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The Herpes Simplex Virus Type 1 Regulatory Protein ICP27 Coimmunoprecipitates with Anti-Sm Antiserum, and the C Terminus Appears To Be Required for This Interaction

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The herpes simplex virus type 1 (HSV-1) immediate-early regulatory protein ICP27 is required for the inhibition of host cell splicing during viral infection and for the reorganization of antigens associated with the small nuclear ribonucleoprotein particles (snRNPs). To determine what effect ICP27 had on splicing proteins that might cause their redistribution, we looked at proteins that were immunoprecipitated with anti-Sm antisera. No significant changes were seen in the migration or amounts of several snRNP common and snRNP-specific proteins from infected cells labeled with [^{35}S]methionine, suggesting that the synthesis of these proteins was not altered by viral infection. However, when cells were labeled with $^{32}P_i$, differences were seen in the phosphorylation of at least two proteins depending on whether ICP27 was expressed. One protein, which had an apparent molecular mass of about 85 kDa, was highly phosphorylated during wild-type HSV-1 infection but much less so during infection with an ICP27 null mutant. The other protein, which migrated at the position of the U1 70-kDa protein and was precipitated with U1-specific antiserum, was also more highly phosphorylated when ICP27 was expressed during infection. Furthermore, a phosphoprotein with an apparent molecular mass of 63 kDa was found to coimmunoprecipitate with anti-Sm antisera during wild-type HSV-1 infection. ICP27 has an apparent molecular mass of 63 kDa, and immunoblot analysis confirmed that ICP27 coimmunoprecipitated with snRNPs. Analysis of mutations throughout the ICP27 protein demonstrated that the region that was required for this interaction was the C terminus of the protein, which includes a cysteine-histidine-rich region that resembles a zinc-finger-like motif. These data suggest that ICP27 interacts with snRNPs during infection and that it fosters changes in the phosphorylation state of at least two proteins that immunoprecipitate with snRNPs, although these studies do not demonstrate whether it does so directly or indirectly.

The splicing of nuclear pre-mRNA requires the recognition of introns and exons by multiple *trans*-acting factors that form splicing complexes termed spliceosomes (18, 43). Among the *trans*-acting factors, the four major small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4/U6, and U5 are essential components of the eukaryotic splicing machinery (18, 36, 43). One function of the snRNPs is to help to define introns and exons by binding to consensus sequences within the pre-mRNA (4). Both snRNA and snRNP proteins contribute to these events (18, 43). The snRNPs contain at least 40 different proteins that can be divided into two classes, the snRNP common proteins and the snRNP-specific proteins (22, 26, 45, 56). The snRNP common proteins, designated B′, B, D1, D2, D3, E, F, and G, are bound to the snRNP particles U1, U2, U5, and U4/U6 (35, 36). Because these eight proteins all react with anti-Sm autoantibodies from patients with systemic lupus erythematosus, the snRNP common proteins are also referred to as Sm proteins (33, 34, 48). The snRNP-specific proteins bind in a particle-specific manner. For example, the U1 70K, U1A, and U1C proteins are unique to the U1 snRNP (2, 6, 48), while up to 11 U2-specific proteins with molecular masses ranging from 35 to 165 kDa have been identified (3).

In addition to the snRNPs, a number of protein factors that are required for spliceosome assembly and splicing have been identified. One of these, U2AF, was identified by its interaction with the polypyrimidine tract at the 3′ splice site (52), which is a prerequisite for binding of the U2 snRNP to the branch point (52, 77, 78). Although U2AF contains a functionally important region that is rich in serine and arginine residues, it is not a member of the SR-splicing factor family (79). SR proteins are a family of pre-mRNA-splicing factors that are characterized by the presence of RNA binding motifs and the SR domain, which consists largely of serine and arginine repeats (51, 73). This family consists of at least six evolutionarily conserved proteins that were first identified as a group by a monoclonal antibody (MAb104) that binds a conserved phosphoepitope (51, 73, 75, 76). They are essential splicing factors, which play critical roles in the initiation of spliceosome assembly on pre-mRNA substrates (16, 17, 30–32). Any one of the six SR protein members can be added to complement a splicing-deficient extract (73); however, recent studies have shown that each member displays distinguishable functions when assayed with pre-mRNAs that can be alternatively spliced (15, 29, 67, 74, 76). The SR domain has been shown to be responsible for protein-protein interactions during spliceosome assembly, and many potential phosphorylation sites are present in the SR domain (1, 19, 20, 30, 72).

Immunofluorescent staining studies have shown that snRNPs and the non-snRNP SR proteins assemble into 20 to 50 highly concentrated regions which display a speckled staining pattern in the nucleus of interphase cells (16, 21, 33, 61–64). It is believed that the nuclear speckles serve as storage sites for

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splicing factors, while splicing occurs on nascent transcripts (18, 63, 81). Splicing factors have been found to redistribute in response to transcription inhibition (9), splicing inhibition (19, 46), and viral infection (5, 8, 13, 28, 37, 49, 53). During infection with herpes simplex virus type 1 (HSV-1), a dramatic redistribution of snRNPs and the SR protein SC35 from their normal speckled staining pattern occurs with the formation of prominent clusters, which appear to condense throughout the nucleus and then migrate to the nuclear periphery as infection proceeds (37, 49, 53). Furthermore, studies by Phelan et al. (49) and in our laboratory (53) demonstrated that an HSV-1 immediate-early regulatory protein, ICP27 (IE63), was essential for the relocalization of the snRNPs and that ICP27 colocalized with the snRNPs. We (23, 25, 54) and others (55) have presented evidence that HSV-1 inhibits host cell splicing and that ICP27 is required for this effect (25). In addition, the C-terminal region of ICP27 that we termed the repressor region, which is required for the inhibitory effects on splicing, is also involved in redistributing snRNPs and SC35 (53). However, splicing inhibition and splicing factor relocalization can be uncoupled, as seen in experiments with a temperature-sensitive (*ts*) mutant in the repressor region. During infections with this *ts* mutant, splicing was not inhibited (23, 25), yet a redistributed staining pattern was seen for both snRNPs and SC35 (53). Furthermore, the *ts* mutant protein colocalized with the snRNPs as did the wild-type protein. Therefore, the redistribution of snRNPs and SC35 correlates with ICP27-mediated impairment of splicing, but these alterations are not sufficient to inhibit host cell splicing because active splicing complexes must still be present. This finding supports the hypothesis that the intranuclear speckles and interchromatin granules serve as storage compartments for splicing factors.

Although splicing inhibition by ICP27 appears to involve mechanisms more complex than simply redistributing splicing factors, we wanted to determine what alterations, if any, were occurring in the splicing antigens that caused their relocalization. Therefore, we looked at proteins that were immunoprecipitated with anti-Sm antisera. No changes were seen in the migration or amounts of several snRNP proteins from HSV-1-infected cells that were labeled with [^{35}S]methionine; however, when cells were labeled with $^{32}P_i$, differences were seen in the phosphorylation of several proteins that precipitated with anti-Sm antisera, depending on whether ICP27 was expressed. Furthermore, because ICP27 colocalized with the snRNPs in immunofluorescent staining studies (49, 53), we wanted to determine whether ICP27 coimmunoprecipitated with anti-Sm antisera. A 63-kDa phosphoprotein was found to immunoprecipitate with anti-Sm antisera in wild-type-infected cells but not during infection with an ICP27 null mutant virus. Immunoblot analysis indicated that the protein was ICP27. In addition, the analysis of mutants throughout ICP27 demonstrated that the region required for this interaction was the C terminus of the protein within the repressor region.

MATERIALS AND METHODS

Cells, viruses, and recombinant plasmids. Vero cells and 293 cells (American Type Culture Collection, Bethesda, Md.) were grown as described previously (53). Cells were infected with wild-type HSV-1 KOS, 27-LacZ, or *ts*LG4 at a multiplicity of infection of 10 as described previously (53). Plasmid pSG130B/S, which contains the wild-type ICP27 gene; plasmids pN6, pS13, pS23, pS1B, pB7, pN2, and pS18, which contain ICP27 insertion mutants; and plasmids pS5 and pH7, which contain ICP27 in-frame deletion mutants, were described previously (24). Plasmid pD2ΔS5, which contains an ICP27 in-frame deletion mutant, and plasmids pTAG and pR1, which contain ICP27 substitution mutants, were described previously (27). Plasmid pS2 used in this study was constructed by inserting a 12-bp *Cla*I linker, CCCATCGATGGG, into a *Sma*I site that occurs at amino acid residue 465 in the ICP27 sequence. The insertion was verified by DNA sequencing.

Transfections. Cells were seeded into 100-mm-diameter tissue culture dishes 12 to 15 h before transfection to achieve 75 to 80% confluence. Lipofectamine (Life Technologies) was used at 50 μl per dish, and the plasmid DNA concentration was 10 μg per dish. The lipofectamine transfection procedure was performed according to the protocol provided by the supplier.

Virus infection and radiolabeling. Cells were mock infected or infected with HSV-1 KOS, 27-LacZ, or *ts*LG4 at the nonpermissive temperature at a multiplicity of infection of 10, as indicated in each experiment. When cells were transfected, infection with 27-LacZ at a multiplicity of infection of 10 was performed 24 h after the transfection. Following virus adsorption for 45 min, the virus inoculum was removed, and the monolayers were rinsed and then overlaid either with 3 ml of methionine-free medium (Irvine Scientific) if [^{35}S]methionine labeling was to be performed or with 3 ml of phosphate-free medium (ICN Biomedicals) if $^{32}P_i$ labeling was to be performed. Incubation at the appropriate temperature was continued for 60 min, at which time [^{35}S]methionine (175 μCi/ml) or $^{32}P_i$ (175 μCi/ml) was added to each dish. Five hours after the addition of the label, the cells were rinsed and harvested as described below.

Preparation of nuclear extracts and immunoprecipitation. Cells that were either mock infected or infected with HSV-1 at the appropriate temperature were chilled on ice, rinsed in chilled phosphate-buffered saline (PBS), and scraped into cold PBS. The cell suspensions were centrifuged, and each cell pellet was resuspended in 1.0 ml of low-salt lysis buffer (10 mM Tris [pH 7.5], 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5% Nonidet P-40, 0.5 mM Pefabloc, and 0.5 mM leupeptin). The cell suspension was passed through a syringe with a 25-gauge needle five times to lyse the cells, and the nuclei were harvested by spinning at 14,000 × *g* in a microcentrifuge for 20 s. The cytoplasmic supernatant was discarded, and the nuclei were resuspended in 1.0 ml of high-salt extraction buffer (PBS [pH 7.5] containing 0.5 M NaCl, 0.5% Nonidet P-40, 0.5 mM Pefabloc, 0.5 mM leupeptin). Proteins were extracted by placing the tubes on an end-to-end rotator at 4°C for 60 min. The extracts were cleared by centrifugation at 14,000 × *g* for 25 min at 4°C. The supernatants were divided into two 500-μl aliquots. To one fraction, 5 μl of anti-Sm antiserum (47) was added. Monoclonal antibody H1113 or H1119 (Goodwin Institute) to ICP27 (5 μl) was added to the other fraction. The tubes were placed on a rotator at 4°C for 60 min. Protein A-Sepharose beads (Pharmacia) were reconstituted in high-salt lysis buffer, and 50 μl of the suspension was added to each tube. The tubes were placed on a rotator for 60 min at 4°C. The antigen-antibody complexes bound to the protein A-Sepharose beads were washed by five rounds of centrifugation and resuspension in high-salt lysis buffer, followed by one wash in PBS without added salt or Nonidet P-40.

Polyacrylamide gel electrophoresis and immunoblotting procedures. Polyacrylamide gel electrophoresis was performed as described previously (58, 59). Electrophoretic transfer of proteins to nitrocellulose was done as described previously (24) except that the transfer took place in a buffer of 20% methanol–25 mM Tris–190 mM glycine (pH 8.5) at 110 mA overnight. Immunoblotting experiments were performed by using enhanced chemiluminescence (ECL; Amersham Lifesciences) with the primary antibodies H1113 and H1119 to ICP27, H1114 to ICP4, and H1112 to ICP0 at dilutions of 1:5,000 and the secondary antibody anti-mouse immunoglobulin whole antibody linked to horseradish peroxidase (Amersham Life Sciences) at a dilution of 1:20,000. Patients' anti-Sm antisera were used at a dilution of 1:5,000, and anti-human whole antibody linked to horseradish peroxidase (Amersham Life Sciences) was used at a dilution of 1:20,000. When membranes were reprobed with different primary antibodies, the membranes were stripped by submergence in stripping buffer, which consisted of 100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), and 62.5 mM Tris-HCl (pH 6.7) at 50°C for 30 min, as directed by the supplier. The membranes were subsequently washed and reprobed according to the protocol provided by the supplier.

Artwork for Fig. 1 to 4, 6, and 7. Autoradiographs were scanned on a Hewlett-Packard ScanJet II CX/T with a transparency adapter. The images were scanned at 300 dots per inch into Adobe Photoshop 3.0 and were exported as TIFF files.

RESULTS

Before beginning the immunoprecipitation experiments, it was necessary to test anti-Sm antisera obtained from patients with systemic lupus erythematosus (47) to determine if there was any cross-reaction with HSV-1 ICP27. Human 293 cells were either mock infected or infected with wild-type HSV-1 KOS, the ICP27 null mutant 27-LacZ (58), or the *ts* mutant *ts*LG4, which reassorts snRNPs even though it does not inhibit splicing (25, 53). Infections with *ts*LG4 were performed at the nonpermissive temperature of 39.5°C. Six hours after infection, the cells were harvested, and Western blot (immunoblot) analysis was performed. Identical nitrocellulose filters were probed with different antibodies (Fig. 1). The monoclonal antibody

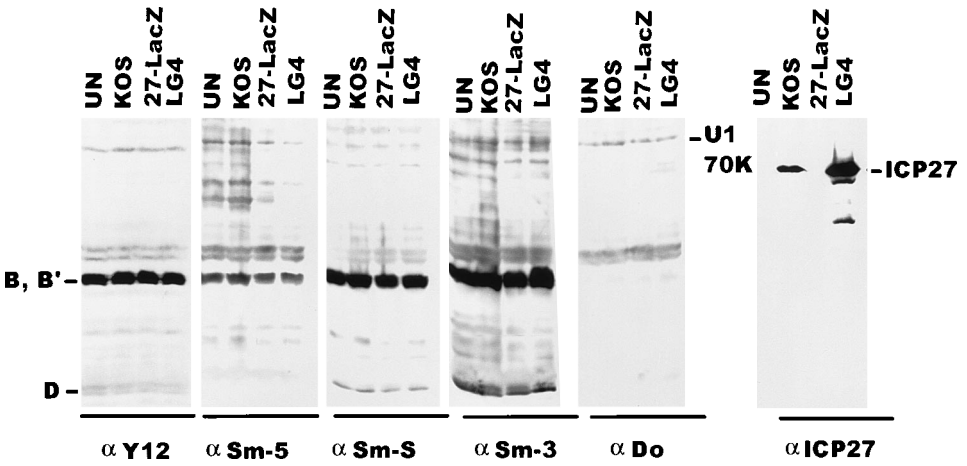

FIG. 1. Immunoblot analysis of proteins that react with anti-Sm antisera. Six identical immunoblots containing samples from mock-infected 293 cells (UN) or cells that were infected for 6 h with HSV-1 KOS, 27-LacZ, or *ts*LG4 at the nonpermissive temperature of 39.5°C were treated with different antibodies as indicated. The Y12 monoclonal antibody is an anti-Sm antibody that reacts predominantly with the 29-kDa B and 28-kDa B′ snRNP common proteins (33). The Sm-5, Sm-S, and Sm-3 antisera were obtained from patients with systemic lupus erythematosus, and these antisera also react predominantly with the common snRNP proteins B′, B, and D (47). The antiserum Do is a patient's antiserum that is specific for U1 RNP and reacts predominantly with the U1 70-kDa protein (34). The anti-ICP27 monoclonal antibody used was H1113. The reacting bands were visualized by ECL.

Y12 reacts predominantly with the B and B′ (29- and 28-kDa, respectively) snRNP proteins, which are found in all snRNPs (33). Patients' antisera, termed Sm-5, Sm-S, and Sm-3, also react predominantly with the B and B′ proteins, with some reactivity seen to the snRNP D protein (16 kDa). This result was expected since anti-Sm autoantibodies precipitate all of the nucleoplasmic snRNPs, as the major immunoreactive Sm proteins are B, B′, and D, which are common to all the snRNPs (48). Some patients with systemic lupus erythematosus or related connective tissue disorders develop autoantibodies that react with particular snRNP proteins (34). The antiserum designated Do was found to react predominantly with U1 RNP polypeptides (34), as can be seen in Fig. 1, in which the U1 70-kDa protein was the predominant band. A band near this size was also seen with the other antisera which have not been reported to react with the U1 70-kDa protein. It is possible that this band may represent a newly identified 69-kDa protein that associates with the Sm core domain of several proteins (22), or alternatively, it may represent some nonspecific cellular background band. The anti-Sm antisera were seen to react with a number of background bands (Fig. 1). Importantly, however, none of the antisera appeared to cross-react with ICP27, indicating that these sera could be used in the immunoprecipitation experiments to examine snRNPs.

No alterations were detected in the synthesis or migration of several snRNP proteins following HSV-1 infection. To address whether changes occurred in splicing antigens as a result of HSV-1 infection, which might account for the redistribution pattern found in immunofluorescent staining studies (37, 49, 53), we performed immunoprecipitation on nuclear extracts with anti-Sm antiserum. The 293 cells were either mock infected or infected with HSV-1 KOS, 27-LacZ, or *ts*LG4 at the nonpermissive temperature. The cells were labeled with [^{35}S]methionine for 5 h beginning 1 h after infection. Nuclear extracts were made in buffer containing 0.5 M NaCl, conditions which are similar to those used in the initial stages of preparing nuclear extracts for in vitro splicing reactions (12, 80). Immunoprecipitation was performed on the extracts under high-salt conditions (0.5 M NaCl) with anti-Sm antiserum Sm-S (Fig. 2). Under these conditions, tightly bound snRNP proteins and proteins that are associated with the snRNPs will precipitate with the anti-Sm antiserum, whereas loosely bound proteins will disassociate from the snRNPs in 0.5 M NaCl (3, 22). The loosely bound splicing proteins include a number of U2-specific snRNP proteins, the snRNP common 69-kDa protein, and the SR family of proteins (3, 22). Following immunoprecipitation, the antigen-antibody complexes were fractionated on an SDS-polyacrylamide gel, and the proteins were then transferred to nitrocellulose. The autoradiograph of the protein gel shows that the U1A protein and the Sm proteins B′, B, and D were labeled equivalently and migrated similarly in both mock- and HSV-1-infected cells (Fig. 2). The U1 70-kDa protein, whose migration varies considerably in different gel systems (22), migrated just below the 68-kDa bovine serum albumin protein marker (Fig. 2). The U1 70-kDa protein does not label efficiently with [^{35}S]methionine (70), however it can be seen that there were several broad bands in the mock-infected sample that may represent different phosphorylated forms of the protein. The U1 70-kDa protein is the only snRNP protein that has been shown to be phosphorylated (70). Two broad bands that could be doublets were also seen in KOS- and *ts*LG4-infected samples, whereas only a single band was clearly distinguishable in the sample from 27-LacZ-infected cells. Several other bands appeared to be equivalent in infected and mock-infected cells, including a doublet with a size of about 200 kDa which may be related to the nuclear-mitotic apparatus protein. This nuclear matrix protein has been shown to colocalize with splicing factors and to coimmunoprecipitate with snRNPs (80). In addition, a number of bands in the HSV-1-infected samples were not seen or were very faint in the mock-infected samples, such as the band marked by the arrow at about 85 kDa. It is not known whether these represent viral proteins or cellular proteins whose synthesis was induced by viral infection.

A faint band with a size of about 63 kDa could be seen in the KOS and *ts*LG4 lanes but not in the mock-infected or 27-LacZ-infected samples. To determine whether this band was ICP27, the nitrocellulose filter was probed with monoclonal antibody H1113, which was visualized by ECL. As the image in the middle of Fig. 2 clearly shows, ICP27 was present in the immunoprecipitated samples from KOS- and *ts*LG4-infected cells. There was also a barely detectable band at this position in the 27-LacZ-infected sample. This likely represents ICP27

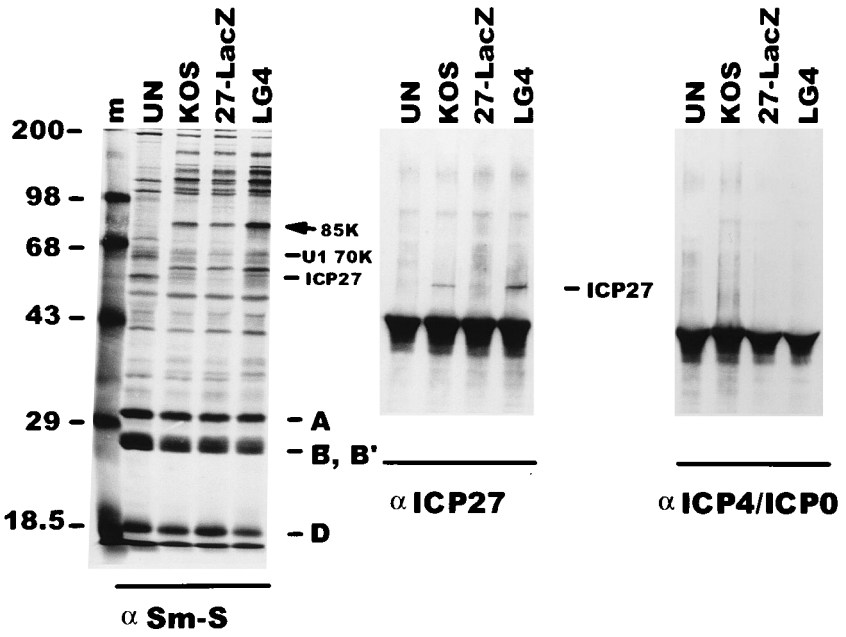

FIG. 2. Immunoprecipitation of [^{35}S]methionine-labeled proteins in uninfected and HSV-1-infected cell extracts with anti-Sm antiserum. 293 cells were either mock-infected (UN) or infected with HSV-1 KOS, 27-LacZ, or *ts*LG4 at the nonpermissive temperature and were labeled with [^{35}S]methionine for 5 h. Immunoprecipitations were performed with nuclear extracts with the patient's anti-Sm antiserum Sm-S. The antigen-antibody complexes were resolved on an SDS-polyacrylamide gel, and the proteins were transferred to nitrocellulose. (Left panel) The blot was first exposed to film for 7 days to obtain the autoradiograph. (Middle panel) Following autoradiography, the blot was treated with ICP27 monoclonal antibody H1113. (Right panel) Following ECL to visualize the bands, the blot was stripped and reprobed with monoclonal antibodies H1114 to ICP4 and H1112 to ICP0. The lane labeled m contains ^{14}C-labeled molecular mass standards, myosin (H-chain), 200 kDa; phosphorylase *b*, 98 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; and β-lactoglobulin, 18.5 kDa. The dark band in the immunoblot in the middle and right panels is heavy chain immunoglobulin G, which reacts with the secondary antibody and is present in the immunoblots because immunoprecipitated proteins were fractionated and transferred. The bands labeled A, B′, and D denote the U1A and snRNP common antigens B′ and D, respectively. The positions of the U1 70-kDa (70K), 85-kDa (85K), and ICP27 proteins are indicated.

expression from a small proportion of wild-type recombinant virus that are generated in some 27-LacZ virus stocks during propagation. Importantly, these data show that wild-type ICP27 protein and the *ts*LG4 mutant protein not only colocalized with snRNPs (53) but also appear to immunoprecipitate with snRNPs. Because several high-molecular-mass bands were present in the samples from virus-infected cells in the ^{35}S autoradiograph, we also probed the blot with monoclonal antibodies to ICP4 (175 kDa) and ICP0 (110-kDa) to determine if they too were associated with snRNPs. As the image on the right shows, neither ICP4 nor ICP0 was present. These data show that HSV-1 infection did not alter the synthesis or migration of several snRNP proteins and further that ICP27 coimmunoprecipitated with snRNPs.

The phosphorylation pattern of two proteins that immunoprecipitate with anti-Sm antisera is altered by HSV-1 infection. To further establish that ICP27 was coimmunoprecipitated with anti-Sm antisera, we labeled cells with $^{32}P_i$. This was done because ICP27 is a phosphoprotein (69) and because the U1 70-kDa protein is the only snRNP protein known to be phosphorylated (70). The SR proteins and splicing factor U2AF are phosphorylated; however, it was not expected that these proteins would remain associated with snRNPs in 0.5 M NaCl. Therefore, it was predicted that the number of immunoprecipitated proteins that could be detected by ^{32}P labeling would be significantly fewer than the number seen in the [^{35}S]methionine-labeling experiment. Thus, ICP27 might be more easily detected. Vero and 293 cells were either mock infected or infected with KOS or 27-LacZ for 1 h, at which time $^{32}P_i$ was added, and the cells were harvested 5 h later. The immunoprecipitated proteins were fractionated on SDS-polyacrylamide gels, and the proteins were transferred to nitrocellulose filters. A band with a size of about 63 kDa could be seen in the KOS-infected 293 cell lane (Fig. 3A). A faint band at this position was also seen on the autoradiograph of the gel with samples from KOS-infected Vero cells, but this band could not be distinguished on the photograph of the gel, in part because of the intensity of the U1 70-kDa band (Fig. 3A). This band was not found in mock- or 27-LacZ-infected cells. To determine if this band was ICP27, the nitrocellulose blot from the 293 samples was probed with the anti-ICP27 monoclonal antibody. As seen in Fig. 3B (right panel), ICP27 was clearly present in the sample from KOS-infected cells, confirming that ICP27 immunoprecipitates with snRNPs.

The autoradiographs of the nitrocellulose blots also showed a protein with a size of about 70 kDa, which is likely the U1 70-kDa protein (Fig. 3A). This protein appeared to be phosphorylated to nearly the same levels in both mock- and KOS-infected samples but was less intensely labeled in the 27-LacZ-infected sample. In addition, a highly phosphorylated band with a size of about 85 kDa was seen in the KOS-infected sample from both Vero and 293 cells, whereas this band could not be seen in the mock- or 27-LacZ-infected samples from Vero cells, and only a very faint band could be seen in this position in the mock- and 27-LacZ-infected samples from 293 cells (Fig. 3A). To eliminate the possibility that the immunoprecipitations were not equally efficient in all samples, thus accounting for the apparent differences in the labeling, the blot with the Vero samples was probed with anti-Sm antiserum Sm-S. As can be seen in the left panel of Fig. 3B, equal amounts of the B and B′ snRNP proteins were recovered, indicating that the differences in the intensities of the bands in

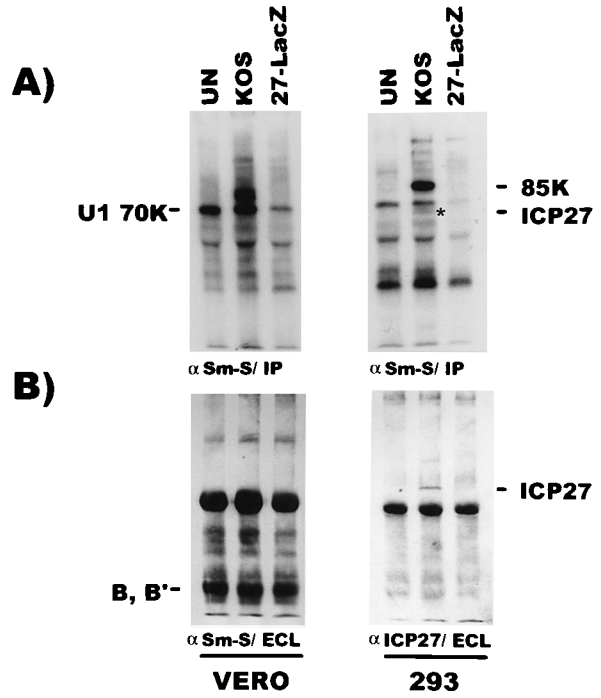

FIG. 3. Immunoprecipitation of ^{32}P-labeled proteins from uninfected and HSV-1-infected nuclear extracts with anti-Sm antiserum. Vero cells and 293 cells were either mock infected or infected with HSV-1 KOS or 27-LacZ and then labeled with $^{32}P_i$ for 5 h. (A) The anti-Sm antiserum Sm-S was used to immunoprecipitate snRNP and snRNP-associated proteins from the nuclear extracts. The antigen-antibody complexes were resolved on SDS-polyacrylamide gels, and the proteins were then transferred to nitrocellulose filters. The autoradiographs were exposed to film for 5 days. (B) After autoradiography, the blot with proteins from Vero cells was treated with anti Sm-S antiserum and the proteins were visualized by ECL. The blot with proteins from 293 cells was treated with anti-ICP27 monoclonal antibody H1113, and the proteins were visualized by ECL. The positions of the U1 70-kDa (70K) protein, the 85-kDa (85K) protein, ICP27, and the B,B′ snRNP common antigen are indicated.

the ^{32}P labeling were not due to differences in the recovery of immunoprecipitated proteins. Therefore, it appeared that phosphorylation of at least two proteins that were immunoprecipitated with anti-Sm antiserum Sm-S was altered as a result of HSV-1 infection.

To address further whether ICP27 is associated with snRNPs and to investigate the phosphorylation of proteins that immunoprecipitate with snRNPs, we performed the immunoprecipitations with two additional anti-Sm antiserum samples and with a U1-specific antiserum sample. In this experiment, 293 cells were either mock infected or infected with KOS, 27-LacZ, or *ts*LG4 at the nonpermissive temperature. A band with a size of 63 kDa was clearly distinguishable in the KOS- and *ts*LG4-infected samples immunoprecipitated with all four antiserum samples that were used (Fig. 4A). This band was not found in the mock- or 27-LacZ-infected samples. To verify that this band was ICP27, the nitrocellulose blots with samples precipitated with anti-Sm-S, Sm-3, and anti-U1 Do were probed with the anti-ICP27 monoclonal antibody (Fig. 3B). In all cases, ICP27 was seen in the KOS and *ts*LG4 samples, confirming that ICP27 coimmunoprecipitates with snRNPs and that this was not a property of one specific antiserum. In addition, this same blot was reprobed with the monoclonal antibody to ICP4, and again, ICP4 was not found in the immunoprecipitated samples (Fig. 4C). Once again, a band with a size of about 85 kDa was highly phosphorylated in both KOS- and *ts*LG4-infected samples with all the antisera used. A less intense band near this position was seen in the mock- and 27-LacZ-infected samples from immunoprecipitations with Sm-S and Sm-3; however, this band could not be definitely distinguished in the corresponding samples precipitated with anti-Do and Sm-5 (Fig. 4A). We do not know the identity of this protein. A protein with a size of about 85 kDa precipitated with Sm-S antiserum in KOS-, 27-LacZ-, and *ts*LG4-infected samples labeled with [^{35}S]methionine, as seen in Fig. 2 (labeled with an arrow). A very faint doublet was also seen in the mock-infected sample. The labeling patterns of this protein with [^{35}S]methionine were more similar in the three samples from infected cells than in these samples. This might indicate that synthesis of this protein is not altered as a result of ICP27 expression during infection but that phosphorylation is altered.

The phosphorylation of the U1 70-kDa protein was seen to vary in different samples. The intensity of this band was greater in the KOS- and *ts*LG4-infected samples than in mock-infected samples that were immunoprecipitated with Sm-3 and Do, whereas the intensities were similar in samples from Sm-S and Sm-5 precipitations. In all cases, the intensity of the U1 70-kDa band was considerably less in the 27-LacZ-infected samples (Fig. 4A). This variation in the phosphorylation of the U1 70-kDa protein was found in a number of different experiments. In all cases, the levels of phosphorylation in the samples from KOS- and *ts*LG4-infected cells were equivalent and significantly greater than those found in 27-LacZ-infected samples. However, the relative levels of phosphorylation in KOS- and *ts*LG4-infected samples were sometimes higher than those in mock-infected samples and were sometimes equivalent. To determine whether phosphorylation overall was altered during infection as a result of ICP27 expression, a fraction of the nuclear extracts that were used for the precipitations with Sm-S serum were immunoprecipitated with the anti-ICP0 monoclonal antibody, because ICP0 is also a phosphoprotein. The intensities of the ICP0 band were similar in KOS-, 27-LacZ-, and *ts*LG4-infected samples, indicating that the differences observed with the proteins that immunoprecipitated with Sm antisera were specific and not due to general changes in phosphorylation during HSV-1 infection where ICP27 is expressed.

The C terminus of ICP27 must be intact for the interaction with snRNPs. We showed previously that the C terminus of ICP27 covering a region from residues 405 to 512 at the end of the protein was required for the inhibition of splicing mediated by ICP27 and for the condensation and redistribution of snRNPs (23, 25, 53). To determine whether this region was also required for the coimmunoprecipitation of ICP27 with anti-Sm antisera, we performed transfections with a series of ICP27 mutants. The positions of the mutations within the ICP27 coding region are represented schematically in Fig. 5. In all cases, 293 cells were transfected with plasmids encoding wild-type or mutant forms of ICP27, and 24 h after transfection, the cells were infected with 27-LacZ to boost expression of ICP27 from the transfected plasmid. The cells were labeled with $^{32}P_i$ 1 h after infection, and cells were harvested 5 h later. The cells were radiolabeled to allow identification of ICP27 in the anti-Sm immunoprecipitations. The immunoprecipitated proteins were transferred to nitrocellulose filters to confirm the identity of ICP27 by detection with the anti-ICP27 monoclonal antibody. In addition, one-half of each nuclear extract was immunoprecipitated with the anti-ICP27 monoclonal antibody to monitor expression of the mutant ICP27 protein in each transfection. The mutants D2ΔS5, R1, TAG, S5, and N6 map to the amino-terminal half of ICP27, as shown in Fig. 5 (24, 27). D2ΔS5 is an in-frame deletion of residues 103 to 178, which includes the major nuclear localization region (40) and

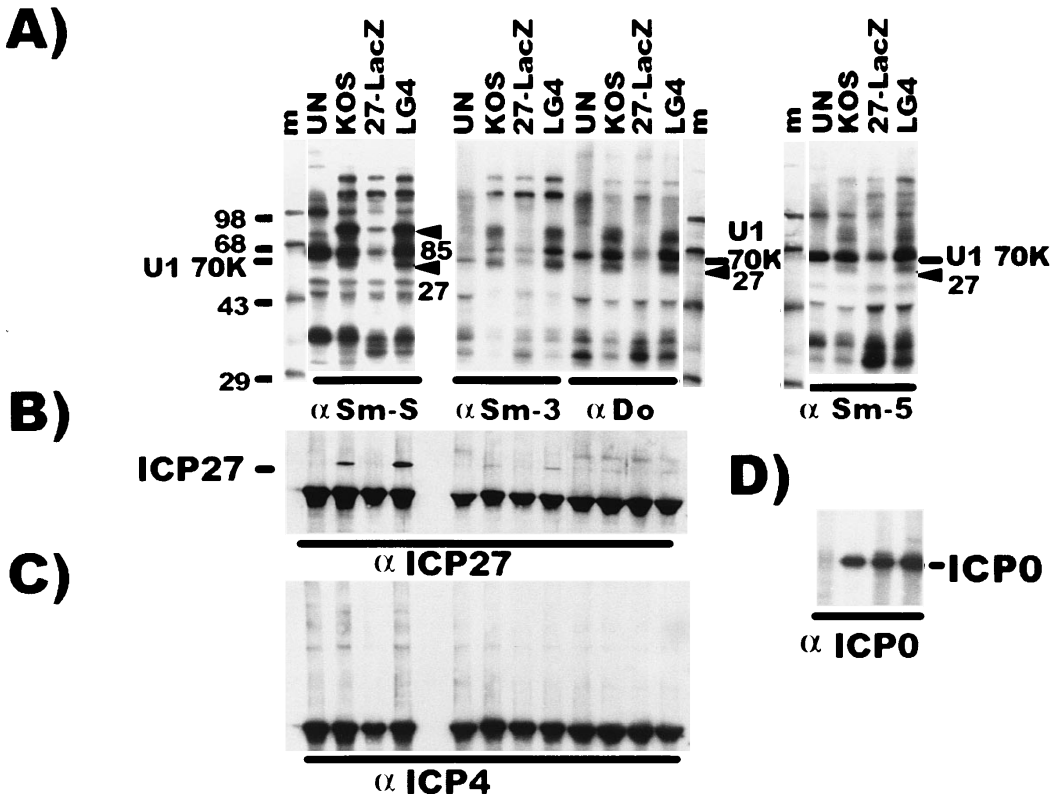

FIG. 4. Immunoprecipitation of ^{32}P-labeled proteins from uninfected or HSV-1-infected cell extracts with patients' antisera. 293 cells were either mock infected or infected with KOS, 27-LacZ, or *ts*LG4 at the nonpermissive temperature as indicated and then labeled with $^{32}P_i$ for 5 h. (A) Nuclear extracts were immunoprecipitated with different antisera. Sm-S, Sm-3, and Sm-5 are anti-Sm antisera that react predominantly with the B′, B, and D snRNP common proteins, respectively, and thus precipitate all of the splicing snRNPs. The Do antiserum is U1 RNP specific. Proteins were transferred to nitrocellulose filters, and the blots were exposed to film for 6 days. The lanes labeled m contain molecular mass standards, as described in the legend to Fig. 3. (B) The Western blots from the immunoprecipitations with anti-Sm-S, Sm-3, and Do were treated with the anti-ICP27 H1113 antibody. The dark bands represent heavy chain immunoglobulin G from the immunoprecipitation. (C) The blots shown in panel B were stripped and reprobed with the anti-ICP4 antibody H1114. (D) A fraction of each lysate used in precipitations with Sm-S antiserum was immunoprecipitated with anti-ICP0 monoclonal antibody H1112. The positions of the U1 70-kDa (70K) protein, the 85-kDa (85K) protein, ICP27, and ICP0 are marked in each panel.

two arginine-rich regions that directly succeed it (27) and which are similar to arginine-rich RNA binding domains in other proteins (7, 57). In the R1 mutant, the first arginine-rich region from amino acids 141 to 151 has been inserted into the D2ΔS5 deletion (27). The TAG mutant contains an insertion of the simian virus 40 large T antigen nuclear localization signal, PKKKRKV, into the site of the D2ΔS5 deletion (27). The S5 mutation contains a 25-amino-acid deletion from res-

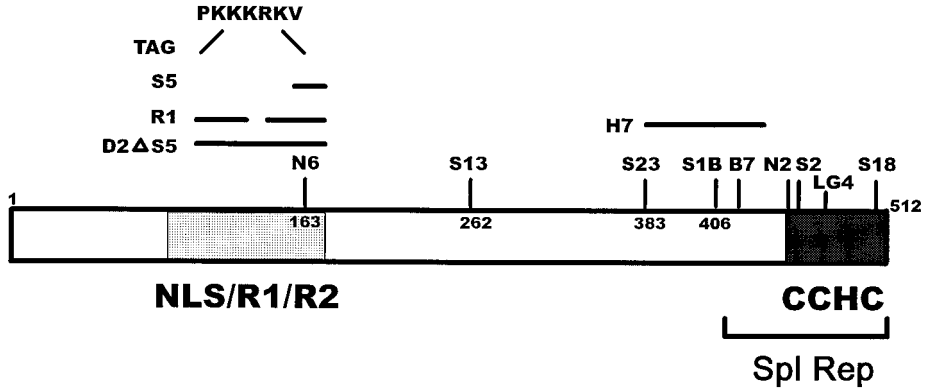

FIG. 5. Schematic representation of ICP27 coding region showing the positions of ICP27 deletion and insertion mutations. The 512-amino-acid coding region of ICP27 is represented. The deleted sequences in mutants D2ΔS5, R1, S5, and H7 are represented as solid horizontal lines. The sequence encoding the nuclear localization region of simian virus 40 T-antigen, PKKKRKV, was inserted into the site of the D2ΔS5 deletion, and the mutant was termed TAG. The positions of the insertions in mutants N6, S13, S23, S1B, B7, N2, S2, and S18 are shown as vertical lines. The position of the base substitution mutation in *ts*LG4 is shown as a shorter vertical line. The numbers under some of the mutants indicate the amino acid residue at which the insertion was made. The region encompassing the major nuclear localization region (NLS) and the two succeeding arginine-rich regions, termed R1 and R2, from amino acids 110 to 171 is also shown. The region spanning the splicing repressor region from amino acids 405 to 512 is bracketed, and the cysteine-histidine-rich zinc-finger-like region is represented as CCHC.

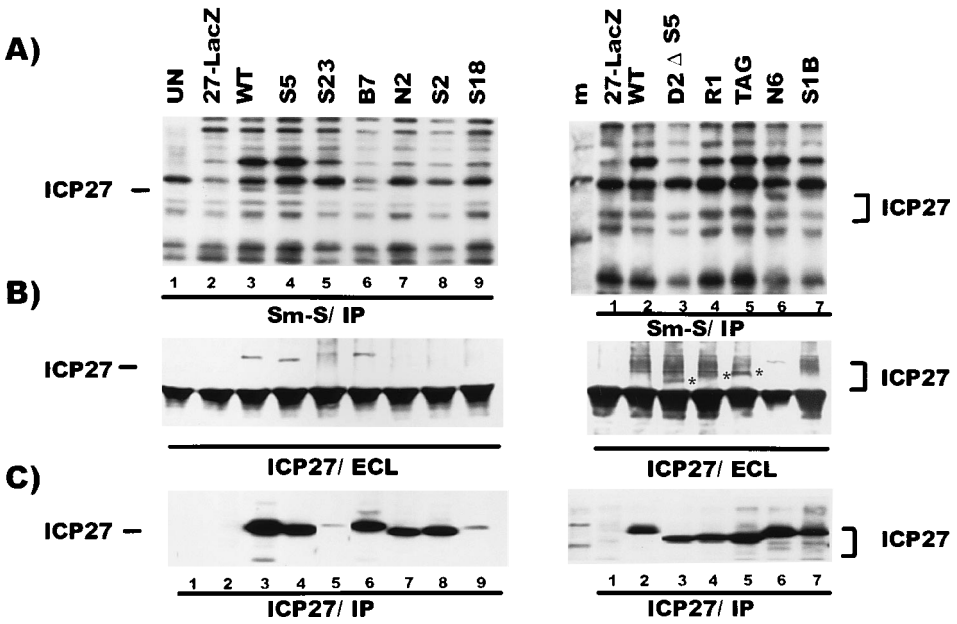

FIG. 6. Analysis of ability of mutant ICP27 proteins to immunoprecipitate with anti-Sm antiserum. 293 cells were transfected with plasmids encoding a series of ICP27 mutants as indicated or with pGEM-1 as a control (lane labeled 27-LacZ). Twenty-four hours after transfection, the cells either were left uninfected (UN) or were infected with 27-LacZ to boost expression of the mutant ICP27 proteins. ^{32}P labeling was done for 5 h, after which the cells were harvested and nuclear extracts were prepared. One-half of each extract was immunoprecipitated with anti-Sm-S, and one-half was immunoprecipitated with anti-ICP27 H1113 and H1119. (A) The Western blots of the anti-Sm-S-immunoprecipitated (IP) proteins were exposed to film for 7 days. (B) The Western blots of the anti-Sm-S-precipitated proteins were treated with anti-ICP27 H1113 (left panel) or H1119 (right panel). (C) The anti-ICP27-immunoprecipitated proteins were fractionated on SDS-polyacrylamide gels and were exposed directly to film without transfer of proteins to nitrocellulose. WT, wild type.

idues 153 to 178, which includes the second arginine-rich region (24, 27). Mutant N6 contains a 4-amino-acid insertion at residue 163, within the second arginine-rich sequence but just outside of the nuclear localization and nucleolar localization regions (24, 40). While D2ΔS5 and R1 were not localized to the nucleus as efficiently as the TAG mutant (27), immunoprecipitation of nuclear extracts from transfections with these mutants showed that the mutant ICP27 protein could be detected in each case when the immunoblot was probed with anti-ICP27 monoclonal antibody H1119 (Fig. 6B). H1119 was used instead of H1113, which was used in previous experiments because the epitope to which H1113 reacts is within the deleted residues (40). The H1119 antibody did not appear to detect ICP27 on immunoblots as efficiently as H1113 because the wild-type ICP27 protein as well as the mutant N6 protein was more readily detectable in the autoradiograph of the blot (Fig. 6A) than in the ECL detection of the immunoblot (Fig. 6B), although both proteins were evident. This was also true for mutant S1B, which contains a 4-amino-acid insertion at residue 406, the start of the splicing repressor region (23, 24). The ICP27 band was evident in the autoradiograph but was more difficult to detect in the immunoblot. The experiment with S1B was repeated with H1113 in the immunoblot detection, and it was confirmed that S1B coimmunoprecipitated with anti-Sm antiserum (data not shown). Mutant S5 also immunoprecipitated with anti-Sm antiserum as seen both in the autoradiograph of the immunoprecipitated proteins (Fig. 6A) and by ECL detection on the immunoblot (Fig. 6B). For this immunoblot, the H1113 antibody was used. Mutants within the activator region of ICP27 spanning residues 260 to 404 were also analyzed. These include S13, with a 4-amino-acid insertion at residue 260; S23, with a 3-amino-acid insertion at residue 383; and H7, which has an in-frame deletion of residues 383 to 450 (24). While the deletion in H7 extends into what we have termed the splicing repressor region (Fig. 5), H7 behaves like an activator mutant in its effects on splicing and the coalescence of snRNPs (24, 53). S13 and S23 were not as efficiently expressed during transfection as many of the other mutants (Fig. 6C and 7C). This was found to be the case in several different experiments and could indicate that these mutant proteins have shorter half-lives than the wild-type protein or other ICP27 mutant proteins. Nevertheless, both the S13 and S23 mutant proteins were seen to immunoprecipitate with anti-Sm antiserum on the autoradiograph of the protein gels (Fig. 6A and 7A) and in the immunoblot treated with anti-ICP27 H1113 (Fig. 6B and 7B). The H7 protein was also found to immunoprecipitate with anti-Sm antiserum (Fig. 7A and B). These data indicate that the amino-terminal half of ICP27, which contains two arginine-rich regions that resemble RNA binding domains, and the activator region of the protein from residues 260 to 404 are not required to be intact for the immunoprecipitation of ICP27 with snRNPs. Furthermore, the efficient immunoprecipitation of mutant S1B at residue 405 and mutant H7, whose deletion extends to residue 450, by anti-Sm antiserum suggests that the region of interaction lies beyond residue 450 in the C terminus of the protein.

The location of the region of interaction was confirmed by the analysis of several mutants in the splicing repressor region in the C terminus of the protein. The mutants B7, which contains a 4-amino-acid insertion at residue 434; N2, which contains a 2-amino-acid substitution and 2-amino-acid insertion at residue 460; S2, which contains a 4-amino-acid insertion at residue 465; and S18, which contains a 4-amino-acid insertion at residue 504, were analyzed. The B7 protein was found to coimmunoprecipitate with snRNPs, as seen in both the autoradiograph of the ^{32}P-labeled proteins (Fig. 6A and 7A) and the immunoblot probed with anti-ICP27 (Fig. 6B and 7B). In contrast, mutants N2, S2, and S18 did not immunoprecipitate

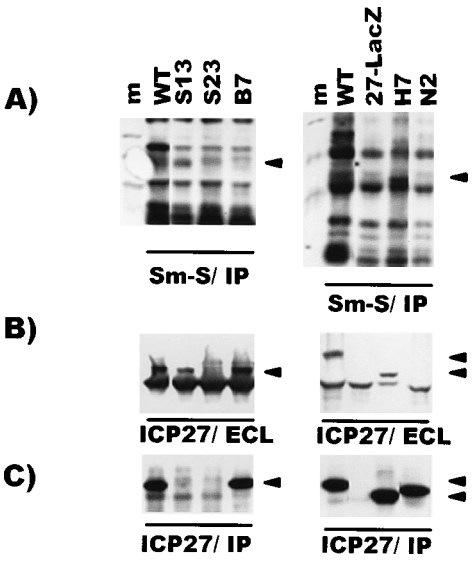

FIG. 7. Analysis of ICP27 mutant proteins that coimmunoprecipitate with anti-Sm antiserum. Transfections were performed with the wild-type ICP27 plasmid, pSG130B/S (WT), with pGEM1 as a control (lane labeled 27-LacZ), or with the mutants indicated. The experimental conditions were as described in the legend to Fig. 6. (A) The anti-Sm-S-precipitated (IP) proteins were transferred to nitrocellulose, and the blot was exposed to film for 6 days. The lanes labeled m contain protein molecular mass standards as described in the legend to Fig. 2. (B) Following autoradiography, the blots shown in panel A were treated with anti-ICP27 H1113 antibody. (C) The anti-ICP27-immunoprecipitated proteins were fractionated on SDS-polyacrylamide gels and were exposed to film without transfer to nitrocellulose filters.

with anti-Sm antiserum as seen in the autoradiographs of the labeled proteins (Fig. 6A and 7A) and in the immunoblot (Fig. 6B and 7B). The S18 protein was not expressed as efficiently as N2 and S2 in this experiment (Fig. 6C). However, the results with N2, S2, and S18 have been reproduced in three different experiments, and coimmunoprecipitation with anti-Sm antiserum was not detected for these three mutants. The insertions in mutants N2, S2, and S18 occur beyond residue 450 in the C terminus of the protein. These data indicate that the region required for the interaction of ICP27 with snRNPs maps to residues beginning after amino acid 450 and extending at least to residue 504 near the end of the protein.

DISCUSSION

Previous studies have shown that the HSV-1 immediate-early regulatory protein ICP27 functions in part at the level of posttranscriptional processing (11, 23, 25, 39, 44, 53, 54, 58). Some of its activities appear to be the inhibition of host cell splicing (23, 25) and the coalescence and redistribution of snRNPs and the splicing factor SC35 (37, 38, 49). In studies on the reassortment of splicing antigens, ICP27 was found to colocalize with the snRNPs in double-labeling experiments (49, 53), and the C-terminal splicing repressor region was shown to be required for the coalescence and for colocalization with snRNPs (53). To determine what alterations might occur to snRNP proteins as a result of ICP27 expression that would cause their redistribution, we immunoprecipitated snRNPs with anti-Sm antisera and analyzed the patterns of the radiolabeled proteins. Differences were not detected in experiments in which [^{35}S]methionine labeling was performed (Fig. 2); however, there were differences in the phosphorylation at least two proteins that coimmunoprecipitated with anti-Sm antiserum when $^{32}P_i$ labeling was performed. One of these proteins, which migrated with an apparent molecular mass of about 85 kDa, was highly phosphorylated during infection with wild-type HSV-1 but was less phosphorylated during 27-LacZ infection or in uninfected cells (Fig. 3 and 4). A protein with about this same size was seen in the [^{35}S]methionine gel, where the amount of labeled protein in KOS- and 27-LacZ-infected samples was similar. This result suggests that the synthesis of this protein was not altered by ICP27 expression but that the phosphorylation was increased. The identity of this protein is not known, and therefore we cannot speculate on how phosphorylation may affect its function. It should also be noted that because we do not know the identity of this protein, phosphorylation of specific residues has not been confirmed by phosphoamino acid analysis. Therefore, it is formally possible that some of the labeling may due to bound ATP or GTP.

The other protein which showed an altered phosphorylation pattern migrated at the position of the U1 70-kDa protein and was precipitated by anti-U1 antiserum Do (Fig. 4). The U1 70-kDa protein is a component of the U1 snRNP that is involved in base pairing at the 5′ splice site. A functional role for the U1 70-kDa protein in splicing has not been demonstrated (50). However, incorporation of a nonhydrolyzable homolog of ATP, ATP-γS, into the isolated U1 snRNP by a kinase yielded a thiophosphorylated product that was resistant to dephosphorylation by phosphatases (66). This resulted in the impairment of the ability of the U1 snRNP to complement splicing in a U1 snRNP-depleted extract (66). Furthermore, overexpression of the arginine-rich carboxy-terminal region of the U1 70-kDa protein, which is the region that is phosphorylated, inhibited splicing and induced a nuclear reorganization of splicing factor SC35 (50). Also, it has been reported that inhibitors of phosphatases can specifically inhibit splicing in vitro (41). It was shown that protein phosphatase activity was not required for efficient assembly of splicing complexes containing each of the U1, U2, U4/U6, and U5 snRNPs; however, phosphatases were required for both catalytic steps of the splicing reaction (41). These findings indicate that reversible protein phosphorylation may play a role in regulating pre-mRNA splicing (41). Additional evidence that phosphorylation plays a role in pre-mRNA splicing is that U1 snRNP-associated kinase activity that could phosphorylate the SR domain in the SR family of splicing factors, and a similar motif consisting of serine and arginine repeats in the U1 70-kDa protein, has been described (71). In addition, a cell cycle-regulated kinase specific for SR proteins has been identified, purified, and cloned (19), and a high level of this kinase can inhibit splicing (20) and cause the disassembly and reassortment of nuclear speckles in immunofluorescent staining experiments (19). It appears then that pre-mRNA splicing can be regulated both positively and negatively by protein phosphorylation (42). Therefore, the differences in phosphorylation in the U1 70-kDa protein and the 85-kDa protein may be significant in the effect of ICP27 on splicing and the reassortment of snRNPs. Although we have found differences in the levels of phosphorylation of these proteins, there was variation in the extent of phosphorylation, and a simple correlation between ICP27 expression, the effects on splicing, and the extent of phosphorylation cannot be drawn from these experiments. For example, although the U1 70-kDa protein and the 85-kDa protein were more highly phosphorylated during KOS infection than during 27-LacZ infection, the level of phosphorylation was also high during *ts*LG4 infection at the nonpermissive temperature. Splicing is inhibited and snRNPs are reassorted in KOS-infected cells (25, 53) but not in 27-LacZ-infected cells. Yet, while snRNPs are reassorted in *ts*LG4-infected cells (53), splicing is not inhibited (25). Perhaps

the higher level of phosphorylation of these two proteins has more of an effect on the intranuclear distribution of snRNPs, as these are generally considered to be storage units for splicing factors rather than sites of active splicing. Because the phosphorylation of splicing proteins appears to be a dynamic process which regulates splicing positively and negatively, it will be necessary to investigate the effect of ICP27 on splicing proteins during in vitro splicing reactions in which purified ICP27 can be added in the absence of other viral factors. In this regard, we have preliminary evidence that the extent of phosphorylation of several proteins in splicing extracts was altered in the presence of ICP27 (82). ICP27 has not been demonstrated to possess kinase activity. However, the migration of ICP4 on an SDS-polyacrylamide gel was altered when ICP4 was expressed in the presence of ICP27 compared with migration in its absence (65). The alteration was consistent with a change in the redox state of ICP4 that would be compatible with a change in the phosphorylation state. At this point it is not known whether ICP27 can affect the phosphorylation of other proteins directly or indirectly by interactions with cellular kinases or phosphatases.

We have also presented evidence that ICP27 interacts with snRNPs and coimmunoprecipitates with anti-Sm antisera. The interaction was specific in that two other HSV-1 regulatory proteins, ICP4 and ICP0, that were not found to colocalize with snRNPs (37, 49) also did not immunoprecipitate with anti-Sm antisera (Fig. 2 and 4). In addition, ICP8, which does not colocalize with snRNPs (53), was not found among the immunoprecipitated proteins when the immunoblot was probed with a monospecific anti-ICP8 antibody (data not shown). We also found that ICP27 was rather tightly associated with the snRNPs because the extraction and immunoprecipitation procedures were performed in 0.5 M NaCl. Under these high-salt conditions, loosely bound splicing proteins are released, including a number of snRNP-specific proteins and the SR proteins (22, 73). Furthermore, it was possible to map the region of ICP27 that was required for the interaction by mutational analysis. The C terminus of the protein from amino acids 450 to at least 504 near the end of the protein was the region required (Fig. 5). This partially overlaps the splicing repressor region, which extends from residues 405 to 512 (23, 24, 53) and includes a cysteine-histidine-rich region that encodes a zinc-finger-like motif (68). The cysteine-histidine-rich region begins at residue 461, with the zinc-finger-like sequence occurring between residues 483 and 508 (68) (Fig. 5). The mutants that were unable to coimmunoprecipitate with the snRNPs included N2, which has an insertion at residue 460 at the start of the cysteine-histidine-rich region; S2, which has an insertion at residue 465; and S18, which has an insertion at residue 504. Interestingly, the mutation in *ts*LG4 is a base substitution that affects amino acid 480, which is also within the Cys-His-rich region (60). The mutation results in a change from an arginine residue in the wild-type protein to a histidine residue in the *ts*LG4 protein. This alteration apparently did not perturb the protein structure sufficiently to interfere with the ability of the *ts*LG4 protein to colocalize with snRNPs in double-staining experiments (53) or to coimmunoprecipitate with the snRNPs as shown here.

It is not known whether the interaction of ICP27 with the snRNPs occurs by protein-protein interactions or by binding to snRNA. The region that was deemed most likely to be involved in RNA binding, namely the two arginine-rich sequences in the amino-terminal half of the protein that resemble RNA binding domains in other proteins (7, 57), was not required for the coimmunoprecipitation with anti-Sm. Deletion of both of these regions in the mutant D2ΔS5, or substitution of these regions with the lysine-rich nuclear localization signal from simian virus 40 T antigen in the mutant TAG, resulted in detectable ICP27 in the anti-Sm-precipitated proteins. However, it is possible that ICP27 could bind to snRNA through the Cys-His-rich region. This region contains a CCHC motif, which we originally described as a zinc-finger-like motif (68) but which also resembles that which has been referred to as a zinc knuckle (10). Zinc fingers and zinc knuckles have been shown to occur in a number of splicing proteins including the U1C protein (45), the human SR splicing factor 9G8 (10), and the SLU7 splicing factor in yeasts (14). These proteins have not yet been shown to bind to RNA.

In summary, we have shown that ICP27 expression during HSV-1 infection affects the phosphorylation of at least two proteins that coimmunoprecipitate with anti-Sm antisera, although the significance of these alterations cannot be directly determined from these studies. We have also shown that ICP27 coimmunoprecipitates with anti-Sm antisera and that the region required for this interaction maps to the C terminus of the protein, which includes a Cys-His-rich region. Further studies will be directed at determining the phosphorylation state of splicing factors during in vitro splicing reactions in the presence and absence of ICP27 to attempt to correlate any changes with the efficiency of splicing in the extract. In addition, we will attempt to determine whether ICP27 binds to snRNA and, if so, if the C terminus of the protein is involved. While these experiments have only begun to uncover the possible mechanism by which ICP27 may influence the process of splicing and the intracellular localization of the splicing factors, important information about splicing regulation may emerge from further probing the action of ICP27.

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