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ICP27 Selectively Regulates the Cytoplasmic Localization of a Subset of Viral Transcripts in Herpes Simplex Virus Type 1-Infected Cells

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Evidence suggests that the herpes simplex virus regulatory protein ICP27 mediates the nuclear export of viral transcripts; however, the extent of this activity during infection is unclear. ICP27 is required for efficient expression of the long, leaky-late *UL24* transcripts, but not for that of the short, early *UL24* transcripts. We found that infection by an *ICP27*-null mutant resulted in undetectable UL24 protein expression, which represented at least a 70-fold decrease relative to that of wild-type virus. Because lack of ICP27 had a greater effect on levels of UL24 protein than on transcripts, we examined its effect on subcellular localization of *UL24* transcripts. In wild-type-infected cells, both short and long *UL24* transcripts fractionated predominantly with the cytoplasm. However, in the absence of ICP27, greater than 50% of long *UL24* transcripts were nuclear, while the percentage of short *UL24* transcripts that were cytoplasmic was not reduced. These results also imply that the short *UL24* transcripts are translated poorly. The effect of ICP27 on cytoplasmic localization of the long *UL24* transcripts did not extend to other transcripts with which it shared a common 3′ end or to other transcripts tested, including *gC* and *UL42*, whose overall expression is highly dependent on ICP27. Thus, the dual effects of ICP27 on mRNA accumulation and cytoplasmic localization are not always linked. These results identify viral transcripts that are dependent on ICP27 for efficient cytoplasmic localization during infection, but they also indicate the existence of ICP27-independent nuclear export pathways that are accessible to many viral transcripts during infection.

Eukaryotic gene expression is a highly complex process in which the multiple stages of transcription and translation are linked, with each stage potentially subject to regulation (reviewed in reference 29). When herpes simplex virus type 1 (HSV-1) infects a cell, viral genes are expressed in an ordered temporal pattern of immediate-early, early, and late genes, which results from the combined activity of viral and cellular factors, including RNA polymerase II (reviewed in reference 42). Like cellular mRNAs, viral mRNAs must be exported from the nucleus via RNA export factors to be translated in the cytoplasm. mRNA export factors are recruited cotranscriptionally and in a splicing-dependent manner to nascent transcripts and direct them to the nuclear pore complex for export (26, 27; reviewed in reference 29). The nuclear export of many cellular mRNAs is mediated by the REF(Aly)/TAP pathway (41; reviewed in reference 8). However, not all mRNAs use this pathway. For example, the intronless transcripts of human immunodeficiency virus type 1 access a CRM1-dependent export pathway through the virally encoded Rev protein (28, 54).

The HSV-1 protein ICP27 (512 amino acids) is a critical regulatory factor mediating the efficient expression of certain early and most late genes (30, 31, 39, 52). ICP27 stimulates viral gene expression at least in part at the level of transcription (23, 30). ICP27 associates with the RNA polymerase II holoenzyme and the viral transcription factor ICP4 (34, 55). It can also function to repress expression from certain viral promoters (46, 49). Through the stimulation of expression of early genes that encode replication proteins, ICP27 promotes viral DNA replication (30, 52). However, ICP27 can also function at the posttranscriptional level. ICP27 has been reported to stimulate the usage of certain polyadenylation [poly(A)] signals (31, 32) and to inhibit host gene expression, in part, by inhibiting splicing (3, 15, 16). ICP27 has been demonstrated to shuttle between the nucleus and cytoplasm, suggesting a role for it in the nuclear export of viral transcripts (38, 45, 47). Consistent with these observations, overexpression of the REF/Aly nuclear export factor increases the ICP27-dependent nuclear export of certain viral transcripts in the context of infection (4), and in *Xenopus laevis* oocyte microinjection experiments ICP27 can stimulate the nuclear export of certain viral transcripts through the REF/Aly cellular mRNA nuclear export pathway (24). However, it is not clear how important this particular activity of ICP27 is to the efficient nuclear export of viral transcripts during infection.

The HSV *UL24* gene encodes a nuclear-associated protein (18, 35) that appears to inhibit cell-to-cell fusion during infection (22, 44, 50). The expression of *UL24* is quite complex (Fig. 1). Six *UL24* transcripts have been identified which originate from three transcription start sites and terminate either at the *UL24* poly(A) signal and exhibit early kinetics (1.4, 1.2, and 0.9 kb; short *UL24* transcripts) or at the *UL26* signal and exhibit leaky-late kinetics (5.6, 5.4, and 5.2 kb; long *UL24* transcripts) (6, 14). The mRNAs originating from both the first and second transcription start site can be translated into full-length UL24 protein (35). In the absence of ICP27, levels of the long *UL24* transcripts are greatly diminished, while levels of the short *UL24* transcripts are affected little, if at all (14).

To investigate further how ICP27 regulates *UL24* gene expression, we examined the effect of an *ICP27*-null mutation on

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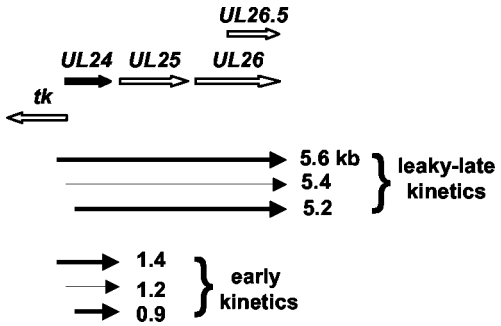

FIG. 1. Diagram of the *UL24* transcription unit. The relative positions and orientations of the *UL24* and flanking ORFs are indicated by the fat arrows at the top of the figure. The six transcripts containing *UL24* sequence that have been identified are illustrated below. The three long transcripts that terminate at the end of *UL26* are expressed with leaky-late kinetics. The three short transcripts that terminate at the end of *UL24* are expressed with early kinetics. Transcripts beginning at the second *UL24* transcription start site are less abundant than those beginning at the other two start sites, which is reflected qualitatively by the relative thickness of the arrows.

UL24 protein expression. These experiments led us to test whether ICP27 functions to regulate expression of *UL24* transcripts at the level of mRNA nuclear export. We have found that ICP27 is required for efficient cytoplasmic localization of the long *UL24* transcripts but not of the short *UL24* transcripts or many other viral mRNAs. Our results provide evidence that translation of the short *UL24* transcripts is inefficient and suggest the existence of multiple nuclear export pathways for viral transcripts during infection, including ICP27-independent pathways.

MATERIALS AND METHODS

Cells and viruses. HSV-1 wild-type strain KOS1.1 (20) and the *ICP27*-null mutant *d*27 (39) were propagated on Vero cells (American Type Culture Collection) or V27 cells (39), respectively, as described previously (5). For analysis of gene expression, cells in 60-mm-diameter dishes were infected at a multiplicity of infection (MOI) of 10 PFU per cell, and the inocula were back-titrated on V27 cells (an ICP27-complementing cell line) to confirm that equal PFU of each virus were used.

Western blotting. The harvesting of lysates from infected cells and Western blotting analysis using rat UL24 antiserum were carried out as described previously (35). Quantification of UL24 signals was done using a standard curve derived from a dilution series of infected cell lysate that was run on the same gel as the experimental samples. The antibody directed against HSV thymidine kinase (TK) protein was generously provided by W. C. Summers (Yale University). The TK signal was detected by chemiluminescence using the SuperSignal West Femto kit (Pierce) according to the manufacturer's instructions.

RNA isolation. RNA was isolated using the Qiagen RNeasy kit following the manufacturer's instructions for adherent cells. To isolate RNA from cytoplasmic and corresponding nuclear fractions, cells were scraped and collected in 1 ml of phosphate-buffered saline. The RNA from the cytoplasmic fractions was isolated following the Qiagen RNeasy protocol for isolation of cytoplasmic RNA. RNA was also isolated from the nuclear pellets, which were resuspended in lysis buffer provided by the kit and processed according to the protocol for isolation of total RNA from cells.

Northern blot hybridization. [^{32}P]dCTP- or [^{32}P]dATP-labeled DNA probes (New England Nuclear) were synthesized by using the DNA Random Primed DNA Labeling kit (Roche) following the manufacturer's instructions. The DNA templates used to make various DNA probes were as follows: for *UL24* (7) and *gB* (7) the templates have been described previously. For VP16 the 993-bp template was synthesized by PCR from viral DNA using the primers 5′ CTAT GTACCATGCTCGATAC and 5′ CGTCTAGCGCGTCGGCA. For *UL42* the 991-bp template was synthesized by PCR using the primers 5′ CGTTTCGCAC GCTGGTTC and 5′ AGGTCGCGAAAGTAACAC. For *gC* the 1,062-bp template was synthesized by PCR using the primers 5′ CGTGTGGTGCGACCGC and 5′ TCAACCGACAGATGTACTC. For *UL26* the 790-bp *Bam*HI fragment containing the *UL26* sequence was isolated from the EcoRIF clone (13) and ligated into pSK+ (Stratagene) (plasmid constructed and graciously provided by David Wensel). The *Bam*HI fragment of this plasmid was subsequently excised and used as a template. All oligonucleotides were obtained from Integrated DNA Technologies. RNA was resolved on a 0.9% denaturing agarose gel (SeaKem) containing formaldehyde in a 3[*N*-morpholino] propanesulfonic acid buffering system (43). Northern blot hybridization was carried out as described previously (14) except for the experiment shown in Fig. 3, where PerfectHyb Plus hybridization solution (Sigma) and Hybond N$^+$ membrane (Amersham Pharmacia) were used. Signals were detected by autoradiography. For quantification, blots were exposed to phosphor storage screens and signals were analyzed using the Personal Molecular Imaging FX system (Bio-Rad) and Quantity One software (Bio-Rad). Background signals were subtracted from each measured experimental value. To visualize particularly faint bands for quantification, the images were transformed to make the bands appear darker (a process that does not alter their measured values), which allowed for their accurate delineation.

RESULTS

ICP27 is required for the expression of UL24 protein. In previous experiments our laboratories found that an *ICP27*-null mutation resulted in a marked decrease in levels of the long *UL24* transcripts that arise from utilization of the *UL26* poly(A) signal but had little, if any, effect on the levels of the short *UL24* transcripts that arise from the use of the *UL24* poly(A) signal (14). We wanted to compare the effect of ICP27 on UL24 protein levels with that on levels of *UL24* transcripts. Vero or V27 cells were either mock infected (Fig. 2, top panel, lanes 1 and 4) or infected with either KOS1.1 (lanes 2 and 5) or with *d*27 (lanes 3 and 6), which contains a null mutation in the *ICP27* gene, and at 15 h postinfection (p.i.) cell lysates were harvested, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and analyzed in Western blot experiments. UL24 was clearly detected in lysates from Vero cells infected with the parental strain, KOS1.1 virus (top panel, lane 2). In contrast, we were unable to detect any UL24 protein in lysates from Vero cells infected with *d*27 virus (lane 3). A standard curve was constructed based on a dilution series of infected cell lysate run on the same gel (lanes 7 to 15), which allowed us to calculate that in this experiment there was at least 70-fold less UL24 expressed in the absence of ICP27 than in its presence. UL24 protein levels were restored to nearly wild-type levels when the *d*27 virus was grown on the ICP27-complementing cell line V27 (lanes 5 and 6), indicating that the defect in UL24 protein levels seen in Vero cells was indeed a consequence of the *ICP27* mutation. As a loading control, the blot was stripped and incubated with an antibody directed against the HSV TK protein (Fig. 2, bottom panel). We found that levels of this viral protein varied less than threefold between samples. We also quantified *UL24* transcript levels in total RNA isolated from cells infected with KOS1.1 or *d*27 (Fig. 3). In this particular experiment there was a 10-fold decrease in levels of *UL24* transcripts (short and long combined) (compare lanes 2 and 3). This effect varied among experiments from as low as 5-fold to as high as 40-fold, although we consistently observed that the effect on levels of the short *UL24* transcripts was quite modest, as opposed to the much more pronounced decrease in levels of the long *UL24* transcripts. Thus, even though close-to-wild-type levels of the short *UL24* transcripts and some, though clearly reduced, levels of the long *UL24* transcripts were ex-

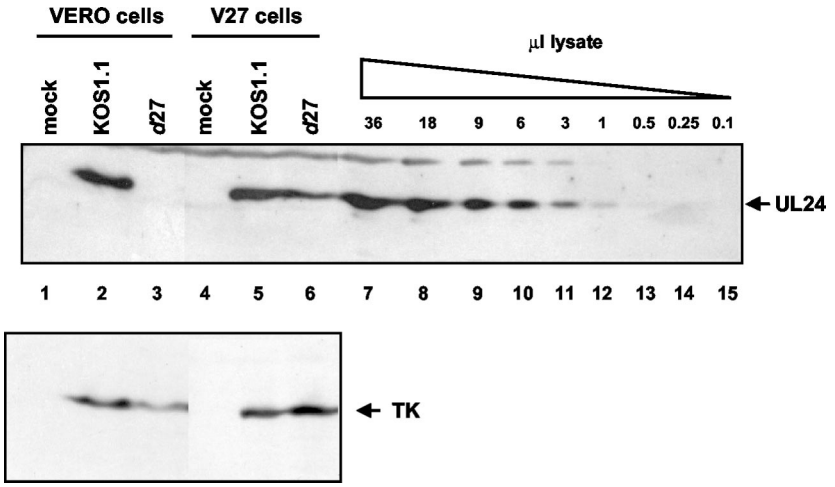

FIG. 2. Western blot showing the effect of an *ICP27*-null mutation on the expression of UL24 protein in infected cells. Vero or V27 cells were either mock infected (lanes 1 and 4) or infected at an MOI of 10 with either KOS1.1 (lanes 2 and 5) or *d*27 (lanes 3 and 6) cells, and total cell lysates were harvested at 15 h p.i. and concentrated 10-fold. A 20-μl aliquot of each concentrated lysate was analyzed by Western blotting with antiserum raised against UL24, the position of which is indicated to the right of the top panel. In lanes 7 to 15, a dilution series of concentrated KOS1.1-infected cell lysate was loaded. The volumes (in microliters) are indicated above the panel. The membrane was subsequently stripped and incubated with an antibody directed against the HSV TK, the position of which is indicated to the right of the bottom panel.

pressed, we were unable to detect any UL24 protein in the absence of ICP27.

ICP27 affects the nucleocytoplasmic distribution of a subset of *UL24* transcripts. Because levels of the short *UL24* transcripts are affected little in cells infected with an *ICP27*-null virus and, although reduced in amount, some long *UL24* transcripts are still detected, we investigated the possibility that the lack of UL24 protein expression was a consequence of a defect in mRNA nuclear export. Vero and V27 cells were either mock infected or infected with KOS1.1 or *d*27. At 13 h p.i. cells were harvested, and RNA was isolated from the corresponding cytoplasmic and nuclear fractions, resolved on an agarose gel, and stained with ethidium bromide (Fig. 4A). As expected, the precursor 45S and 32S rRNAs were detected only in the nuclear and not in the cytoplasmic fractions. Likewise, the 28S and 18S rRNAs, which are processed in the nucleus and then quickly exported to the cytoplasm (9), were predominantly in the cytoplasmic fractions. Thus, we concluded that the fractionation into, and subsequent isolation of RNA from, the cytoplasmic and nuclear fractions was successful. Nucleic acids from this gel were transferred to nitrocellulose for analysis by Northern blotting and probed for *UL24* sequences. The blot presented in Fig. 4B is overexposed so that it is possible to clearly see the long *UL24* transcripts in the *d*27 samples. The migration of the *UL24* long transcripts, particularly in the cytoplasmic lanes, is altered because of the comigration of the broad band of 28S rRNA (Fig. 4A). However, despite these complications Northern blot analysis allowed us to differentiate between and quantify (see Materials and Methods) the short and long *UL24* transcripts. Where percentages of nuclear and cytoplasmic transcripts are reported, 100% represents the total amount of transcript detected in both fractions together, which varied from transcript to transcript.

In Vero cells infected with the parental strain KOS1.1 (Fig. 4B, lanes 3 and 4), most of both the long and short *UL24* transcripts were cytoplasmic. However, in Vero cells infected with *d*27 (lanes 5 and 6), we observed different effects of the lack of ICP27 on the intracellular distribution of the long and the short *UL24* transcripts. In Vero cells infected with *d*27, the fraction of 1.4-kb *UL24* transcripts, which are the most clearly visible of the short *UL24* transcripts, that was cytoplasmic was slightly more than that in KOS1.1-infected cells (87 versus 76%). (In this experiment we also observed increased levels of short *UL24* transcripts in Vero cells infected with *d*27 com-

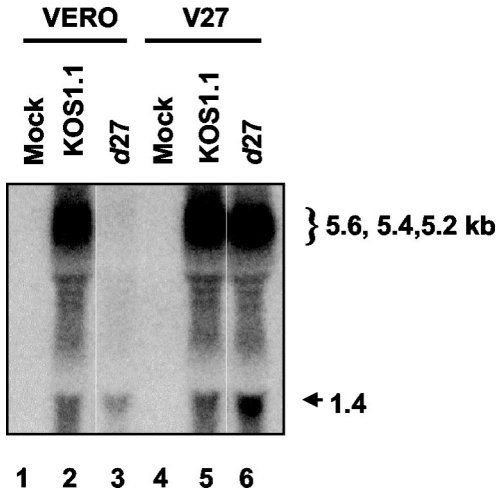

FIG. 3. Effect of an *ICP27*-null mutation on the expression of *UL24* transcripts in infected cells. Vero or V27 cells were either mock infected (lanes 1 and 4) or infected at an MOI of 10 with either KOS1.1 (lanes 2 and 5) or *d*27 (lanes 3 and 6) cells, and at 13 h p.i. total RNA was isolated. RNA was analyzed by Northern blot hybridization with a probe corresponding to the *UL24* sequence. The position of the 1.4-kb short *UL24* transcript is indicated by an arrow to the right of the panel. The position of the broad band corresponding to the 5.2-, 5.4-, and 5.6-kb-long *UL24* transcripts is indicated by a bracket to the right of the panel.

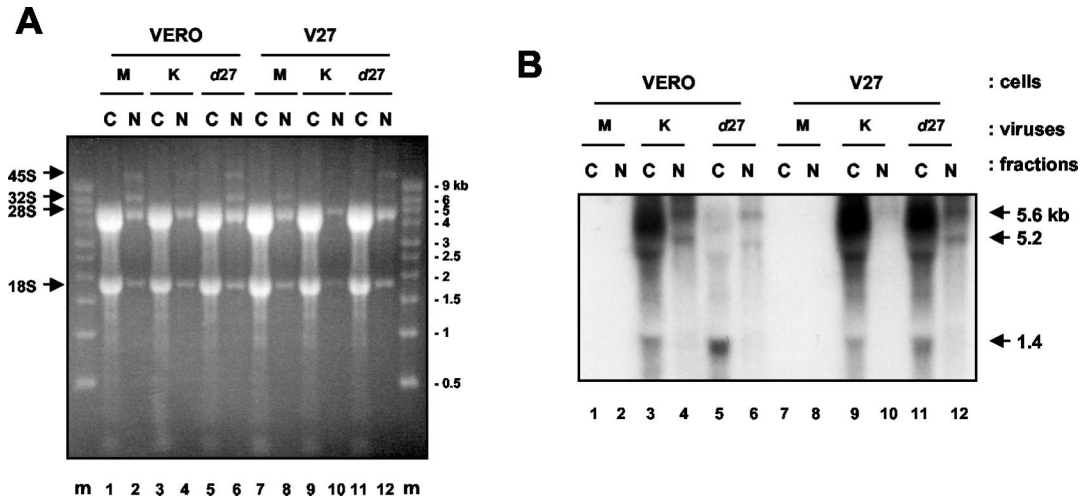

FIG. 4. ICP27 affects the cytoplasmic versus nuclear distribution of the long, leaky-late *UL24* transcripts. Vero cells (lanes 1 to 6) or V27 cells (lanes 7 to 12) were either mock infected (M) (lanes 1, 2, 7, and 8) or infected with KOS1.1 cells (lanes 3, 4, 9, and 10) or the *ICP27*-null virus *d*27 (lanes 5, 6, 11, and 12). (A) RNA was isolated from nuclear (N) and cytoplasmic (C) fractions at 13 h p.i., resolved on a denaturing formaldehyde agarose gel, and stained with ethidium bromide to allow for visualization of the major rRNA species. The positions of the various rRNAs are indicated by arrows to the left of the panel. RNA molecular mass markers (Ambion) were resolved in the first and last lanes of the gel (lanes m). The sizes of the markers are located to the right of the panel. (B) Nucleic acids from the agarose gel were analyzed by Northern blot hybridization for *UL24* transcripts, the positions of which are indicated by arrows to the right of the panel. The bands corresponding to the long *UL24* transcripts in the cytoplasmic fractions are distorted due to the comigration of the 28S rRNA.

pared to KOS1.1. This was also observed for the early transcripts *ICP8* [data not shown] and *tk* [reference 10 and data not shown]. These modest increases likely reflect the role of ICP27 in DNA replication and in the transition from early to late gene expression [30, 31, 39]. Presumably, in the absence of ICP27, early gene expression is not down-regulated efficiently, leading to somewhat higher levels of expression of the early, short *UL24* transcripts at late times in infection.)

In contrast, the percentage of the remaining long *UL24* transcripts that was cytoplasmic dropped from 70% in KOS1.1-infected Vero cells to 47% in *d*27-infected cells. This defect in cytoplasmic localization was highly reproducible. Furthermore, when the *d*27 virus was grown on the ICP27-complementing cell line V27 (lanes 11 and 12), cytoplasmic localization of the majority of long *UL24* transcripts was restored. Although the defect in cytoplasmic localization of the long *UL24* transcripts in *d*27-infected cells was not complete, these results show that during infection ICP27 is important for the efficient cytoplasmic localization of the long *UL24* transcripts, but not of the short *UL24* transcripts. In addition, the observation that levels of long *UL24* transcripts in the nucleus are reduced in *d*27-infected cells compared to wild-type-infected cells suggests a defect in synthesis of these transcripts.

Interestingly, we also observed a slight increase in the cytoplasmic localization of both the short and long *UL24* transcripts (to 96 and 89%, respectively) when levels of ICP27 were unusually high as a result of infecting an ICP27-complementing cell line (V27) with an ICP27-competent virus (KOS1.1) (52) such as seen in Fig. 4B (compare lanes 3 and 4 to lanes 9 and 10), an observation that we have made for several other viral transcripts analyzed (see subsequent figures).

The effect of ICP27 on the nucleo-cytoplasmic distribution of the long *UL24* transcripts does not correlate with utilization of the *UL26* poly(A) signal. One possible explanation for the differential effect of an ICP27 defect on the levels and subcellular distribution of various *UL24* transcripts is that the long *UL24* transcripts utilize the *UL26* poly(A) signal while the short *UL24* transcripts utilize the poly(A) signal immediately 3′ to the *UL24* open reading frame (ORF). The long *UL24* transcripts (5.6, 5.4, and 5.2 kb) share the *UL26* poly(A) signal with the *UL25* (4.2 kb), *UL26* (2.4 kb), and *UL26.5* (1.4 kb, not to be confused with the 1.4-kb *UL24* transcript) transcripts (Fig. 5A). We therefore investigated whether the effects of ICP27 on expression and nucleocytoplasmic distribution of the long *UL24* transcripts were also observed for those transcripts with which it shares a poly(A) signal. We stripped the Northern blot shown in Fig. 4 and probed it with sequences contained within *UL26.5*, which would hybridize to the six transcripts illustrated in Fig. 5A. We found that levels of *UL26.5*, *UL26*, and *UL25* transcripts (Fig. 5B) were reduced somewhat in *d*27-infected cells (less than twofold). However, the percentages of cytoplasmic *UL26.5* and *UL26* transcripts were similar in *d*27-infected Vero cells compared to KOS1.1-infected Vero cells (76 to 70% and 84 to 89%, respectively). In this experiment it was not possible to clearly differentiate between the long *UL24* and the *UL25* transcripts. However, the *UL25* transcripts have been reported to be more abundant than any of the long *UL24* transcripts (17). Therefore, because greater than 50% of the long *UL24* transcripts from cells infected with *d*27 were nuclear (Fig. 4B), the observation that the 4.2-kb signal was stronger in the cytoplasmic fraction than in the nuclear one in Fig. 5B implied that the *UL25* transcripts were efficiently localized to the cytoplasm in cells infected with the *d*27 virus. Thus, it appears that the 3′ end of the long *UL24* transcripts was not sufficient to mediate ICP27-dependent cytoplasmic localization.

Effect of ICP27 on the nucleocytoplasmic distribution of other viral transcripts. ICP27 is reported to associate prefer-

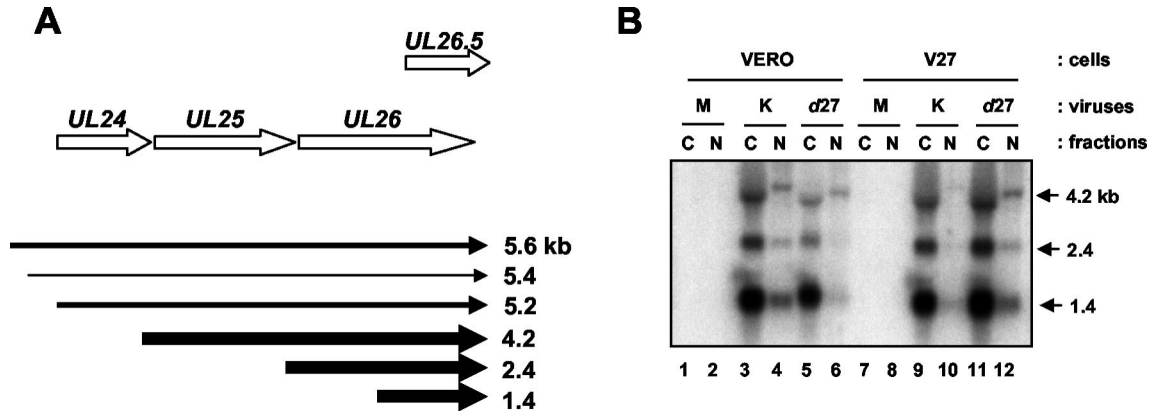

FIG. 5. The 3′ UTR of the long *UL24* transcripts is not sufficient to confer ICP27-dependent cytoplasmic localization. (A) Diagram illustrating the six 3′ coterminal transcripts that utilize the *UL26* polyadenylation signal. The 5.6-, 5.4-, and 5.2-kb transcripts represent the long *UL24* transcripts, the 4.2-kb transcripts originate from the *UL25* promoter, the 2.4-kb transcripts originate from the *UL26* promoter, and the 1.4-kb transcript corresponds to *UL26.5*. (B) The membrane analyzed in Fig. 4 was stripped and hybridized with a radioactive probe corresponding to the *UL26.5* sequence. The positions of the *UL25* (4.2-kb), *UL26* (2.4-kb), and *UL26.5* (1.4-kb) transcripts are indicated by arrows to the right of the panel. M, mock; K, KOS1.1; N, nuclear; C, cytoplasmic.

entially with certain viral mRNAs in the cytoplasmic compartment of cells (45). ICP27 interacts with REF (4, 24), a component of the REF(Aly)/TAP cellular mRNA nuclear export pathway, and stimulates the nuclear export of certain viral transcripts in *Xenopus* oocyte microinjection experiments (24). Furthermore, overexpression of REF through transient transfection of mammalian cells stimulates the nuclear export of certain viral transcripts during infection in an ICP27-dependent manner (4). We therefore asked what effect the absence of ICP27 had on the cytoplasmic-to-nuclear ratio of some of these viral transcripts during a typical infection in cell culture. The blot from Fig. 4 was stripped and reprobed for *gB* transcripts, which have been reported to become more cytoplasmic upon overexpression of REF in an ICP27-dependent manner (4). Total levels of *gB* transcripts were approximately twofold lower in cells infected with *d*27 than in cells infected with the parental virus KOS1.1 (Fig. 6A). To our surprise, we did not observe a meaningful difference in the ratio of cytoplasmic to nuclear *gB* transcripts between the KOS1.1- and *d*27-infected Vero cells (83 to 17% and 82 to 18%, respectively). We did note, however, that similar to what we observed for *UL24* transcripts, there was a slight increase in the cytoplasmic localization of *gB* transcripts when levels of ICP27 were unusually high, as a result of infecting an ICP27-complementing cell line (V27) with an ICP27-competent virus (KOS1.1) (93%) (compare lanes 3 and 4 to lanes 9 and 10).

We also examined transcripts that contain the *VP16* ORF. In *Xenopus* oocyte experiments, coinjection of recombinant ICP27 stimulated the nuclear export of synthetic *VP16* transcripts (24). We used a probe corresponding to sequences within the *VP16* ORF to probe the same membrane analyzed in the previous figures. This probe detected three coterminal transcripts corresponding to transcripts originating at the *UL48* (*VP16*) promoter (1.7 kb), the *UL49* promoter (3.2 kb), and the *UL49.5* promoter (3.8 kb) (36) (Fig. 6B). Levels of the *UL48* and *UL49* transcripts were similar in *d*27- and KOS1.1-infected cells, although levels of *UL49.5* transcripts were clearly reduced in the absence of ICP27. Once again, we were surprised to find that *VP16* transcripts localized efficiently to the cytoplasm in Vero cells infected with either KOS1.1 or *d*27 (lanes 3 to 6). The band corresponding to the *UL49.5* (3.8-kb) transcripts was distorted in the cytoplasmic fraction due to comigration of the 28S rRNA; however, it does appear that there was a modest increase in the relative percentage of 3.8-kb transcripts present in the nuclear fraction of *d*27-infected Vero cells compared with the KOS1.1-infected cells (30 and 19%, respectively). The significance of this observation is difficult to ascertain, although it could be consistent with a role for ICP27 in the nucleocytoplasmic distribution of transcripts containing VP16 sequences. Similar to what we observed previously, there appeared to be a modest increase in cytoplasmic localization of each of these transcripts when the wild-type virus was grown on the ICP27-complementing cell line (compare lanes 3 and 4 to lanes 9 and 10).

We decided to examine a transcript that is known to be highly dependent on ICP27 for its synthesis in case there was a link between a dependency on ICP27 for expression and a dependency on ICP27 for efficient cytoplasmic localization. Thus, we analyzed *gC*, whose transcription has been demonstrated to be highly dependent on ICP27 (23, 40). As expected, in the absence of ICP27 levels of *gC* transcripts were greatly reduced, in this particular experiment greater than 50-fold (Fig. 6C) and, thus, in order to visualize them the blot was overexposed. However, despite this critical dependency on ICP27 for expression, those *gC* transcripts that were expressed were predominantly cytoplasmic. Therefore, it would appear that a dependency on ICP27 for synthesis is not necessarily indicative of a dependency for cytoplasmic localization.

Effect of ICP27 on the nucleocytoplasmic distribution of transcripts at earlier times in infection. We considered the possibility that the reason we did not detect a dependency on ICP27 for the cytoplasmic location of any transcripts tested other than the long *UL24* transcripts was related to the relatively late time point we had chosen. This choice had been made to ensure that we would be able to detect the reduced levels of the long *UL24* transcripts in *d*27-infected cells well

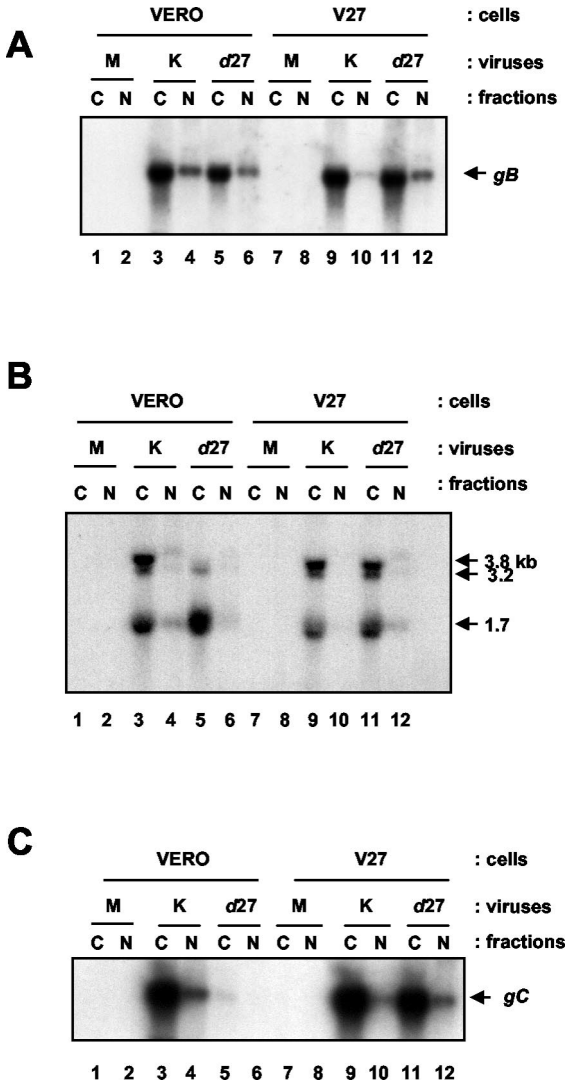

FIG. 6. Effect of ICP27 on the cytoplasmic localization of *gB*, *VP16*, and *gC* transcripts. (A) The membrane analyzed in Fig. 4 was stripped and hybridized with a radioactive probe corresponding to the *gB* sequence. The position of the *gB* transcripts is indicated to the right of the panel. (B) The membrane in Fig. 4 was stripped and hybridized with a *VP16*-specific probe (*UL48*). The positions of the 1.7-kb transcripts originating at the *UL48* promoter, the 3.2-kb transcripts originating at the *UL49* promoter, and the 3.8-kb transcripts originating at the *UL49.5* promoter are indicated to the right of the panel. (C) The membrane in Fig. 4 was stripped and hybridized with a *gC*-specific probe. The position of the *gC* transcripts is indicated to the right of the panel. Abbreviations for all three panels: M, mock; K, KOS1.1; N, nuclear; C, cytoplasmic.

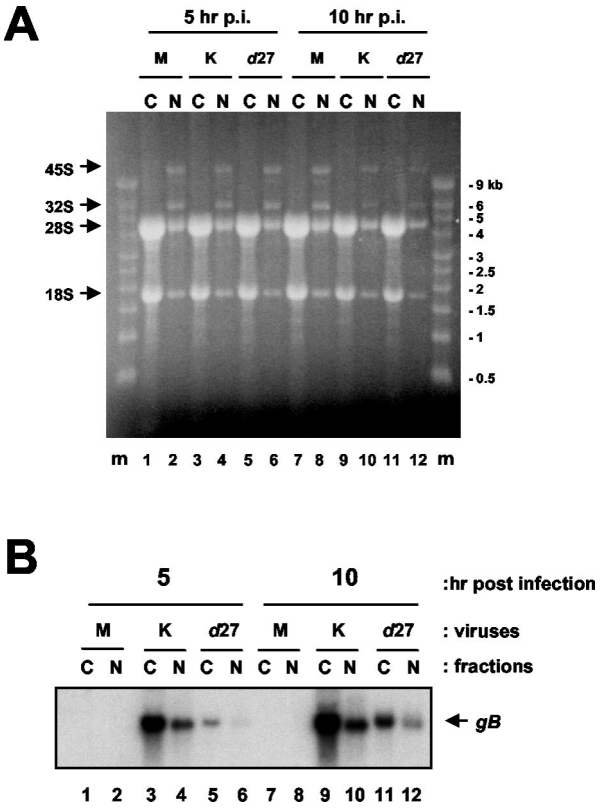

FIG. 7. Effect of ICP27 on the cytoplasmic localization of *gB* transcripts at earlier times in infection. (A) Vero cells were either mock infected (lanes 1, 2, 7, and 8) or infected with KOS1.1 (lanes 3, 4, 9, and 10) or the *ICP27*-null virus *d*27 (lanes 5, 6, 11, and 12). (A) At 5 h (lanes 1 to 6) or 10 h (lanes 7 to 12) p.i., total RNA was isolated from nuclear (N) and cytoplasmic (C) fractions, resolved on a denaturing formaldehyde agarose gel, and stained with ethidium bromide. The positions of precursor 45S and 32S rRNAs and fully processed 28S and 18S rRNAs are indicated by arrows to the left of the panel. RNA molecular mass markers are resolved in the first and last lanes of the gel (lanes m). The sizes of the markers are located to the right of the panel. (B) The membrane was hybridized with a *gB*-specific probe. The position of *gB* transcripts is indicated by an arrow to the right of the panel. M, mock; K, KOS1.1; N, nuclear; C, cytoplasmic.

enough to draw conclusions about their cytoplasmic versus nuclear localization. However, the experiments showing a stimulation of nuclear export upon overexpression of REF used a time point of 6 h p.i. (4). Therefore, we either mock infected cells or infected Vero cells with KOS1.1 or *d*27, and at 5 and 10 h p.i. total RNA was isolated from nuclear and cytoplasmic fractions. Once again, the ethidium bromide-stained gel indicated that the fractionation was successful, that the RNA isolated was of a high quality, and that similar amounts of RNA were loaded for each sample (Fig. 7A). We probed the corresponding Northern blot for *UL24* transcripts. In the presence of ICP27, most of the *UL24* transcripts were predominantly cytoplasmic; however, we could not compare the ratios of cytoplasmic to nuclear RNAs in Vero cells infected with *d*27 because, as expected, levels of these transcripts were barely detectable at these early times and could not be quantified with confidence (data not shown). We next stripped and probed the blot for *gB* transcripts (Fig. 7B). At these earlier time points, we did detect a greater effect on overall levels of *gB* transcripts such that they were reduced 5- to 10-fold in the absence of ICP27. However, we did not observe a dependency on ICP27 for the efficient cytoplasmic localization of *gB* transcripts at either 5 or 10 h p.i. (Fig. 7B). In each combination of viruses and cells tested, between 75 and 82% of *gB* transcripts were present in the cytoplasmic fractions. We also stripped and reprobed the blot for *UL26* transcripts (Fig. 8A). At these earlier times, levels of transcripts in the *d*27 fraction were too low to quantify accurately, but upon visual inspection it was

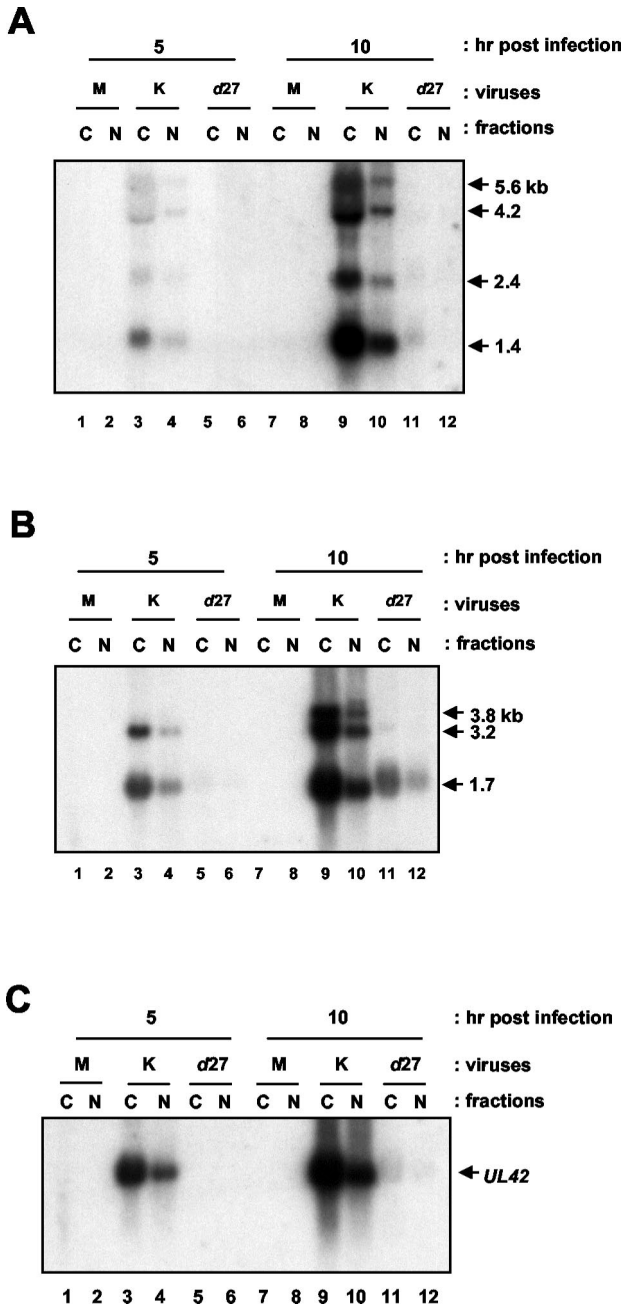

FIG. 8. Effect of ICP27 on the cytoplasmic localization of various viral transcripts at earlier times in infection. (A) The membrane in Fig. 7 was stripped and hybridized with a *UL26*-specific probe. The positions of the *UL26.5* (1.4-kb), *UL26* (2.4-kb), *UL25* (4.2-kb), and *UL24* (5.6-kb) transcripts are indicated to the right of the panel. (B) The membrane in Fig. 7 was stripped and hybridized with a *VP16* (*UL48*)-specific probe. The positions of the *UL48* (1.7-kb), *UL49* (3.2-kb), and *UL49.5* (3.8-kb) transcripts are indicated to the right of the panel. (C) The membrane in Fig. 7 was stripped and hybridized with a *UL42*-specific probe. The position of the *UL42* transcripts is indicated to the right of the panel. M, mock; K, KOS1.1; N, nuclear; C, cytoplasmic.

clear that for the *UL26.5* transcripts the band in the lane corresponding to the cytoplasmic fraction at 10 h p.i. was more intense than in the corresponding nuclear fraction (Fig. 8A, compare lanes 11 and 12). Thus, it appears that there was no major defect in cytoplasmic localization of *UL26.5* transcripts in the absence of ICP27 expression during infection.

We next probed the blot for *VP16* transcripts (Fig. 8B). Levels of all transcripts were clearly reduced at these earlier times in the absence of ICP27. At 5 h p.i. levels of transcripts were too low to be clearly detected in Vero cells infected with *d*27 lanes (lanes 5 and 6). At 10 h p.i., the *UL48* (1.7-kb) transcripts were detectable in Vero cells infected with either KOS1.1 or *d*27; however, they were efficiently localized to the cytoplasm in both the absence and presence of ICP27 (lanes 9 to 12).

Finally, we looked at *UL42* transcripts as an example of an early gene whose expression is highly dependent on ICP27 (52). The blot was overexposed so that we could detect the low levels of transcripts in the *d*27 samples, at least at 10 h p.i. (Fig. 8C). Here again, although the dependency on ICP27 for expression was clearly observed, in that there was an approximately 50-fold reduction in levels of *UL42* transcripts in Vero cells infected with *d*27 compared to that with the parental strain, the remaining transcripts were still predominantly cytoplasmic, suggesting that even at earlier times in infection, transcripts whose expression is highly dependent on ICP27 can be efficiently localized to the cytoplasm of infected cells in the absence of this regulatory protein.

In this experiment we observed a slight retardation in the electrophoretic mobility of several different viral transcripts from Vero cells infected with *d*27 compared to that with wild-type virus (Fig. 5B, 7B, and 8A to C). These effects are similar to the effect that has been seen for *gC* transcripts in transfected cells with or without ICP27 (37). Further study will be required to determine the significance of these observations; however, one possible explanation might be modifications in length of poly(A) tails, similar to what has been observed for the α-globin gene during HSV-1 infection (10) and consistent with previous reports that ICP27 affects polyadenylation of viral transcripts (32).

Thus, we have found that for those genes whose expression is dependent on ICP27, this stimulatory effect is more pronounced at earlier than at late times in infection. However, transcripts whose expression is dependent on ICP27, even highly dependent, are not necessarily dependent on ICP27 for their efficient cytoplasmic localization. Of those transcripts we have analyzed, only the long *UL24* transcripts exhibited a clear defect in cytoplasmic localization in the absence of ICP27.

DISCUSSION

Regulation of gene expression can occur at multiple levels simultaneously, which can complicate the interpretation of results obtained from mutational analyses of multifunctional proteins such as ICP27. Despite sharing common transcription start sites, the long and short *UL24* transcripts are affected differently by *ICP27* defects and, thus, they are very useful for investigating the function of this regulatory factor. Conversely, the differential effect of ICP27 on the short and long *UL24* transcripts can be exploited to further our understanding of the role of the different *UL24* transcripts during infection.

The effect of ICP27 on levels of UL24 protein. ICP27 is crucial for the expression of UL24 protein, because we were unable to detect any UL24 protein (>70-fold decrease) in the

absence of ICP27 expression during infection. Both the long and short *UL24* transcripts are expressed during productive infection; however, we have now identified several instances where the expression of UL24 protein does not correlate with levels of the short *UL24* transcripts but rather with levels of the long *UL24* transcripts that encode multiple genes. For example, inhibition of viral DNA replication has little effect on levels of the short *UL24* transcripts; however, levels of UL24 protein are reduced by fivefold (35), which is similar to the sixfold reduction in levels of the long *UL24* transcripts observed under such conditions (6, 14; A. Pearson and D. Coen, unpublished data). Similarly, here we report that the absence of ICP27 had little effect on the levels and cytoplasmic localization of the short *UL24* transcripts, but levels of UL24 protein were reduced greater than 70-fold. These results suggest that the short *UL24* transcripts are poorly translated. This observation raises the question of whether translation of the short *UL24* transcripts is aborted somehow, or whether these transcripts ever associate with ribosomes. It is interesting that in a large-scale analysis of polyadenylated, ribosome-associated transcripts corresponding to this region of the viral genome, the long *UL24* transcripts, but not the short *UL24* transcripts, were detected (17). We have previously found evidence suggesting that there is antisense regulation of the short but not of the long *UL24* transcripts by overlapping *tk* mRNA (7), which may contribute to the down-regulation of translation of the short *UL24* transcripts, perhaps, for example, by double-stranded RNA-mediated RNA editing (reviewed in references 2 and 25). We cannot rule out the possibility that translation of the short *UL24* transcripts is cell-type dependent. However, it is also possible that the short *UL24* transcripts may play a non-protein-coding regulatory role or that inhibiting their translation is important. That said, it is not clear what function, if any, the short *UL24* transcripts may play during infection.

Possible importance of late expression of UL24. As reviewed above, translation of the short *UL24* transcripts, which are expressed with early kinetics, appears to be inhibited. ICP27 helps promote the transition from early to late gene expression (31). Thus, a requirement for ICP27 in the expression of UL24 protein and other late proteins may serve to delay their expression until late times in infections. Kinetics of expression can have an impact on pathogenesis, as has been demonstrated for VP5 (ICP5) (51). *UL24* shares several features with viral genes that have been demonstrated to play a role in egress of newly synthesized viral particles (18, 35), such as *gK* (*UL53*) (12, 21) and *UL20* (1, 53). If indeed UL24 functions at the stage of membrane fusion during viral egress, or at some other late stage in the viral life cycle, then perhaps it is advantageous for the virus to delay expression of UL24 until the assembly of viral capsids is well under way.

Differential regulation of the short and long *UL24* transcripts by ICP27. ICP27 regulates gene expression at multiple levels. Our results indicate that in addition to regulating the expression level of the long *UL24* transcripts (14), ICP27 regulates the expression of *UL24* at the level of mRNA cytoplasmic localization. Notwithstanding the formal possibility that in the absence of ICP27 the long *UL24* transcripts that are cytoplasmic have a shorter half-life than those present in the nucleus, given the extensive literature on ICP27 and nuclear export, for the remaining discussion we will assume that our results were due to effects on nuclear export.

It is not obvious what features of the long *UL24* transcripts differentiate them from the short *UL24* transcripts with regard to ICP27-regulated nuclear export. Given that the long and short *UL24* transcripts share common transcription start sites, it would appear that promoter elements alone are not mediating this difference. Similarly, because the long *UL24* transcripts and the *UL25*, *UL26*, and *UL26.5* transcripts share a common 3′ end, it would also appear that elements shared with these latter transcripts are not sufficient to mediate ICP27-dependent nuclear export of the long *UL24* transcripts. Perhaps it is a combination of sequence elements that affect the nuclear export of these transcripts. Links between different aspects of RNA metabolism are well documented (reviewed in reference 29). Thus, 5′ elements of *UL24* may specifically direct transcripts that utilize the *UL26* poly(A) signal, but not the *UL24* signal, to a nuclear export pathway distinct from that of the other transcripts we have analyzed.

One possible difference between the long and the short *UL24* transcripts may lie in a portion of the 3′ untranslated region (3′ UTR) of *UL24*. Depending on where the exact start site of transcription for *UL25* is located (it has not, to our knowledge, been mapped yet), there may be sequences between it and the cleavage and polyadenylation site of the *UL24* short transcripts further upstream that would be unique to the long *UL24* transcripts. Regardless, our results with *UL24* transcripts do not support a model whereby ICP27 functions to compensate for a missing RNA element in HSV transcripts (24), because the long *UL24* transcripts that do apparently depend on ICP27 for their efficient nuclear export would appear to contain all of the sequences found within the short *UL24* transcripts that are efficiently exported from the nucleus independent of ICP27. It is possible, however, that depending on the cells being used for the experiments, the pathways and mechanisms being studied could be different.

ICP27-dependent HSV mRNA nuclear export. We have found that ICP27 is required for efficient nuclear export of long *UL24* transcripts. There is evidence that ICP27 associates with the RNA nuclear export factor REF and that under certain experimental conditions ICP27 can function in the nuclear export of several late viral transcripts (4, 24). Our observation that artificially high levels of ICP27 resulting from the infection of ICP27-complementing cells with an ICP27-competent virus seemed to correlate with small increases in the cytoplasmic localization of several viral transcripts appears to mirror the results obtained from experiments looking at the stimulatory effect of overexpressing REF on nuclear export of viral transcripts (4). The long *UL24* transcripts, which exhibit a defect in cytoplasmic localization in the absence of ICP27, may access the ICP27/REF pathway (4, 24), or they may access a different nuclear export pathway that is regulated indirectly by ICP27. For example, the expression of ICP27 may bolster the activity of a nuclear export pathway that is accessed by the long *UL24* transcripts in some manner, and in the absence of ICP27 this pathway is less active.

ICP27-independent HSV mRNA nuclear export. Regardless of the mechanism of ICP27-dependent nuclear export, we must invoke the existence of another export pathway to explain the residual cytoplasmic localization of the long *UL24* transcripts

in the absence of ICP27 and that of all the other HSV transcripts that were efficiently localized to the cytoplasm under those conditions. Even transcripts such as *gB*, whose nuclear export is stimulated by the overexpression of REF in an ICP27-dependent manner (4), and *VP16*, whose nuclear export in *Xenopus* oocytes is stimulated by the coinjection of recombinant ICP27 protein (24), were efficiently localized to the cytoplasm during infection with either an ICP27-competent or an *ICP27*-null virus. One possible explanation for the different results regarding the importance of ICP27 in the cytoplasmic localization of viral transcripts may be the use of different cells in the experiments. Nevertheless, our results demonstrate that during infection of Vero cells ICP27-independent nuclear export pathways do exist for viral transcripts, including late and intronless transcripts. One obvious candidate is the CRM1 pathway that mediates nuclear export of human immunodeficiency virus intronless transcripts (11). HSV replication is inhibited by leptomycin B, a pharmacological inhibitor of the CRM1 nuclear export pathway, and the cytoplasmic localization of several viral transcripts is reduced upon treatment of cells with this drug (33, 48). Although there is evidence against ICP27-mediated nuclear export functioning through the CRM1 pathway (4, 24), the evidence does not rule out the possibility that viral transcripts access a CRM1-nuclear export pathway independent of ICP27. Interestingly, *ICP27* mutations can confer resistance to leptomycin B (33). This effect might conceivably be due to a gain of ICP27 function that can compensate for the loss of CRM1 function. Another possibility is that one of the newly identified TAP-binding proteins, 9G8 and SRp20, may be mediating the nuclear export of viral transcripts though the TAP nuclear export pathway (19).

Like many biological systems, it appears that there is redundancy built into the system of HSV mRNA nuclear export. The relative importance of the possible different pathways may be dependent on the cellular environment. The *UL24* transcription unit has a ready-made set of transcripts with various combinations of promoters and 3′ ends, making it a particularly good system with which to study gene regulation during HSV infection. Thus, one can distinguish between promoter-dependent and -independent events. Similarly, the relative importance of 3′ UTRs can also be investigated in the context of similar promoter elements. This system will be particularly useful in the study of multifunctional regulatory factors such as ICP27. ICP27 clearly has, at a minimum, dual roles in viral gene expression including (i) the stimulation of transcription of at least some true late genes and (ii) increased cytoplasmic localization of some transcripts. This work raises the need to look at more early and late genes to better understand the gene-specific role of ICP27 in promoting transcription of viral genes and their cytoplasmic accumulation. In addition, the possible effect of cell type on the role of ICP27 during infection must be investigated.

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