Identification of Herpes Simplex Virus RNAs That Interact Specifically with Regulatory Protein ICP27 in Vivo*

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Marcus Sokolowski‡§, James E. Scott‡, Robert P. Heaney‡, Arvind H. Patel¶, and J. Barklie Clements‡‡

From the ‡Division of Virology, Institute of Biomedical Life Sciences and ¶Medical Research Council Virology Unit, Institute of Virology, Church Street, University of Glasgow, Glasgow G11 5JR, Scotland, United Kingdom

Herpes simplex virus type 1 (HSV-1) protein ICP27 has an essential regulatory role during viral replication, in part by post-transcriptional control of gene expression, and has a counterpart in all herpes viruses sequenced so far. Although much is known about the functions of this signature herpesvirus protein, little is known about its RNA binding capabilities; ICP27 interacts with specificity for a subset of intronless HSV-1 RNAs and poly(G), through its RGG box. We performed an in vivo yeast three-hybrid screen of an HSV-1 genomic library, searching for ICP27 interacting RNAs. Comparable with a yeast genomic screen, 24 of 55 single inserts mapped to antisense strands of HSV-1 transcribed regions or non-transcribed regions. The 31 HSV-1 sense RNAs identified were 35 to 225 nucleotides in length and interacted with preferred specificity for ICP27 as compared with an unrelated RNA-binding protein. They map to 10 monocistronic and 10 polycistronic transcripts of all kinetic classes and represent 28 open reading frames encoding predominantly essential viral proteins with roles in viral DNA replication and virion maturation. Several studies show regulatory effects by ICP27 on the majority of these transcripts, consistent with its regulation of the early-late switch in the HSV-1 life cycle. Deletion of the ICP27 RGG box and the ICP27 M15 mutation, both lethal in virus, abolished or severely reduced the ICP27-RNA interactions, indicating their biological relevance. The study facilitates continued study of gene regulation by ICP27 by further defining its interactions with viral RNAs.

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‡Present address: Capio Diagnostics, Malarsjukhuset, SE-631 88 Eskilstuna, Sweden.

§Recipient of a postdoctoral scholarship from the Swedish Foundation for International Co-operation in Research and Higher Education. To whom correspondence should be addressed: Inst. of Virology, University of Glasgow, Church St., Glasgow G11 5JR, Scotland, UK. Tel.: 44-141-330-4037; Fax: 44-141-337-2236; E-mail: b.clements@vir.gla.ac.uk.

¶Recipient of a postdoctoral scholarship from the Swedish Foundation for International Co-operation in Research and Higher Education. To whom correspondence should be addressed: Inst. of Virology, University of Glasgow, Church St., Glasgow G11 5JR, Scotland, UK. Tel.: 44-141-330-4037; Fax: 44-141-337-2236; E-mail: b.clements@vir.gla.ac.uk.

†The abbreviations used are: HSV-1, herpes simplex virus type 1; aa, amino acids; β-gal, β-galactosidase; RGG, arginine- and glycine-rich; KH, K homology; S.D., synthetic dropout; AD, activation domain; IRE, iron responsive element; IRP1, iron regulatory protein 1; ORF, open reading frame; IE, immediate-early; wt, wild type.

In a lytic infection with herpes simplex virus type 1 (HSV-1),1 the viral genes are expressed in a cascade-like fashion and are subject to regulation (1, 2). The ~80 genes have been grouped into one of three kinetic classes based on their tempo-
A specific ICP27-interacting RNA binding site has not been identified to date, and it is unknown whether other HSV-1 mRNAs may interact with ICP27. In the present study, we address these points by using a yeast three-hybrid system (reviewed in Ref. 43). This approach is the first to identify in vivo ICP27 interactions against a library of HSV-1 RNAs of various lengths that represent the entire viral genome.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The following plasmids have been described elsewhere: pACTII-CAN, pIIIA/MS2-2 (44), pM15 (38), pMD4-5 (35) (kind gifts from S. Rice), pH1/MS2-IRE, pADIRP1 (45), and pG130 (46). pAD27 was generated from a 25-base PCR amplification of a 10-amino acid N-terminal truncated form of the ICP27 open reading frame (ORF) from plasmid pG130 using oligonucleotides ICP27a/S (5'-GGCAGCTTCATCGCGGCTGACCG3') and ICP27a/A (5'-CGAGATCTCTTAAAACGGGGGTTGCAATAAAAATAT3'), which introduced BglII restriction enzyme recognition sites (underlined) at both ends of the ORF. The PCR product was digested with BglII and ligated to pACTII-CAN digested with BglII and treated with calf intestinal alkaline phosphatase, resulting in pAD27. pAD27 encodes a fusion protein containing the GAL4 activation domain fused to ICP27 aa 10 to 512, followed by the GAL4 activation domain fused to ICP27 aa 10 to 512 containing the M15 mutation (38) was PCR-amplified from plasmid pM15 and cloned into pACTII-CAN as outlined above. To generate pADARGG, pADARGG/KH2/3, and pADARGG/KH1/2/3, various 10-aa N-terminal truncated ICP27 fragments, also having the RGG box (aa 138–152) internally deleted, were PCR amplified from plasmid pMD4-5 (35) using ICP27a9/S (3'-ATTAACCAAGGGGTTGCAATAAAAATAT3') and ICP27a/KH2/3 (5'-GGAGATCTCTTAGTGACATCTTGCCGACCG3') or ICP27a/S (3'-ATTAACCAAGGGGTTGCAATAAAAATAT3') and ICP27a9/KH1/2/3 (5'-GGAGATCTCTTGCCGACCG3') (45), respectively. The PCR products were digested with BglII and ligated to pACTII-CAN digested with BglII, and treated with calf intestinal alkaline phosphatase, resulting in pADARGG, pADARGG/KH2/3, and pADARGG/KH1/2/3. Plasmid structures were confirmed by digestion with restriction enzymes and DNA sequencing.

**Construction of HSV-1 Genomic DNA Plasmid Library**—The source of HSV-1 strain T757 genomic DNA was a set of five previously described cosmids (47), whose inserts overlap and represent the entire viral genome (cosmids 6, 14, 28, 48, and 56; a kind gift from Dr. A. J. Davison). Cosmids were digested with various combinations of 11 different restriction enzymes, partially or to completion, and the ends were treated with calf intestinal alkaline phosphatase, resulting in pAD27. PCR products were digested with BglII and ligated to pACTII-CAN digested with BglII, and treated with calf intestinal alkaline phosphatase, resulting in pADARGG, pADARGG/KH2/3, and pADARGG/KH1/2/3. Plasmid structures were confirmed by digestion with restriction enzymes and DNA sequencing.

**RESULTS**

**Screening for HSV-1 RNAs Interacting with ICP27 Protein**—ICP27 protein interacts with various RNAs in vitro (23–25) and with several HSV-1 RNAs during lytic infection (7). A yeast three-hybrid system was utilized to examine interactions of ICP27 protein with HSV-1 RNAs; modified from its original presentation (45), this system has enabled the isolation of unknown RNAs from a genomic background that interact with a known RNA-binding protein (reviewed in Ref. 43).

This yeast three-hybrid system detects physical RNA-protein interactions upon the in vivo assembly of two protein hybrids and a fusion RNA, forming a complex capable of transactivating promoters of the lacZ and HIS3 reporter genes. The first protein hybrid component, a LexA-MS2 coat protein fusion, was stably expressed in yeast strain L40 (45). An HSV-1 genomic plasmid DNA library was constructed to provide the fusion RNA component. Gel-purified HSV-1 DNA fragments were ligated upstream of the bacteriophage MS2 coat protein binding sites, in the RNA pol III promoter-driven RNA expression plasmid pIIIA/MS2-2. A total of \( \times 10^4 E. coli \) transformants was generated, and sequence analysis of more than twenty clones revealed that inserts varied in length from 20 to 200 nucleotides and corresponded to different locations on the HSV-1 genome. This confirmed the construction of a small-insert library, similar to that used to screen the *Saccharomyces cerevisiae* genome (48), and having a >15-fold theoretical coverage of the entire HSV-1 genome. To generate the second protein hybrid component, the ICP27 ORF was inserted in-frame with the GAL4 activation domain (AD) in plasmid pACTII-CAN, generating plasmid pAD27 (resembling plasmid p502CAD used in yeast two-hybrid screens; see Refs. 28 and 30); because inclusion of ICP27 aa 1 to 9 fused to the GAL4 AD transactivates certain yeast promoters unspecifically in a yeast two-hybrid system (28, 30), an N-terminal truncated ICP27 ORF (aa 10 to 512) was used.

The HSV-1 plasmid library was transformed into yeast strain L40, which had been pre-transformed with plasmid activity of resulting diploids using a liquid assay and confirmed by analysis of β-gal activity in the presence of ICP27 mutants.

**Assays of β-Gal Activity**—Using the X-gal filter assay, lawns of yeast transformants were grown on appropriate S.D. plates. Yeast transformants were lifted onto Whatman (grade 5) filter paper, freeze-thawed once in liquid nitrogen, and placed onto Whatman 3MM paper pre-soaked in Buffer Z (60 mM NaHPO₄, 40 mM Na₂HPO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol, pH 7.0) containing 300 μg/ml X-gal. Filters were examined for development of blue color after incubation for 3 h at 30 °C.

To assay β-gal activity quantitatively, the liquid assay was used with chlorophenol red β-galactopyranoside as substrate (49, 50). Briefly, cultures were grown in appropriate S.D. medium overnight, followed by reincubation of fresh cultures and regrowth to \( A_{600} \). Cells were pelleted, washed in appropriate enzyme buffer once, and freeze-thawed three times in between liquid nitrogen and 37 °C. Enzyme buffer containing CPRG substrate was added, and following color development, the reactions were stopped to yield optical density values in the linear range of the assay. Measurements below one unit (in reactions stopped after 2 h of color development) were not in the linear range of the assay and were referred to as <1 unit.

**Yeast Cell Extracts and Western Blotting**—Cultures of R40 cells were grown in appropriate S.D. medium overnight, followed by reincubation of fresh cultures and regrowth to \( A_{600} \). Cells (100 ml) were added to an equal volume of ice and then pelleted, washed, and suspended in 400 μl 10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 10 mM KF, 0.2% Triton X-100, 2% glycerol, 1 mM phenylmethylsulfonyl fluoride, Sigma protease inhibitors (1:1000), pH 7.5). Glass beads (20 μl, 425–600 μm; Sigma) were added, and samples were vortexed four times each for 30 s followed by 30 s on ice. Debris was removed by full speed centrifugation in a microfuge, and 30-μl samples of lysates were separated by SDS-PAGE followed by Western blotting using a mixture of mouse monoclonal anti-ICP27 antisera H1113 and H1119 (Goodwin Institute for Cancer Research) as described previously (28, 30).
pAD27. Growth of transformants was performed on solid S.D. medium lacking leucine and histidine and containing 3-aminotriazole, selecting for plasmid pAD27 and activation of the HIS3 reporter gene, respectively. The transformation yielded >5 × 10^5 yeast transformants, of which >10^3 white colonies grew on the S.D. medium 6 days post-transformation (Fig. 1).

By analysis of activation of the lacZ reporter gene of colonies picked at random, using a filter lift assay, we isolated 334 transformants that had β-gal activity (Fig. 1).

Having isolated 334 colonies that activated expression of both reporter genes (LacZ/His^+), we investigated whether activation of the lacZ reporter gene was dependent on the

Fig. 1. Yeast three-hybrid screen of an HSV-1 genomic RNA expression library, examining ICP27-specific interactions. A flow chart, in which the number of transformants that pass (vertical arrows) or fail (horizontal arrows) five major selection steps, is indicated. The determined phenotypes of transformants at each selection step are indicated. 1, a limited number of white His^+ colonies was picked at random from the initial large scale transformation; 2, transformants were tested for expression of the lacZ reporter gene; 3, His^+LacZ^- transformants were made to lose the pAD27 plasmid and then tested for the loss of lacZ expression, eliminating protein-independent RNA activators; 4, pAD27, pA-DIRP1 and pACTII-CAN were reintroduced into the LacZ^- colonies by mating and cells analyzed for ICP27-specific activation of lacZ expression; 5, library plasmid DNAs were isolated, sequenced, and analyzed, followed by selection of single insert RNAs that map to HSV-1 transcribed genes.
GAL4 AD-ICP27 protein hybrid. Thus, transformants were grown in rich medium without selection, causing some cells to lose the pAD27 plasmid, followed by growth on S.D. medium lacking uracil to select for those cells that retained the library plasmid but had lost the pAD27 plasmid. These colonies were analyzed for loss of β-gal activity as compared with the initially isolated LacZ/His transformants (which contained all three hybrid components). Of the 334 transformants analyzed, 113 showed β-gal activity in the absence of the GAL4 AD-ICP27 protein hybrid (Fig. 1), suggesting that they activated the lacZ reporter gene in a ICP27-independent manner. Similar RNA activators were reported in a screen of a yeast DNA library (48), in which 84% of the LacZ/His transformants showed this property as compared with 34% observed here.

The remaining 221 transformants, which showed loss of β-gal activity upon loss of the GAL4-ICP27 protein hybrid, were then mated with R40 (opposite mating type of L40), pre-transformed with plasmids pAD27, pADIRP1 (expressing a GAL4 AD-IRP1 protein hybrid), or pACTII-CAN (expressing the GAL4 AD alone). Analysis of β-gal activity by the filter lift assay showed that 68 single transformants had a restored activation of the lacZ reporter gene in a 1CP27-independent manner. Similar RNA activators were reported in a screen of a yeast DNA library (48), in which 84% of the LacZ/His transformants showed this property as compared with 34% observed here.

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The identified HSV-1 RNAs Interact Specifically with ICP27 Protein in Vivo—First, we wished to characterize the use of a quantitative, liquid β-gal assay. As a positive control, we used the well characterized high affinity interaction between the iron responsive element (IRE) RNA and IRP1 (45, 48). Plasmid pIII/MS2-IRE in strain L40 was mated with strain R40, con-
Table I

Identification of HSV-1 RNAs interacting specifically with the ICP27 protein

| Gene, kinetic class | Isolate no. | Length(nt) | Genomic position | β-Gal +ICP27 units | % β-gal +ICP27 relative | β-Gal + IRP1 units |
|---------------------|-------------|------------|------------------|-------------------|------------------------|-------------------|
| ICP4                | α           | 69         | 95               | 130251c           | 6.8–19                 | 36 ± 15           | 3.6–4.9           |
| ICP4                | α           | 16         | 50               | 125892c           | 14–26                  | 58 ± 8            | 2.9–3.8           |
| ICP4                | α           | 17         | 36               | 1258003c          | 4.6–6.3                | 16 ± 1            | <1                |
| ICP4                | α           | 247        | 71               | 1288005c          | 8.0–16                 | 34 ± 7            | <1                |
| ICP4                | α           | 332        | 196              | 127879c           | 10–16                  | 38 ± 1            | <1                |
| ICP4                | α           | 75         | 148              | 127879c           | 3.8–11                 | 20 ± 9            | <1                |
| UL5u                | β           | 324/16.2   | 66               | 15333c            | 7.3–7.7                | 23 ± 8            | <1                |
| UL29                | β           | 309        | 108              | 59581             | 13–28                  | 59 ± 13           | <1–2.1            |
| UL30                | β           | 82         | 61               | 64180             | 9.6–11                 | 31 ± 6            | <1                |
| UL30                | β           | 5          | 72               | 66092             | 4.4–13                 | 24 ± 11           | <1                |
| UL30                | β           | 231        | 100              | 66092             | 14–19                  | 52 ± 26           | <1–1.8            |
| UL39                | β           | 13.2       | 104              | 88443             | 4.8–7.0                | 17 ± 1            | <1                |
| UL42                | β           | 3          | 62               | 93504             | 8.2–13                 | 31 ± 1            | <1                |
| UL49                | β           | 8          | 40               | 106100c           | 4.0–4.1                | 12 ± 4            | <1                |
| US3                 | β           | 4          | 72               | 135702            | 11–19                  | 44 ± 4            | <1                |
| US3                 | β           | 242        | 87               | 135702            | 8.2–16                 | 35 ± 6            | <1                |
| UL6a                | γ           | 212        | 63               | 15022             | 7.1–12                 | 28 ± 2            | <1                |
| UL13                | γ           | 12         | 160              | 27101c            | 8.4–14                 | 8 ± 4             | <1                |
| UL15i               | γ           | 23         | 35               | 32178             | 8.8–16                 | 36 ± 5            | <1                |
| UL15u               | γ           | 21         | 209              | 28826             | 2.3–9.0                | 15 ± 9            | <1                |
| UL16                | γ           | 274        | 41               | 30757c            | 13–21                  | 50 ± 2            | <1                |
| UL19                | γ           | 19/85/185  | 122              | 38856c            | 8.4–15                 | 34 ± 4            | 1.8–2.5           |
| UL19                | γ           | 251        | 159              | 38802c            | 5.1–8.6                | 20 ± 1            | <1–1.8            |
| UL32u               | γ           | 55         | 225              | 69322c            | 10–12                  | 34 ± 14           | <1                |
| UL36                | γ           | 281.2      | 47               | 77886c            | 6.8–18                 | 35 ± 13           | <1                |
| UL48                | γ           | 334        | 36               | 104979c           | 1.4–4.6                | 8 ± 4             | <1                |
| UL49Au              | γ           | 300        | 40               | 106100c           | 6.6–8.6                | 23 ± 2            | <1                |
| IPC54.5             | γ           | 258        | 139              | 1306              | 2.1–6.8                | 12 ± 6            | <1                |

* HSV-1 transcribed genes in which the identified RNAs are encoded, with expression kinetics for the genes as described previously (55, 58).
* Position of the 5’-end of each RNA isolate in the HSV-1 17 strain, genome. For IPC54.5 and IPC4, nucleotide numbers refer to one of the inverted repeats.
* Inter-assay range, β-gal activity in the presence of the GAL4 AD-ICP27 or −IRP1 protein hybrids.
* Mean ± S.E. percent (%) β-gal activity in the presence of GAL4 AD-ICP27, relative to the amount of units produced from the positive IRE-IRP1 control. The results are derived from at least three independent experiments.

To efficiently compare the β-gal activities from different experiments, we calculated the percent (%) units obtained in each experiment in relation to the units obtained with the positive IRE-IRP1 control included in each experiment (see Table I and Fig. 3). Four of the HSV-1 RNA that showed strongest activation of the lacZ gene in the presence of ICP27 protein (RNAs 16, 309, 231, and 274) had a mean β-gal activity from 50 to 58% (inter-assay ranges from 13 to 26 units; see Table I and Fig. 3) as compared with the IRE-IRP1 interaction (defined here as 100% β-gal activity; inter-assay range of 27 to 44 units). The remaining 27 RNAs showed a spectrum of β-gal activity from 8 to 50% of the IRE/IRP1 interaction, with inter-assay ranges from 1.4 to 19 units. Generally, high β-gal activity is indicative of a high affinity RNA-protein interaction in this system. However, RNAs that activate lacZ expression at low levels (e.g. 12 and 334; see Table I) could have this property, because they contain partial high affinity ICP27-interacting motifs, presented to ICP27 protein in a sub-optimal flanking context. Interestingly, members of the five sets of RNAs that overlapped at similar genomic sites (19/85/185 and 251, 352 and 75, 17 and 247, 4 and 242, 5 and 231; see Fig. 1) showed similar levels of β-gal activity for each site (see Table I and Fig. 3). We concluded that all identified HSV-1 RNAs significantly activated the lacZ reporter gene in the presence of ICP27 protein, reflecting the result of physical RNA-protein interactions occurring in vivo.

All the HSV-1 RNAs displayed at least 2-fold higher β-gal activity in the presence of plasmid pAD27 as compared with pADIRP1 in all experiments (Table I), strongly suggesting that all RNAs interacted with preferred specificity for ICP27 protein. And 23 of the 31 HSV-1 sense RNAs had <1 unit activity in the presence of the GAL4 AD-IRP1 protein hybrid, demonstrative of no interactions between these RNAs and IRP1 protein. Furthermore, although six of the identified HSV-1 RNAs (231, 309, 19/85/185, and 251) showed elevated levels of β-gal activity with the GAL4 AD-IRP1 protein hybrid (<1 to 2.5 units), these levels suggested insignificant or weak interactions.
The results demonstrated that 29 RNAs had a high specificity in interacting with ICP27 protein. In contrast, two HSV-1 RNAs (16 and 69) showed β-gal activity in the presence of the GAL4 AD-IRP1 protein hybrid, at levels that clearly exceeded the background of the assay (from 2.9 to 4.9 units; see Table I), indicating that these RNAs also had specificity for IRP1 protein, albeit to a lower extent as compared with ICP27 protein (Table I). In summary, we concluded that all HSV-1 RNAs identified and isolated in the yeast-three hybrid screen interacted with ICP27 protein in vivo with a high degree of specificity.

The ICP27-RNA Interactions Depend on the Presence of the RGG Box and Are Disrupted by a C-terminal Mutation, Correlating RNA Binding with ICP27 Regulatory Function—In previous studies, the RGG box in the ICP27 protein (aa 138–152) was necessary for interaction with mRNA (12, 25) and for ICP27-regulatory functions during lytic HSV-1 infection (12, 14, 35, 39). Similarly, a previously reported substitution mutation in the overlapping KH-like motif 3 (tsR480H) resulted in a ts lethal phenotype during HSV-1 lytic infection (39). We wished to characterize the ICP27 protein structures involved in binding to the HSV-1 sense RNAs identified here, as a final test of specificity and to examine their biological relevance. All 31 library plasmids in strain L40 were mated with strain R40, which had been pre-transformed with plasmids pADΔRGG (RGG box deleted; see Fig. 4A), pADM15 (substitution mutations P465L and G466E, in the overlapping KH-like motif 3/Sm homology domain; see Refs. 38 and 39 and Fig. 4A), pADΔRGG/KH23 (RGG box, KH-like motifs 2 and 3 deleted; see Fig. 4A), and pADΔRGG/KH1/23 (RGG box, KH-like motifs 1, 2, and 3 deleted; see Fig. 4A). In parallel, we isolated diploids containing the IRE-IRP1 interaction, serving as a positive control. The resulting diploids were subject to measurement of β-gal activity by the liquid assay, in at least three independent experiments.

The deletion of the ICP27 RGG box (Fig. 4A) resulted in β-gal activities of <1 unit for 22 HSV-1 RNAs, corresponding to less than 4.7 to 34% of the levels produced in the presence of wt protein (see Table II and Fig. 4B). This indicated that the RGG box was a necessary determinant for the interaction of ICP27 protein with these RNAs. Another five RNAs (247, 4, 212, 23, and 274) produced β-gal activities between 1.1 to 2.5 units (7.3 to 20% of wt levels; see Table II), and the remaining RNA (19/85/185) consistently produced β-gal above 2 units (range of 2.1 to 3 units, 24% of wt levels; see Table II) in the presence of RGG-deleted ICP27 protein. These results indicated that weak interactions could occur between these last six RNAs and ICP27 protein in the absence of the RGG box, although RNA binding was significantly reduced compared with wt ICP27.

Further deleting the C-terminal parts of the already RGG box-deleted ICP27 protein (removing the KH-like motifs; see Fig. 4A) resulted in β-gal activities (Table III) for all RNAs at levels similar to those observed in RGG-deleted ICP27 alone, confirming the importance of the RGG box in the interaction with all RNAs identified here.

Interestingly, two C-terminal amino acid substitution mutations (M15), which map to the overlapping KH-like motif 3/Sm homology domain (38, 39), also had detrimental effects on the interaction of ICP27 protein with most RNAs, albeit to a somewhat lower extent compared with the RGG box deletion (Table II). Table II shows that the M15 mutation resulted in below or around 1 unit of β-gal being produced for 7 RNAs (247, 324/16.2, 13.2, 12, 21, 251, 300) whereas the remaining 21 RNAs interacted weakly with M15-mutated ICP27 protein (range of 1.1 to 3.2 units). The mean percent (%) β-gal activity produced in the presence of wt ICP27 protein, RGG box-deleted ICP27 protein, or ICP27 containing mutation M15, for each RNA in

![Figure 3](image-url)
Fig. 4. Disrupted interactions between the identified HSV-1 RNAs and mutated ICP27 protein, containing a RGG box deletion or mutation M15. A, schematic illustration of ICP27 protein and its mutants expressed as GAL4 AD fusion proteins from the indicated plasmids. B, Western immunoblot, using anti-ICP27 antisera, showing expression of GAL4 AD-ICP27 fusion proteins in extracts of strain R40. Lane 1 and lane 2, cells without or with empty vector respectively; lane 3, from cells containing pAD27; lane 4, from cells containing pADM15; lane 5, from cells containing pADΔRGG; lane 6, from cells containing p502CD; lane 7, from uninfected BHK cells; lane 8, from BHK cells infected with HSV-1 virus strain 17.

Table II

Effect of the RGG box deletion and C-terminal mutation M15 on ICP27-RNA interactions

For the second and fourth columns, β-gal activity in the presence of the GAL4 AD-ICP27/ΔRGG or -ICP27/M15 protein hybrids. For the third and fifth columns percent (%) β-gal activity relative to wt ICP27; [mean percent β-gal activity for each RNA in the presence of the GAL4 AD-ICP27/ΔRGG or -ICP27/M15 protein hybrids, relative to units produced from the positive IRE - IRP1 control (see Table I)], multiplied by 100. The results are derived from at least three independent experiments.

| Isolate no. | β-Gal +pADΔRGG units | β-Gal +pADΔRGG relative to WT | β-Gal +pADM15 units | β-Gal +pADM15 relative to WT |
|-------------|----------------------|-------------------------------|---------------------|-------------------------------|
| 69          | <1                   | 1.2-1.7                       | 16                  | 1.6-2.1                       |
| 16          | <1                   | 1.2-1.7                       | 15                  | 1.6-2.1                       |
| 17          | <1                   | 1.2-1.7                       | 24                  | 1.6-2.1                       |
| 247         | 1.1-1.5              | 1.2-1.7                       | 14                  | 1.6-2.1                       |
| 352         | <1                   | 1.2-1.7                       | 9.6                 | 1.6-2.1                       |
| 75          | <1                   | 1.2-1.7                       | 29                  | 1.6-2.1                       |
| 324/16.2    | <1                   | 1.2-1.7                       | 12                  | 1.6-2.1                       |
| 309         | <1                   | 1.2-1.7                       | 6.1                 | 1.6-2.1                       |
| 82          | <1                   | 1.2-1.7                       | 10                  | 1.6-2.1                       |
| 5           | <1                   | 1.2-1.7                       | 10                  | 1.6-2.1                       |
| 231         | <1                   | 1.2-1.7                       | 8.1                 | 1.6-2.1                       |
| 13.2        | <1                   | 1.2-1.7                       | 8.9                 | 1.6-2.1                       |
| 3           | <1                   | 1.2-1.7                       | 12                  | 1.6-2.1                       |
| 8           | <1                   | 1.2-1.7                       | 22                  | 1.6-2.1                       |
| 4           | 1.2-1.7              | 1.2-1.7                       | 22                  | 1.6-2.1                       |
| 242         | <1                   | 1.2-1.7                       | 14                  | 1.6-2.1                       |
| 212         | 1.2-1.7              | 1.2-1.7                       | 17                  | 1.6-2.1                       |
| 12          | <1                   | 1.2-1.7                       | 38                  | 1.6-2.1                       |
| 23          | 1.2-1.7              | 1.2-1.7                       | 15                  | 1.6-2.1                       |
| 21          | <1                   | 1.2-1.7                       | 18                  | 1.6-2.1                       |
| 274         | 1.2-1.7              | 1.2-1.7                       | 11                  | 1.6-2.1                       |
| 19/85/185   | 2.1-3.0              | 1.2-1.7                       | 24                  | 1.6-2.1                       |
| 251         | <1                   | 1.2-1.7                       | 15                  | 1.6-2.1                       |
| 55          | <1                   | 1.2-1.7                       | 22                  | 1.6-2.1                       |
| 281.2       | <1                   | 1.2-1.7                       | 44                  | 1.6-2.1                       |
| 334         | <1                   | 1.2-1.7                       | 12                  | 1.6-2.1                       |
| 300         | <1                   | 1.2-1.7                       | 34                  | 1.6-2.1                       |

Table III

Effect of C-terminal truncations in RGG box-deleted ICP27 protein

For the second and fourth columns, β-gal activity in the presence of the GAL4 AD-ICP27/ΔRGG/KH2/3 or -ICP27/ΔRGG/KH1/23 protein hybrids. For the third and fifth columns percent (%) β-gal activity relative to wt ICP27; [mean percent β-gal activity for each RNA in the presence of the GAL4 AD-ICP27/ΔRGG/KH2/3 or -ICP27/ΔRGG/KH1/23 protein hybrids, relative to units produced from the positive IRE - IRP1 control (see Table I)], multiplied by 100. The results are derived from at least three independent experiments.

| Isolate no. | β-Gal +pADΔRGG/KH2/3 units | β-Gal +pADΔRGG/KH2/3 relative to WT | β-Gal +pADΔRGG/KH1/23 units | β-Gal +pADΔRGG/KH1/23 relative to WT |
|-------------|-----------------------------|-------------------------------------|------------------------------|-------------------------------------|
| 69          | <1                          | 1.2-1.7                             | 16                           | 1.2-1.7                             |
| 16          | <1                          | 1.2-1.7                             | 16                           | 1.2-1.7                             |
| 17          | <1                          | 1.2-1.7                             | 24                           | 1.2-1.7                             |
| 247         | 1.1-1.5                     | 1.2-1.7                             | 14                           | 1.2-1.7                             |
| 352         | <1                          | 1.2-1.7                             | 14                           | 1.2-1.7                             |
| 75          | <1                          | 1.2-1.7                             | 29                           | 1.2-1.7                             |
| 324/16.2    | <1                          | 1.2-1.7                             | 12                           | 1.2-1.7                             |
| 309         | <1                          | 1.2-1.7                             | 6.1                          | 1.2-1.7                             |
| 82          | <1                          | 1.2-1.7                             | 15                           | 1.2-1.7                             |
| 5           | <1                          | 1.2-1.7                             | 15                           | 1.2-1.7                             |
| 231         | <1                          | 1.2-1.7                             | 10                           | 1.2-1.7                             |
| 13.2        | <1                          | 1.2-1.7                             | 16                           | 1.2-1.7                             |
| 3           | <1                          | 1.2-1.7                             | 16                           | 1.2-1.7                             |
| 8           | <1                          | 1.2-1.7                             | 22                           | 1.2-1.7                             |
| 4           | 1.2-1.7                     | 1.2-1.7                             | 22                           | 1.2-1.7                             |
| 242         | <1                          | 1.2-1.7                             | 14                           | 1.2-1.7                             |
| 212         | 1.2-1.7                     | 1.2-1.7                             | 17                           | 1.2-1.7                             |
| 12          | <1                          | 1.2-1.7                             | 38                           | 1.2-1.7                             |
| 23          | 1.2-1.7                     | 1.2-1.7                             | 15                           | 1.2-1.7                             |
| 21          | <1                          | 1.2-1.7                             | 18                           | 1.2-1.7                             |
| 274         | 1.2-1.7                     | 1.2-1.7                             | 11                           | 1.2-1.7                             |
| 19/85/185   | 2.1-3.0                     | 1.2-1.7                             | 24                           | 1.2-1.7                             |
| 251         | <1                          | 1.2-1.7                             | 15                           | 1.2-1.7                             |
| 55          | <1                          | 1.2-1.7                             | 22                           | 1.2-1.7                             |
| 281.2       | <1                          | 1.2-1.7                             | 44                           | 1.2-1.7                             |
| 334         | <1                          | 1.2-1.7                             | 12                           | 1.2-1.7                             |
| 300         | <1                          | 1.2-1.7                             | 34                           | 1.2-1.7                             |
| 258         | <1                          | 1.2-1.7                             | 12                           | 1.2-1.7                             |

Table 2

Interaction of ICP27 with HSV-1 RNAs

We examined for expression of GAL4 AD-ICP27 fusion protein hybrids, relative to units produced from the positive IRE - IRP1 control (see Table I), multiplied by 100. The results are derived from at least three independent experiments.

to WT

extenuation by deleting the RGG box as compared with the introduction of the C-terminal M15 mutant (Table II).

We examined for expression of GAL4 AD-ICP27 fusion pro-
tains in strain R40 by Western blotting cell extracts using antibodies directed against ICP27 protein. Plasmid pAD27 contains ICP27 aa 10–512 fused to 130 aa from the C terminus of the GAL4 AD, with a calculated size of around 70 kDa. Compared with the cells with or without empty vector, cell extracts transformed with vectors expressing the ICP27 fusion proteins contained clearly visible bands of the appropriate size (Fig. 4B, compare lanes 1 and 2 with lanes 3–6). And cells transformed with p502 CAD, used by us in previous yeast two-hybrid screens (28, 30), gave a slightly larger fusion protein band (Fig. 4B, lane 6) consistent with expression of ICP27 aa 10–512 fused to the slightly 160-aa GAL4 DNA-binding domain in vector pAS2–1. We concluded that the specificities of the newly identified HSV-1 RNA-ICP27 protein interactions had been confirmed. The RGG box was a necessary determinant for strong interaction of ICP27 protein with all the HSV-1 sense RNAs studied here. Furthermore, integrity of the overlapping KH-like motif S/m homology domain of ICP27 protein was also of importance for RNA binding. Because the RGG box deletion and M15 mutant both have been shown to abolish post-transcriptional regulatory functions of ICP27 (12, 14, 21, 35, 39), the results indicated that these regulatory functions correlated with the interaction of ICP27 and the HSV-1 sense RNAs identified here.

**DISCUSSION**

The RNA-ICP27 protein interactions reported here arose from an extensive selection procedure, similar to the prototypic screen of a yeast DNA library performed in a search for the well established RNA binding site of Snpl protein (48). We estimated that >10^9 white His+ colonies appeared 5 days after the initial large scale transformation (of which >334 colonies also had a LacZ+ phenotype; see Fig. 1), compared with 250 colonies in the screen of the yeast genome (with 83 colonies having a LacZ+ phenotype) (48). Furthermore, only 34% of the 334 His+ / LacZ+ colonies found here acted as protein-independent RNA activators compared with 84% of the 83 His+ /LacZ+ found in the yeast screen. At the final step of the screen, we found that 24 of 68 RNAs mapped to antisense strands of HSV-1 transcribed regions (19 isolates) or non-transcribed regions (five isolates). This is comparable with the results of the yeast genome screen, where four of nine identified Snpl-interacting RNAs corresponded to regions not transcribed in vivo (48). Here, 50% of the final isolates contained RNAs that mapped to sense regions representing, because of duplications, 48% of identified RNA sites; no antisense sites were duplicated. Comparable with our results, in the yeast screen, 56% of the final isolates contained RNAs that mapped to sense regions, representing 40% of identified RNA sites. In contrast to the yeast screen, 13 RNAs were expressed as hybrid RNAs, generated by ligation of two or three HSV-1 genomic fragment inserts during library construction, as may be expected to occur during blunt-end cloning. The construction of a small insert library from HSV-1 cDNAs, using optimized ligation conditions, would eliminate such false positives and subsequently make the screening process more efficient. Seventeen of the 31 sense RNAs identified each corresponded to an equal number of unique locations in the HSV-1 genome and were isolated only once (Fig. 2). In contrast, two sets of identical RNAs were isolated two and three times (324/16.2 and 19/85/185; see Fig. 2), respectively. Furthermore, five sets of overlapping RNAs were isolated (17 and 247, 352 and 75, 5 and 231, 4 and 242, 19/85/185 and 251) that covered five HSV-1 genomic regions, respectively (Fig. 2). These results are comparable with the previous yeast screen where the known high affinity RNA partner for Snpl protein and other interacting RNAs were isolated only once whereas two other RNAs were isolated two and four times, respectively (48). A final demonstration of the specificity of the interactions identified here was the loss of RNA binding when using the unrelated RNA-binding protein IRP1 and upon deletion of the ICP27 RGG box. Apparently, the interactions of ICP27 with the HSV-1 RNAs identified did not involve other HSV-1 gene products and were a result of direct physical RNA-protein contacts occurring in vivo.

The identified RNAs show a high degree of heterogeneity in primary sequence, making it difficult to identify an RNA motif(s) interacting specifically with ICP27 by, for example, sequence alignments. Presently, G residues appear to be involved in specific ICP27-RNA interactions. The ICP27 RGG box has been shown to have enhanced specificity for poly(G) in vitro (25). Similarly, all 31 HSV-1 RNAs identified here also contain poly(G) repeats, with each RNA containing multiple (G)2 repeats, 23 RNAs containing one or more (G)4 motifs, and 15 RNAs having at least one (G)4 motif. In contrast, the MS2-RNA, which did not interact with ICP27 protein, encodes only two (G)2 repeats. We do not know whether the identified RNAs form similar higher order structures, which may expose in bulges or loops certain nucleotides crucial for specific ICP27 interaction. The regulatory fragile X mental retardation protein, FMR-1, was shown to interact with higher order RNA G quartets through its RGG box domain (51, 52). Similarly, all of

**Interaction of ICP27 with HSV-1 RNAs**

**TABLE IV**

**HSV-1 transcripts containing newly identified ICP27 RNA binding sites**

| Isolate no. | Transcript | Kinetic class | Functional role |
|-------------|------------|---------------|-----------------|
| 60, 16, 17, 247, 352, 75 | ICP4 | α | Broad-range transcriptional trans-activator, E |
| 324/16.2 | UL45 | β | Part of replication complex, NE/E |
| 309 | UL29 | β | ssDNA binding protein, E |
| 82, 5, 231 | UL30 | β | DNA pol subunit, E |
| 13.2 | UL39/40 | β | Ribonucleotide reductase, NE |
| 3 | UL42 | β | DNA pol subunit, E |
| 8, 300 | UL49/49.5 | β/γ | Tegument protein, NE |
| 4, 242 | US3 | β | Protein kinase, NE |
| 212 | UL6 | γ | DNA cleavage/packaging, E |
| 12 | UL13 | γ | Protein kinase, NE |
| 21, 23 | UL15 | γ | DNA packaging, E |
| 274 | UL16/17 | γ | Tegument/DNA packaging, NE/E |
| 185/85/19, 251 | UL19/20 | γ | Major capsid protein/viral encyctosis, E/NE |
| 55 | UL31/32 | γ | Nuclear phosphoprotein/DNA packaging, E |
| 281.2 | UL36 | γ | Tegument protein, E |
| 334 | UL45 | γ | Tegument protein, IE trans-activator, E |
| 258 | ICP94.5 | γ | Neurovirulence, NE |

* HSV-1 mRNA transcripts to which the identified RNA-isolate(s) map, with expression kinetics (55, 58).
* Functional role in the HSV-1 life cycle of the proteins encoded by the transcripts as summarized in Ref. 3.
the RNAs identified here each contain multiple, interspersed G repeats in close proximity to each other, indicating a possible capacity of forming stable G quartets. It is tempting to speculate that higher order RNA structures such as G quartets may mediate high affinity binding to the ICP27 RGG box. It would be interesting to evaluate the cationic dependences of ICP27-RNA interactions in vitro assays, as G quartets can form in the presence of K+ or Na+ but not Li+.

Because the RNAs identified here were only 35 to 225 nucleotides in length (Table I), this now enables more detailed mapping of the RNA structure(s) capable of interacting specifically with ICP27 protein. For example, one may examine the RNA residues in contact with ICP27 protein by using RNase T1 protection, complemented by studies of the apparent affinities of the ICP27-RNA interactions and to mutants thereof by in vitro RNA mobility shift or filter binding assays. Furthermore, several of the RNA sites identified may bind ICP27 protein cooperatively in the context of the full-length mRNA, forming one combined high affinity site embedded in a larger higher order structure. It would be of interest to examine whether a combination of the identified RNA binding sites in, say, ICP4, in the context of the full-length mRNA, may result in a increased overall avidity for ICP27 protein. This could be performed by using a modified three-hybrid system based on RNA expression driven by RNA pol II (54), enabling the analysis of ICP27 interactions with long transcripts in vivo.

Some of the HSV-1 transcription units are comprised of 3′ co-terminal nested mRNA, in which the smallest monocistronic transcript shares a poly(A)+ site with longer overlapping polycistronic RNAs (reviewed in Ref. 55). The accepted wisdom is that the virus proteins are translated from the 5′ most ORF of polycistronic RNAs; however it is presently unknown whether downstream ORFs are translated. The 31 ICP27 interacting sense RNAs map to 10 monocistronic and 10 polycistronic transcripts that represent 28 ORFs. The ORFs encode viral proteins that display a broad range of predominantly essential functions, with roles in DNA replication, DNA processing, and virion maturation (Table IV). Intriguingly, several studies show regulatory effects by ICP27 protein on the majority of these transcripts, consistent with ICP27 acting as an essential regulator of the early-late switch in the HSV-1 life cycle (reviewed in 3, 6). For example, ICP27 has been shown to have regulatory effects on ICP4 mRNA translatability (8, 37), nucleocytoplasmic transport of UL15, UL17, and UL48 mRNAs (9, 11, 39) and on the cytoplasmic levels of the UL30, UL29, UL42, and UL5 mRNAs (53). Furthermore, our results show that the RGG box is a necessary determinant for interaction with all the HSV-1 RNAs identified here, in line with previous studies demonstrating the importance of the RGG box for ICP27-regulated functions (12, 14, 25, 39). We also show that the ICP27 mutation M15 (38) severely reduces ICP27 binding to HSV-1 RNA. The two-aa substitution mutation M15 has detrimental effects on ICP27 regulatory functions (21, 28), and the integrity of the protein C terminus was important for ICP27-mediated nucleocytoplasmic transport (39). Both mutations are lethal in virus. Because of the apparent correlation between the RNA-ICP27 interactions identified here and the previously shown ICP27 regulatory functions, we consider the HSV-1 transcripts described here to be potential targets for ICP27-mediated post-transcriptional regulation and are investigating regulatory effects of ICP27 protein on mRNAs containing the novel RNA binding sites identified.

Sandri-Goldin’s laboratory (12) showed that IPC27 protein can form UV light-induced cross-links with seven HSV-1 intronless mRNAs from all kinetic classes but not with the UL15 and ICP0 intron-containing RNAs, and the ICP27 RGG box mediated UV cross-linking to its own RNA (12). We also demonstrate interaction with intronless RNAs from all kinetic classes, mediated in all cases by the RGG box, and, additionally, we isolated two RNAs that mapped to the 5′-untranslated region first exon and to the intron of the UL15 transcript. We only verify the interaction with ICP4 RNA that encodes the essential virus transcription trans-activator, which we show contains up to four different sites of interaction with ICP27 protein. In vitro cross-linking assays are qualitative in nature, reflecting the relative abundances of viral transcripts. We may have missed the identification of HSV-1 RNAs because of screening an incomplete genomic set of RNA binding sites or as >584 white His+ colonies generated in the initial large scale transformation (Fig. 1) were not studied further. ICP27 may have altered binding specificity in virus-infected cells, or full-length transcripts exhibit different binding affinities compared with the shorter RNAs identified here.

Few studies have been reported to date using the three-hybrid system to identify natural RNA ligands by screening a genomic RNA library with an RNA-binding protein activation domain fusion as bait (43). This approach parallels in vitro reiterative selection methods, including SELEX and genomic SELEX (56, 57), and is the first to identify new ICP27-interacting HSV-1 RNAs at the level of the entire HSV-1 genome, whereas previous studies have tested selected RNAs or total cellular poly(A)+ mRNA (12, 23–25, 39). The results further strengthen the function of ICP27, a protein that interacts with a broad range of HSV-1 RNAs through its RGG box. Identification of new ICP27 specific RNA binding sites may help in the dissection of the post-transcriptional regulatory mechanisms of ICP27 during the different phases of HSV-1 lytic infection. The results help in the progress toward a more detailed understanding of the formation of specific RNA-ICP27 protein contacts that could enable the design of antiviral drugs against HSV-1.

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