Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 6694–6699, June 1999
Biochemistry

Regulation of eukaryotic protein synthesis: Selective influenza viral mRNA translation is mediated by the cellular RNA-binding protein GRSF-1

YOUNG WOO PARK*, JEFFREY WILUSZ†, AND MICHAEL G. KATZE*‡§

*Department of Microbiology, School of Medicine, and ‡Regional Primate Research Center, University of Washington, Seattle, WA 98195; and †Department of Microbiology and Molecular Genetics, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, NJ 07103

Edited by Robert Palese Perry, Fox Chase Cancer Center, Philadelphia, PA, and approved April 19, 1999 (received for review January 5, 1999)

ABSTRACT **To better understand regulation of eukaryotic protein synthesis, we studied cellular and viral mRNA translation in influenza virus-infected cells. Influenza virus infection results in a dramatic shut-off of cellular protein synthesis that is concomitant with selective viral mRNA translation. Earlier work showed that these events are mediated by viral and/or cellular factors binding to the 5′ untranslated region (5′ UTR) of viral mRNAs. To identify trans-acting cellular proteins responsible for selective viral protein synthesis, we employed the yeast three-hybrid system. Using the 5′ UTR of the influenza virus nucleocapsid protein (NP) mRNA as bait, we identified the cellular RNA-recognition motif containing RNA-binding protein G-rich sequence factor 1 (GRSF-1) as a positive-acting translational regulatory factor. The *in vivo* yeast assay revealed GRSF-1 specifically bound to the NP 5′ UTR but not select NP 5′ UTR mutants or cellular RNA 5′ UTRs. These data were confirmed by gel shift assays using recombinant GRSF-1. Importantly, recombinant GRSF-1 specifically stimulated translation of a NP 5′ UTR-driven template in cell-free translation systems. Furthermore, translation efficiency of NP 5′ UTR-driven templates was reduced markedly in GRSF-1-depleted HeLa cell extracts, but restored in GRSF-1-reconstituted extracts. GRSF-1 also stimulated translation of an NP 5′ UTR-driven template in HeLa cell extracts that were depleted of essential factors by addition of RNA oligonucleotides representing the viral 5′ UTR RNA. Taken together, these data document the functional demonstration of a cellular protein binding to influenza virus RNAs and, importantly, suggest that influenza virus may recruit GRSF-1 to the 5′ UTR to ensure preferential translation of viral mRNAs in infected cells.**

The control of mRNA translation has become recognized increasingly as an important component of gene regulation (for recent reviews see ref. 1). Translation of eukaryotic mRNAs into proteins is a complex process that is subjected to multiple controls at the levels of initiation, elongation, and termination. The initiation step of protein synthesis is the most widely studied. Moreover, the initiation of mRNA translation most often is the target of regulation in both cellular and viral systems (1–7). Such regulation often is mediated through the interaction of trans-acting cellular factors, which interact with the mRNA 5′ leader or untranslated region (UTR). The best understood example of mRNA regulation by 5′ UTR–RNA–protein interactions is the ferritin system in which ferritin mRNA translation is blocked by the binding of the iron regulatory protein (IRP) to the 5′ UTR iron-responsive element (IRE) (8–10).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

Eukaryotic viruses, including the picornaviruses and influenza viruses, because of their dependence on the host cell protein synthesizing machinery, have provided excellent models to understand the control of mRNA translation. Picornaviruses, perhaps the best understood viral system, are unique in that their mRNA contains an internal ribosome entry site (IRES) located within the 5′ UTR that is more than 700 nt in length and contains multiple ATGs and an extensive secondary structure (11, 12). Host or cellular mRNAs cannot be translated in infected cells because of the virally induced inactivation of the cellular cap-binding complex (13). Several host factors, which bind to the 5′ UTR and may play a functional role, have been identified, including the autoantigen La, the polypyrimidine tract-binding protein, PTB, the poly (rC)-binding protein 2 (PCBP2) (14–16), and the eIF4G homologue poly(A)-interacting protein (PAIP) (17).

Inside an influenza virus-infected cell, as in a picornavirus-infected cell, there is a dramatic shift from cellular to viral protein synthesis. However, influenza virus mRNAs, in contrast to the picornaviruses, are translated by a cap-dependent mechanism that does not involve IRES elements (18). Indeed, all influenza virus mRNAs contain the cellular cap and 10–14 nucleotides of host mRNA sequences at the very 5′ end because of "cap stealing" during viral mRNA transcription (19). Further, the viral 5′ UTRs are comparatively unremarkable in structure. The mRNA leaders contain ordinary 20- to 50-nt sequences (depending on the gene), with little apparent secondary structure and no upstream AUGs. Despite its relative simplicity, the influenza virus mRNA 5′ UTR is both necessary and sufficient to direct selective translation (18, 20). Previous studies have identified a number of host and viral factors, which interact with the viral mRNA, potentially to regulate viral protein synthesis (21). In the current report, we utilized the yeast three-hybrid system to identify and clone genes whose product specifically interacts with the influenza virus mRNA 5′ UTR.

MATERIALS AND METHODS

The Three-Hybrid Library Screen. The yeast strains L40-coat and R40-coat (the same genotype as L40-coat except for *MAT*α) and plasmids pIII/MS2–1, pAD-IRP1, and pIII/MS2-IRE were generous gifts from Dhruba J. SenGupta (Univ. of Washington) (22). The plasmid pIII/MS2-NP, which expresses an MS2-NP 5′ UTR, was constructed by inserting a double-

This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: UTR, untranslated region; IRE, iron-responsive element; PTB, polypyrimidine tract-binding protein; GST, glutathione *S*-transferase; NP, nucleocapsid protein; NS, nonstructural protein; RRM, RNA-recognition motif; GRSF-1, G-rich sequence factor 1; SEAP, secreted embryonic alkaline phosphatase; 5-FOA, 5-fluoroorotic acid.

§To whom reprint requests should be addressed at: Department of Microbiology, Box 357242, University of Washington, Seattle, WA 98195. e-mail: honey@u.washington.edu.

stranded oligonucleotide (prepared by annealing the synthetic oligonucleotides 5′-CCGGGAGCAAAAGCAGGGTAGATAATCACTCACTGAGTGACATCAAAATCG-3′ and 5′-GGCCCGATTTTGATGTCTCTCAGTGAGTGATTATCTAC CCTGCTTTTGCTC-3′) into the *Xma*I site on plasmid pIII/MS2–1. The plasmid pIII/MS2-NP-A, which contains a mutant form of the nucleocapsid protein (NP) 5′ UTR (called NP-A), was constructed with the synthetic oligonucleotides 5′-CCGGGGTAGATAATCACTCACTGAGTGACATCAAAATCG-3′ and 5′-GGCCCGATTTTGATGTCTCTCAGTG-AGTGATTATCTACC-3′.

The plasmid pIII/MS2-NP (*URA3*) was introduced into yeast L40-coat by transformation. Two hundred micrograms of the HeLa cell two-hybrid cDNA library cloned in pGAD-GH (called pAD-cDNAs) was transformed into L40-coat/NP. Double-transformants (Trp^+, Leu^+, Ura^+) were plated on −Trp, −Ura, −Leu, −His plates containing 3 mM 3-aminotriazole (AT). Resulting His^+ protoprophs represent putative positive colonies in which an *HIS3* reporter gene is activated by either interaction between bait RNA and target protein (RNA-dependent) or a target protein alone (RNA-independent). To select RNA-dependent potential positives that required the MS2-NP 5′ UTR hybrid RNA for activation of an *HIS3* reporter gene, the positives were treated with 5-fluoroorotic acid (5-FOA), in which RNA-dependent His^+ protoprophs were sensitive to 5-FOA (unpublished data). Approximately 2,000 His^+ protoprophs, grown on −Trp, −Ura, −Leu, −His plates containing 3 mM 3-AT were transferred onto 5-FOA plates and master plates. Two hundred and thirty 5-FOA-sensitive colonies were identified on master plates and counter-screened with the mutant bait RNA, NP-A, by using a yeast-mating assay. The RNA-dependent His^+ protoprophs on the master plates, which contained two plasmids, pIII/MS2-NP and individual pAD-cDNA, were transferred onto −Trp, −Leu plates. Each colony was replica-plated onto −Trp, −Leu plates containing 5 mM 5-FOA. The resulting colonies were mated to R40-coat/NP-A (*MATα*, Trp^+, Leu^-, Ura^+) (23). Diploids were selected by replica-plating onto −Trp, −Ura, −Leu plates. Each diploid then was assayed for β-galactosidase activity by direct measurement of enzyme activity using *o*-nitrophenyl β-D-galactopyranoside as a substrate.

***In Vitro* Transcription.** The ^{32}P-labeled riboprobes were synthesized as described previously (21). The single-stranded ribooligonucleotides for competition assays were synthesized by using a MAXIscript T7 Kit. For capped mRNA transcripts, plasmid DNA was linearized with an appropriate, proper restriction enzyme and transcribed with T7 RNA polymerase by using a T7 mMesSAGE mMACHINE (Ambion, Austin, TX) kit in the presence of cap and a trace amount of [^{32}P]UTP.

Mobility-Shift Assays. Mobility-shift assays and supershift assays were performed as described previously (21) by using recombinant G-rich sequence factor 1 (GRSF-1) purified from *Escherichia coli* (24) or cell extracts prepared from HeLa cell monolayers (21). Hybridoma supernatant containing anti-GRSF-1 was used for supershift assays.

Purification of GST-GRSF-1. *E. coli* HB101 transformed with the plasmid pGEX2TZQ-2.7 (24) was cultured and induced with 1 mM isopropyl β-D-thiogalactoside. Glutathione *S*-transferase (GST) fusion proteins were incubated with glutathione-agarose beads (Sigma) and were eluted with the elution buffer (50 mM Tris·HCl, pH 8.0/5 mM reduced glutathione).

***In Vitro* Translation.** The *Hin*dIII-*Bst*EII fragments, containing the T7 promoter followed by influenza viral NP 5′ UTR, were amplified by PCR from pBC/CMV/(NP)SEAP (20) by using the primers 5′-AAAAAGCTTAATACGACTCACTATAGGGAGCAAAAGCAGGGTAG-3′ and 5′-GTCGGTGACCATGATTTTGATGTCACTCAGT-3′. Fragments were subcloned between *Hin*dIII and *Bst*EII sites on pSP-luc+NF (Promega), yielding plasmid pSP-NP. Plasmid pSP-NP-A was constructed in the same way by using primers 5′-AAAAAGCTTAATACGACTCACTATAGGGGTAGATAATCACTCACTGAG-3′ and 5′-GTCGGTGACCATGATTTTGATGTCACTCAGT-3′. For plasmid pSP-SEAP, the synthetic, double-stranded DNA fragments (5′-AGCTTAATACGACTCACTATAGGGCGCCTCGCCGCTCTCCGACTGCTTCCAGACATG-3′ and 5′-GTGACCATGTCTGGAAGCTGTCGGAGAGCGGCGAGGCGCCCTATAGTGAGTCGTATTA-3′), which contain the T7 promoter followed by secreted embryonic alkaline phosphatase (SEAP) 5′ UTR, were inserted into the *Hin*dIII and *Bst*EII sites on pSP-luc+NF. The *Bst*EII–*Eco*RI fragment containing the "firefly" luciferase-coding region of pSP-NP-A was replaced with the "sea pansy" luciferase-coding region that was amplified by PCR from pRL-CMV by using the primers 5′-GCTAGCCACCATGGTCACCACTTCGAAAGTT-3′ and 5′-CGCTCTAGAATTCTTATTGTTACTT-3′ and digested with *Bst*EII and *Eco*RI, yielding plasmid pRL-NP-A. To make capped mRNAs, plasmids were linearized with *Eco*RI and were transcribed with T7 RNA polymerase by using a T7 mMesSAGE mMACHINE kit (Ambion) in the presence of cap and a trace amount of [^{32}P]UTP.

Cell-free translation extracts from suspension HeLa cells, which were infected with influenza strain A/PR/8/34 (25), were prepared as described previously (26) without micrococcal nuclease treatment. For immunodepletion of GRSF-1, protein A-agarose beads were coated with IgG molecules by using GRSF-1-specific ascites fluid (27). For *in vitro* translation, 125 ng of capped template RNAs was preincubated in the absence (elution buffer for the GST fusion protein) or presence of 0.2 μg of GST-GRSF-1 (or GST-PTB/U1A) for 10 min at 30°C and incubated further with HeLa cell extract. Samples then were analyzed for luciferase activity by using a Dual-Luciferase Reporter Assay System (Promega).

RESULTS

Three-Hybrid Cloning of the NP 5′ UTR-Binding Protein, GRSF-1. We demonstrated previously that sequences within the 5′ UTR of the NP or nonstructural protein (NS) mRNA (20) mediated the selective translation of influenza virus mRNAs. Homology between the 5′ UTR of the NP and NS mRNA is limited to the first 14 nt of the 5′ end, of which a 12-nt sequence (referred to as region A) is conserved among the eight influenza viral mRNAs (Fig. 2*A*). In addition, we demonstrated that region A of the NP 5′ UTR specifically binds to a set of cellular proteins (21). Thus, we attempted to clone cDNAs encoding RNA-binding proteins that specifically interact with region A by utilizing the yeast three-hybrid system (22). RNA–protein interactions in this system are detected by the transcriptional activation of the dual-reporter system consisting of *HIS3* and *lacZ* placed under the control of the GAL promoter, brought about when a target protein binds to bait RNA.

To clone a cellular protein that specifically interacts with region A of the NP 5′ UTR, we designed two separate hybrid RNA baits, MS2-NP 5′ UTR (wild-type bait RNA) and MS2-NP-A (mutant bait RNA). MS2-NP 5′ UTR contains full-length influenza virus NP 5′ UTR to detect potential NP 5′ UTR-binding proteins, whereas MS2-NP-A contains the NP 5′ UTR lacking region A, to counter-screen against proteins that bind to other regions of the 5′ UTR (Fig. 1*A*). A plasmid encoding wild-type bait RNA and an uninfected HeLa cell cDNA library fused to the GAL4 activation domain were consecutively introduced into yeast L40-coat. From ≈2,000 3AT-resistant colonies, 230 RNA-dependent candidates were selected based on their 5-FOA sensitivity. These clones then were tested for binding specificity to region A by using a mating assay. One expressed an RNA-binding protein that interacted

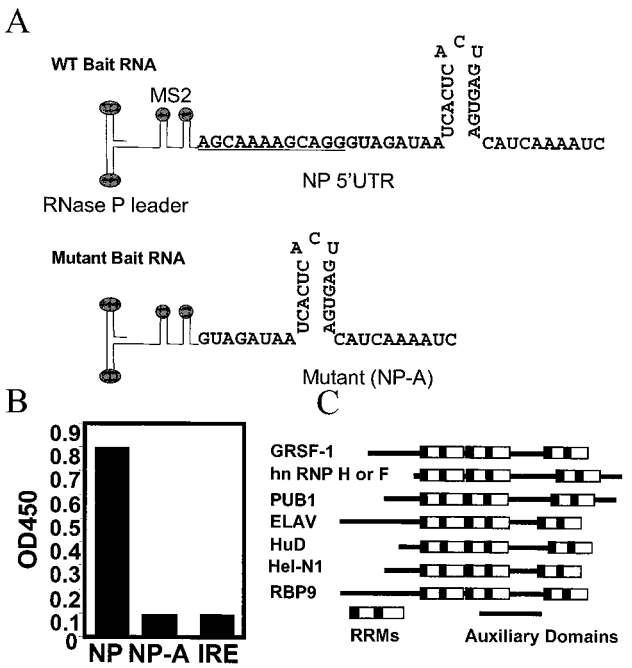

FIG. 1. Yeast three-hybrid analysis reveals that GRSF-1 binds to influenza virus NP 5′ UTR. (*A*) Sequence and computer-predicted structure of the wild-type and mutant bait RNAs. The 5′ UTR of influenza viral NP mRNA or its 12-nt deletion mutant (NP-A) was placed at the 3′ end of the tandem MS2 RNAs. (*B*) Specific interaction of clone 149 with the wild-type bait RNA. The plasmid isolated from clone 149 was reintroduced into yeast strain L40-coat with the plasmid expressing the wild-type (NP), mutant (NP-A), or negative control (IRE) bait RNA. Yeast double transformants were assayed for β-galactosidase activity by direct measurement of enzyme activity. (*C*) Diagrammatic representation of a partial listing of RNA-binding proteins that share similar domain organization to the RRM-containing GRSF-1 protein is presented.

with the NP 5′ UTR, but not with NP-A or IRE RNA [a negative control RNA (22)] (Fig. 1*B*). The HeLa cDNA encoding this protein was isolated, and a BLAST database search revealed a 100% match to the human cDNA encoding GRSF-1. GRSF-1 is an RNA-binding protein with three RNA-recognition motifs (RRM) and is a member of the RRM-containing superfamily (Fig. 1*C*). Until the current study, the function of this RNA-binding protein remained unknown (24).

GRSF-1 Binds to the 12-nt Conserved Region and Its Flanking Sequence in the 5′ UTR of Influenza Viral NP and NS mRNAs. To demonstrate a direct interaction between GRSF-1 and region A of the NP 5′ UTR, an *in vitro* mobility-shift assay was performed by using recombinant GRSF-1. Highly purified recombinant GRSF-1 formed a major complex with the wild-type NP 5′ UTR, but not with the NP 5′ UTR lacking region A (NP-A) (Fig. 2*B*), thus confirming the yeast three-hybrid data. Similarly, when the region A sequences were inverted (A_{inv}) rather than deleted, interactions with GRSF-1 were diminished. If GRSF-1 were involved with selective viral mRNA translation, one would expect that it not interact with the 5′ leader from a cellular RNA. For this purpose we selected the 5′ UTR of SEAP. Neither the native SEAP 5′ UTR nor SEAP+A, which consisted of the region A appended to the SEAP 5′ UTR, formed a complex with GRSF-1 (Fig. 2*B*, lanes 8 and 9). We next examined whether sequences adjacent to region A were required for the GRSF-1/NP 5′ UTR complex formation by preparing various mutant riboprobes lacking other regions of the NP 5′ UTR (Fig. 2*A*). Mutant NP-B failed to form a stable complex with GRSF-1 (Fig. 2*B*, lane 3). In contrast, mutant NP-C interacted with

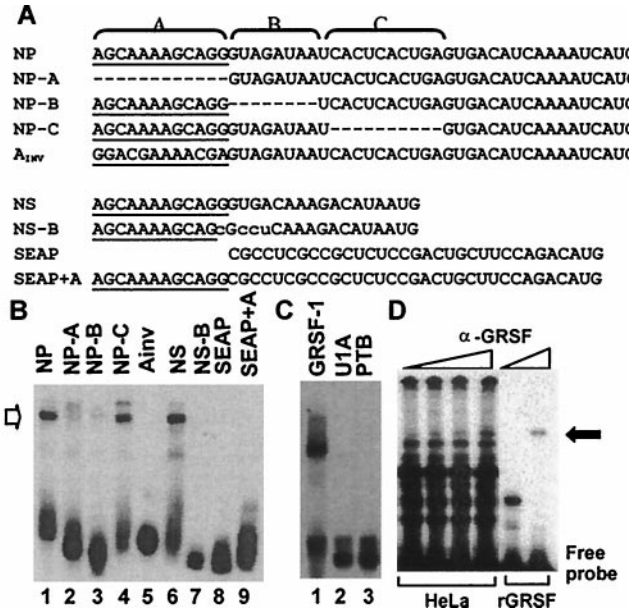

FIG. 2. GRSF-1 interacts with specific sequences within the influenza virus mRNA 5′ UTR as detected by gel mobility-shift analysis. (*A*) Sequences of the *in vitro* transcribed RNA transcripts used as probes in gel mobility-shift assays. Underlined sequences represent the conserved 12-nt sequences found on all influenza virus type A mRNAs. The 5′ UTR of NP mRNA was divided into three regions (regions A, B, and C) as depicted across the top. The name of each transcript is indicated on the left side of its sequence. A_{INV} is a derivative of NP, in which region A is reversed. The NS 5′ UTR is shown along with a sequence of the substitution mutant NS-B (mutated bases are shown in lowercase letters). Below are shown the sequences of the SEAP 5′ UTR along with the SEAP 5′ UTR appended to region A (SEAP+A). (*B*) Recombinant GRSF-1 (0.050 μg) purified from *E. coli* was incubated in the presence of the nonspecific competitor, heparin (0.125 mg/ml), with the various probes indicated across the top, as described under *Materials and Methods*. (*C*) The RRM-containing RNA-binding purified proteins U1A and PTB (0.050 μg) were incubated with the NP 5′ UTR probe as specificity controls (for details, see *B*). (*D*) HeLa S10 extract (200 μg, *Left*) or recombinant GRSF-1 (rGRSF-1) (0.050 μg, *Right*) was incubated with the NP 5′ UTR in the presence of increasing amounts of monoclonal anti-GRSF-1 for supershift analysis. The resulting RNA–protein complexes were resolved on a native polyacrylamide gel. The GRSF-1-RNA complex (open arrow on the left) and its antibody complex (solid arrow on the right) are indicated.

GRSF-1 as strongly as wild-type NP 5′ UTR (Fig. 2*B*, lane 4). We also examined the ability of the 5′ UTR of influenza viral NS mRNA to bind GRSF-1. Consistent with our previous results that the NS 5′ UTR also mediates viral mRNA-selective translation (20), NS 5′ UTR also formed a stable complex with GRSF-1, whereas mutant NS-B, containing a random 6-nt substitution in the region B, failed to bind (Fig. 2*B*, lanes 6 and 7). These data, taken together, suggest that both regions A and B of the NP and NS 5′ UTRs are necessary for binding to GRSF-1, but that region A alone is not sufficient to promote GRSF-1 binding. As an additional test of specificity, we analyzed the interaction between the NP 5′ UTR and two additional RRM containing RNA-binding proteins. In contrast to GRSF-1, neither the PTB nor the U1A proteins bound to the viral 5′ UTR (Fig. 2*C*). Finally, we validated that GRSF-1, present with multiple proteins within a crude HeLa cell lysate, also bound to the NP 5′ UTR by using supershift analysis and GRSF-1-specific mAb (Fig. 2*D*).

GRSF-1 Mediates Selective Translation of Influenza Viral NP 5′ UTR-Driven Templates. Having established binding of GRSF-1 to specific regions of the influenza viral 5′ UTRs, it was essential to test whether GRSF-1 played a role in the selective translation of influenza virus mRNAs. For this

analysis, we established a dual-luciferase reporter system that consisted of NP 5′ UTR-driven firefly (NP-LUC) and NP-A-driven sea pansy luciferase (NP-A-rLUC). Enzyme activity of these luciferases can be measured separately because of their different substrate requirements. This allowed us to examine whether GRSF-1 was able to preferentially enhance translation of an NP 5′ UTR-driven template over a mutant NP 5′ UTR-driven template in a single reaction. These experiments were performed by using non-micrococcal nuclease-treated, influenza virus-infected, HeLa cell cell-free extracts to best approximate events that would occur inside an influenza virus-infected cell. The mRNA templates were translated simultaneously in the absence or presence of GST-GRSF-1 fusion protein. Highly purified recombinant GRSF-1 (Fig. 3*A*) enhanced translation of NP-LUC mRNA more than 5-fold, but only slightly enhanced translation of the mutant NP-A-rLUC (Fig. 3*B*). The minor effects of translation of NP-A-rLUC mRNA by GRSF-1 could be explained by a weak interaction between GRSF-1 and the NP-A, which was detected in a gel-shift assay (Fig. 2*B*, lane 2). As a specificity control, a parallel experiment was performed by using two other cellular RRM-containing RNA-binding proteins, recombinant PTB fused to GST and purified U1A. As predicted from the binding data, neither GST-PTB nor U1A stimulated the translation of NP-LUC or NP-A-rLUC (Fig. 3*B*). Importantly, the stability of template mRNAs was not markedly effected by incubation with or without exogenous GST-GRSF-1 (Fig. 3*C*). These results provide evidence that GRSF-1 selectively enhances the translation of the wild-type but not region A mutant NP 5′ UTR-driven templates.

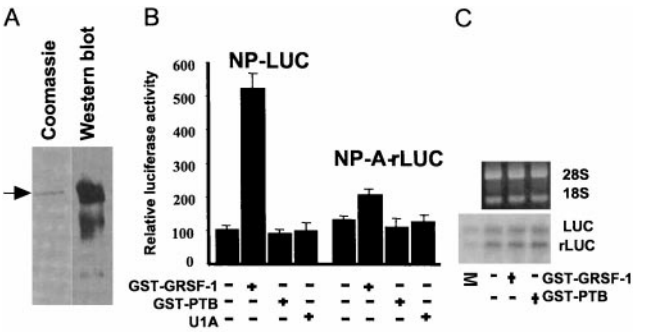

FIG. 3. GRSF-1 selectively enhances translation of the influenza viral NP 5′ UTR-driven template. (*A*) GST-GRSF-1 fusion proteins (0.200 μg) were visualized by Coomassie staining or Western blotting. The arrow indicates the intact GST-GRSF-1 fusion protein. (*B*) The NP 5′ UTR-driven (NP-LUC) and mutant NP 5′ UTR-driven (NP-A-rLUC) templates (0.125 μg each) were translated in a HeLa extract in the absence or the presence of GST-GRSF-1 (0.200 μg). GST-PTB fusion protein (0.200 μg) and U1A (0.200 μg) again were utilized as specificity controls. After 45 min at 30°C, translation products were assayed by using a Dual-Luciferase Reporter Assay System (Promega). Values are the mean ± SD of three experiments per group. A scintillation counter was used to measure luciferase activity. Counts per minute (cpm) were produced by calculating the square root of measured cpm minus background cpm. We arbitrarily assigned a value of 100 to the control NP-luciferase reaction that, in this case, represented an average value of 3,560 cpm of luciferase activity per μl of HeLa extract. Other relative luciferase activity values were calculated relative to this number. (*C*) GST-GRSF-1 does not affect stability of template RNAs. Aliquots of translation products in *B* were extracted with phenol and phenol/chloroform, and RNAs were fractionated by formaldehyde-agarose gel electrophoresis. After electrophoresis, gels were stained with ethidium bromide to visualize ribosomal RNAs (18S and 28S, *Upper*) for internal controls. Template RNAs, which had been radiolabeled with trace amounts of ^{32}P, then were visualized on x-ray film after gels were dried. LUC and rLUC indicate the firefly luciferase and the sea pansy luciferase RNA, respectively. M indicates mixture of an aliquot of the starting material of templates only.

The role of GRSF-1 in the selective translation of viral NP 5′ UTR-driven template was examined further in GRSF-1 immunodepletion/reconstitution experiments using influenza virus-infected HeLa cell extracts. Interestingly, HeLa cells appeared to express a complex series of isoforms of GRSF-1, which were detected by two different mAbs (24) (Fig. 4*A*). The second fastest migrating protein, which has a predicted molecular mass of approximately 48 kDa, comigrated with recombinant GRSF-1 expressed in bacteria. The identity of the different GRSF-1-related proteins are unknown but potentially may arise from posttranslational modification and/or differential splicing (24). Endogenous GRSF-1 proteins were depleted from HeLa cell extracts with protein A-agarose beads coated with IgG molecules specific to GRSF-1. As a control, HeLa cell extracts were treated under the same conditions with protein A-agarose beads coated with normal mouse IgG. Western blotting analysis indicated a nearly complete depletion of GRSF-1 proteins except for the smallest isoform (Fig. 4*A*). Subsequent passages over the GRSF-1 IgG column did not result in complete elimination of the smallest GRSF-1

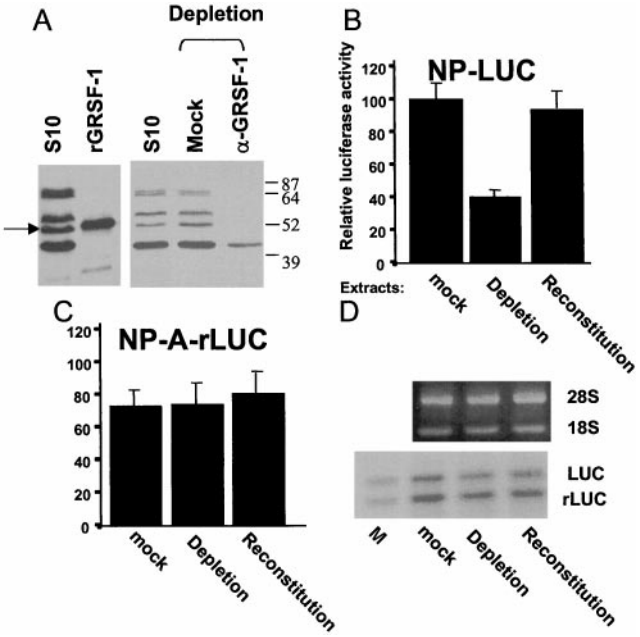

FIG. 4. Immunodepletion of GRSF-1 compromises translation of influenza virus 5′ UTR-driven chimeric mRNA translation, whereas GRSF-1 reconstitution restores mRNA translation. (*A*) HeLa S10 extracts (S10) were incubated with protein A-agarose beads coated with GRSF-1-specific IgG molecules (anti-GRSF-1) or with normal mouse IgG molecules (Mock) at 4°C for 4 hr. Depleted extracts then were centrifuged briefly, and the supernatant was examined by Western blotting. The GRSF-1 isoforms in the S10 starting material (100 μg) and the migration of the recombinant GRSF-1 (50 ng) are shown on the left. The GRSF-1 proteins patterned after depletion are shown on the right. (*B* and *C*) The mock-depleted, GRSF-1-depleted, or GRSF-1-reconstituted extracts were used for the cell-free translation of wild-type and mutant NP 5′ UTR-driven mRNA translation [NP-LUC (*B*) and NP-A-rLUC (*C*)]. For the GRSF-1-reconstituted extracts, 0.200 μg of GST-GRSF-1 fusion protein was added. After 45 min at 30°C, translation products were assayed by using a Dual-Luciferase Reporter Assay System (Promega). In this experiment, 100 was equivalent to an average of 2,340 cpm of luciferase activity per μl of HeLa extract. Mock, Depletion, or Reconstitution at the bottom indicates the mock-depleted, GRSF-1-depleted, or GRSF-1-reconstituted extract, respectively. Values are the mean ± SD of three experiments per group. (*D*) GST-GRSF-1 does not affect stability of template RNAs. Using aliquots (12 μl) of translation products (*B* and *C*), template RNA stability was tested as described in Fig. 3*C*. LUC and rLUC indicate the firefly luciferase and the sea pansy luciferase RNA, respectively. M indicates mixture of an aliquot of the starting-material templates.

species (data not shown). Each extract then was used for cell-free translation of NP (5′ UTR)-LUC and NP-A (5′ UTR)-rLUC in the absence or presence of recombinant GST-GRSF-1. Translation efficiency of the NP-LUC was reduced ≈60% in a GRSF-1-depleted extract compared with a mock-depleted extract (Fig. 4*B*). We were unable to completely eliminate NP 5′ UTR-driven translation through GRSF-1 depletion. It may be that other cellular or viral factors in the lysate can compensate for the lack of GRSF-1 in the extracts. It is also possible that the residual translation was mediated by the remaining GRSF-1 isoform. More critically, however, the translational inhibition resulting from immunodepletion was brought back to basal levels by the addition of GRSF-1. However, it is important to note that we did not succeed in enhancing translation above the original basal levels (e.g., compare with the 5-fold stimulation above basal levels shown in Fig. 3*B*). These data suggest that other essential factors may have been depleted along with GRSF-1 and that addition of GRSF-1 alone was not sufficient to cause stimulation beyond basal levels. Thus, we cannot rule out that other cellular factors also are required for specific viral mRNA translation. Significantly, however, neither depletion nor reconstitution of GRSF-1 affected translation efficiency of mutant NP-A-rLUC (Fig. 4*C*). As before, the differences in mRNA translation were not due to differences in mRNA levels (Fig. 4*D*).

RNA Oligonucleotide Competition Experiments Independently Demonstrate a Role for GRSF-1 in Selective Viral mRNA Translation. To obtain further evidence that GRSF-1 mediates selective translation of a NP 5′ UTR-driven template through their physical interaction, we performed RNA oligoribonucleotide-competition experiments. Sequestration of GRSF-1 (and other viral/cellular factors) in a HeLa cell extract by competitor 5′ UTR RNAs should affect the translation of the viral and cellular 5′ UTR-driven templates. We also reasoned that events in these "translationally compromised" extracts might represent even better the events occurring in an influenza virus-infected cell. We transcribed competitor RNAs representing the following 5′ UTRs: the cellular SEAP, viral NP wild type, viral mutant NP-A, and viral mutant NP-C RNAs (see Fig. 2*A*). We then compared the translation of the cellular-driven SEAP-5′ UTR-LUC and the viral-driven NP-5′ UTR-LUC chimeric mRNAs in the presence or absence of these RNA 5′ UTR "sense" transcripts. The translation of the SEAP-LUC mRNA was inhibited by excess amounts of the NP, NP-A, and SEAP 5′ UTR RNA competitors (Fig. 5*A*). One can speculate that all the RNA transcripts inhibited translation of the cellular 5′ UTR-driven mRNA because of a general sequestration of required translation factors. In contrast to SEAP-LUC translation, translation of the NP 5′ UTR-driven template (NP-LUC) was inhibited only by the NP and NP-C RNA transcripts but not by competitor NP-A or SEAP 5′ UTR RNAs (Fig. 5*B*). These data strongly suggest that GRSF-1 is required for efficient viral 5′ UTR-driven translation because only RNA competitors, such as the NP and NP-C 5′ UTRs (that sequester GRSF-1), are inhibitory.

If our hypothesis were correct, then addition of GRSF-1 to a translation extract, compromised by RNA transcripts that bind GRSF-1, should restore translation of NP-LUC to basal levels. On the other hand, GRSF-1 addition to extracts, whose translation was compromised by RNA transcripts that cannot bind or sequester GRSF-1, would have no effect on the cellular 5′ UTR SEAP-LUC translation. As predicted, although both NP-LUC and SEAP-LUC translation were inhibited by the presence of excess NP5′ UTR, GRSF-1 addition restored NP-LUC mRNA translation but could not restore SEAP-LUC mRNA translation (Fig. 5*C*). Collectively, these data provide additional evidence that GRSF-1 can discriminate between cellular and viral 5′ UTR chimeric mRNAs such that only the latter are up-regulated positively.

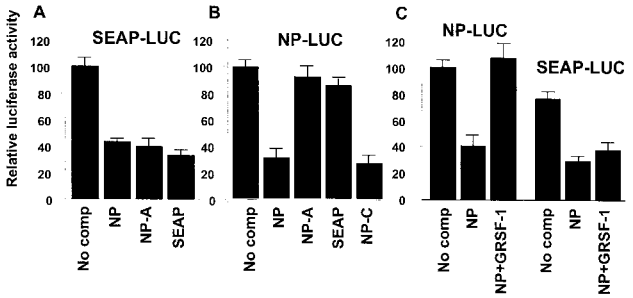

FIG. 5. RNA oligonucleotide-competition experiments confirm a GRSF-1-positive translational regulatory function. (*A* and *B*) RNA oligonucleotide-competition experiments. Template cellular 5′ UTR SEAP-LUC (*A*) or viral 5′ UTR NP-LUC (*B*) was translated in an influenza virus-infected HeLa cell extract in the absence or presence of various competitors as indicated below each bar. Competitor RNAs were added at 200 × molar ratio to template. The nucleotide sequences of competitors are shown in Fig. 2*A*. An arbitrary value of 100 represented 4,215 cpm SEAP-LUC of luciferase activity per μl of HeLa cell extract in *A* and 4,520 cpm of NP-LUC luciferase activity per μl of HeLa cell extract in *B*. (*C*) Effects of GRSF-1 reconstitution in RNA oligonucleotide-compromised extracts. Either template NP-LUC or SEAP-LUC was translated in a virus-infected HeLa extract in the absence (No comp) or presence of competitor NP 5′ UTRs (NP) or in the presence of competitor NP 5′ UTRs plus 0.200 μg of GST-GRSF-1 (NP+GRSF-1) as indicated below each bar. After 45 min at 30°C, translation products were assayed by using a Luciferase Assay System. A value of 100 represented 4,240 cpm of NP-LUC luciferase activity per μl of HeLa cell extract in *C*. Other values then were determined relative to this standard. Values throughout are the mean ± SD of three experiments per group.

In closing, it is important to point out that the mutant viral and cellular chimeric mRNAs were, at times, translated with similar translational efficiency to the wild-type viral mRNA chimera in the noncompromised, virus-infected extracts. It was only after depletion of GRSF-1 and possibly other factors, either by immunodepletion or RNA oligonucleotide addition, that more dramatic differences in translational efficiencies were observed. This suggests that our *in vitro* extracts, *per se*, did not mimic precisely what occurred in an influenza virus-infected cell unless a more naturally "competitive," factor-limiting environment was introduced. Under these conditions, only the wild-type viral-driven chimeric mRNAs were translated selectively.

DISCUSSION

A clue to the complex mechanisms underlying selective influenza virus mRNA translation first arose from our study in which we found that the 5′ UTR was necessary and sufficient to direct select viral mRNA translation (18, 20). We subsequently identified mainly unknown cellular proteins that bound to specific regions of the 5′ UTR of viral but not cellular mRNAs (21). Additional insights into the mechanisms of translational regulation came from studies that found that the influenza virus-induced host-cell shut-off might be due to the partial inactivation of eIF4E (28). Also relevant are reports that the specific stimulation of viral protein synthesis may be due to the virally encoded NS1 by as yet unknown mechanisms (29, 30). We now propose that the cellular RRM-containing RNA-binding protein, GRSF-1, interacts with specific sequences within the viral 5′ UTR to selectively promote viral mRNA translation. GRSF-1 was identified first by using a Northwestern cloning strategy and a G-rich RNA element as a probe (24). Based on sequence homologies to a *Dictyostelium* ribosomal large protein subunit, the investigators suggested that GRSF-1 might play a role in mRNA translation. Indeed, other RRM-containing proteins, including poly(A)-binding protein (PABP) (31–33), PTB (34–37), and the ELAV gene

product (38), all have been shown to play a role in the regulation of protein synthesis.

We do not yet know how GRSF-1 promotes selective mRNA translation. Recent work on another RRM-containing RNA-binding protein, PABP (31–33), has shown that, in yeast, PABP interacts with the eukaryotic initiation factor, eIF-4G, to mediate the ability of the poly(A) tail to stimulate translation *in vitro*. This association is not essential *in vivo* unless the function of eIF-4E is compromised. In a mammalian system, the Sonenberg laboratory (17) has found that PABP interacts with a novel eIF-4G homologue, PAIP, to stimulate mRNA translation. In a recent relevant report, Gallie found that hsp101 heat-shock protein enhanced cap-dependent translation of the tobacco mosaic virus 5′ UTR-driven luciferase RNA (39). This hsp 101-mediated enhancement required both eIF-3 and eIF-4G. It is tempting, therefore, to speculate that GRSF-1 also interacts with eukaryotic initiation factors to efficiently recruit ribosomes to viral mRNAs and stimulate mRNA translation. We also cannot rule out a possible translational regulatory role for other cellular RNA-binding proteins or, certainly, the viral NS1 protein, given that the latter interacts with the viral 5′ UTR (21) and appears to regulate viral mRNA translation (29, 30).

GRSF-1 does not function solely to stimulate the translation of influenza virus mRNAs. An untranslated region database search, UTRdb (40), identified several non-influenza virus mRNA 5′ UTRs that contain homology to the GRSF-1-binding region present on the NP 5′ UTR. The best match (13 of 14 nt) was to the 5′ UTR sequences of an isoform of the cellular transcription factor, NF-E2 (41). Intriguingly, the 5′ leaders of both hsp40 and hsp70 molecular chaperone mRNAs also contained homology to the GRSF-1-binding site. The latter takes on added significance in that we have demonstrated recently that both hsp40 and hsp70 are involved in the regulation of the $P58^{IPK}$/PKR regulatory pathway (42, 43). $P58^{IPK}$ is a cellular inhibitor of PKR that is activated by influenza virus to prevent excessive phosphorylation of eIF-2α and a global shutdown of protein synthesis. Hsp40 likely functions as a negative regulator of $P58^{IPK}$ whereas hsp70 forms a trimeric complex with both hsp40 and $P58^{IPK}$ and may contribute to the refolding and inactivation of PKR. It therefore would be advantageous if these chaperone mRNAs were translated efficiently during compromised cellular protein synthesis. It will be of considerable interest to directly determine whether GRSF-1 can regulate translation of these stress proteins and transcription factor in influenza virus-infected cells.

This work was supported by National Institutes of Health Grants GM-56434 (J.W.) and AI-22646, RR-00166, and AI-41629 (M.G.K.).

1. Mathews, M. B. (1996) in *Interactions Between Viruses and the Cellular Machinery for Protein Synthesis*, eds. Hershey, J., Mathews, M. B. & Sonenberg, N. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 505–548.
2. Ehrenfeld, E. (1996) in *Initiation of Translation by Picornavirus RNAs*, eds. Hershey, J., Mathews, M. B. & Sonenberg, N. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 549–574.
3. Hinnebusch, A. G. (1996) in *Translational Control of GCN4: Gene-Specific Regulation by Phosphorylation of eIF2*, eds. Hershey, J., Mathews, M. & Sonenberg, N. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 199–244.
4. Katze, M. G. (1996) in *Translational Control in Cells Infected with Influenza Virus and Reovirus*, eds. Hershey, J. W. B., Mathews, M. B. & Sonenberg, N. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 607–630.
5. Merrick, W. C. & Hershey, J. W. B. (1996) in *The Pathway and Mechanism of Eukaryotic Protein Synthesis*, eds. Hershey, J., Mathews, M. & Sonenberg, N. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 31–70.
6. Schneider, R. J. (1996) in *Adenovirus and Vaccinia Virus Translational Control*, eds. Hershey, J., Mathews, M. B. & Sonenberg, N. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 575–606.
7. Sonenberg, N. (1996) in *mRNA 5′ Cap-Binding Protein eIF4E and Control of Cell Growth*, eds. Hershey, J., Mathews, M. & Sonenberg, N. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 245–270.
8. Rouault, T. A., Klausner, R. D. & Harford, J. B. (1996) in *Translational Control of Ferritin,* eds. Hershey, J. W. B., Mathews, M. B. & Sonenberg, N. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 335–362.
9. Rouault, T. A., Hentze, M. W., Caughman, S. W., Harford, J. B. & Klausner, R. D. (1988) *Science* **241,** 1207–1210.
10. Walden, W. E., Daniels, M. S., Brown, P. H., Gaffield, L., Russell, D. A., Bielser, D., Bailey, L. C. & Thach, R. E. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9503–9507.
11. Belsham, G. J. & Sonenberg, N. (1996) *Microbiol. Rev.* **60,** 499–511.
12. Pelletier, J. & Sonenberg, N. (1988) *Nature (London)* **334,** 320–325.
13. Etchison, D., Milburn, S. C., Edery, I., Sonenberg, N. & Hershey, J. W. (1982) *J. Biol. Chem.* **257,** 14806–14810.
14. Jang, S. K., Pestova, T. V., Hellen, C. U. T., Witherell, G. W. & Wimmer, E. (1990) *Enzyme* **44,** 292–309.
15. Svitkin, Y. V., Meerovitch, K., Lee, H. S., Dholakia, J. N., Kenan, D. J., Agol, V. I. & Sonenberg, N. (1994) *J. Virol.* **68,** 1544–1550.
16. Gamarnik, A. V. & Andino, R. (1997) *RNA* **3,** 882–892.
17. Craig, A. W., Haghighat, A., Yu, A. T. & Sonenberg, N. (1998) *Nature (London)* **392,** 520–523.
18. Garfinkel, M. S. & Katze, M. G. (1992) *J. Biol. Chem.* **267,** 9383–9390.
19. Krug, R. M., Alonso-Caplen, F., Julkunen, I. & Katze, M. G. (1989) in *Expression and Replication of the Influenza Virus Genome*, ed. Krug, R. M. (Plenum, New York), Vol. **77,** 89–152.
20. Garfinkel, M. S. & Katze, M. G. (1993) *J. Biol. Chem.* **268,** 22223–22226.
21. Park, Y. W. & Katze, M. G. (1995) *J. Biol. Chem.* **270,** 28433–28439.
22. SenGupta, D. J., Zhang, B., Kraemer, B., Pochart, P., Fields, S. & Wickens, M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 8496–8501.
23. Finley, R. L. J. & Brent, R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 12980–12984.
24. Qian, Z. & Wilusz, J. (1994) *Nucleic Acids Res.* **22,** 2334–2343.
25. Katze, M. G., Chen, Y. T. & Krug, R. M. (1984) *Cell* **37,** 483–490.
26. Molla, A., Paul, A. V. & Wimmer, E. (1991) *Science* **254,** 1647–1651.
27. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 300–310.
28. Feigenblum, D. & Schneider, R. J. (1993) *J. Virol.* **67,** 3027–3035.
29. Enami, K., Sato, T. A., Nakada, S. & Enami, M. (1994) *J. Virol.* **68,** 1432–1437.
30. de la Luna, S., Fortes, P., Beloso, A. & Ortín, J. (1995) *J. Virol.* **69,** 2427–2433.
31. Tarun, S. Z. J. & Sachs, A. B. (1995) *Genes Dev.* **9,** 2997–3007.
32. Tarun, S. Z. J. & Sachs, A. B. (1996) *EMBO J.* **15,** 7168–7177.
33. Tarun, S. Z. J., Wells, S. E., Deardorff, J. A. & Sachs, A. B. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 9046–9051.
34. Borovjagin, A., Pestova, T. & Shatsky, I. (1994) *FEBS Lett.* **351,** 299–302.
35. Ali, N. & Siddiqui, A. (1995) *J. Virol.* **69,** 6367–6375.
36. Kaminski, A., Hunt, S. L., Patton, J. G. & Jackson, R. J. (1995) *RNA* **1,** 924–938.
37. Niepmann, M., Petersen, A., Meyer, K. & Beck, E. (1997) *J. Virol.* **71,** 8330–8339.
38. Antic, D. & Keene, J. D. (1998) *J. Cell Sci.* **111,** 183–197.
39. Wells, D. R., Tanguay, R. L., Le, H. & Gallie, D. R. (1998) *Genes Dev.* **12,** 3236–3251.
40. Dalphin, M. E., Brown, C. M., Stockwell, P. A. & Tate, W. P. (1998) *Nucleic Acids Res.* **26,** 335–337.
41. Pischedda, C., Cocco, S., Melis, A., Marini, M. G., Kan, Y. W., Cao, A. & Moi, P. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3511–3515.
42. Melville, M. W., Hansen, W. J., Freeman, B. C., Welch, W. J. & Katze, M. G. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 97–102.
43. Melville, M. W., Tan, S.-L., Wambach, M., Song, J., Morimoto, R. I. & Katze, M. G (1999) *J. Biol. Chem.* **274,** 3797–3803.