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The C-Terminal Repressor Region of Herpes Simplex Virus Type 1 ICP27 Is Required for the Redistribution of Small Nuclear Ribonucleoprotein Particles and Splicing Factor SC35; However, These Alterations Are Not Sufficient To Inhibit Host Cell Splicing

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Herpes simplex virus type 1 infection results in a reorganization of antigens associated with the small nuclear ribonucleoprotein particles (snRNPs), resulting in the formation of prominent clusters near the nuclear periphery. In this study, we show that the immediate-early protein ICP27, which is involved in the impairment of host cell splicing and in the changes in the distribution of snRNPs, is also required for reassorting the SR domain splicing factor SC35. Other viral processes, such as adsorption and penetration, shutoff of host protein synthesis, early and late gene expression, and DNA replication, do not appear to play a role in changing the staining pattern of splicing antigens. Furthermore, the C-terminal repressor region of ICP27, which is required for the inhibitory effects on splicing, also is involved in redistributing the snRNPs and SC35. During infection or transfection with five different repressor mutants, the speckled staining pattern characteristic of uninfected cells was seen and the level of a spliced target mRNA was not reduced. Infections in the presence of activator mutants showed a redistributed snRNP pattern and a decreased accumulation of spliced target mRNA. Moreover, two arginine-rich regions in the N-terminal half of ICP27 were not required for the redistribution of snRNPs or SC35. Substitution of these regions with a lysine-rich sequence from simian virus 40 large-T antigen resulted in a redistribution of splicing antigens. Unexpectedly, a repressor mutant with a *ts* phenotype showed a redistributed staining pattern like that seen with wild-type infected cells. During infections with this *ts* mutant, splicing was not inhibited, as shown in this and previous studies, confirming its repressor phenotype. Furthermore, both the mutant and the wild-type protein colocalized with snRNPs. Therefore, the redistribution of snRNPs and SC35 correlates with ICP27-mediated impairment of host cell splicing, but these alterations are not sufficient to fully inhibit splicing. This indicates that active splicing complexes are still present even after dramatic changes in the organization of the snRNPs.

Splicing of nuclear pre-mRNA occurs in complexes called spliceosomes, which consist of the small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4, U5, and U6; several non-snRNP splicing factors; and pre-mRNA (19). At the cellular level, the localization of these components has been elucidated by using immunocytochemical procedures. Studies using immunofluorescent staining with anti-snRNP or anti-snRNA antibodies showed a diffuse nuclear staining superimposed upon 20 to 50 highly concentrated regions which displayed a speckled staining pattern (20, 39, 40, 47, 62–65). Electron microscopic and three-dimensional reconstruction techniques have shown that the snRNPs are distributed within a network composed of interchromatin granules and perichromatin fibrils that extends between the nucleolar surface and the nuclear lamina envelope (9, 64). Many of the elements required for splicing also have been shown to be associated with interchromatin granules and perichromatin fibrils. These include heterogeneous nuclear RNP (hnRNP) proteins (12) and the non-snRNP splicing factors SC35 (17) and U2AF (8), as well as snRNP proteins (12, 20, 47, 64, 65) and U1 and U2 snRNAs (7, 29). In addition, nascent RNA polymerase II transcripts (9, 13) and specific pre-mRNAs (28, 70, 71) have been found to be associated with these nuclear components. These data suggested that the speckled regions are more than sites of snRNP storage or assembly and may actually correspond to complexes directly involved in pre-mRNA splicing. Additional support for this was presented in studies of the nuclear organization of pre-mRNA processing components in cells infected with adenovirus (4, 30). A redistribution of cellular splicing factors, RNA polymerase II, and hnRNP proteins to sites of viral RNA transcription was observed (30). The finding that a significant number of splicing factors appeared to have been recruited from their normally speckled distribution to colocalize with new sites of active transcription further suggested that the speckled staining pattern represents complexes directly involved in splicing. In contrast, different conclusions were drawn from a study in which active pre-mRNA splicing sites were identified by localizing the nascent spliced mRNA of specific genes (74). It was found that splicing occurred at the sites of transcription which were not coincident with intranuclear speckles, leading those authors to conclude that the nucleus is not compartmentalized with respect to transcription and pre-

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mRNA splicing (74). Using immunofluorescent staining of splicing factors in adenovirus-infected cells, Bridge et al. (5) similarly concluded that splicing factors are not recruited to sites of virus transcription but that there is a dynamic organization of splicing factors in infected cells.

A large number of adenovirus transcripts are spliced. A very different situation exists with herpes simplex virus type 1 (HSV-1), where only a few of its more than 70 transcripts are spliced (69). Evidence has been presented that HSV-1 inhibits host cell splicing (22, 24, 56, 57). Martin et al. (41) looked at the distribution of snRNPs and hnRNPs following infection with HSV-1. They observed a striking redistribution of the snRNPs from their speckled staining pattern, resulting in the formation of prominent clusters which appeared to condense throughout the nucleus and then migrate to the periphery as infection proceeded. The examination of the distribution of an HSV-1 protein involved in transcriptional activation (ICP4) showed no correlation with the distribution of the snRNPs, which suggested that splicing factors are not recruited to sites of active HSV-1 transcription as had been inferred for adenovirus infection (30). Furthermore, Phelan et al. (50) demonstrated that an HSV-1 immediate-early protein, ICP27 (IE63), was essential for the relocalization of snRNPs and that ICP27 colocalized with the redistributed snRNPs. Martin et al. (41) and Phelan et al. (50) postulated that the reassortment of splicing antigens may result in the inhibition of splicing during HSV-1 infection. While this conclusion is compatible with the fact that few HSV-1 transcripts require splicing, we wanted to ascertain the biological significance of the redistribution of snRNPs and particularly to determine if this phenomenon was related to the impairment of host cell splicing by HSV-1. Previous studies in our laboratory showed that host cell splicing is inhibited through the action of ICP27 (22, 24, 56).

In this investigation, we monitored the distribution of snRNPs and the essential non-snRNP SR domain splicing factor SC35 (16, 17, 73) after infection with HSV-1. We found that a redistribution in the staining pattern of SC35 also occurred. The redistribution of snRNPs and SC35 from the speckled pattern to a few prominent globular clusters was unlike effects seen after inhibition of transcription in uninfected cells (7). The relocalization was also unrelated to viral DNA replication, or early or late gene expression. In accord with the results of Phelan et al. (50), there was a correlation between ICP27 expression and the redistribution of splicing factors. We attempted to relate these findings on the reorganization of cellular splicing factors to effects on host cell splicing. Our previous studies showed that the C-terminal region of ICP27, which we termed the repressor region (23), was required for the inhibitory effects on splicing (22, 56). In this study, we monitored the localization of snRNPs and SC35 in cells infected with a variety of ICP27 mutants and measured the accumulation of a spliced target mRNA (chloramphenicol acetyltransferase) (CAT) during infections with these mutants. Activator mutants (23) behaved like the wild type. In addition, the arginine-rich sequences that follow the N-terminal major nuclear localization signal (26, 43), while similar to RNA binding regions in other proteins (3, 10, 25, 33, 37, 38, 45), were not required for the effects on the pattern of snRNPs. Substitution of the R-rich regions with a heterologous lysine-rich sequence resulted in the reassortment of splicing factors. In contrast, infections or transfections with mutants carrying insertions in the C-terminal cysteine-histidine-rich repressor region of ICP27 showed a staining pattern identical to that seen in uninfected cells and spliced CAT mRNA levels were not decreased, implicating this region in both effects. However, a different result was found with a viral temperature-sensitive mutant, *ts*LG4, which contains a base substitution within the repressor region (61). While spliced mRNA levels were not decreased in infections with this mutant, the staining pattern of snRNPs and SC35 was condensed and redistributed. Furthermore, the *ts*LG4 ICP27 protein colocalized with the snRNPs, as did the wild-type protein. These data show that the redistribution of splicing factors correlates with host cell splicing inhibition, and both effects appear to be mediated by ICP27 through its C-terminal repressor region. However, the changes in the localization of snRNPs and SC35 within the nucleus do not appear to be sufficient to inhibit splicing, suggesting that active splicing complexes are still present. This result would support the hypothesis that the intranuclear speckles and interchromatin granules serve as storage compartments for splicing factors rather than as centers of splicing.

MATERIALS AND METHODS

Cells and viruses. Vero cells were grown on glass coverslips for 16 to 20 h in Eagle's minimal essential medium with 3% fetal calf serum, 3% newborn calf serum, and 3% calf supreme (GIBCO). The cell line 2-2, which contains the wild-type ICP27 gene, and Stu15 and Sst26, which contain the ICP27 mutants S1B and S23, respectively (23), were described previously (60, 61). The cell lines TAG, S13, and S18 were constructed by cotransfecting Vero cells with the plasmids pTAG (26), pS13 (23), and pS18 (23) with pFeLTR-neo (60, 61), which encodes the gene for neomycin resistance. Colony selection and characterization of the resulting cell lines were performed as described elsewhere (26). Cell lines harboring ICP27 mutants were grown on coverslips as described above except that 750 μg of G418 per ml was included in the medium. Cells were infected with wild-type HSV-1 KOS, 27-LacZ, or *ts*LG4 at a multiplicity of infection of 10 as described previously (60, 61). The UV inactivation of KOS was performed as described previously (22), and the multiplicity of infection was calculated on the basis of the viral titer before inactivation.

Immunofluorescent staining. Cells were fixed at the appropriate time for each experiment with freshly prepared 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature. After being rinsed three times in PBS, cells were permeabilized in PBS containing 0.5% Nonidet P-40 for 5 min. The cells were rinsed three times in PBS containing 1% newborn calf serum. In general, coverslips were rinsed three times in PBS containing 1% newborn calf serum before the addition of each antibody or reagent. The cells were treated with a 1:100 dilution of donkey serum for 30 min to block nonspecific interactions. Cells were again rinsed and reacted with the primary antibody. Monoclonal anti-Sm antibody Y12 (39) was used at a dilution of 1:1,000; anti-SC35 (hybridoma supernatant) (17) was used at a dilution of 1:50; anti-ICP27 monoclonal antibodies, H1113 (1) and H1119, and anti-ICP8 monoclonal antibody, H1115, were used at a dilution of 1:400; and patient anti-Sm antisera (44) were used at a dilution of 1:500. Monoclonal antibodies to ICP27 and ICP8 were obtained from the Goodwin Institute for Cancer Research. In the single-labeling experiments, primary antibodies were adsorbed to cells for 30 min and then cells were rinsed. Biotinylated goat anti-mouse or goat anti-rabbit antibody (Amersham) at a 1:100 dilution was added to cells for 30 min. Following rinsing, a 1:100 dilution of streptavidin-conjugated fluorescein (Amersham) was added to the cells for 30 min. Cells were rinsed three times in PBS containing 1% newborn calf serum and twice in PBS without serum before being mounted on the coverslips in PBS. In the double-labeling experiments, patient anti-Sm antiserum was added to cells, which were then rinsed before the addition of a 1:100 dilution of biotinylated goat anti-human immunoglobulin G (Amersham). Cells were rinsed and then treated with a 1:100 dilution of streptavidin-conjugated Texas red (Amersham). After 30 min, cells were rinsed and a 1:400 dilution of anti-ICP27 or anti-ICP8 monoclonal antibody was added to cells. Rinsed cells were incubated with a 1:50 dilution of fluorescein-linked anti-mouse antibody (from sheep; Amersham). Cells were washed as described above and mounted on coverslips in PBS. Cells were examined with a Nikon UFX-II epifluorescent microscope with a ×100 objective lens with a numerical aperture of 1.25.

Transfection, RNA isolation, and ribonuclease protection assays. The plasmid pTK-CAT-5′S-SV LPA was transfected into Vero cells and ICP27 mutant cell lines by using lipofectin reagent (Life Technologies) as described previously (26, 56). Sixteen hours after transfection, cells were infected with HSV-1 KOS, *ts*LG4, or 27-LacZ as indicated in the legend to Fig. 8, and RNA was isolated from the infected cultures 6 h later as described elsewhere (56). RNase protection experiments were performed by using an antisense RNA probe which spans the 16S-19S simian virus 40 (SV40) splice sites as previously described (56).

Artwork for Fig. 8. The autoradiographs were scanned on a Hewlett-Packard ScanJet II CX/T with a transparency adapter. The images were scanned at 600 dpi into Adobe Photoshop 3.0 and were exported in tagged image file format.

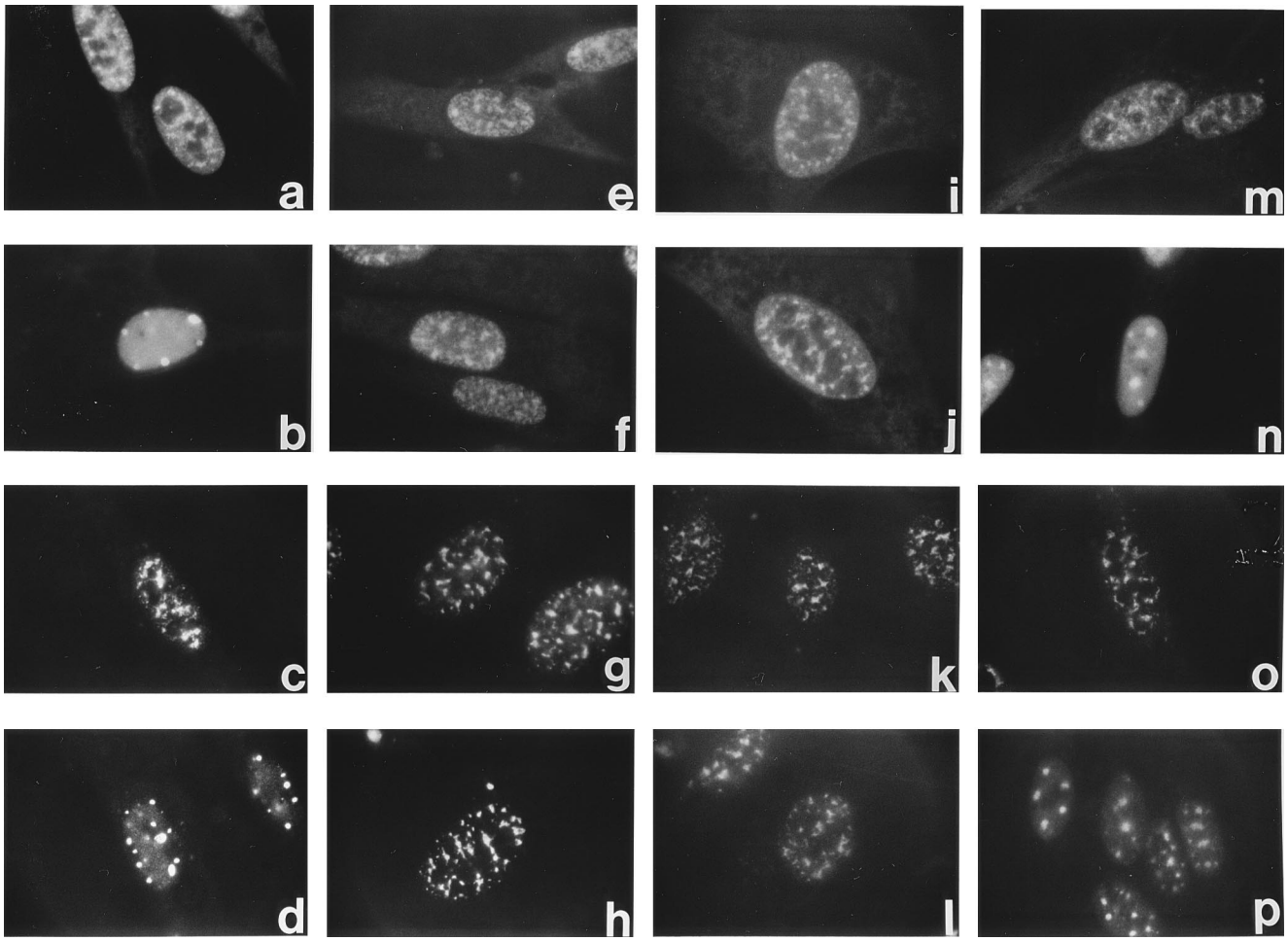

FIG. 1. Effect of HSV-1 infection compared with effects of inhibitors of transcription, protein synthesis, and HSV-1 DNA replication on the staining pattern of snRNPs and SC35. Vero cells were either not treated with chemical inhibitors (column 1, a to d), treated with 10 μg of actinomycin D per ml to inhibit transcription (column 2, e to h), treated with 100 μg of cycloheximide per ml to inhibit protein synthesis (column 3, i to l), or treated with 300 μg of phosphonoacetic acid per ml to inhibit HSV-1 DNA replication (column 4, m to p). Cells in row 1 (a, e, i, and m) and row 3 (c, g, k, and o) were uninfected. Cells in row 2 (b, f, j, and n) and row 4 (d, h, l, and p) were infected with HSV-1 KOS. Infecting virus was added 1 h after addition of the inhibitors. Virus adsorption for 1 h and virus infection for 6 h were performed in the presence of the inhibitors. Cells in rows 1 and 2 were stained with anti-Sm antibody Y12. Cells in rows 3 and 4 were stained with anti-SC35 antibody.

RESULTS

ICP27 expression is required for the redistribution of snRNPs and SC35 during HSV-1 infection. Indirect immunofluorescence studies by Phelan et al. (50), using a monoclonal antibody specific for the U2 snRNP antigen B″, demonstrated that ICP27 expression was required for the redistribution of snRNPs into condensed intranuclear structures termed interchromatin granules. In those experiments, Phelan et al. (50) noted that snRNP redistribution caused by HSV-1 infection resembled the effect seen after inhibition of transcription in uninfected cells (7). Because HSV-1 infection also results in the decreased transcription of many cellular genes (22, 59), we first determined what other aspects of HSV-1 infection might contribute to the reorganization of the splicing components. Uninfected cells and cells infected with wild-type HSV-1 strain KOS were treated with chemical inhibitors of RNA synthesis (actinomycin D), protein synthesis (cycloheximide), and viral DNA replication (phosphonoacetic acid). Cells were stained with the monoclonal antibody Y12, which reacts predominantly with the 28-kDa polypeptide B, B′ found in the U1, U2, U4/U6, and U5 snRNPs (39), or with a monoclonal antibody to the SR domain essential splicing factor SC35 (17). Cells were treated with actinomycin D for 1 h and then were mock infected or infected with HSV-1 KOS for 6 h in the presence of the inhibitor. Some condensation of the normally speckled distribution of the snRNPs was seen (Fig. 1; compare panel a with panels e and f), although condensation of the discrete, punctate staining of SC35 (17) was less apparent (Fig. 1, compare panel c with panels g and h). However, the condensation observed in the snRNP staining was very different from the dense clusters which were observed at the periphery of the nuclei in KOS-infected cells which were not treated with inhibitors (Fig. 1b and d). In those cells, a striking redistribution was seen for both snRNPs and SC35. Another effect of HSV-1 infection, inhibition of host protein synthesis (14, 66, 67), was also examined. Treatment with cycloheximide had little effect on the staining pattern of snRNPs or SC35 (Fig. 1i to l). This experiment also showed that de novo viral gene expression was required for the redistribution of snRNPs, because cells infected with HSV-1 KOS in the presence of cycloheximide (Fig. 1j and l) were similar to mock-infected cells (panels a, c, i, and k). This indicates that functions present in the HSV-1 virion, like VP16 (αTIF) and the virion host shutoff function *vhs* (UL41), are not involved in the effects seen on splicing com-

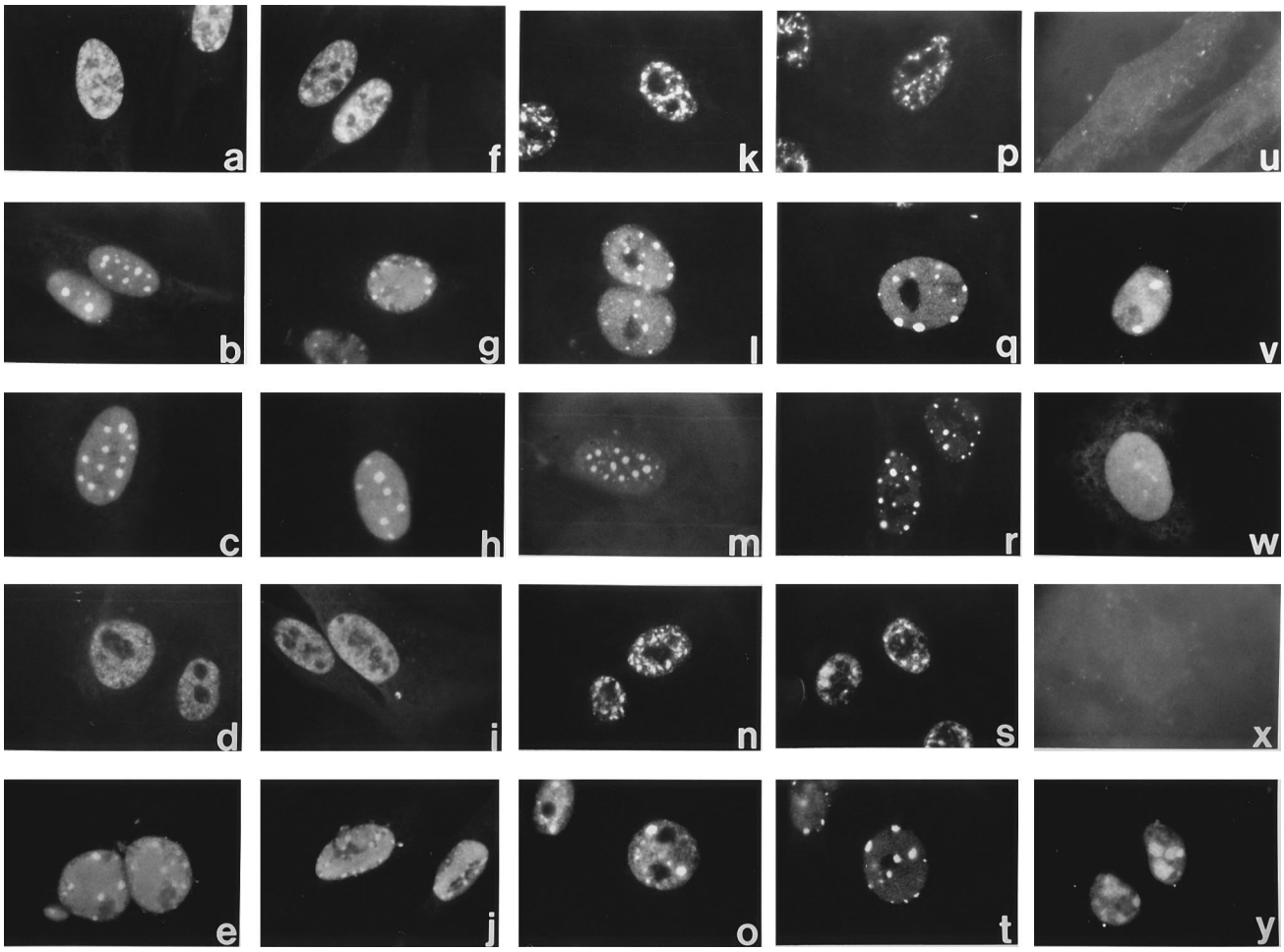

FIG. 2. Immunofluorescent staining pattern of snRNPs and splicing factor SC35 in cells infected with wild-type HSV-1 compared with those infected with the ICP27 viral mutant 27-LacZ. The changes in the distribution of snRNPs from the pattern seen in uninfected cells stained with anti-Sm Y12 monoclonal antibody (a and f) and anti-SC35 (k and p) were monitored at early and late times after HSV-1 infection. KOS-infected Vero cells were fixed at 2.5 h (b and l) or 8 h (g, q, and v) after infection. Similarly, cells infected with *ts*606 at 39°C were fixed at 2.5 h (c and m) and 8 h (h, r, and w) after infection. Vero cells infected with 27-LacZ for 2.5 h (d and n) and 8 h (i, s, and x) are shown, as are cells of the ICP27-complementing line 2-2 infected with 27-LacZ for 2.5 h (e and o) and 8 h (j, t, and y). Cells shown in columns 1 and 2 (a to e and f to j, respectively) were stained with monoclonal antibody Y12. Cells in columns 3 and 4 (k to o and p to t, respectively) were stained with anti-SC35 antibody. Cells in column 5 (u to y) were stained with anti-ICP27 monoclonal antibody H1113.

ponents, because although these proteins can function in the presence of cycloheximide, no changes occurred. We also examined the role of viral DNA replication in the reassortment of splicing factors because de Bruyn Kops and Knipe (11) demonstrated that the distribution of cellular replication sites was altered in HSV-1-infected cells such that it corresponded to the pattern of viral prereplicative sites. Martin et al. (41) also reported that HSV-1 infection causes the collapse of the host chromatin into internal aggregates and aggregates along the nuclear envelope. Therefore, we wanted to determine whether the formation of viral replicative structures was correlated with the redistribution of snRNPs. KOS-infected cells treated with the HSV-1 DNA synthesis inhibitor phosphonoacetic acid showed the clustering and reassortment of snRNPs (Fig. 1n) and SC35 (panel p) that was seen with untreated infected cells (panels b and d). These results showed that the redistribution of splicing components occurs independently of viral DNA replication.

Next, we looked at the involvement of viral gene products, specifically at ICP27 expression, in this phenomenon. Cells were infected with wild-type HSV-1 KOS; with *ts*606 (54), a *ts* mutant defective in ICP4, the major transcriptional activator of HSV-1 gene expression; or with an ICP27 null mutant termed 27-LacZ (60) in which the *lacZ* gene was inserted into the ICP27 locus. In addition to being stained with anti-snRNP and anti-SC35 antibodies, cells were stained with the anti-ICP27 monoclonal antibody H1113 (1) to monitor ICP27 expression. In this experiment, cells were infected for 2.5 or 8 h to monitor the reassortment through the early and later stages of infection. The condensation of snRNPs and SC35 was seen to occur in wild-type KOS-infected cells by 2.5 h after infection (Fig. 2b and l), and this was followed by the movement of these globular, condensed regions to the periphery of the nuclei by 8 h after infection (panels g and q). A similar condensation was seen during infection with *ts*606, the ICP4 mutant (Fig. 2c, h, m, and r). Only immediate-early proteins are efficiently expressed when ICP4 is defective, suggesting that an immediate-early protein other than ICP4 is required for the redistribution of snRNPs and SC35. That this is the case was shown in infections with 27-LacZ, where ICP27 was not expressed (Fig. 2x). The staining pattern of the snRNPs and SC35 was indistinguishable from that seen in uninfected cells even 8 h after

infection (Fig. 2; compare panels a, f, k, and p, showing uninfected cells, with panels d, i, n, and s, showing 27-LacZ-infected cells). In fact, the staining pattern remained unchanged even 24 h after infection (data not shown). In contrast, when the complementing cell line 2-2 (60) was infected with 27-LacZ, the staining pattern found with KOS-infected cells was again seen (Fig. 2e, j, o, and t). The 2-2 cell line is stably transfected with the wild-type ICP27 gene, which is induced upon infection with 27-LacZ (Fig. 2y). Thus, in the absence of ICP27 expression, no change in the staining pattern was observed, while the changes first described by Martin et al. (41) and later by Phelan et al. (50) were seen whenever ICP27 was expressed for both snRNPs and the non-snRNP factor SC35. Furthermore, the results of infection with *ts*606, infection in the presence of cycloheximide, and infection in the presence of phosphonoacetic acid showed that ICP4 is not required for the redistribution, and neither are virion-associated proteins, viral early or late gene products, or viral DNA replication.

ICP27 expression is sufficient for the redistribution of snRNPs and SC35. During infection with the ICP4 mutant *ts*606, three other immediate-early proteins besides ICP27 are expressed. Therefore, it is possible that ICP27 could act with one of these other viral products. To address whether ICP27 alone could cause the changes seen in the distribution of snRNPs, an experiment using UV-inactivated virus was performed. HSV-1 gene expression is greatly reduced following infection with UV-inactivated virus because of the damage to the viral template. However, the virion-associated proteins, like VP16 and the *vhs* protein, can still function. Infection of Vero cells with UV-inactivated virus, where only the input HSV-1 virion-associated proteins were present, showed no change in the staining pattern of snRNPs (Fig. 3c) or SC35 (panel h) compared with patterns obtained with uninfected cells (panels a and f). Under these conditions, ICP27 was not expressed (Fig. 3m). In contrast, when the complementing cell line 2-2 was infected with UV-inactivated virus, ICP27 was expressed from the cell line (Fig. 3n and o), with the result that the condensation of splicing structures was again seen in the staining of snRNPs (panels d and e) and SC35 (panels i and j). The formation and redistribution of clusters, while clearly evident, were not as pronounced as those observed with KOS-infected cells (Fig. 3b and g). This could mean that while ICP27 alone is sufficient to cause the formation of the snRNP clusters, other viral products may contribute to this process. It is also possible that the total amount of ICP27 expressed may be important to cause redistribution. ICP27 levels were found to be about twofold lower in 2-2 cells infected with UV-inactivated virus than they were in KOS-infected Vero cells by immunoblot analysis (data not shown).

The N-terminal arginine-rich regions of ICP27 are not necessary for the redistribution of snRNPs. To determine what regions of the ICP27 protein were involved in altering the distribution of snRNPs and SC35, we examined the localization of these factors during infection with a variety of ICP27 mutants defective in different functions of the protein. These studies utilized a series of G418-resistant cell lines which were stably transfected with each mutant (26, 61). Infection of these cell lines with the viral null mutant 27-LacZ results in the expression of the mutant form of ICP27 resident in the cell line as the only source of ICP27 present during infection (26, 61). First, a mutant termed TAG, altered in the amino-terminal half of the protein, was examined. The region encompassing two arginine-rich sequences, similar to those found in RNA-binding domains of other proteins (3, 10, 25, 33, 37, 38, 45), was targeted because of the possibility that ICP27 may interact with an snRNA in the snRNPs to cause the redistribution. The first arginine-rich region, RRGRRRGRGRGG, from residues 141 to 151, resembles the RGG motif found in nucleolar proteins, hnRNP proteins, and the SmD snRNP protein, where it is thought to be involved in RNA processing (3, 33, 53). The second R-rich region, PRRRAPRTNR, encompasses residues 162 to 171. Both of the R-rich regions of ICP27 bear resemblance to the R-rich RNA-binding motifs and nuclear localization domains in HIV Tat and Rev (6, 27).

The TAG mutant has a deletion of residues 103 to 178, including the major nuclear localization region (43) and both of the succeeding R-rich regions (26). A synthetic oligonucleotide encoding the SV40 large-T antigen nuclear localization signal (31, 32), PKKKRKV, was inserted into the site of the deletion (26). This insertion resulted in the efficient nuclear localization of ICP27 (Fig. 4i) (26). The formation of snRNP clusters and the condensation of staining of SC35 seen to occur in KOS-infected cells (Fig. 4b and e) were observed following infection of the TAG cell line with 27-LacZ (panels c and f). The SV40 nuclear localization signal has not been implicated in RNA binding and it differs from the R-rich regions of ICP27 in being rich in lysine residues, yet splicing antigens were reassorted in the presence of this ICP27 mutant protein. This result indicates that the R-rich regions of ICP27 are not required for the effects on snRNPs seen.

The C-terminal repressor region of ICP27 is required for the redistribution of splicing factors. Previously, we showed that the region between amino acids 260 and 405 was involved in the activation function of ICP27 (23) but not in effects on splicing (22, 56), whereas the C-terminal region from amino acids 406 to 512, termed the repressor region, was required for the inhibitory effects on splicing (22, 56). Two cell lines containing activator mutants and two cell lines containing repressor mutants were analyzed for their effects on the localization of splicing components following infection with 27-LacZ. The activator mutant S13 contains an in-frame insertion of 4 amino acids at residue 262 (23, 26), the start of the activator region. Both snRNPs and SC35 were clustered and relocalized in 27-LacZ infections of the S13 cell line (Fig. 5A, panels c and f). Similarly, in Sst26-infected cells, the clustering and redistribution were also seen (Fig. 5B, panels l and o). Sst26 contains an insertion of 3 amino acids at residue 383 (23, 61).

The cell line Stu15 contains the mutant S1B, which has a 4-amino-acid insertion at the start of the repressor region (23, 61). Infection of cell line Stu15 with 27-LacZ resulted in a staining pattern indistinguishable from that seen in mock-infected Stu15 cells (Fig. 6A; compare uninfected cells in panels a and d with 27-LacZ-infected cells in panels c and f). Similarly, S18 cells infected with 27-LacZ (Fig. 6B, panels l and o) showed the speckled staining pattern characteristic of uninfected cells (Fig. 6B, panels j and m). S18 contains a 4-amino-acid insertion at residue 504, which is 8 amino acids from the end of the protein (23). These results indicate that the repressor region of ICP27, which is required for the inhibition of splicing, also must be intact for the redistribution of splicing components to occur.

The effect of ICP27 on splicing can be separated from its effect on the relocalization of snRNPs. We also examined the staining pattern of snRNPs and SC35 during infections with the viral temperature-sensitive mutant, *ts*LG4. This mutant has a base substitution at residue 480 within the repressor region (61). Unexpectedly, the formation of condensed snRNP clusters that were redistributed was seen in *ts*LG4 infections performed at the nonpermissive temperature (Fig. 7c and f). This was not due to heat stress because the characteristic speckled pattern of the snRNPs (Fig. 7a) and the discrete punctate pattern of SC35 (panel d) were observed for uninfected cells

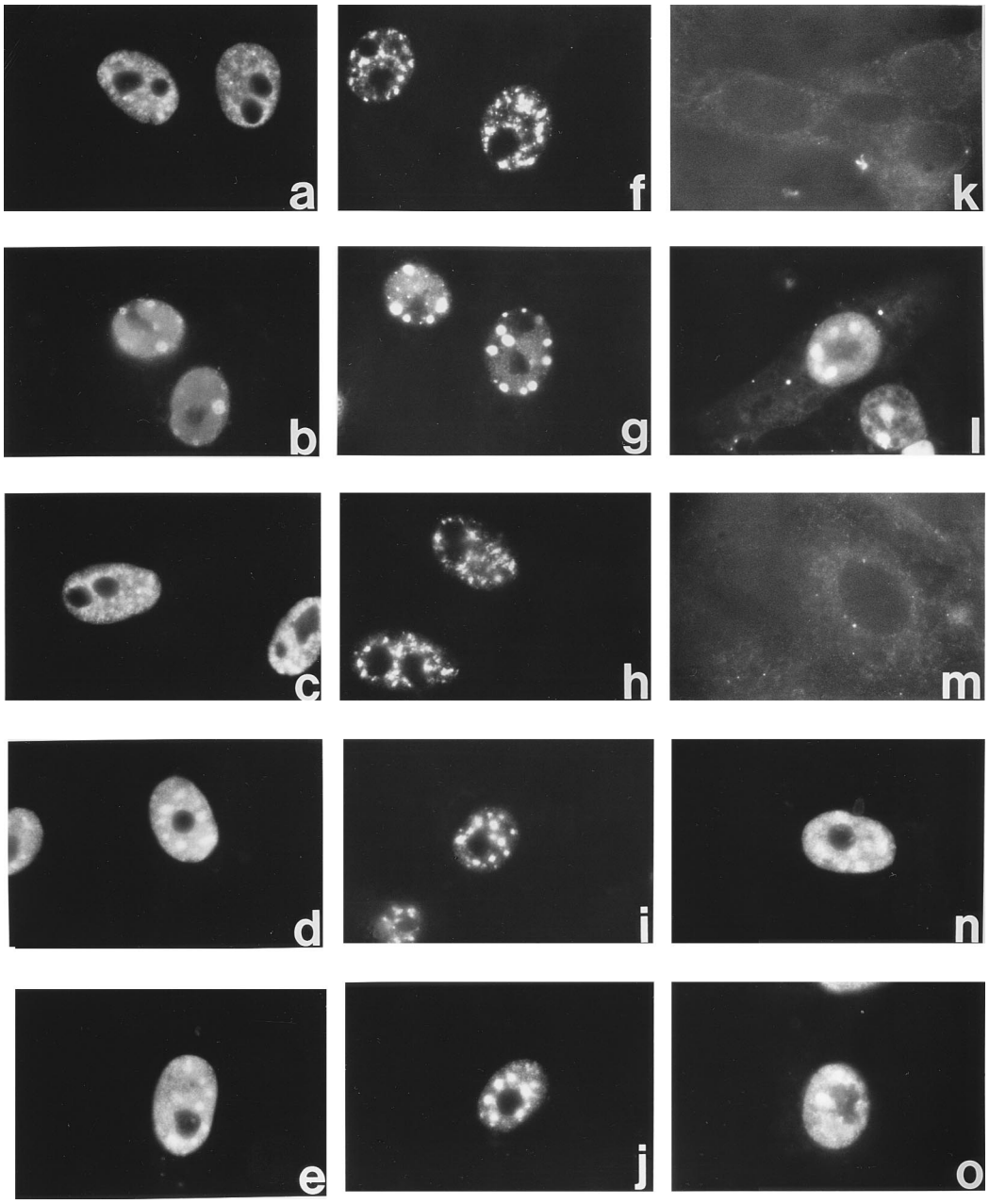

FIG. 3. ICP27 appears to be able to cause the condensation of splicing factors in the absence of other de novo synthesized viral gene products. To determine whether ICP27 expression was sufficient for the effects seen on snRNPs and SC35, an experiment was performed in which HSV-1 KOS was inactivated by UV irradiation (22) and then was used to infect Vero cells or 2-2 cells, which can be induced to express ICP27. Infections were performed for 6 h. (a, f, and k) Uninfected Vero cells; (b, g, and l) wild-type KOS-infected cells; (c, h, and m) Vero cells infected with UV-inactivated KOS. ICP27 was not expressed during infection of Vero cells with UV-inactivated virus (m). 2-2 cells infected with UV-inactivated virus are shown in panels d, i, and n and e, j, and o. The cells in column 1 (a to e) were stained with Y12 antibody. Cells shown in column 2 (f to j) were stained with anti-SC35 antibody. Cells in column 3 (k to o) were stained with anti-ICP27 antibody H1113.

that were incubated at 39°C along with the *ts*LG4 (panels c and f) and KOS-infected (panels b and e) cultures. Previous studies with this viral mutant showed that the accumulation of spliced cellular mRNAs was not decreased and precursor mRNA did not accumulate relative to levels in infections with KOS (22). Furthermore, nuclear extracts from cells infected with *ts*LG4 at the nonpermissive temperature were competent in splicing a pre-mRNA substrate (24). Therefore, *ts*LG4 behaved as a repressor mutant with respect to effects on splicing, yet the prominent condensation of snRNPs was observed.

To determine whether the inhibitory effects of ICP27 on splicing correlated with the alterations in the distribution of snRNPs for the other repressor and activator mutants analyzed, we measured the amount of a spliced target mRNA in mutant-infected cells. The plasmid pTK-CAT-5′S-SV LPA (56), which contains a modified 16S-19S intron from SV40 (48) in the 5′ noncoding region, was transfected into Vero cells or into ICP27 mutant cell lines. Sixteen hours later cells were infected, and 6 h later RNA was isolated. RNase protection assays were performed with a probe spanning the intron and

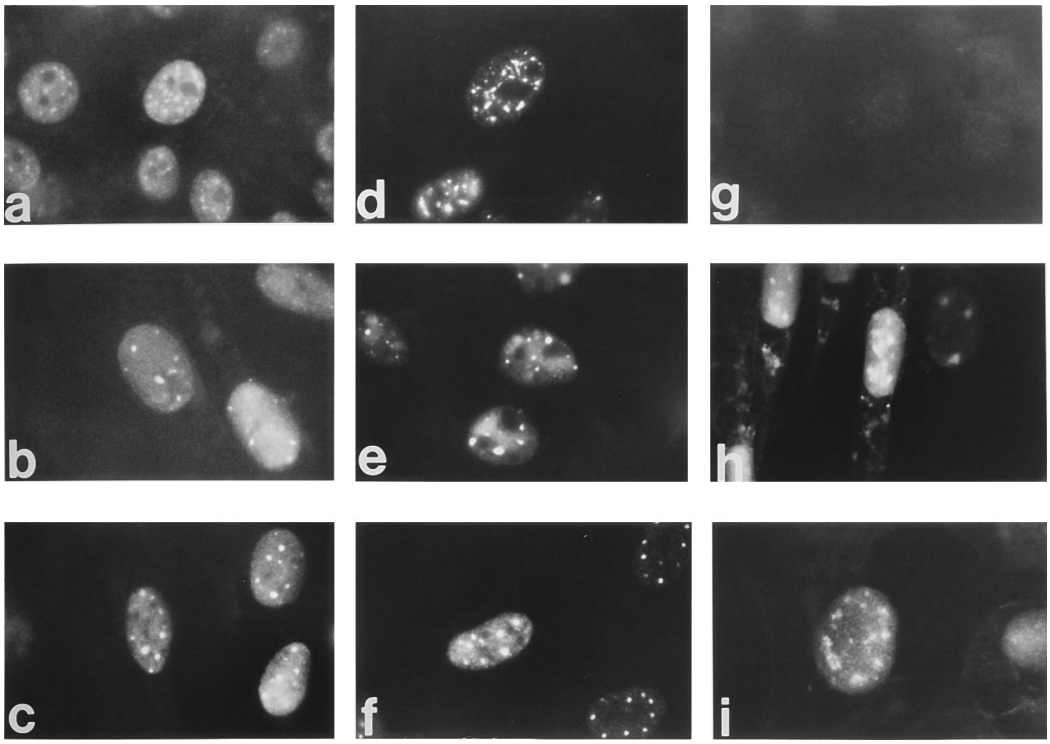

FIG. 4. The R-rich regions in the amino-terminal half of ICP27 are not necessary for the redistribution of snRNPs. The cell line TAG contains an ICP27 gene with a deletion of residues 103 to 178, including both R-rich regions, and has an insertion of the lysine-rich nuclear localization signal from SV40 large-T antigen (26, 32). (a, d, and g) Uninfected TAG cells; (b, e, and h) KOS-infected TAG cells; (c, f, and i) staining of TAG cells infected with 27-LacZ. Cells in column 1 (a to c) were stained with Y12 antibody. Cells in column 2 (d to f) were stained with anti-SC35 antibody. Cells in column 3 (g to i) were stained with anti-ICP27 monoclonal antibody H1119.

the first 250 nucleotides of the CAT exon. Two alternatively spliced products were expected, a 310-nucleotide protected product and an alternatively spliced, less abundant 375-nucleotide product. Unspliced CAT pre-mRNA (450 nucleotides) is difficult to detect in transfection assays because unspliced RNA is rapidly degraded in the nucleus. Little CAT mRNA was detected in uninfected cells because the constitutive level of expression from the thymidine kinase (TK) promoter is low in the absence of HSV-1 transactivators (Fig. 8A, lane 1). Infection with *ts*LG4 at the nonpermissive temperature or with 27-LacZ, where ICP27 was not expressed, resulted in the accumulation of about 10 times more spliced CAT mRNA than was seen in the presence of wild-type ICP27 in KOS-infected Vero cells (Fig. 8A, lanes 2 to 4). This result is in agreement with previous results seen with *ts*LG4 infections (22, 24) and further indicates that *ts*LG4 displays a repressor phenotype with respect to effects on splicing. Infection of the S13 cell line with 27-LacZ showed that CAT mRNA levels were reduced as much as they were in KOS infections or infections of the cell line 2-2, which contains the wild-type ICP27 gene (Fig. 8C). In contrast, 27-LacZ infections of the repressor mutant cell line S18 showed no reduction in spliced CAT RNA levels (Fig. 9b). The levels of spliced cellular mRNA for the repressor cell line Stu15 and the activator cell line Sst26 have been reported previously (22), and the results were in accord with their repressor and activator phenotypes. Therefore, except with *ts*LG4, the staining pattern of the snRNPs correlated with the effect of ICP27 on spliced mRNAs.

Wild-type ICP27 and mutant *ts*LG4 protein colocalize with snRNPs. Phelan et al. (50) reported that ICP27 colocalized with redistributed snRNPs. To determine whether ICP27 expressed during nonpermissive *ts*LG4 infections was also associated with snRNPs, we performed double-labeling experiments. Cells were stained with human anti-Sm antiserum (44) and with the monoclonal antibody H1113 to ICP27. The speckled staining pattern of the snRNPs was seen in mock-infected cells with the human anti-Sm antiserum (Fig. 9a), as was previously seen with monoclonal antibody Y12. The same field of mock-infected cells did not show staining with the monoclonal antibody to ICP27 (Fig. 9e). Cells infected with KOS showed the redistribution of snRNP clusters (Fig. 9b). Some of the same pattern was seen with ICP27 antibody staining on the same field of cells (Fig. 9f). Many of the discrete clusters corresponded to those sites seen with the anti-Sm antibody. However, there were additional sites of ICP27 staining which were not directly located with the snRNPs and the staining seen in these additional sites was more diffuse. This result indicates that some of the ICP27 present in the infected cell appears to colocalize with snRNPs. This was also the case for the mutant form of ICP27 expressed during infection at the nonpermissive temperature with *ts*LG4 (Fig. 9c and g). A similar colocalization was observed, along with additional sites of ICP27 staining. As a control, KOS-infected cells were stained with anti-Sm antibody and a monoclonal antibody (H1115) to ICP8, the major DNA-binding protein of HSV-1, which is involved in DNA replication. ICP8 is found associated with HSV-1 DNA replication complexes (11). The staining pattern of ICP8 was quite distinct from that observed with the snRNPs (Fig. 9d and h). The large concentrated areas of staining were primarily in regions where snRNPs were not seen, confirming that HSV-1 DNA replication complexes are not associated with snRNPs. Similar results were obtained when ICP4 was

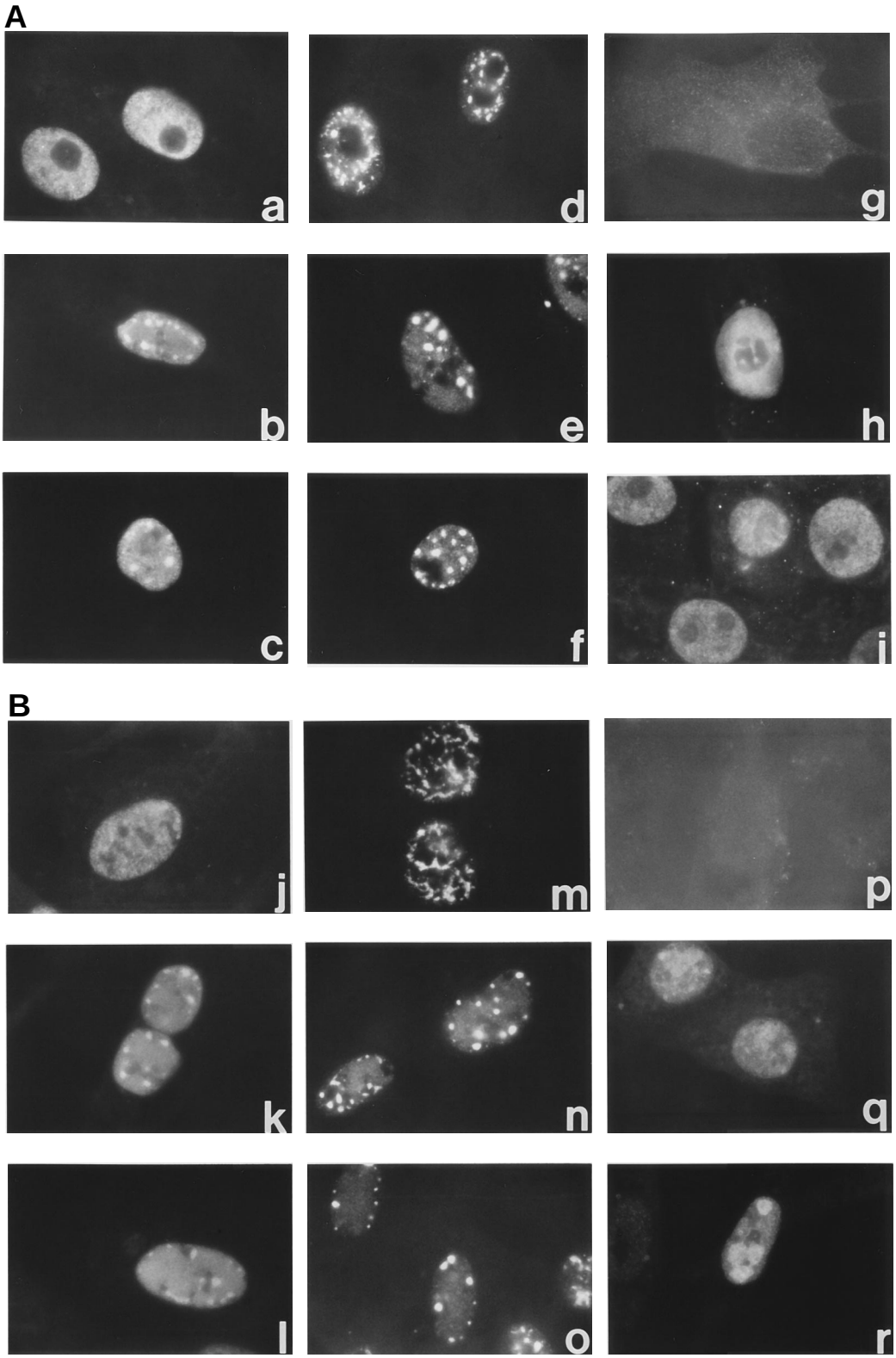

FIG. 5. Staining of splicing factors during infections in the presence of two ICP27 activator mutants. (A) Cell line S13 contains an ICP27 gene with an insertion at the start of the activator region (23, 26). Uninfected (a, d, and g), KOS-infected (b, e, and h), and 27-LacZ-infected (c, f, and i) S13 cells are shown. (B) Cell line Sst26 contains an ICP27 gene with an insertion near the end of the activator region (61). Uninfected (j, m, and p), KOS-infected (k, n, and q), and 27-LacZ-infected (l, o, and r) Sst26 cells are shown. Cells in column 1 were stained with Y12 antibody. Cells in column 2 were stained with anti-SC35 antibody. Cells in column 3 were stained with anti-ICP27 monoclonal antibody H1113.

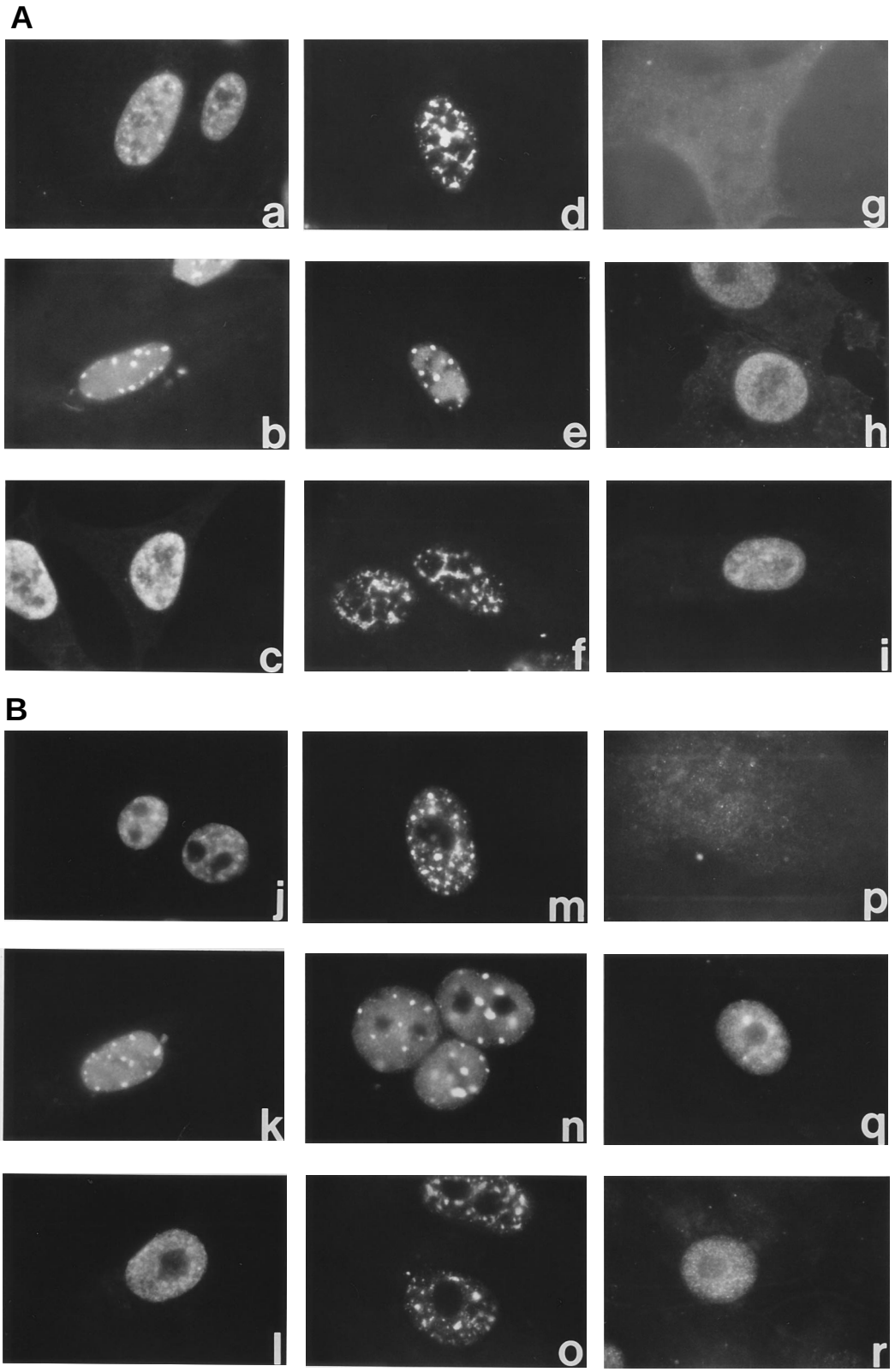

FIG. 6. Infections in the presence of two ICP27 repressor mutants showed no changes in the distribution of snRNPs or SC35. (A) Cell line Stu15 has an ICP27 gene with an insertion at what we have defined as the start of the repressor region (22). Uninfected (a, d, and g), KOS-infected (b, e, and h), and 27-LacZ-infected (c, f, and i) Stu15 cells are shown. (B) Cell line S18 has an ICP27 gene with an insertion 8 amino acids from the C terminus of the protein (23). Uninfected (j, m, and p), KOS-infected (k, n, and q), and 27-LacZ-infected (l, o, and r) S18 cells are shown. Cells in column 1 were stained with Y12 antibody. Cells in column 2 were stained with anti-SC35 antibody. Cells in column 3 were stained with anti-ICP27 monoclonal antibody H1113.

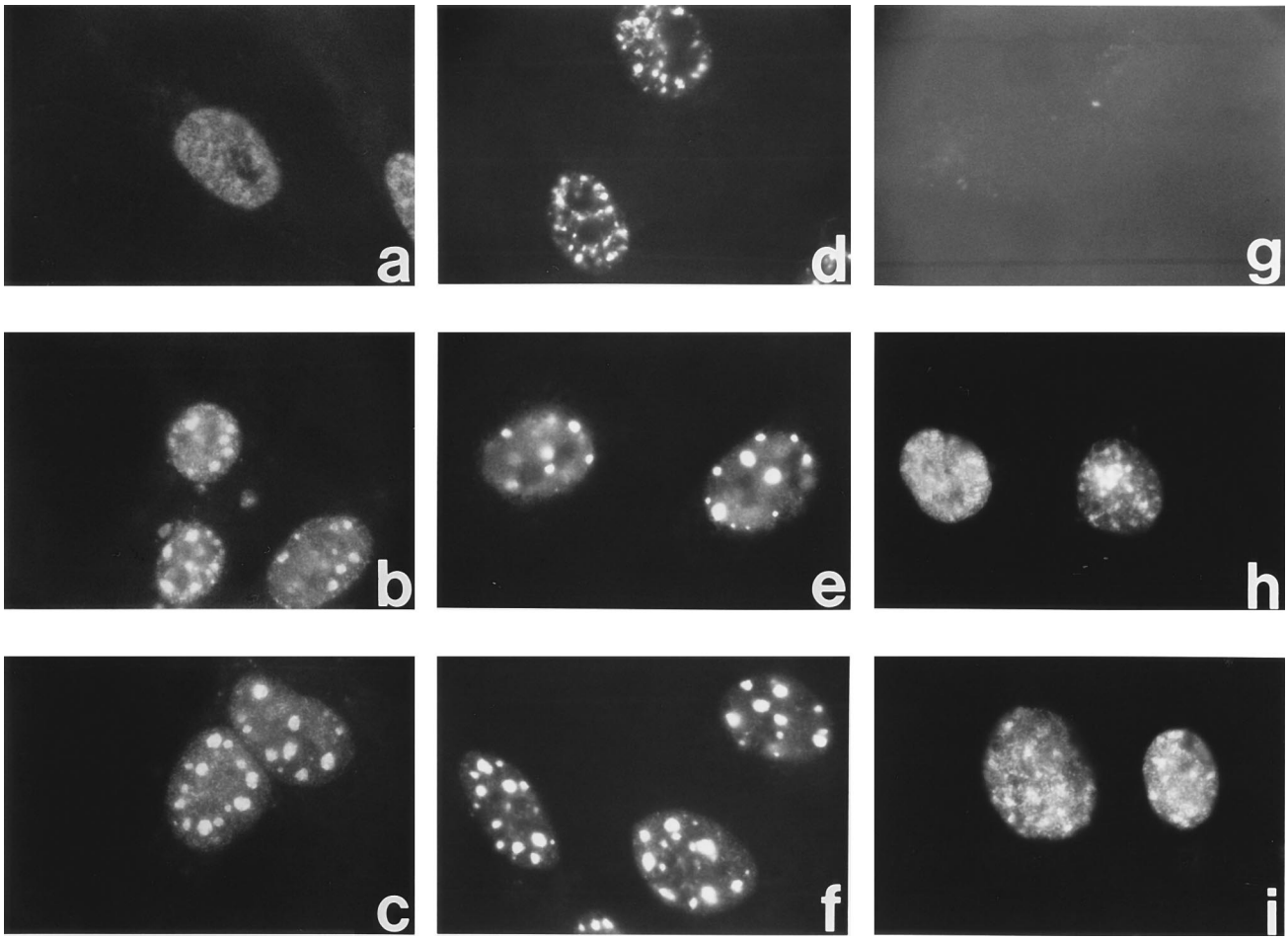

FIG. 7. Infection with *ts*LG4, whose lesion is within the repressor region, resulted in the redistribution of snRNPs and SC35. The ICP27 mutant *ts*LG4 has a base substitution within the repressor region (61). Vero cells infected with *ts*LG4 at 39°C, the nonpermissive temperature, are shown after being stained for snRNPs (c) and SC35 (f). KOS-infected cells (b and e) and uninfected cells (a and d) were also incubated at 39°C. Cells in column 1 were stained with Y12, while cells shown in column 2 were stained with anti-SC35. Staining of ICP27 with H1113 was performed on cells shown in column 3.

stained with monoclonal antibody H1114. Areas of ICP4 staining did not coincide with snRNP clusters (data not shown). In contrast, ICP27 appears to be associated with snRNPs.

To determine the localization of ICP27 in cells where snRNPs were not clustered and redistributed, we analyzed several additional ICP27 repressor mutants in double-staining experiments. Cells were transfected with plasmids expressing wild-type or mutant forms of ICP27. Transfections, rather than infections, of stable cell lines were performed to avoid potential interactions of ICP27 with other viral regulatory proteins (46, 72, 75, 76) and to address the question of whether ICP27 alone could induce the condensation of snRNPs, as suggested by the experiment shown in Fig. 3 and as reported by Phelan et al. (50). The formation of snRNP clusters was seen in cells transfected with wild-type ICP27 (Fig. 10a) and with the mutant H7 (panel b). H7 contains an in-frame deletion of 66 amino acids from residues 382 to 449 and behaves like an activator mutant (23), although the deletion extends into the delineated repressor region (22). The same field of cells transfected with the wild type or H7 plasmids showed that staining of ICP27 was largely concentrated over the areas in which the snRNP clusters were localized (Fig. 10, compare panels f and g with panels a and b), although as in KOS-infected cells (Fig. 9), there were additional, more diffuse areas of ICP27 staining. In contrast, cells transfected with repressor mutants B7, N2, and S2 (23) showed the characteristic speckled and diffuse staining pattern of snRNPs seen in uninfected cells (Fig. 10c to e). The staining of ICP27 was also diffusely localized throughout the nucleus (Fig. 10h to j). These results demonstrate that ICP27 alone can induce the condensation of snRNP clusters and that ICP27 colocalized with the snRNP clusters, although as also seen in the UV inactivation experiment shown in Fig. 3, the effect is less pronounced than that seen with infected cells. The extent of condensation was similar to that seen by Phelan et al. (50) in transfections with wild-type ICP27-expressing plasmid. This may mean that while ICP27 is sufficient for the coalescence of the snRNP staining pattern, other viral factors may contribute to this process to achieve the more dramatic redistribution seen during infection.

DISCUSSION

We have analyzed the distribution of snRNPs and the non-snRNP essential splicing factor SC35 following infection with HSV-1. In accord with results previously reported by Martin et al. (41), a dramatic redistribution of these splicing components was seen. In addition, Phelan et al. (50) reported that ICP27 was responsible and sufficient for the relocalization of snRNPs.

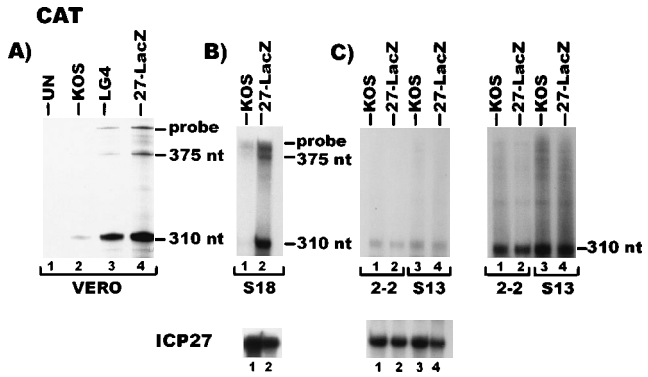

FIG. 8. RNase protection studies to analyze the accumulation of spliced products in cells expressing ICP27 mutants. Vero cells and the cell lines used in the immunofluorescence experiments were transfected with pTK-CAT-5′S-SV LPA (56), which contains an intron with alternative 3′ splice sites inserted into the 5′ noncoding region of the CAT gene. Sixteen hours later, cells were infected with wild-type KOS, *ts*LG4 (at the nonpermissive temperature), or 27-LacZ as indicated above the lanes. Six hours after infection, RNA was isolated and analyzed by RNase protection with a CAT antisense probe which spans the intron and 220 nucleotides of the CAT coding sequences (56). The favored spliced product is 310 nucleotides, and the less abundant, alternative product is 375 nucleotides. (A) Vero cells were left uninfected (UN) or infected with KOS, *ts*LG4, or 27-LacZ as indicated. (B) KOS or 27-LacZ infections were performed with cell line S18. As shown in the lower gel, protections were also performed with a 260-nucleotide antisense RNA probe which encompasses the sequences from amino acids 190 to 260 within the ICP27 coding region to monitor ICP27 expression. (C) KOS or 27-LacZ infections were performed with cell lines 2-2 and S13. The gel on the right is a longer exposure of the CAT RNase protection gel on the left, done to show that amounts of CAT RNA were equivalent in KOS and 27-LacZ infections of cell lines 2-2 and S13. To ensure that equivalent amounts of total RNA were analyzed in each case, portions of each sample were fractionated in agarose and the ethidium bromide staining patterns of 28S and 18S rRNAs were compared (data not shown). nt, nucleotides.

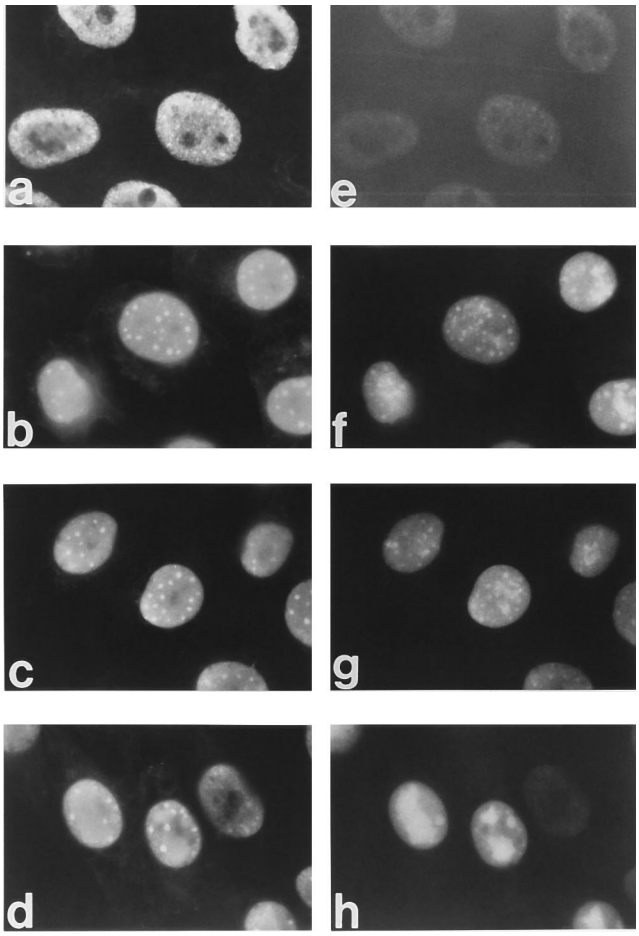

FIG. 9. Colocalization of ICP27 with snRNPs. A double-labeling experiment was performed with Vero cells infected with KOS or with *ts*LG4. The snRNPs were stained with anti-Sm patient antiserum which was bridged to Texas red through biotinylated mouse anti-human immunoglobulin G and streptavidin (column 1, a to d). ICP27 was stained with the monoclonal antibody H1113 and fluorescein-conjugated anti-mouse serum (e, f, and g). ICP8 was stained with the monoclonal antibody H1115 and fluorescein-conjugated anti-mouse serum (h). In each case, the same field of cells was photographed for Texas red fluorescence and for fluorescein by using different Nikon filters. (a and e) Uninfected cells; (b and f) KOS-infected cells stained with anti-Sm serum and anti-ICP27; (c and g) cells infected with *ts*LG4 (at the nonpermissive temperature) and stained with anti-Sm serum (c) and anti-ICP27 (g); (d and h) KOS-infected cells stained with anti-Sm serum (d) and anti-ICP8 (h).

We also demonstrated that ICP27 was the only viral product required for the coalescence of snRNPs (Fig. 10) and, further, that the C-terminal repressor region of the protein was involved in this process (Fig. 6 and 10). Because we previously presented evidence that HSV-1 infection inhibits host cell splicing, and that ICP27 is required for this inhibition of splicing (22, 24), we were particularly interested in determining if the effects on snRNP clustering and distribution were related to the inhibition of splicing. First, we eliminated the possibility that other effects of viral infection may have a role in the condensation and reassortment of snRNPs. We found that the redistribution of snRNPs by HSV-1 infection was distinct from alterations seen when transcription was blocked in uninfected cells (7) (Fig. 1). Redistribution also was not related to inhibition of protein synthesis, expression of virion-associated proteins during viral adsorption or entry, expression of viral early and late genes, or the formation of viral DNA replication complexes. The only correlation that was found was with the expression of ICP27 (Fig. 2), and ICP27 expression was sufficient to cause the condensation of snRNPs into clusters (Fig. 3 and 10).

A correlation between ICP27 expression and the redistribution of splicing factors suggests that these alterations could result in the inhibition of host cell splicing by disrupting active cellular splicing complexes. This would be advantageous to a virus that does not require splicing of the majority of its transcripts. For example, it could permit the relocalization of RNA polymerase II and other cellular transcription factors to sites of HSV-1 transcription. This has been reported to occur in adenovirus infection (30). In this case, snRNPs and hnRNPs appeared to be reassorted and recruited to sites of adenovirus transcription where RNA polymerase II was also relocated. However, more recent studies have contradicted this finding, casting doubt on the hypothesis that the intranuclear speckles observed by staining represent sites of active splicing (74). Specifically, it was found that a monoclonal antibody to the splicing factor SC35, used in the adenovirus study by Jimenez-Garcia and Spector (30), cross-reacted with the adenovirus 72-kDa major DNA-binding protein (5, 74). Therefore, what appeared to be relocalization of splicing factor SC35 to sites of adenovirus transcription and DNA replication actually represented staining of the adenovirus DNA-binding protein, which is required for DNA replication (5, 74). With HSV-1, the snRNPs do not appear to relocalize around sites of HSV-1 transcription. This conclusion is based upon results by Martin et al. (41) and Phelan et al. (50), who showed that two transcriptional activators of HSV-1, ICP4 and ICP0, do not colocalize with the snRNPs. Furthermore, we showed that the redistribution of snRNPs was unrelated to HSV-1 DNA rep-

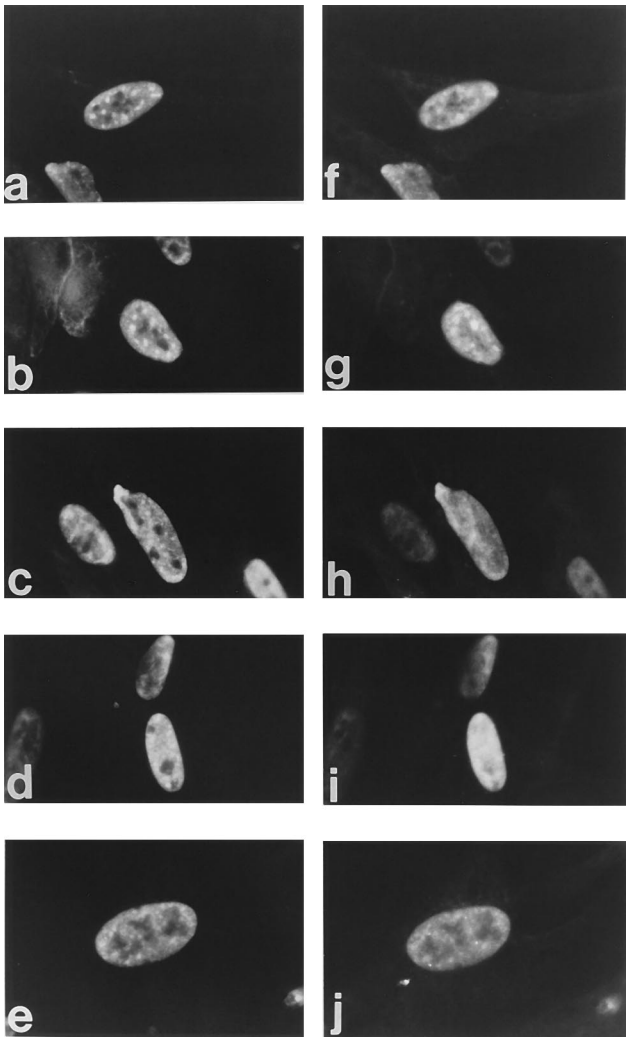

FIG. 10. ICP27 alone can cause coalescence of snRNPs and can colocalize with the clusters. To determine if ICP27 in the absence of all other viral products can cause the coalescence of snRNPs, transfections were performed with plasmids expressing wild-type or mutant forms of ICP27. Double staining was performed on the transfected cells by using patient anti-Sm serum (column 1, a to e) and monoclonal antibody anti-ICP27 (column 2, f to j) as described in the legend to Fig. 9. In each set, the same field of cells was photographed for Texas red fluorescence and for fluorescein staining. (a and f) Cells transfected with the wild-type ICP27 plasmid pSG130B/S (58); (b and g) cells transfected with the mutant H7, which behaves like an activator mutant (23); (c and h) cells transfected with the repressor mutant B7 (22, 23); (d and i) cells transfected with repressor mutant N2 (23); (e and j) results from transfection with repressor mutant S2 (23).

lication (Fig. 1) or to the distribution of ICP8, an essential DNA-binding protein involved in replication complex formation (Fig. 9). Because HSV-1 transcription and DNA replication are likely to be physically associated, especially during late gene expression, this further indicates that the reassortment of splicing components is not related to HSV-1 transcription. We also found that unlike the situation with adenovirus, the staining of hnRNP proteins A, C, and L did not appear to be altered up to 8 h after HSV-1 infection, lending further support to this conclusion (21).

The spatial organization of pre-mRNA processing in the nucleus remains controversial. A number of recent reports, however, suggest that there is a dynamic organization of splicing factors on the nascent RNA and that the nuclear speckles, coiled bodies, and interchromatin granules likely represent storage sites for splicing proteins, which are subsequently recruited to sites of transcription where splicing occurs (2, 5, 15, 18, 34, 36, 52, 74). What then is the significance of the redistribution of the snRNP staining pattern that occurs following HSV-1 infection? A logical hypothesis would be that moving splicing complexes away from sites of viral transcription and replication would result in an inhibition of host cell splicing. However, this assumption is based upon the hypothesis that the intranuclear speckles represent centers of active splicing. Recent findings indicate that interchromatin granule clusters most likely represent sites of factor assembly or storage. For example, interchromatin granule clusters increase in size and splicing factor content following microinjection into living cells of oligonucleotides or antibodies that inhibit splicing in vitro (49). This is the same effect as seen in HSV-1-infected cells, where splicing is also inhibited. However, the converse is not necessarily the case; that is, the coalescing and reassortment of snRNPs do not always result in the inhibition of splicing. Results presented here from the analysis of a repressor mutant, *ts*LG4, where the effect of ICP27 on splicing can be separated from its effect on snRNP distribution suggest that the mechanism of splicing inhibition is more complex than this. During infection with *ts*LG4, snRNPs were redistributed in a pattern indistinguishable from that seen with wild-type KOS infections. Yet, this mutant does not inhibit splicing (Fig. 8) (22, 24). The lesion in *ts*LG4 is a base substitution, whereas the other repressor mutants analyzed had insertions of 3 or 4 amino acids. The change to the *ts*LG4 protein was less disruptive, and this mutant apparently retains the ability to alter the distribution of snRNPs; however, it has lost the ability to inhibit splicing relative to that of the wild-type protein. This would indicate that while the redistribution of snRNPs may be part of the process involved in inhibiting host cell splicing, additional interactions are required. This also indicates that while the splicing factors visible by these staining techniques have been completely displaced from their original location in uninfected cells, active splicing complexes must still be present.

Another role for the interchromatin granules or clusters that become enlarged during HSV-1 infection has been suggested (2). Besse et al. (2) monitored the synthesis and maturation of HSV-1 transcripts by in situ hybridization with biotinylated viral DNA probes. It was found that a large viral region that contained RNA polymerase II molecules was induced following infection. This region likely corresponds to centers of HSV-1 DNA replication and transcription (11). This viral region contained only trace amounts of U1 and U2 snRNA, which is consistent with the low frequency of HSV-1 transcripts that require splicing. However, poly(A) RNA was found to accumulate over the large clusters of interchromatin granules that contained splicing factors. Those authors postulated that the interchromatin granules, or condensed snRNPs, in addition to being involved in spliceosome assembly, might serve as a transient storage site for viral RNA, possibly a sorting site for regulating transport to the cytoplasm (2). Carter et al. (9) also found poly(A)-rich transcripts in discrete internal nuclear regions on analyzing cellular transcripts. They similarly concluded that RNA transport could radiate from these centers. These studies would suggest another role for the interchromatin granules besides splicing factor storage, namely, a role as centers to direct RNA transport. Therefore, the condensation of these structures upon HSV-1 infection could have functional significance apart from the impairment of splicing. Namely, these centers, when moved to the periphery of the nucleus, could guide the movement of a large number of newly transcribed viral mRNAs to the cytoplasm. This would suggest that

this redistribution would play an important regulatory role in HSV-1 infection. The fact that repressor mutants of ICP27, which fail to reassort the snRNPs, are also defective in viral late gene expression (23, 42, 51) would support that theory.

Studies to discern the mechanism by which ICP27 inhibits host cell splicing and causes the disruption of snRNPs are continuing in our laboratory. The results with the TAG mutant that lacks both R-rich regions yet redistributes snRNPs suggest that binding to snRNA does not occur through the RGG-like region (3) in the N-terminal half of the protein. This does not exclude the possibility of RNA binding through the zinc-finger-like region encoded in the C terminus (68). Recent studies in our laboratory have shown that ICP27 not only colocalized with snRNPs but also coimmunoprecipitated with anti-Sm antibody (55). The C terminus is also required for this interaction. Elucidation of the action of ICP27 may uncover important findings concerning splicing mechanisms and provide new insights into the dynamic interactions that occur in the compartmentalization of splicing factors in the nucleus.

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