation in infected cells is a saturable process, suggests that ICP0 serves to increase the level of mRNA accumulation at low multiplicities of infection when RNA is rate limiting for gene expression. By boosting the level of mRNA during low-multiplicity infection, ICP0 exerts a positive effect on viral growth. At high multiplicities, an activity associated with the infection process per se but independent of ICP0 apparently raises the level of mRNA until a point is reached where the rate-limiting step for gene expression is independent of the level of mRNA (i.e., translation becomes rate limiting). At this point, ICP0 is no longer required for efficient gene expression.

How does ICP0 activate transcription? The findings presented in this paper demonstrate that ICP0 functions at the level of transcript accumulation and, more specifically, at or before initiation of transcription. Available evidence concerning the physical and functional properties of ICP0 supports this hypothesis, suggesting that ICP0 is a transcriptional activator with properties not unlike the transcriptional regulatory proteins of other DNA-containing viruses.

By definition, transcriptional activators function to increase the rate of initiation of mRNA synthesis through binding to specific *cis*-acting DNA sequences as well as to components of the transcriptional complex. Although ICP0 alone increases the rate of initiation of mRNA synthesis, as demonstrated in these studies, it appears to do so without binding to specific DNA sequences. Notably, bona fide viral transcriptional activators that do not themselves bind to specific DNA sequences are well documented. For example, HSV-1 VP16, the virion-associated activator of IE gene expression, activates transcription through interactions with cellular DNA binding proteins (2, 29, 33, 42, 49, 64). Moreover, E1A, which is incapable of binding directly to DNA, activates gene expression indirectly through interactions with sequence-specific DNA binding proteins (14). It may be that ICP0 also activates gene expression

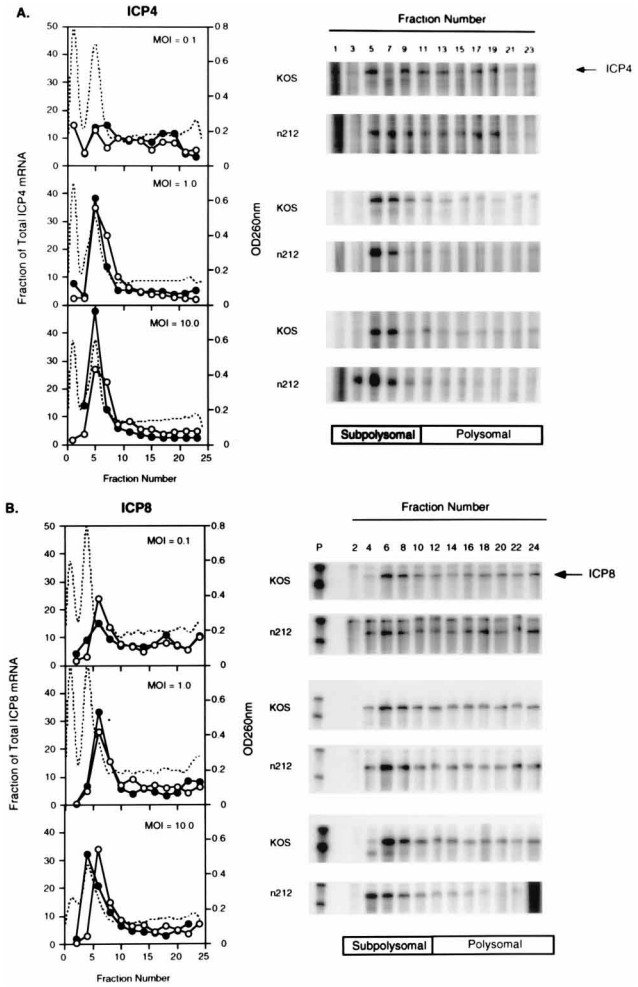

FIG. 8. Distribution of polyribosomes on ICP4 and ICP8 mRNA in *n*212- and KOS-infected cells. Vero cells (4×10^7) were infected with 0.1, 1.0, or 10.0 PFU/cell of KOS (●) or *n*212 (○). At 6 hpi cells were harvested and cytoplasmic mRNA was isolated and fractionated by velocity sedimentation on 5 to 20% sucrose gradients (12 ml). Fractions (0.5 ml) were collected from the top of the gradient and absorbance at 260 nm was measured (dotted lines). The mRNA in each fraction was quantitated by RNase protection assay with probes specific for ICP4 (A) or ICP8 (B) message. The subpolysomal pool contained 40S, 60S, and 80S ribosomal subunits as determined by ethidium bromide staining of the agarose gels run prior to Rnase protection assay and from the absorbance profiles of the fractions at 260 nm. The polysomal pool constituted the remaining fractions. The graphs represent the amount of ICP4 or ICP8 mRNA in each fraction relative to the total amount of mRNA isolated.

indirectly through interactions with other viral or cellular proteins. Indeed, ICP0 cooperatively activates gene expression with ICP4, a sequence-specific DNA binding protein which is itself a transcriptional activator. Moreover, ICP0 interacts physically with ICP4 in vitro (69). Based on these findings and on its ability to stimulate mRNA synthesis, ICP0 can be regarded as a bona fide transcriptional activator.

Other properties of ICP0, however, suggest that its broad transcription-activating function is more complex. HSV-1 replicates in both dividing and nondividing cells, and it is ICP0 that confers cell cycle independence on HSV-1 replication (6). This property of ICP0 and its ability to activate transcription suggest that ICP0 can either substitute for or activate cell-cycle-regulated activities that in turn activate transcription. Consistent with this hypothesis is the observation that a cellular activity expressed maximally in Vero cells after release from growth arrest in the G_0/G_1 phase of the cell cycle can complement the low-multiplicity growth phenotype of ICP0 null mutants (6). This same activity, or one expressed at a similar time in cells of neural lineage, can specifically activate HSV-1 gene expression in transient-transfection assays (50). The ability of ICP0 to colocalize with and redistribute proteins in nuclear substructures (ND10) (3, 36, 37) involved in cell growth regulation may reflect, in part, the mechanism by which ICP0 influences cell cycle controls (13, 23, 31, 37, 68). The existence of cellular activities able to complement ICP0 null mutants implies that these activities create a specific cellular environment conducive to activation of viral gene expression in response to induction of cell-cycle-regulated factors and suggests that ICP0 may induce these cell-cycle-specific activities. Thus, in addition to its role as a transcriptional activator, ICP0 may regulate the activities of cellular transcription factors in a manner similar to the way cells regulate transcription factors after stimulation of signal transduction pathways, e.g., through mechanisms involving protein phosphorylation and modulation of protein stability by targeted ubiquitination as suggested by Everett (et al. 14a). Such a mechanism for activating cellular activities may be critical during reactivation from latency when initiation of viral gene expression in the absence of ICP0 and other viral factors is required to initiate viral replication. Efforts are currently under way to determine whether the cellular activities that substitute for ICP0 act at the level of mRNA synthesis, as one would predict.

TABLE 2. Rate of protein synthesis in *n*212-infected cells relative to that in KOS-infected cells[a]

MOI (PFU/cell)	Rate			
	Expt 1	Expt 2	Expt 3	Mean (SD)
0.1	1.5	1.1	2.6	1.7 (0.8)
1.0	1.2	0.9	0.7	0.9 (0.2)
10	0.9	0.9	0.4	0.7 (0.8)

[a] Vero cells were infected with KOS or *n*212 at the indicated multiplicities of infection (MOI). At 3 hpi, the medium was removed, and fresh medium supplemented with [^{35}S]methionine or [^{35}S]methionine containing 75 μM unlabeled methionine was added. At 2-min intervals the cells were harvested and TCA-precipitable counts were measured. The relative rates of protein synthesis in KOS- and *n*212-infected cells were calculated from the differences in the slopes of samples containing 75 μM unlabeled methionine and samples containing only [^{35}S]methionine. These data were used to calculate the ratio of protein synthesis rates in *n*212-infected cells to those in KOS-infected cells according to a previously described method (41). This method provides an estimate of protein synthesis rates independent of amino acid pool sizes. A value of 1.0 corresponds to an equal rate of protein synthesis.

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