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The RGG Box Motif of the Herpes Simplex Virus ICP27 Protein Mediates an RNA-Binding Activity and Determines In Vivo Methylation

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ICP27 is an essential herpes simplex virus type 1 nuclear regulatory protein that is required for efficient viral gene expression. Although the mechanism by which ICP27 regulates genes is unknown, a variety of evidence suggests that it functions posttranscriptionally, and recent studies indicate that it is an RNA-binding protein. Previously, we noted that a short arginine- and glycine-rich sequence in ICP27 (residues 138 to 152) is similar to an RGG box motif, a putative RNA-binding determinant found in a number of cellular proteins (W. Mears, V. Lam, and S. Rice, J. Virol. 69:935–947, 1995). In the present study, we have further investigated ICP27's association with RNA and examined the role of the RGG box in RNA binding. We find that ICP27 binds efficiently to RNA homopolymers composed of poly(G) and weakly to poly(U) RNA homopolymers. Poly(G) binding activity maps to the N-terminal 189 residues of ICP27 and requires the RGG box sequence. Using a northwestern blotting assay, we demonstrate that the RGG box alone (residues 140 to 152) can mediate RNA binding when attached to a heterologous protein. As many cellular RGG box proteins are methylated on arginine residues, we also investigated the in vivo methylation status of ICP27. Our results demonstrate that ICP27 is methylated in herpes simplex virus-infected cells. Methylation is dependent on the presence of the RGG box, suggesting that one or more arginine residues in the RGG box sequence are modified. These data demonstrate that ICP27 displays the characteristics of an RGG box-type RNA-binding protein.

Herpes simplex virus type 1 (HSV-1) is a common human alphaherpesvirus which establishes life-long infections in affected individuals. Its effects range from asymptomatic infections to serious, life-threatening illnesses. HSV-1 is also an important research tool, serving as a model system with which to study the fundamental pathways used by herpesviruses to replicate in their host cells. Our laboratory is particularly interested in using HSV-1 to characterize the mechanisms by which herpesviruses regulate the expression of their genes during lytic infections.

Much is already known about the pattern of gene expression in cells lytically infected with HSV-1 (reviewed in reference 35). After the virus enters the cell, its double-stranded DNA genome is transported to the cell nucleus, where it is transcribed by the host RNA polymerase II. The approximately 80 viral genes are similar in structure to cellular genes, with the exception that most do not possess intron sequences. During infection, the genes are expressed in a highly regulated fashion and have been grouped into three general categories based on the temporal order of, and requirements for, their expression. Immediate-early (IE, also called α) genes are the first to be expressed and encode several regulatory proteins, including ICP0, ICP4, ICP22, and ICP27. The transcription of IE genes can occur in the absence of viral protein synthesis and is stimulated by VP16, a virus-encoded virion protein. The next genes to be expressed are the delayed-early (DE, also called β) genes. Transcription of these genes requires the prior synthesis of IE proteins, in particular ICP4. For the most part, the DE genes encode proteins involved in viral DNA replication. The last genes to be expressed are the late (L, also called γ) genes. Like DE gene expression, L gene expression requires prior viral protein synthesis and, in addition, is stimulated by viral DNA replication. The majority of L genes encode viral structural proteins. Concomitant with the activation of viral genes in HSV-1-infected cells, shutoff of cellular gene expression also occurs. Host gene shutoff (reviewed in reference 41) is a complex phenomenon involving the down-regulation of transcription, the inhibition of mRNA splicing (10), and the induced degradation of cellular mRNAs.

The mechanisms controlling viral and cellular gene expression during HSV-1 infection have been the subject of numerous investigations. One important fact which has emerged from these studies is that the HSV-1 IE proteins play critical roles in gene regulation. Our laboratory is particularly interested in the IE protein ICP27. This polypeptide is composed of 512 residues, migrates on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as a 63-kDa protein, and is localized predominantly to the cell nucleus (1, 15, 22). ICP27 is posttranslationally modified by phosphorylation (1, 46), although the function and sites of phosphorylation are unknown. Genetic studies indicate that ICP27 is absolutely essential for virus growth (21, 36). One phenotype of HSV-1 ICP27 null mutants is that they are deficient in the expression of many L proteins and their corresponding mRNAs (21, 23, 31, 36). A second phenotype is that they show a 5- to 20-fold reduction in the level of viral DNA synthesis (31). ICP27's effects on late gene activation and DNA replication may be due to distinct functions of the protein, as they can be differentially affected by mutations in the ICP27 gene (31–33). Recent work suggests that ICP27 stimulates DNA replication indirectly, by enhancing the expression of some of the DE mRNAs which encode DNA replication proteins (23, 44). Thus, ICP27 appears to mediate at least two separable functions which together enhance the production of both DE and L mRNAs. In addition, ICP27 contributes to the shutoff of host gene expres-

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sion, as cellular protein synthesis (36) and mRNA levels (9) are greater in ICP27 mutant infections than in wild-type (WT) HSV-1 infections.

It is not yet known if ICP27's effects on viral and cellular genes occur at the transcriptional level, the posttranscriptional level, or both. However, a variety of evidence suggests that ICP27 mediates at least some of its effects through changes to the cell's pre-mRNA processing machinery. One effect appears to be an inhibition of pre-mRNA splicing. This was first noted in transfection assays, in which it was found that ICP27 can inhibit the expression of cotransfected target genes which possess introns (39). Furthermore, ICP27 can partially inhibit in vitro splicing in nuclear extracts (10) and can alter the intranuclear distribution of splicing factors in vivo (29, 38). In addition to affecting splicing, ICP27 may also modulate pre-mRNA polyadenylation. The specificity of in vitro poly(A) site utilization is altered in HSV-1-infected cells (25), and this effect is dependent on a functional ICP27 (24). Moreover, ICP27's effects on polyadenylation may be responsible for its ability to stimulate certain target genes in cotransfection assays, as the responsive sequences map to the 3′ segment of the target genes, which includes their poly(A) sites (5, 39). Recent data reported by McGregor et al. (23) support the hypothesis (39) that ICP27 increases the efficiency with which weak poly(A) sites are utilized.

The evidence for posttranscriptional regulation by ICP27 suggests the possibility that the protein interacts with RNA in infected cells. Consistent with this possibility, two recent studies have demonstrated that ICP27 can interact with RNA molecules in vitro (3, 13). Previously (26), we noted that ICP27 possesses a sequence which resembles an RGG box, a putative RNA-binding motif found in a number of cellular nuclear proteins involved in mRNA and rRNA metabolism (4, 14, 20). ICP27's RGG box sequence is composed of 15 consecutive arginine and glycine residues and maps to residues 138 to 152. It is required for ICP27's nucleolar localization, possibly reflecting an in vivo RNA-binding activity (26). It is also essential for its biological function, as a recombinant HSV-1 which encodes an RGG box-minus ICP27 fails to replicate efficiently in cultured cells (26). The goal of this study was to further investigate ICP27's in vitro interaction with RNA and to explore the role of the RGG box motif in RNA binding.

MATERIALS AND METHODS

Plasmids. Plasmid constructs for in vitro transcription-translation of ICP27 were constructed by using the vector pCITE-1 (Novagen). To clone the WT ICP27 gene, pCITE-1 was first modified by the introduction of an *Sst*I site into its polylinker sequence. This was done by cleaving pCITE-1 with *Acc*I, filling in the DNA ends with the Klenow fragment of *E. coli* DNA polymerase, and inserting an *Sst*I linker. This plasmid was termed pCITE-SstI. To generate pCITE-27, encoding WT ICP27, the following steps were performed. A plasmid containing the ICP27 gene, pM27 (32), was digested with *Drd*I, which cuts just upstream of ICP27's initiation codon. The resulting ends were made blunt by using T4 DNA polymerase. The DNA was then digested with *Sst*I, which cleaves downstream of ICP27's termination codon. The 2.0-kb *Drd*I-*Sst*I fragment containing the ICP27 gene was then cloned into *Bal*I-*Sst*I-digested pCITE-SstI. An analogous procedure was used to engineer pCITE-d4-5, in this case using plasmid pMd4-5 (26) instead of pM27. pCITE-d4-6 was constructed by ligation of the 1.5-kb *Xho*I-*Sst*I ICP27 gene-containing fragment from pM6 (32) to the 4.4-kb *Xho*I-*Sst*I vector fragment of pCITE-d4-5. pCITE-d5-6 was constructed by ligating the 1.6-kb *Dra*III-*Sst*I fragment of pMd5-6 (16) to the 4.0-kb vector fragment of pCITE-d4-6 which is obtained after complete *Sst*I digestion and partial *Dra*III digestion. pCITE-M11, pCITE-M15, and pCITE-M16 were constructed by substitution of the 1.2-kb *Sal*I-*Sst*I ICP27 gene fragments of pM11, pM15, and pM16 (32) into pCITE-27 in place of the WT ICP27 gene fragment. The ICP27 molecules encoded by pCITE-27 and the derivatives described above have three additional N-terminal residues (Met-Ala-Val) compared with WT ICP27. To construct pCITE-262C, pBS27 (33) was digested with *Sal*I, and the DNA ends were made blunt by using the Klenow enzyme. After cleavage with *Sst*I, the 1.2-kb fragment was cloned into *Bal*I-*Sst*I-cleaved pCITE-SstI. pCITE-262C encodes a protein having an N-terminal methionine residue followed by residues 262 to 512 of ICP27. Heterogeneous nuclear ribonucleoprotein particle (hnRNP) protein C1 was produced by in vitro transcription-translation using plasmid pHC12 (43), obtained from G. Dreyfuss (University of Pennsylvania).

Plasmids for bacterial expression of glutathione *S*-transferase (GST)–ICP27 fusion proteins were constructed by using the vectors pGEX-SstI and pGEX-BglII, which are modified forms of pGEX-5X-3 (Pharmacia). To create pGEX-SstI, the *Not*I site in pGEX-5X-3 was converted to an *Sst*I site by digesting pGEX-5X-3 with *Not*I, filling in the DNA ends with the Klenow fragment, and inserting an *Sst*I oligonucleotide linker. pGEX-BglII was made in a similar fashion except that a *Bgl*II linker was used. To generate pGEX-27, the 2.0-kb *Drd*I-*Sst*I ICP27 gene fragment obtained from pM27 was cloned into the 4.9-kb *Sma*I-*Sst*I vector fragment of pGEX-SstI. This plasmid encodes a GST-ICP27 fusion protein consisting of GST, 12 linker residues, and all 512 residues of WT ICP27. pGEX-d4-5 was constructed by ligating the 1.7-kb *Dra*III-*Sst*I fragment of pMd4-5 to the 5.2-kb *Dra*III-*Sst*I vector fragment of pGEX-27. Plasmid pGEX-RGG was constructed as follows. First, oligonucleotide-directed mutagenesis (Altered Sites system; Promega) was used to engineer a *Bgl*II site in plasmid pM4 (32). This resulted in plasmid derivative pM4-5B, which contains an *Xho*I site at codons 138 and 139 of the ICP27 gene and a *Bgl*II site at codons 153 and 154. pM4-5B was cleaved with *Xho*I and *Bgl*II, and the small *Xho*I-*Bgl*II fragment was cloned into the 4.9-kb *Sal*I-*Bgl*II vector fragment of pGEX-BglII to produce pGEX-RGG. The polypeptides encoded by pGEX-5X-3 and pGEX-RGG differ only at their C termini: GST contains the sequence -SSGRIVTD, which is replaced by -GRRGRRRGRGRGGPDLPAAS in GST-RGG (the underlined residues correspond to residues 140 to 152 of ICP27).

The plasmids used to generate RNA probes for the northwestern blotting assays, pBSSV40PA and pBSIFNPA (3), were provided by Charles Brown and L. P. Perera (National Institutes of Health).

Expression of in vitro-translated ICP27. For in vitro transcription-translation, plasmids were first linearized at appropriate restriction sites. pHC12 was cleaved with *Nsi*I, whereas pCITE-27, pCITE-d4-5, pCITE-d4-6, pCITE-d5-6, and pCITE-262C were cleaved with *Ecl*136II. C-terminally truncated forms of ICP27 were produced by linearization of plasmids as follows: pCITE-d4-5 was digested with *Xho*I to generate the N137 template; pCITE-27 was digested with *Nco*I to generate the N189 template; pCITE-27 was digested with *Sal*I to generate the N261 template; pCITE-M11 was digested with *Xho*I to generate the N339 template; pCITE-27 was digested with *Stu*I to generate the N405 template; and pCITE-M16 was digested with *Xho*I to generate the N487 template. RNA transcripts were produced by in vitro transcription of the linearized plasmids by using the RIBOMAX system (Promega) according to the manufacturer's directions. Transcription was carried out with T7 RNA polymerase (Bethesda Research Laboratories), with the exception of pHC12, which was transcribed by using SP6 RNA polymerase (Promega). After transcription, RNAs were precipitated by the addition of ammonium acetate to 2.5 M and resuspended in water. In vitro translation was carried out in a rabbit reticulocyte lysate (Promega) in the presence of 800 μCi of [^{35}S]methionine-cysteine (DuPont NEN) per ml as recommended by the manufacturer.

RNA homopolymer binding assays. Binding of in vitro-translated protein to ribonucleotide homopolymers or single-stranded DNA (ssDNA) was carried out as described by Kiledjian and Dreyfuss (14). Briefly, 10^5 cpm of trichloroacetic acid-precipitatable translated protein was made up to a final volume of 0.5 ml with binding buffer (10 mM Tris [pH 7.6], 2.5 mM $MgCl_2$, 0.5% Triton X-100, NaCl of the appropriate concentration). To this, 50 to 60 μl of washed agarose beads containing attached ribonucleotide homopolymer (Pharmacia or Sigma) or ssDNA (Pharmacia) was added. The mixture was incubated for 60 min at 4°C on a rocking platform. The beads were pelleted by centrifugation for 1 min in a microcentrifuge and washed five times with binding buffer. Protein was eluted from the beads by boiling in SDS-PAGE sample buffer and analyzed by SDS-PAGE on 12.5 or 15% gels. Radioactive bands were visualized and quantitated by autoradiography and phosphor image analysis (Fuji).

Expression and purification of GST fusion proteins. GST fusion proteins were expressed in *Escherichia coli* BL21. Individual transformants were grown overnight at 37°C in medium containing 100 μg of ampicillin per ml. The overnight cultures were diluted 1:10 in a total volume of 100 ml and grown at 37°C until the optical density (A_{600}) reached 1.1 to 1.4 units. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM, and induction was allowed to proceed for 2 h. The cells were pelleted at 4°C and resuspended in 5 ml of phosphate-buffered saline (PBS), and lysozyme (Sigma) was added to 1 mg/ml. Lysis was allowed to proceed on ice for 60 min. The cell lysate was spun at 5,000 × *g* for 10 min at 4°C, and the resulting pellet was quick-frozen in a dry ice-isopropanol bath. After thawing, the pellet was resuspended in 5 ml of PBS containing 0.1% sodium deoxycholate (Sigma). After a 10-min incubation on ice, $MgCl_2$, Triton X-100, and DNase I were added to final concentrations of 8 mM, 1%, and 10 μg/ml, respectively. Incubation was continued for 30 min on ice with gentle agitation. The mixture was then spun at 10,000 × *g* for 10 min at 4°C, and the supernatant was retained. To purify GST and GST fusion proteins, 5 ml of supernatant was incubated with 100 μl of prewashed glutathione-Sepharose 4B beads (50% slurry; Pharmacia) for 30 min at 4°C. The beads were then washed three to four times in PBS. Bound protein was eluted at room temperature with three to four 50-μl washes of elution buffer (50 mM Tris-HCl [pH 8.0], 10 mM glutathione). Purified protein preparations were stored at −70°C until needed. The protein concentration of each preparation was measured by the Bradford

assay (Bio-Rad). The preparations were also analyzed by Western blotting (immunoblotting) using monoclonal antibodies directed against GST (Pharmacia) or ICP27 (H1113; Goodwin Institute for Cancer Research, Plantation, Fla.).

Northwestern blotting assays. ^{32}P-labeled riboprobes for the northwestern blotting experiments were generated by in vitro transcription. To generate templates for the beta interferon (IFN-β) and simian virus 40 (SV40) RNA probes, pBSIFNPA and pBSSV40PA were linearized with *Eco*RI. To generate a template for the IFN-β antisense probe, pBSIFNPA was linearized with *Hin*dIII. One microgram of each DNA template was transcribed in vitro, using T3 RNA polymerase (for the IFN-β and SV40 probes; Promega) or T7 RNA polymerase (for the IFN-β antisense probe) in the presence of 50 μCi of [α-^{32}P]CTP (400 Ci/mmol; DuPont NEN). After transcription, the samples were phenol-chloroform extracted and ethanol precipitated twice, using 2.5 M ammonium acetate as the precipitating salt. The final RNA pellet was resuspended in RNase-free TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Approximately 8×10^5 cpm of each probe was analyzed by electrophoresis in a 5% denaturing acrylamide-urea gel.

The northwestern blotting assays were carried out as described by Brown et al. (3). Briefly, 10 or 40 μg of purified GST (expressed from *E. coli* cells harboring pGEX-5X-3) or GST fusion proteins was electrophoresed on SDS–15% acrylamide gels and electrophoretically transferred to nitrocellulose filters. The transferred proteins were renatured in situ for 12 h at 4°C in binding buffer (10 mM Tris-HCl [pH 7.6], 50 mM NaCl, 1 mM EDTA, 1× Denhardt's solution). The filters were incubated at room temperature for 8 h in 15 ml of binding buffer containing 1.7×10^7 cpm of ^{32}P-labeled RNA probe. The blots were individually washed three times in binding buffer for 10 min at room temperature and analyzed by autoradiography.

Cells, viruses, and infections. Vero (African green monkey kidney) cells were propagated in Dulbecco modified Eagle medium containing 5% heat-inactivated fetal bovine serum (GIBCO). HSV infections were carried out at a multiplicity of infection of 10 PFU per cell as described previously (34). Strain KOS1.1 (12) was the WT strain of HSV-1 used. HSV-2 (strain G) was obtained from the American Type Culture Collection (Rockville, Md.). The HSV-1 ICP27 mutants *d*27-1, *n*263R, *n*406R, *d*1-2, *d*3-4, and *d*4-5 have been described elsewhere (26, 31, 33). The HSV-1 mutant alleles *d*5-6 and *d*1-5 were constructed by using engineered *Xho*I sites in the HSV-1 ICP27 gene (32), and recombinant viruses bearing these alleles were isolated (16). *d*1-5 and *d*5-6 encode ICP27 molecules missing amino acid residues 13 to 153 and 154 to 173, respectively.

In vivo methylation assay. Metabolic labeling of methylated proteins was performed as described by Liu and Dreyfuss (19). At 5 h postinfection (hpi), medium was removed from the infected cells and replaced with medium containing 100 μg of cycloheximide per ml and 40 μg of chloramphenicol per ml. After 30 min, the cells were rinsed twice with methionine-free Dulbecco modified Eagle medium (GIBCO) and then incubated for 3 h in methionine-free Dulbecco modified Eagle medium containing 100 μg of cycloheximide per ml, 40 μg of chloramphenicol per ml, and 45 μCi of L-[*methyl*-^{3}H]methionine (DuPont NEN) per ml. In some experiments, 100 μM anisomycin was used in place of cycloheximide. After labeling, the cells were rinsed in PBS containing 50 μg of *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) per ml and 100 μg of phenylmethylsulfonyl fluoride per ml and lysed in modified radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl [pH 8.0], 50 μg of TLCK per ml, 100 μg of phenylmethylsulfonyl fluoride per ml) for 30 min on ice. The cell lysate was transferred to a microcentrifuge tube, and insoluble material was pelleted at $10{,}000 \times g$ for 10 min at 4°C. The supernatant was transferred to a new tube and analyzed immediately or stored at −70°C.

Immunoprecipitations. Immunoprecipitations were carried out with a mixture of H1113 and H1119, two mouse monoclonal antibodies specific for ICP27 (Goodwin Institute for Cancer Research). To carry out the immunoprecipitations, 0.8 μl of each antibody was added to 400 μl of cell lysate, and the samples were incubated at 4°C on a rocking platform. After 30 min, 80 μl of a 1:40 dilution of rabbit anti-mouse immunoglobulin G (heavy and light chains; Jackson ImmunoResearch Laboratories Inc.) was added as a bridging antibody. After a further 30-min incubation, 200 μl of prewashed *Staphylococcus aureus* cells (Pansorbin; Calbiochem), resuspended in modified RIPA buffer (10% [wt/vol]), was added, and the incubation was continued for another 30 min. The cells were pelleted and washed four times in modified RIPA buffer. Bound proteins were eluted by heating the cells for 10 min at 85°C in SDS-PAGE sample buffer. Immunoprecipitates were analyzed by electrophoresis on SDS–12.5% acrylamide gels. For fluorographic enhancement, gels were impregnated with 1.0 M sodium salicylate before drying. Immunoprecipitates were also analyzed by immunoblotting using a 1:1,000 dilution of the H1119 antibody. Immunoreactive proteins were detected by enhanced chemiluminescence (ECL detection kit; Amersham).

RESULTS

ICP27 binds to poly(G) RNA homopolymers. Recent studies by Brown et al. (3) and Ingram et al. (13) have demonstrated that ICP27 can bind to RNA molecules in vitro. However, it is not known what sequence or structural determinants in RNA are recognized by ICP27. To gain insight into ICP27's RNA-binding specificity, we tested its ability to interact with various RNA homopolymers in vitro (42). Such homopolymer binding assays have been used by many investigators to characterize the RNA-binding potential and target specificity of putative RNA-binding proteins (2, 8, 14, 28, 40). For the binding studies, full-length ICP27 was expressed by in vitro translation in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine-cysteine. The radiolabeled protein was incubated with agarose beads to which RNA homopolymers [poly(A), poly(C), poly(G), or poly(U)] were covalently attached. The binding reactions and subsequent washing steps were carried out at several NaCl concentrations ranging between 0.1 and 1.0 M. After washing, protein was eluted from the beads and analyzed by SDS-PAGE and phosphor image analysis. ICP27 bound to poly(G) ribopolymers, with significant binding of the input material occurring up to an NaCl concentration of 0.5 M (Fig. 1A and B). ICP27 also bound weakly to poly(U) homopolymeric RNA at 0.1 and 0.25 M NaCl. In contrast, ICP27 did not bind to poly(A) or poly(C). In control experiments, in vitro-

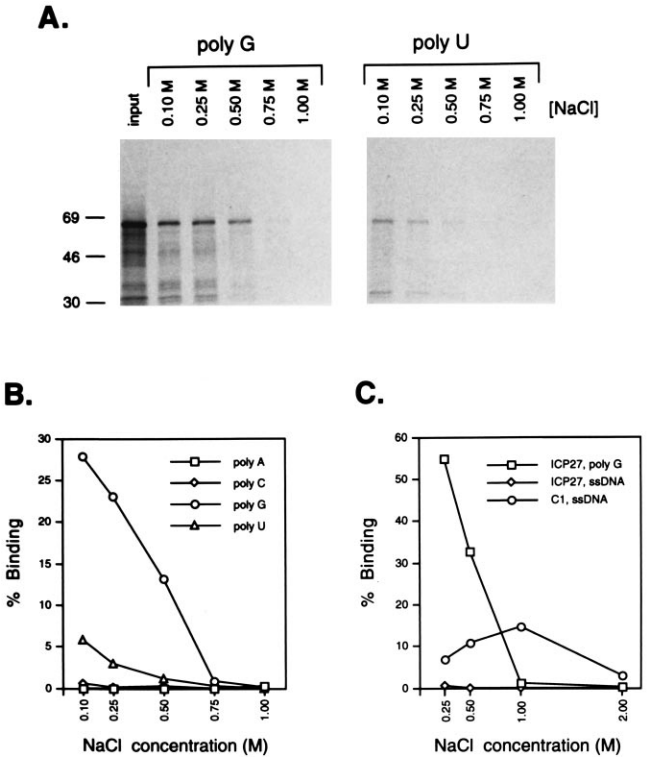

FIG. 1. Binding of in vitro-translated ICP27 to RNA homopolymers. (A and B) Binding of ICP27 to RNA homopolymers. ICP27, produced by in vitro translation in the presence of [^{35}S]methionine-cysteine, was incubated with RNA homopolymers [poly(A), poly(C), poly(G), or poly(U)] attached to agarose beads. Binding and washing were carried out at various NaCl concentrations ranging from 0.1 to 1.0 M. Bound protein was eluted and analyzed by SDS-PAGE, autoradiography, and phosphor image analysis. (A) Binding of ICP27 to poly(G) and poly(U) ribopolymers. The lane labeled "input" contains an aliquot of the translation mixture used in the binding reaction. The other lanes show protein bound at various salt concentrations. Equivalent fractions of the input and bound samples were loaded on the gel to facilitate the determination of percent binding. The positions of molecular weight markers are indicated at the left (molecular masses in kilodaltons). (B) Quantitation of ICP27 binding to RNA homopolymers at different NaCl concentrations. The percentage of input ICP27 bound at each NaCl concentration was determined by phosphor image analysis. (C) Comparison of ICP27's binding to poly(G) RNA and ssDNA. In vitro-translated ICP27 or hnRNP C1 were bound at various NaCl concentrations to agarose beads containing either poly(G) RNA or ssDNA. Analysis and quantitation of binding were carried out as for panels A and B.

translated hnRNP C1 bound avidly to poly(U) agarose beads up to an NaCl concentration of 1.0 M (data not shown), consistent with previous results (42). From the results of these experiments, we conclude that ICP27 binds with moderate avidity to poly(G) RNA homopolymers and weakly to poly(U) homopolymers.

Previously, Vaughn et al. demonstrated that ICP27 purified from infected cells can bind to a ssDNA-agarose column and be eluted at relatively high salt (45). We therefore examined ICP27's interaction with ssDNA in our binding assay. To do this, in vitro-translated ICP27 was incubated with ssDNA- or poly(G)-agarose beads at NaCl concentrations ranging from 0.25 to 2.0 M. As a control, in vitro-translated hnRNP C1, previously demonstrated to interact with ssDNA (30), was also tested for ssDNA binding. After binding and washing, protein was eluted from the beads and analyzed by SDS-PAGE and phosphor imaging (Fig. 1C). ICP27 bound to poly(G) as before but demonstrated little if any binding to ssDNA. In contrast, hnRNP C1 bound to the ssDNA up to an NaCl concentration of 1.0 M. We conclude that under the conditions of our binding assay, in vitro-translated ICP27 binds to poly(G) RNA but not to ssDNA.

Role of the RGG box in poly(G) binding. To identify the sequences in ICP27 which are responsible for poly(G) binding, several N- or C-terminally truncated forms of ICP27 were expressed by in vitro translation and tested in the binding assay at an NaCl concentration of 0.25 M NaCl (Fig. 2). A fragment corresponding to the C-terminal half of ICP27, 262C, did not bind efficiently to poly(G) (<10% of WT ICP27 binding). This result suggests that the N-terminal half of ICP27 is required for strong binding. Several C-terminally truncated forms of ICP27 (N487 to N137) were also studied. Significant poly(G) binding (>50% of WT binding) was observed for the mutants N487, N405, N339, N261, and N189. Interestingly, the N189 protein showed enhanced binding compared with the WT protein. The only mutant in this series which did not bind efficiently to poly(G) was N137, which was similar to 262C in its low binding (~10% of the WT level). Overall, these data demonstrate that the major determinant of poly(G) binding maps to the N-terminal 189 residues of ICP27. Additionally, the data suggest that sequences important for RNA binding are located between residues 137 and 189, since N189 but not N137 bound strongly to poly(G). This region of ICP27 contains the RGG box motif, which maps to residues 138 to 152.

To determine whether the RGG box or nearby sequences are required for ICP27's interaction with poly(G), three ICP27 deletion proteins (Fig. 3A) were expressed by in vitro translation and tested in the poly(G) binding assay. The *d*4-5 mutant lacks residues 138 to 152 and thus contains a precise deletion of the RGG box sequence. The *d*5-6 mutant lacks residues 153 to 174; this deletion removes a second arginine-rich sequence designated R2 by Hibbard and Sandri-Goldin (11). Mutant *d*4-6 lacks both the RGG box and R2 sequences. The poly(G)-binding activities of these mutants, as well as WT ICP27, were measured at 0.1, 0.25, and 0.5 M NaCl (Fig. 3B and C). Both the *d*4-5 and *d*4-6 proteins were defective in poly(G) binding, showing less than 5% of the WT level of binding at 0.25 and 0.5 M NaCl. These two mutants differed slightly at 0.1 M NaCl, in that *d*4-5 mutant bound weakly whereas *d*4-6 did not bind to an appreciable extent. In contrast to *d*4-5 and *d*4-6, the *d*5-6 mutant bound to poly(G) almost as efficiently as the WT protein, although its binding was reduced approximately twofold compared with that of the WT protein at 0.5 M NaCl. Together, these data indicate that the RGG box motif, but not the arginine-rich sequence immediately C-terminal to it (R2), is required for efficient poly(G) binding.

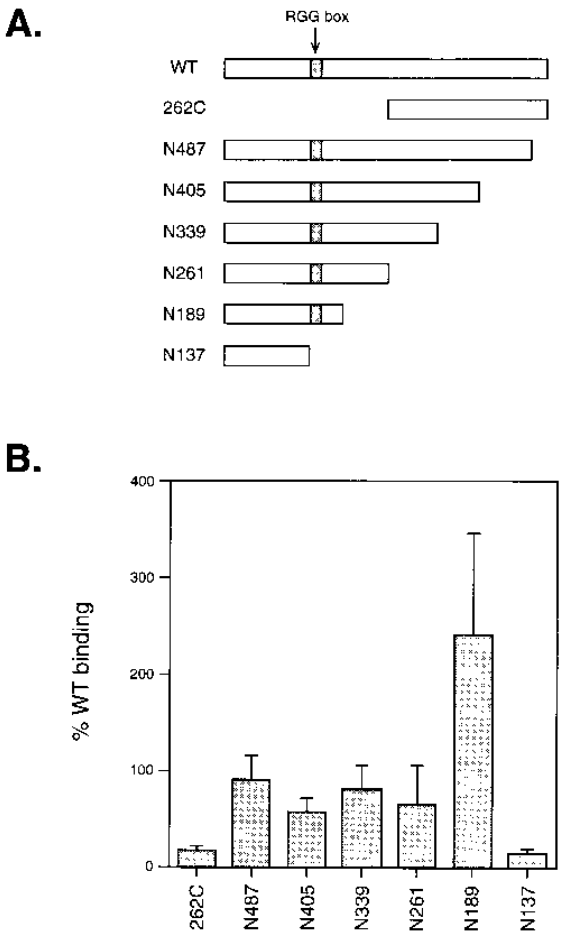

FIG. 2. Poly(G) binding by ICP27 truncation mutants. (A) Schematic diagram of ICP27 N- and C-terminal truncation mutants produced by in vitro translation. The RGG box sequence is indicated. (B) Binding of truncation mutants to poly(G). The truncation mutants were tested in the poly(G) binding assay at an NaCl concentration of 0.25 M. The values given are the percent binding relative to a WT ICP27 control. The values for each mutant are means of four to nine independent binding assays; the error bars indicate standard deviations. The mean value of WT ICP27 binding to poly(G) in these experiments was 37.5%.

Direct interaction of ICP27's RGG box with RNA. Brown et al. (3) recently used a northwestern blotting assay to demonstrate that ICP27 is an RNA-binding protein. These investigators showed that a bacterially expressed GST-ICP27 fusion protein can bind to >600-nucleotide-long RNA probes corresponding to sequences from the 3′ ends of the c-*myc* or IFN-β gene. The binding appeared to be target specific, as GST-ICP27 did not bind to a similar-size RNA corresponding to sequences from the 3′ end of the SV40 early region. To determine whether ICP27's RGG box plays a role in this RNA interaction, we tested several GST fusion proteins for the ability to bind to the β-RNA probe used by Brown et al. (3). The proteins tested were (i) GST; (ii) GST-ICP27, a fusion having all of ICP27 tagged to GST; (iii) GST–*d*4-5, a fusion identical to GST-ICP27 but lacking the RGG box; and (iv) GST-RGG, a fusion containing only the RGG box sequence (residues 140 to 152) joined to GST. The proteins were expressed in *E. coli*, purified by glutathione-Sepharose affinity chromatography, and analyzed by SDS-PAGE and Coomassie blue staining (Fig. 4A). Although the GST and GST-RGG preparations yielded major bands of the expected molecular sizes (lanes 2 and 5,

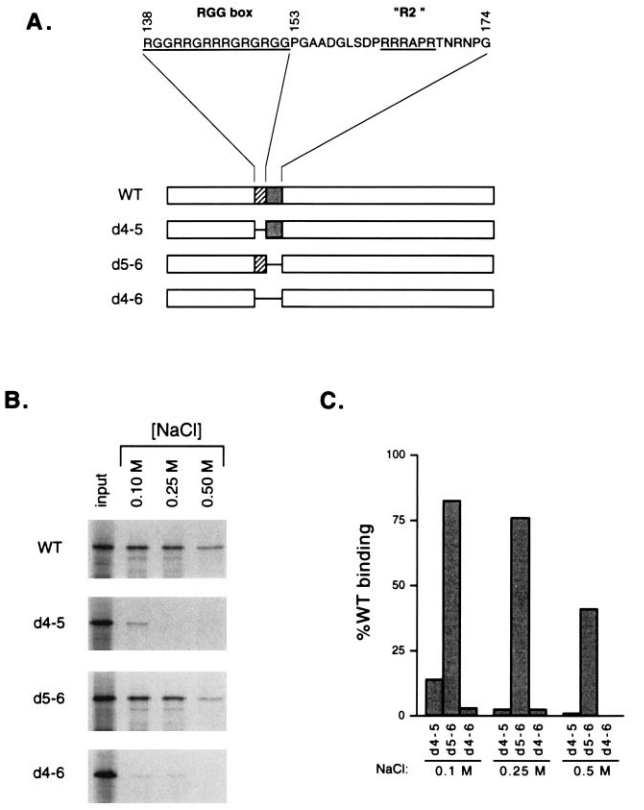

FIG. 3. Poly(G) binding by ICP27 in-frame deletion mutants. (A) Schematic diagram of ICP27 in-frame deletion mutants. The sequence of residues 138 to 174 of ICP27 is shown at the top. Below, the open bars represent ICP27, while the cross-hatched and gray bars represent the RGG box and R2 regions, respectively. The lines connecting bars indicate in-frame deletions. (B) Poly(G) binding by in-frame deletion mutants. WT ICP27 or in-frame deletion mutants were expressed by in vitro translation and tested in the poly(G) binding assay at the NaCl concentrations indicated. Equivalent fractions of the input and bound fractions were analyzed by SDS-PAGE and autoradiography. (C) Quantitation of poly(G) binding by ICP27 deletion mutants. The data from panel B were quantitated by phosphor image analysis. Poly(G) binding of each deletion mutant is presented as a percentage of WT ICP27 binding at the same NaCl concentration.

respectively), much of the protein in the GST-ICP27 and GST–*d*4-5 preparations (lanes 3 and 4) migrated as bands that were smaller than expected, possibly as a result of proteolysis during expression and/or purification. However, there was some apparently full-length GST-ICP27 and GST–*d*4-5 in each preparation, migrating at the expected molecular masses of ~95 and ~93 kDa, respectively. Western blot analysis of the GST-ICP27 and GST–*d*4-5 preparations demonstrated that the putative full-length molecules as well as most of the smaller species reacted with both an anti-GST monoclonal antibody and H1113, a monoclonal antibody directed against an N-terminal epitope in ICP27 (data not shown).

For the northwestern blotting assay, GST or the GST fusion proteins were subjected to SDS-PAGE, electrophoretically transferred to nitrocellulose filters, and renatured in situ. Identical filters were incubated with three different ^{32}P-labeled RNA probes. One probe was the ~650-nucleotide IFN-β gene probe previously shown to form a complex with GST-ICP27 (Fig. 4B, lane 2). As controls for specificity, two other probes were used: an antisense version of the IFN-β probe (lane 1) and an ~840-nucleotide RNA corresponding to the 3′ end of the SV40 early region (lane 3), previously shown not to form a complex with GST-ICP27 (3). After binding, the northwestern filters were washed and analyzed by autoradiography. The results (Fig. 4C) indicate that the RGG box is both required and sufficient for interaction with RNA, as both GST-ICP27 and GST-RGG, but not GST or GST–*d*4-5, efficiently bound to RNA molecules. However, we did not observe the specificity reported by Brown et al. (3), as all three probes bound comparably to GST-ICP27 and GST-RGG. Although the IFN-β probe appeared to bind slightly more efficiently to GST-ICP27 in this experiment than did the SV40 probe, this was not observed in a repeat experiment (data not shown).

Two major bands in the GST-ICP27 preparation formed complexes with RNA (Fig. 4C, lane 2). These corresponded not to full-length GST-ICP27 but to the two truncated forms of GST-ICP27 indicated by the squares in Fig. 4A. However, a

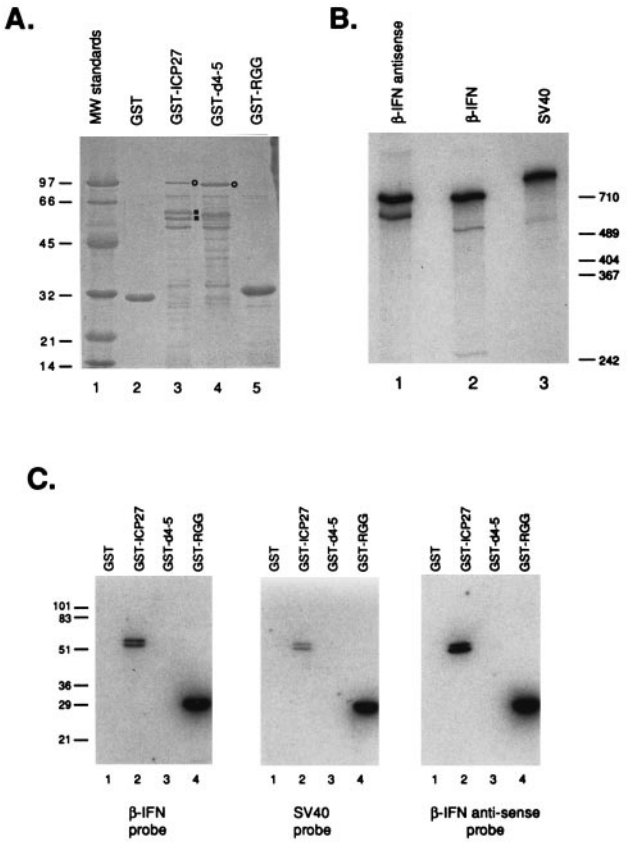

FIG. 4. RNA binding by GST-ICP27 fusion proteins. (A) Purification of GST fusion proteins. GST or GST-ICP27 fusion proteins were expressed in *E. coli* and purified by affinity chromatography on glutathione-Sepharose. Ten micrograms of each preparation was analyzed by SDS-PAGE and Coomassie blue staining. The molecular masses of the molecular weight (MW) marker proteins are indicated at the left in kilodaltons. The circles to the right of lanes 3 and 4 indicate the positions of full-length GST-ICP27 and GST–*d*4-5. The squares to the right of lane 3 indicate truncated GST-ICP27 products which interact strongly with RNA. (B) ^{32}P-labeled RNA probes. RNA probes for the northwestern assay were generated by in vitro transcription in the presence of [α-^{32}P]CTP. An aliquot of each probe was analyzed by 5% urea-acrylamide gel electrophoresis and autoradiography. Lane 1, antisense version of the IFN-β gene 3′-end probe; lane 2, IFN-β gene 3′-end probe; lane 3, SV40 early region 3′-end probe. The positions and nucleotide sizes of markers are indicated at the right. The expected size of the IFN-β probes is 650 nucleotides; that of the SV40 probe is 840 nucleotides (3). (C) Binding of GST-ICP27 and GST-RGG to RNA. Ten-microgram aliquots of GST fusion preparations were electrophoresed on an SDS–15% polyacrylamide gel, blotted to nitrocellulose, and incubated with ^{32}P-labeled RNA probes as described by Brown et al. (3). An autoradiograph of the washed blot is shown. The molecular masses of protein standards are indicated at the left in kilodaltons.

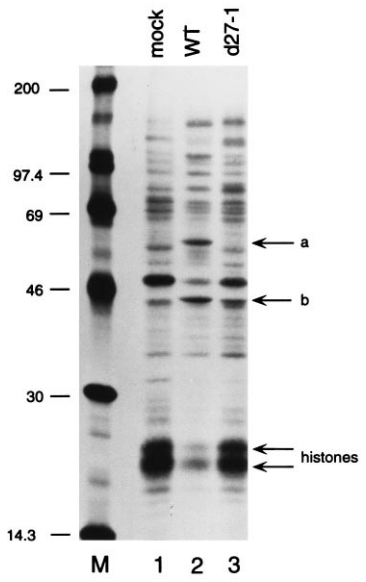

FIG. 5. Protein methylation in HSV-1-infected Vero cells. Mock- or HSV-1-infected Vero cells were treated with translation inhibitors (100 μg of cycloheximide per ml and 40 μg of chloramphenicol per ml) at 5 hpi. Thirty minutes later, the cells were labeled for 3 h with 45 μCi of L-[*methyl*-^{3}H]methionine per ml in the presence of the same translation inhibitors. The cells were scraped and lysed in RIPA buffer, and the lysates were electrophoresed on an SDS–15% polyacrylamide gel. A fluorograph of the gel is shown. Lane M, protein standards; lane 1, mock-infected cells; lane 2, WT HSV-1-infected cells; lane 3, *d*27-1-infected cells. At the left, the molecular masses of the protein standards are indicated in kilodaltons. At the right, bands corresponding to the two major methylated proteins in WT HSV-infected cells (a and b) and the positions of cellular histones are indicated.

repeat experiment in which 40 μg of protein, rather than 10 μg, was analyzed on the northwestern blot demonstrated that the full-length GST-ICP27, but not full-length GST–*d*4-5, could also bind to the IFN-β and SV40 RNA probes (data not shown).

Two important conclusions can be drawn from these experiments regarding the role of ICP27's RGG box in RNA binding. First, the RGG box is required, in the context of both intact and truncated GST fusion proteins, for interaction with RNA molecules. Second, the RGG box sequence alone can mediate RNA binding, since GST-RGG efficiently binds to RNA.

In vivo methylation of ICP27. The experiments described above suggest that ICP27 is an RGG box-type RNA-binding protein, since its RGG box sequence can mediate RNA binding. A characteristic of many cellular RGG box proteins is that they are targets for posttranslational modification by protein arginine methyltransferases, which dimethylate arginine residues to produce N^G-N^G-dimethylarginine (19, 27). The function of this unusual modification is not known. To determine whether ICP27 is methylated in vivo, we carried out a metabolic labeling experiment to specifically identify methylated proteins (19). The assay is based on the fact that the methyl donor for protein methylation, *S*-adenosylmethionine, acquires its methyl group from free methionine in the cell. Thus, if translation is blocked, methylated proteins can be specifically labeled by incubating cells in L-[*methyl*-^{3}H]methionine. To carry out the methylation assay, Vero cells were mock infected, infected with WT HSV-1, or infected with *d*27-1, an ICP27 deletion mutant (31). At 5 hpi, the cells were treated with cycloheximide and chloramphenicol to inhibit protein synthesis. Thirty minutes later, the cells were labeled with L-[*methyl*-^{3}H]methionine for 3 h in the presence of the translation inhibitors. The labeled proteins were examined by SDS-PAGE and fluorography (Fig. 5). Three lines of evidence indicate that the majority of labeling is due to in vivo methylation, not incorporation of methionine via protein synthesis: (i) the pattern of labeled proteins is dramatically different from that obtained when cells are labeled with [^{35}S]methionine in the absence of translation inhibitors (31, 33); (ii) control experiments demonstrated that the cycloheximide-choramphenicol treatment inhibited overall translation levels approximately 20-fold (not shown); and (iii) treatment with a more stringent inhibitor of protein synthesis, anisomycin, led to a nearly identical pattern of labeled proteins (not shown).

The pattern of protein methylation in WT HSV-1-infected cells was quite distinct from that of the mock-infected cells (Fig. 5, lanes 1 and 2). First, the labeling of several cellular proteins was markedly reduced after HSV-1 infection. In particular, the labeling of histones was significantly inhibited. Moreover, this effect requires ICP27, as histone methylation was not inhibited in the *d*27-1 infection (lane 3). Second, several methylated proteins were either induced or enhanced in WT-infected cells. We noted two prominent bands, labeled a and b in Fig. 5. These proteins may correspond either to viral proteins which are methylated or to cellular proteins whose methylation is induced upon infection.

Although the identity of band b was not investigated further, we suspected that band a might correspond to ICP27, since it has approximately the same apparent molecular mass (~63 kDa) and is absent from the ICP27 null mutant infection (Fig. 5; compare lanes 2 and 3). To determine whether band a corresponds to ICP27, a second experiment was performed. Vero cells were mock infected or infected with WT HSV-1, WT HSV-2, *d*27-1, or *d*4-5 (26). *d*4-5 is a defective HSV-1 ICP27 mutant which contains the same RGG box deletion shown in Fig. 3A. The infected cells were labeled with L-[*methyl*-^{3}H]methionine as before, and the cell lysates were subjected to immunoprecipitation using a mixture of two anti-ICP27 monoclonal antibodies. Analysis of the fluorograph (Fig. 6A) indicated that both HSV-1 and HSV-2 ICP27 are methylated proteins, since they efficiently label with L-[*methyl*-^{3}H]methionine. Furthermore, comparison of the immunoprecipitated

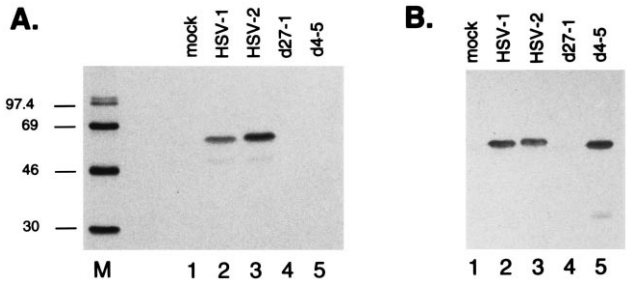

FIG. 6. In vivo methylation of ICP27. Vero cells were mock infected (lane 1) or infected with WT HSV-1, WT HSV-2, *d*27-1, or *d*4-5 (lanes 2 to 5, respectively). The cells were labeled from 5.5 to 8.5 hpi with L-[*methyl*-^{3}H]methionine as described in the text. Cell lysates were subjected to immunoprecipitation using a mixture of two monoclonal antibodies specific for ICP27 (H1113 and H1119). (A) Labeling of ICP27 with L-[*methyl*-^{3}H]methionine. Aliquots of the immunoprecipitates were subjected to SDS-PAGE (15% gel) and analyzed by fluorography. A 40-h exposure is shown. Molecular weight markers are shown in lane M (sizes in kilodaltons). (B) Presence of ICP27 in the immunoprecipitates. Aliquots of the immunoprecipitates were subjected to SDS-PAGE (15% gel), and the proteins were electrophoretically transferred to nitrocellulose. The filter was subjected to Western blot analysis using H1119 antibody. Antigen detection was performed with an enhanced chemiluminescence detection system; a 1-s exposure is shown.

HSV-1 ICP27 with the total labeled lysate (not shown) demonstrated that ICP27 corresponds to band a seen in Fig. 5.

Interestingly, no labeled ICP27 was present in the *d*4-5 immunoprecipitate (Fig. 6A, lane 5), suggesting that the RGG box is required for in vivo methylation of ICP27. To demonstrate that the *d*4-5 protein was present in the immunoprecipitate, we subjected the immunoprecipitates to Western blot analysis (Fig. 6B). The *d*4-5 protein was indeed present, in amounts comparable to those of the WT protein (compare lanes 2 and 5). Therefore, the RGG box is required for ICP27's in vivo methylation. Moreover, this experiment confirms the validity of the methylation assay, as the WT and *d*4-5 ICP27 proteins contain equal numbers of methionine residues yet label distinctly with L-[*methyl*-^{3}H]methionine.

Requirement for the RGG box in in vivo methylation. The foregoing experiment demonstrates that the RGG box sequence is required for ICP27's methylation but does not exclude the possibility that other regions also are involved. To systematically determine which portions of ICP27 are required for in vivo methylation, we used several HSV-1 ICP27 mutants which contain nonsense or in-frame deletion mutations in the ICP27 gene (Fig. 7A). Infected Vero cells were labeled with L-[*methyl*-^{3}H]methionine, and ICP27 proteins were subjected to immunoprecipitation. To confirm that the ICP27 mutant proteins were expressed and immunoprecipitated, the samples were first subjected to Western blot analysis (Fig. 7B). With one exception, all of the mutant proteins were present in quantities similar to that of WT ICP27. The exception was the *n*406 protein, which was present in somewhat lesser amounts. To determine whether the proteins were methylated, the immunoprecipitates were analyzed by SDS-PAGE and fluorography (Fig. 7C). Although the *n*406 protein was present in lower amounts, it was clearly methylated (lane 9), as was the *n*263R protein (lane 8). This result indicates that methylation occurs predominantly or exclusively on the N-terminal half of ICP27. Examination of several N-terminal in-frame deletion mutant proteins gave further information about the sequences in ICP27 required for methylation. The *d*1-2 and *d*5-6 proteins were efficiently methylated (lanes 3 and 6, respectively), indicating that residues 12 to 63 and 154 to 173 are not required. The *d*3-4 protein (lane 4) was weakly labeled, indicating that the engineered deletion in *d*3-4 lowers the efficiency of, but does not completely prevent, ICP27 methylation. The only two mutant proteins which did not appear to be methylated to any appreciable extent were those encoded by *d*4-5 and *d*1-5, both of which lack the RGG box motif. These data demonstrate that the RGG box is a critically important determinant of ICP27's in vivo methylation.

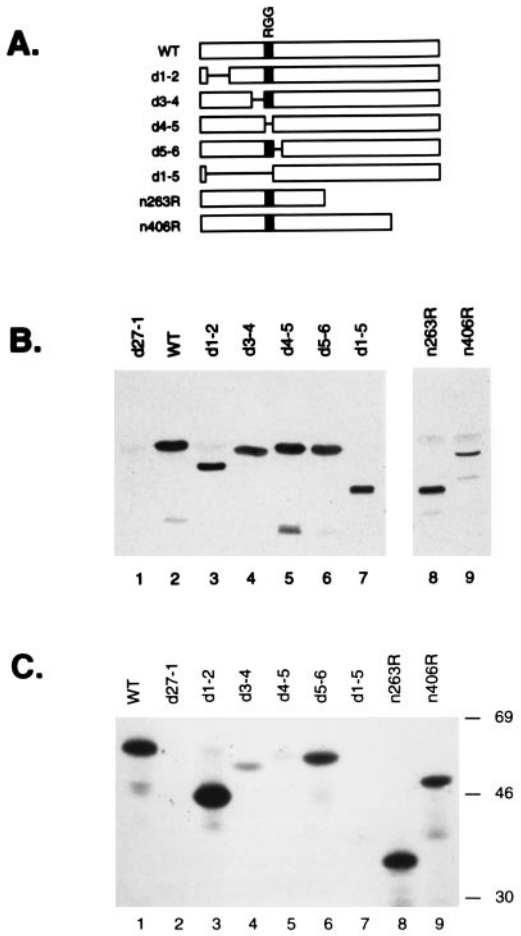

FIG. 7. The RGG box is required for ICP27 methylation. (A) ICP27 proteins expressed by HSV-1 ICP27 mutants. Bars represent ICP27 coding segments; lines connecting bars indicate in-frame deletions. The RGG box is indicated by a stippled box. (B and C) In vivo methylation assay. Cells were infected with WT HSV-1, *d*27-1, or the ICP27 mutants shown in panel A. Cells were labeled with L-[*methyl*-^{3}H]methionine, and anti-ICP27 immunoprecipitations were performed as described in the text. (B) Western blot detection of ICP27 molecules in immunoprecipitates. Aliquots of the immunoprecipitates were subjected to Western analysis using ICP27-specific monoclonal antibodies. Antigen detection was by enhanced chemiluminescence; a 5-s exposure is shown. (C) Labeling of ICP27 with L-[*methyl*-^{3}H]methionine. Aliquots of the immunoprecipitates were subjected to SDS-PAGE and fluorography. A 6-day exposure is shown. The positions of molecular weight standards are shown at the right in kilodaltons.

DISCUSSION

ICP27's RGG box and RNA binding. In this work, we show that ICP27 binds to RNA homopolymers composed of poly(G). This finding is consistent with two recent studies which have also demonstrated that ICP27 can interact with RNA in vitro (3, 13). We used both the poly(G) binding assay and the northwestern blotting assay described by Brown et al. (3) to identify the sequences in ICP27 which mediate RNA binding. Both sets of experiments implicate ICP27's RGG box motif, which maps to residues 138 to 152. In the poly(G) binding assay, there is a strict correlation between the presence of the RGG box and RNA binding. In addition, the RGG box is required in the context of an otherwise intact ICP27 molecule for association with poly(G). In the northwestern assay, the RGG box is required for RNA interaction and alone can mediate RNA binding when attached to a heterologous protein. Together, our data demonstrate that ICP27's RGG box is an RNA-binding domain that can mediate interaction with both poly(G) RNA homopolymers and more complex RNA sequences.

Our results differ from those of Brown et al. (3) in that we do not observe the same RNA target specificity in the northwestern blotting assay. Brown et al. found that GST-ICP27 could associate with RNAs derived from the 3′ ends of the IFN-β and c-*myc* genes but not with an RNA derived from the 3′ end of the SV40 early region. In contrast, we find that GST-ICP27 fusion binds efficiently to both the IFN-β and SV40 probes as well as to an antisense version of the IFN-β probe. We note that Ingram et al. also failed to find evidence for sequence-specific RNA-binding by ICP27 (13). A complicating factor in our experiments is that the major RNA-binding polypeptides in our GST-ICP27 preparation are approximately 25 to 30 kDa smaller than the full-length protein. However, truncation of

our GST-ICP27 is unlikely to explain the conflicting results, as the GST-ICP27 fusion protein of Brown et al. (3) is also ~30 kDa smaller than expected and thus likely corresponds to a similar proteolytic fragment. The discrepancies in binding results thus leave open the question of ICP27's RNA target specificity. However, the fact that ICP27 binds efficiently to poly(G) RNA raises the possibility that G-rich sequences play a role in the recognition of natural RNA targets by ICP27.

The RGG box motif, found in a number of cellular RNA-binding proteins, is one of several known RNA-binding domains (4, 14, 20). It is defined as an arginine- and glycine-rich sequence that usually contains closely spaced RGG repeats interspersed with other, often aromatic amino acids (4). The minimal size of a functional RGG box RNA-binding sequence is unknown, but most RGG boxes are larger than the 15 residues which comprise ICP27's RGG box. In this work, we have shown that 13 residues of ICP27's RGG box, GRRGRRRG RGRGG, are sufficient to mediate RNA binding when attached to a heterologous protein, GST. To our knowledge, this is the smallest RGG-like sequence which has been shown to bind to RNA. The GST-RGG molecule may therefore be a useful tool for defining the specific amino acid residues which interact with RNA.

Two observations suggest that the C terminus of ICP27 may inhibit RNA binding by the RGG box. First, the C-terminally truncated N189 polypeptide displays significantly enhanced poly(G) binding compared with the WT protein. Second, C-terminally truncated forms of GST-ICP27 appear to be more efficient RNA-binding proteins than full-length GST-ICP27, although the full-length molecule binds RNA to some extent. These observations raise the possibility that the C-terminal region of ICP27 negatively regulates the RNA-binding activity of the RGG box. However, it is also possible that enhanced RNA binding by truncated ICP27 molecules represents an artifactual effect of mutagenesis.

ICP27's RGG box and protein methylation. Several cellular proteins involved in nuclear RNA metabolism, including fibrillarin (18), nucleolin (17), and hnRNP A1 (47), are methylated on arginine residues. The predominant modified residue in these proteins is the asymmetrically dimethylated derivative N^G-N^G-dimethylarginine (DMA). The function of this protein modification is unknown. Recently, Liu and Dreyfuss found that in addition to hnRNP A1, many other hnRNP proteins are methylated in vivo (19). In addition, these investigators partially purified a protein arginine *N*-methyltransferase from HeLa cells, using unmodified recombinant hnRNP A1 as a substrate. The enzyme activity appeared to be specific for RGG box sequences and modified arginine residues to DMA and N^G-monomethylarginine. Moreover, in vitro, the enzymatic activity methylated the same spectrum of hnRNP proteins that are methylated in vivo. These results suggest that many hnRNP proteins, and possibly other RGG box proteins, are methylated on their RGG box motifs by a common cellular enzyme.

On the basis of the foregoing information, we investigated the methylation status of ICP27 in HSV-1-infected cells. Our results demonstrate that ICP27 is one of the major methylated proteins in infected cells, at least under the conditions of our labeling assay. Furthermore, ICP27's methylation correlates with and requires the presence of the RGG box sequence. Residues 109 to 137, just N terminal to the RGG box, may also play a role in methylation, as the *d*3-4 protein was only weakly labeled in the in vivo methylation assay. However, it is worth noting that the *d*3-4 mutation, in addition to deleting codons 109 to 137, also alters the first two codons of the RGG box, changing them from RG to LE. This modification to the RGG box could also explain the reduced methylation of the *d*3-4 protein. Overall, our results indicate that the RGG box is critical for methylation and suggest that it is the RGG box motif itself which is the target of methylation. If so, then the modified residue is arginine, as this is the only residue in the RGG box sequence known to accept methylation (6). However, further experiments will be required to directly map the site(s) of methylation and to identify the modified residue(s).

What role, if any, could RGG box methylation play in the biological function of ICP27? An intriguing possibility is that methylation regulates ICP27's association with RNA. Methylation of arginine's guanidinium group would not alter the strong positive charge of the arginine side chain, but the bulky methyl groups could sterically affect RNA binding. In addition, arginine methylation might alter hydrogen bonding interactions between arginine residues and RNA. It is thus conceivable that arginine methylation could alter the overall level, or target specificity, of ICP27's binding to RNA. It is noteworthy that the enzymatic activity thought to be responsible for RGG box methylation is not found in *E. coli* (19). Since bacterially expressed GST-ICP27 and GST-RGG bind RNA, methylation is probably not required for RNA binding. It is also of interest that the RGG box methyltransferase is present in a rabbit reticulocyte lysate (19). Thus, it is possible that the ICP27 used in our poly(G) binding assays is methylated to some extent. Since there are specific inhibitors available for protein methyltransferases, it may be possible to produce methylated and unmethylated ICP27 by in vitro translation for side-by-side comparison in RNA-binding experiments.

Possible in vivo RNA binding by ICP27's RGG box. We have previously shown that the RGG box is important for ICP27's biological functions, as an HSV-1 mutant which encodes an RGG box-minus ICP27 fails to replicate in Vero cells (26). The studies described herein demonstrate that ICP27's RGG box motif can function in vitro as an RNA-binding domain. It is tempting to speculate that ICP27's RGG box also mediates RNA binding activity in vivo. Previously, we suggested that the nucleolar localization of ICP27, which depends on the RGG box, reflects an in vivo association with abundant RNAs in the nucleolus (26). However, the physiologically relevant RNA target, or targets, of ICP27 are not necessarily nucleolar. Given ICP27's effects on pre-mRNA splicing, possible targets might be one or more of the small nuclear RNAs (snRNAs) associated with spliceosomal snRNPs. In this regard, it is interesting that at least a small fraction of ICP27 interacts with U snRNPs during infection (37). Another possibility is that ICP27 functions as an hnRNP protein, binding to nascent pre-mRNA in the nucleus (7). Indeed, our work shows that ICP27 shares characteristics with several cellular hnRNP proteins, in that it contains an RGG box RNA-binding domain and is methylated. In this scenario, ICP27's association with pre-mRNAs, perhaps in combination with cellular hnRNP proteins, could lead to modified hnRNP complexes on viral or cellular transcripts. Such novel complexes might determine alternate pathways of mRNA polyadenylation, splicing, or export from the nucleus. This model could explain how a single viral protein can affect multiple and seemingly diverse aspects of pre-mRNA metabolism.

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