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HnRNP A1 Alters the Structure of a Conserved Enterovirus IRES Domain to Stimulate Viral Translation

Michele Tolbert¹, Christopher E. Morgan¹, Marvin Pollum¹, Carlos E. Crespo-Hernández¹, Mei-Ling Li², Gary Brewer², and Blanton S. Tolbert¹

¹Department of Chemistry, Case Western Reserve University, Cleveland, OH

²Department of Biochemistry and Molecular Biology, Rutgers Robert Wood Johnson Medical School, Piscataway New Jersey

Abstract

Enteroviruses use a type I IRES structure to facilitate protein synthesis and promote genome replication. Type I IRES elements require auxiliary host proteins to organize RNA structure for 40S ribosomal subunit assembly. Heterogeneous nuclear ribonucleoprotein A1 stimulates Enterovirus 71 (EV71) translation in part through specific interactions with its stem loop II (SLII) IRES domain. Here, we determined a conjoined NMR-SAXS structure of the EV71 SLII domain and a mutant that significantly attenuates viral replication by abrogating hnRNP A1 interactions. Native SLII adopts a locally compact structure wherein stacking interactions in a conserved 5'-AUAGC-3' bulge preorganize the adjacent helices at nearly orthogonal orientations. Mutating the bulge sequence to 5'-ACCCC-3' ablates base stacking in the loop and globally reorients the SLII structure. Biophysical titrations reveal that the 5'-AUAGC-3' bulge undergoes a conformational change to assemble a functional hnRNP A1-RNA complex. Importantly, IRES mutations that delete the bulge impair viral translation and completely inhibit replication. Thus, this work provides key details into how an EV71 IRES structure adapts to hijack a cellular protein and it suggests the SLII domain is a potential target for antiviral therapy.

Graphical abstract

Correspondence: Blanton S. Tolbert, Phone: 216-368-0605, bst18@case.edu.

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Accession numbers

Atomic coordinates, chemical shifts and restraint files for SLII²²³¹ and SLII^{CCC} have been deposited in the RCSB PDB under accession numbers 5V16 and 5V17, respectively.

Supplemental Information

Supplementary data are available online.

Introduction

Non-polio human enteroviruses (EV) infect millions of people worldwide and in the US alone, infections account for tens of thousands of hospitalizations per year [1–3]. In most cases, EV manifest as mild flu-like symptoms; however, persistent infection in children can lead to severe cardiovascular and neurological complications, paralysis, respiratory failure and even death [4, 5]. Recent global outbreaks of Enterovirus 71 and D68 have reinvigorated interest in understanding molecular mechanisms of EV pathogenesis. Currently, there is no broad-spectrum vaccine or antivirals to impair viral progression. This is of particular concern because EV can persist in the central nervous system and other organs for extended periods.

Enteroviruses belong to the *picornaviridae* family, which includes poliovirus, coxsackievirus and hepatitis A. Like all picornaviruses, the genomes of EV consist of a positive-sense RNA approximately 7,500 nucleotides long that carries out a division of labor to facilitate protein synthesis and genome replication [6, 7]. The 5' untranslated region (5' UTR) is the most phylogenetically conserved portion of the EV genome; it folds into a structured RNA element composed of six stem loops (Fig 1A). The first stem loop (SLI) directs RNA strand synthesis whereas stem loops II–VI promote cap-independent translation by internally recruiting the 40S ribosomal subunit via a type I IRES [6]. Efficient recruitment of 40S requires canonical eukaryotic initiation factors (eIFs) and host RNA binding proteins collectively known as IRES trans-acting factors (ITAFs).

There is a paucity of structural and mechanistic details as to how ITAFs associate with type I IRES elements to stimulate viral translation. Most of what is known derives from *in vitro* reconstitution studies whereby binding sites of eIFs and the ITAFs, PTB1 and PCBP2, have been mapped using chemoenzymatic methods [8–11]. From these studies, a general view of how these factors promote internal ribosome assembly on type I IRES structures has been

outlined. Intriguingly, there is disagreement between the ITAFs required for *in vitro* translation initiation and those that contribute to viral replication by affecting protein synthesis [12–15]. These discrepancies likely reflect the complexities of viral translation within the cellular environment as well as other functions carried out by the IRES [15, 16].

For EV71, proteomic analysis revealed more than a dozen ITAFs that interact with its 5′-UTR of which at least four (hnRNP A1, AUF1, HuR, and Ago2) bind directly to the SLII IRES domain to either stimulate or repress translation [13, 17–20]. Of the ITAFs that bind SLII, members of the hnRNP A/B family are critical for viral translation and replication since siRNA depletion of hnRNP A1 and its homolog hnRNP A2 significantly attenuates EV71 titers [13]. Moreover, we previously demonstrated that the N-terminal domain (UP1 for unwinding protein 1) of hnRNP A1 interacts specifically with SLII at a conserved bulge and hairpin loop. Mutating the 5′-AUAGC-3′ bulge sequence to 5′-ACCCC-3′ disrupts an apparent 2:1 binding *in vitro*, reduces translation efficiency *in vivo*, and suppresses EV71 replication by ~5 log units [20]. Thus, these collective results clearly indicate that the interaction between hnRNP A1 and SLII is critical for EV71 replication despite it not being classified as a common ITAF [8].

In this study, we provide new details into the physicochemical determinants by which hnRNP A1 interacts with the EV71 iReS by determining the solution structures of its native SLII domain and the SLII 5′-ACCCC-3′ bulge mutant using a conjoined NMR and small angle x-ray scattering (SAXS) approach. Comparison of the structures shows that the 5′-AUAGC-3′ bulge determines the overall topology of unbound SLII wherein the bulge residues adopt an internally stacked conformation. Using multiple biophysical methods, we demonstrate that hnRNP A1 alters the conformation of the bulge to assemble a complex that correlates with viral translation. Thus, our data provides key mechanistic insights into how the EV71 IRES element commandeers hnRNP A1 and it indicates the SLII bulge is a promising candidate for antiviral therapy.

Results

Solution structure of the phylogenetically conserved EV SLII IRES domain

Using full genome alignments of all available EV71 sequences, we previously observed that the SLII IRES domain folds into a conserved structure composed of a 5-nt bulge and a 6-nt hairpin loop [20]. Here, we expanded the phylogenetic comparison to include all human enterovirus strains, for which complete genomic sequences (n=88) are available. Analysis of the alignments reveals general features of SLII that have been conserved throughout the evolution of enteroviruses. Notably, the bulge and hairpin loop residues, which are not under selective pressures to maintain Watson-Crick base pairs, show a high level of sequence conservation (Fig 1A). The consensus motif of the hairpin loop is NA/YNCCA (where N is any nt and Y is U or C), which agrees with earlier alignments using only EV71 strains [20]. Despite some variability in the size of the bulge, a clear AYAGN signature is observed. Consistent with its phylogenetic conservation, mutations within the central 5′-UAG-3′ motif of EV71 SLII impair viral replication [20]. Thus, SLII is a functional unit within the EV IRES that positions conserved residues in unpaired elements of secondary structure.

To investigate if SLII adopts a defined tertiary fold, we determined the solution NMR structure of a construct derived from Ev71 strain 2231 (SLII²²³¹) along with a bulge mutant that replaces the 5'-UAG-3' motif with 5'-CCC-3' (SLII^{CCC}). Figure 1C shows the ¹H-¹H NOESY spectrum of SLII²²³¹ prepared with selective ²H-rNTPs (see Experimental section). Analysis of the spectrum shows NOE patterns throughout both helices that are consistent with A-form geometry. Notably, sequential and long-range (H2-H1') NOEs are observed for residues located in the bulge and hairpin loops, indicating that these elements fold into stable structures in solution (Figs 1C, S1 and S2). Identical NOE cross peaks are detected for the helices and hairpin loop of SLII^{CCC}; however, a clear break in the sequential NOE pattern occurs in the central C135 and C136 dinucleotide of the bulge (Fig S3). Collectively, the NOESY data indicate that native SLII²²³¹ adopts a well-defined structure with stacking interactions in the bulge and hairpin loops. Some of the bulge interactions are disrupted when the central 5'-UAG-3' motif is mutated to 5'-CCC-3', however.

Structures of SLII²²³¹ and SLII^{CCC} were determined using a conjoined NMR-SAXS approach (Figs 2 and 3). Table S1 summarizes the experimental restraints used during structure refinement along with overall quality statistics. SLII²²³¹ and SLII^{CCC} structures are well determined by the experimental data, with overall rmsd values of 0.5 Å and 1.2 Å, respectively. The predicted helical segments of SLII²²³¹ and SLII^{CCC} fold into approximate A-form geometries with Watson-Crick base pairs adjacent to the bulge and hairpin loops. The hairpin loop in both RNAs adopt identical structures as evidenced by the small difference in rmsd (0.14 Å), indicating the bulge mutation does not affect the more distal structural element. Interestingly, the hairpin adopts a well-defined structure wherein A148 and C149 stack on the 5' side, prior to a turn between residues C149 and A150. Residues 150–153 continue to stack along the 3' side onto the adjacent C147:G154 base pair. While no experimental hydrogen bonds were observed for the hairpin loop, NOEs are detected between A148H2–C149H1' and A153H2–G154H1'. Similar NOE interactions were observed for 5'-AAUCCA-3' and 5'-AAACCA-3' apical loops of SLII domains derived from poliovirus [21]. The NOEs lead to placement of the Watson-Crick edge of A148 next to the Watson-Crick edge of A153.

In contrast to the hairpin loop, the bulge of SLII²²³¹ and SLII^{CCC} form very different structures (Fig 4). The central AG dinucleotide in SLII²²³¹ stack inward with both nucleobases adopting the *syn* conformation as evidenced by intense H8-H1' NOE cross peaks and a downfield C8 chemical shift for G137 (Fig S4). The inward-stacked conformation of the AG dinucleotide does not readily appear to be stabilized by canonical hydrogen bonding interactions. Residues flanking the central AG dinucleotide also participate in base stacking interactions. The SLII²²³¹ bulge is compact, which leads to an approximate 91.5° ± 3.6° bend angle between the adjacent helices (Fig 4). By comparison, the bulge of SLII^{CCC} folds into a more open conformation wherein residues C135–C136 do not stack. The more open bulge results in the backbone taking an extended topology, leading to a wider angle (99.6° ± 3.2°) between the adjacent helices (Fig 4). In sum, SLII²²³¹ adopts a well-defined structure wherein tertiary interactions within the conserved 5'-AUAGC-3' bulge define its overall topology. Mutating the central 5'-UAG-3' motif to 5'-CCC-3' leads to a topological reorientation of the adjacent helices and an elongation of the overall

molecule as determined by a comparison of the respective pair distance distribution plots (Fig S5).

Complete thermodynamic profile of the hnRNP A1-SLII²²³¹ interaction

We previously demonstrated that UP1 interacts with SLII²²³¹ to form an apparent 2:1 complex [20]. Mutating the bulge 5'-UAG-3' motif to 5'-CCC-3' results in a 1:1 complex that correlates with an inhibition of viral replication. Given the functional importance of the SLII²²³¹ bulge loop, we endeavored to characterize the complete thermodynamic signature (ΔG , ΔH , $\Delta T \Delta S$ and ΔC_p) of its interaction with hnRNP A1 so as to gain insights into the energetic contributions to specificity. Figure 5A shows calorimetric titrations of UP1 into SLII²²³¹ carried out from 283 K to 293 K. The processed isotherms indicate UP1 stably binds SLII²²³¹ at two independent sites over the temperature range examined; however, the extracted thermodynamic parameters for the low affinity site were less reliable under the conditions used for the titration therefore they are not discussed further. Binding of UP1 to the high-affinity site (bulge loop) is driven by a large favorable change in total enthalpy and opposed entropically (Table 1). The binding event shows that ΔH decreases linearly with an increase in temperature and that this enthalpic temperature dependence is compensated by an increase in $-\Delta T \Delta S$, resulting in the ΔG of binding being nearly temperature independent (Fig 5B). Such thermodynamic signatures result from negative changes in heat capacity (ΔC_p). Indeed, the magnitude of ΔC_p for UP1 binding to the bulge loop is $-1044 \pm 21 \text{ cal mol}^{-1} \text{ K}^{-1}$. By comparison, a recent thermodynamic study using the isolated RRM1 domain of hnRNP A1 titrated with a 5'-UUAGGU-3' oligo over a 283–308 K temperature range reports a ΔC_p value of only $-130 \pm 27 \text{ cal mol}^{-1} \text{ K}^{-1}$ [22]. The significant difference in the observed ΔC_p values measured for the minimal RRM1-(5'-UUAGGU-3') system and the larger UP1-SLII²²³¹ complex likely represent distinct modes of molecular recognition whereby the folded SLII²²³¹ structure and RRM2 make additional contributions to binding not captured in model systems. It is nontrivial to interpret the complete molecular origins of ΔC_p without a high-resolution structure of the UP1-SLII²²³¹ complex; however, a general consensus from protein-DNA studies is that a large negative ΔC_p , with reported ranges from -360 to $-950 \text{ cal mol}^{-1} \text{ K}^{-1}$, is a thermodynamic signature for specific biomolecular recognition [23, 24]. By analogy, we therefore conclude that the thermodynamic data provides a comprehensive description of the energetic contributions to specific hnRNP A1-SLII²²³¹ recognition, wherein the bulge loop represents the high-affinity and primary binding surface. A thorough analysis of the molecular origins of the negative ΔC_p values measured here awaits a high-resolution structure of the UP1-SLII²²³¹ complex.

The SLII bulge structure is necessary for EV71 replication

Since the SLII bulge is a conserved EV IRES structural element (Fig 1), we endeavored to investigate its functional contribution by preparing bicistronic luciferase reporter plasmids that contain the native 5'UTR or a mutant wherein the SLII²²³¹ bulge is deleted. The plasmids were used to *in vitro* transcribe bicistronic RNAs, RLuc-(EV71)5'UTR-FLuc, which were transfected into HeLa cells. The bicistronic reporter RNAs contain the EV71 5'UTR flanked by the Renilla luciferase (RLuc) and Firefly luciferase (FLuc) open reading frames (Fig 5C). Translation of RLuc is cap-dependent, whereas translation of FLuc is driven by the EV71 IRES. We also prepared a bicistronic transcript with the EV71 IRES in

the antisense direction (AS), which was used as a negative control in the assays. Figure 5C shows that the EV71 IRES activity was reduced significantly (>5 fold) in cells transfected with the bicistronic reporter transcript wherein the SLII²²³¹ bulge is deleted, indicating the bulge structure is required for viral translation. By comparison, cells transfected with the bicistronic reporter containing the native IRES showed comparable levels of RLuc and FLuc activity, as expected. Also as expected, FLuc activity was not detected with the IRES in the antisense (AS) orientation; this indicates that synthesis of FLuc is solely IRES-dependent.

To determine if the bulge structure is a key determinant in viral replication, the SLII²²³¹ bulge deletion was introduced into the infectious clone of EV71 and the full-length viral RNA was transfected into Rhabdomyosarcoma (RD) cells. RNA from the wild-type infectious clone served as a positive control. Virus released into medium was harvested 2 days after transfection and the titer was determined by plaque assay on Vero cells. The titer of native virus is about 10⁶ pfu/ml; however, there was no plaque formation on cells transfected with viral RNA harboring the SLII²²³¹ bulge deletion (Fig S6). This indicates that deletion of the bulge is lethal to the virus. Collectively, these results confirm that the SLII²²³¹ bulge structure is a critical determinant for virus translation and replication.

Binding of hnRNP A1 to SLII²²³¹ changes the bulge conformation

To gain mechanistic insights into how hnRNP A1 recognizes the SLII²²³¹ bulge, we performed a series of NMR, fluorescence, and SAXS experiments with the UP1 domain. Since UP1 binds two independent sites on SLII²²³¹, NMR and SAXS experiments were performed below stoichiometric ratios needed to saturate the second lower affinity site. Figure 6A shows a TROSY-HSQC titration (C8–H8 region) of UP1 into a ¹³C(A)-labeled SLII²²³¹ construct. Correlation peaks for A136, A157, and A159 of SLII²²³¹ completely disappear in the bound state, whereas the remaining adenosines are only slightly perturbed. These observations indicate that the primary UP1 binding interface localizes to the bulge (A136) and in the vicinity of the upper helix (A157 and A159, Fig S7). Of note, the intensity of the correlation peaks for A157 and A159 in the unbound state are considerably weaker than A136, which indicates that binding of UP1 leads to a significantly larger chemical shift perturbation of A136.

As an independent probe to map the UP1 binding site, we next performed steady state fluorescence titrations using a SLII²²³¹ construct wherein A136 was replaced with 2-aminopurine (2AP-SLII²²³¹). Comparison of the ¹H imino chemical shifts of 2AP-SLII²²³¹ and unmodified SLII²²³¹ verify that the modification does not alter the global structure (not shown). Figure 6B shows that the fluorescence intensity of 2AP-136 increases significantly (>5 fold) as a function of UP1 concentration. The increase in 2AP fluorescence is consistent with a change in the bulge conformation wherein A136 goes from a stacked environment to one that is more exposed. As a control, we also replaced A157 with 2AP and repeated the titration. A157 was selected as a control because it is located distal to the bulge in an uninterrupted helix and substitution of A157 with 2AP should maintain the AU base pair, albeit with a slightly different conformation [25]. At UP1 concentrations below that required to fully saturate the bulge binding site, the fluorescence intensity of 2AP-157 only increased by ~2 fold; however, a steep increase in 2AP-157 fluorescence intensity is observed as UP1

binds to its second lower affinity site on SLII²²³¹ (Fig S8). These observations indicate that UP1 changes the structure of the SLII²²³¹ bulge loop and likely destabilize the upper helix. However with respect to the latter point, it has been shown that 2AP substitution into DNA duplexes slightly perturbs local conformation and this effect can propagate to non-nearest neighboring base pairs [25, 26].

To visualize how UP1 might interact with the SLII²²³¹ bulge, we determined a structural model using a combined experimental and computational approach. Models of the complex were generated using the docking program HADDOCK[27, 28] and scored against experimental SAXS profiles. RNA restraints for modeling were obtained from the NMR and fluorescence titrations, whereas the binding surface on UP1 was modeled according to the crystal structure of the protein in complex with a 5'-AGU-3' oligomer[29]. The modeled binding mode also agrees with recent biophysical and high-throughput sequencing equilibrium (HTS-EQ) binding data that shows RNA targets compete for the same surface on UP1[30]. Briefly, we calculated 1000 rigid body models of the UP1-SLII²²³¹ complex and selected the 200 most favorable models for semi-flexible water refinement. We next filtered the models based on their mutual agreement with SAXS data and HADDOCK scores. The selected models were then subjected to a final refinement in AMBER[31] (see materials and methods) that included restraints to allow A136 and G137 to rotate out of the bulge and dock into the UP1 nucleobase pocket as observed in the crystal structure[29]. Application of these restraints is consistent with the NMR and fluorescence data, which show that the conformational dynamics of the bulge changes upon binding. AMBER simulations were performed for 20 ns with coordinates written every 10 ps, resulting in 2,000 unique models. Final filtering against the SAXS curve resulted in three models that gave the best agreement ($\chi^2 < 1.5$) with the experimental scattering data (Fig 6C).

In the models generated, UP1 docks onto the bulge loop through its RRM1 domain and inter-RRM linker (Fig 6D). Consistent with UP1 docking onto SLII²²³¹, a clear difference in the pair distance distribution plot was detected for the complex compared to unbound SLII²²³¹, leading to extra density in the molecular reconstruction of the UP1-SLII²²³¹ complex (Fig S9). Analysis of the final models show comparable binding sites on SLII²²³¹; however, UP1 engages the bulge through multiple orientations, hinting at a degree of structural plasticity in how UP1 orients relative to the bulge (Fig 6D). In all cases, the models reveal the β 23 loop of RRM1 makes contacts with the upper helix, either through its major groove or the phosphodiester backbone. This interaction likely explains the chemical shift perturbations observed for A157 and A159, which are part of the upper helix. Collectively, our biophysical results and HADDOCK-SAXS models suggest that UP1 binds site specifically to the SLII²²³¹ bulge to alter its conformation.

SLII²²³¹ bulge conformational dynamics revealed by ultrafast transient absorption spectroscopy

To explore if the bulge undergoes rapidly interconverting conformations in the free state and if UP1 alters the dynamics of these conformations, we carried out ultrafast transient absorption experiments on free 2AP-SLII²²³¹ and when bound to UP1. Ultrafast transient absorption reports on dynamical processes that occur on a fs-ns timescale and as such allow

correlations between structure and population dynamics. The quenching of the excited singlet (S_1) state population in 2AP is widely recognized to occur by electron transfer when 2AP is stacked with another nucleic acid base,[32, 33] and the photophysical aspects of the charge transfer mechanism, as well as the origin of the multiphasic decay lifetimes, have been discussed in detail elsewhere[34–38]. Of importance to this work, the transient absorption signal reports on the distance-dependent charge recombination dynamics that follows the charge transfer quenching event between the 2AP and its adjacent nucleobases[37]. Moreover, the transient absorption technique has been shown to be a valuable tool for probing conformational distributions in RNA-protein complexes[36].

Figure 7 shows that the transient absorption signal in free 2AP-SLII²²³¹ decays in three different time scales. Approximately 68% of the initial population decays in 510 fs (τ_1), whereas about 26% and 7% of the residual population decays in 17 ps (τ_2) and >3 ns (τ_3), respectively (Table S2). We believe that the longer component is due to a small fraction of misfolded RNA and in our fitting analyses the lifetime of this species was fixed to 10 ns (see materials and methods), a value consistent with that of isolated 2AP nucleoside in aqueous solution[39]. The distribution of lifetimes extracted from the transient decay signals may be understood by invoking a basic distance-dependent charge recombination model[38, 40] according to which 2AP in the bulge undergoes charge recombination with the closest nucleobase at a rate that depends exponentially on their mutual separation. Conformations in which 2AP and the adjacent nucleobases are oriented closer together undergo hundreds of femtoseconds charge recombination (τ_1), whereas those oriented in a way that increase the interbase distance undergo intermediate, picosecond dynamics (τ_2). Finally, those conformations in which the interbase distance between the 2AP and the nucleobases is longer than the critical distance for charge recombination (assumed to be ~ 10 Å), exhibit similar nanosecond dynamics as the free 2AP nucleoside in solution (τ_3). Therefore, the closely stacked structures give rise to the ultrafast decay lifetime, whereas the picosecond lifetime represents distributions of 2AP conformations that experience on average longer interbase distances.

Interestingly, the addition of UP1 to 2AP-SLII²²³¹ with increasing molar ratios (1:0.2 and 1:0.5) also results in three different population distributions; however, only the magnitude of the second lifetime increases with addition of UP1, the first and last lifetimes remain unchanged within the experimental uncertainty of the measurement (Table S2). The magnitude of the second lifetime correlates linearly with the increase in the steady-state fluorescence intensity observed upon addition of equivalent amounts of UP1 (Fig S10). This correlation can be understood as a UP1 induced increase in the interbase distance between 2AP and the adjacent nucleobases, which proportionately increases the magnitude of the charge recombination lifetime and decreases the charge quenching that occurs in 2AP steady-state fluorescence. Thus, our collective results from ultrafast transient absorption spectroscopy suggest the bulge loop samples, on average, two stacked conformations with different lifetimes (τ_1 and τ_2) and that UP1 preferentially binds the less stacked conformer (τ_2).

Discussion

Given their limited coding capacity, viruses use multiple strategies to subvert host factors. One such strategy, executed by some RNA viruses, is to use conserved IRES elements to bypass cap-dependent translation. IRES elements are grouped into four types that depend on the degree to which they require auxiliary host proteins to internally recruit the ribosome [41, 42]. Type I IRES elements are known to associate with multiple host RNA binding proteins; however, in most cases the mechanistic outcomes of those interactions are poorly understood. The IRES element of EV71 interacts with more than a dozen RNA binding proteins that either stimulate or repress ribosome recruitment [17]. Functional studies have demonstrated that its SLII IRES domain alone binds to at least four host proteins and a small viral derived RNA, indicating SLII is a central hub in coordinating virological functions.

In this study, we endeavored to understand the physicochemical basis by which hnRNP A1 associates with the SLII²²³¹ IRES domain. To that end, we solved the high-resolution structure of native SLII²²³¹ and a mutant form that inhibits viral replication by disrupting a critical interaction with hnRNP A1. We further characterized the hnRNP A1-SLII²²³¹ complex through detailed biophysical studies. Unbound SLII²²³¹ adopts a compact and bent shape in solution, wherein the high-affinity hnRNP A1 binding site (bulge) exists in an internally stacked conformation. In this geometry, the bulge organizes the adjacent helices at approximate orthogonal orientations. Mutating the central 5'-UAG-3' bulge motif to 5'-CCC-3' ablates the stacking interactions, which in turn leads to a more open and elongated structure while preserving the conformation of the hairpin loop.

Interestingly, domain II of the HCV IRES contains a 5-nt 5'-AACUA-3' bulge that adopts a similar internally stacked structure [43, 44]. Unlike the EV71 IRES, the HCV IRES directly recruits the 40S subunit and a recent high-resolution structure reveals the bulge orients the apical loop of domain II so that it specifically contacts ribosomal proteins uS7 and uS11 located in the E site [43]. Moreover, small-molecule inhibitors of HCV impair translation efficiency by binding directly to the 5'-AACUA-3' bulge, which in turn induces a change in the global conformation of domain II [45].

By comparison, the internally stacked conformation of the SLII²²³¹ bulge indicates that it must undergo a conformational change to facilitate hnRNP A1 assembly. Evidence for the bulge conformational change manifests in the UP1-SLII²²³¹ thermodynamic profile, whereby a large negative C_p (-1044 ± 21 cal Mol⁻¹ K) is observed. Large negative heat capacity changes have been documented for other protein-NA systems wherein the formation of adaptive surfaces that facilitate stereochemical complementarity is a physicochemical hallmark [23]. To achieve surface complementarity, the SLII²²³¹ bulge adapts its conformation to allow the central AG dinucleotide to fit inside the UP1 nucleobase pocket [29]. At present, we cannot distinguish between an induced fit versus a conformational capture mechanism; however, the steady state fluorescence titrations with 2AP-SLII²²³¹ shows that the bulge rearranges in response to UP1. Results from the ultrafast transient absorption experiments indicate that the conformational dynamics of the bulge are complicated, wherein the stacking environment of A136 is heterogeneous. Binding of UP1 appears to alter the conformational landscape without shifting the distribution of existing

conformers; instead the protein selectively influences the conformer that contributes to the longer ps (τ_2) component. A similar phenomenon was recently observed for metal-ion binding to the Prohead RNA loop whereby it was determined that binding occurs via an induced fit mechanism between dynamic ensembles [46]. In the case of UP1-SLII²²³¹, additional studies are needed to understand the mechanism by which UP1 gains access to the bulge. Nevertheless, the current data shows that the SLII²²³¹ bulge adopts different structures in its free and UP1 bound states.

The structural models of the UP1-SLII²²³¹ complex offer some insights into the overall shape complementarity of the UP1-SLII²²³¹ complex. Consistent with the large favorable change in enthalpy ($\Delta H = -50 \text{ kcal mol}^{-1}$ at 298 K) that signifies the high-affinity binding event, UP1 docks onto the bulge via an extended interface. This interface provides multiple contact points for favorable, non-covalent interactions. Along those lines, the models suggest the $\beta 23$ loop of RRM1 contributes to binding affinity since it is positioned to interact with the upper helix either through the major groove or the phosphodiester backbone. While definitive evidence of this interaction and others awaits a high-resolution structure, the models provide an excellent opportunity to further explore structure-function relationships.

Biological Implications

The mechanisms by which EV71 uses to initiate translation via its type I IRES remain enigmatic. Biochemical reconstitution experiments show that a single ITAF, PCPB2, along with several eIFs are necessary and sufficient to internally recruit the ribosome [8]; however, a growing body of cellular evidence indicate that efficient viral translation and replication require multiple host RNA binding proteins, executing their functions through interactions with the viral 5'-UTR [13, 18, 19, 47–49]. It is important to not overlook these discrepancies but instead attempt to better characterize 5'-UTR RNA elements and protein-IRES interactions that exhibit virological function. With the emerging knowledge, the molecular mechanisms that contribute to the various functions of the viral 5'-UTR will be revealed.

Stem loop II is a critical domain in enteroviruses since mutations within conserved sequences impair viral replication whereas others associate with virulence [20, 50–52]. The 3D NMR structure of isolated SLII²²³¹ demonstrates that it folds into a stable stem loop consistent with phylogenetic analysis [20]. As part of an ongoing study, we have also confirmed by DMS-MaPseq [53] that SLII²²³¹ adopts an identical secondary structure within the context of a larger EV71 IRES fragment (unpublished results) and a related structure was chemically mapped for domain II of Cocksackievirus B3 [54]. These observations suggests that at least part of SLII²²³¹ is not involved in long-range base pair interactions that would otherwise preclude its binding to cellular or viral factors. Instead, SLII²²³¹ contains local internal structure within its 5-nt bulge loop that sequesters its core 5'-UAG-3' motif. Phylogenetic analysis indicates the bulge is under evolutionary pressure to maintain its size and sequence (Fig 1). Thus, the SLII²²³¹ bulge loop is a conserved structural element that is necessary for viral translation and replication (Fig 5C and S6).

As mentioned above, SLII²²³¹ acts as a central hub by interacting with several host proteins and vsRNA1 to differentially modulate viral translation. Binding of hnRNP A1 to SLII²²³¹

stimulates translation but this interaction is competed by AUF1, which acts to repress translation [13, 19, 20]. Crystal structures and comprehensive binding studies indicate that hnRNP A1 specifically recognizes an exposed 5'-UAG-3' motif [29, 30, 55]. Given the requirement to have the bulge 5'-UAG-3' motif available for hnRNP A1 recognition, why then would the unbound SLII²²³¹ structure predominantly populate an internally stacked conformation? The answer to this question may very well pertain to the central role that SLII²²³¹ plays in coordinating virological functions. If the EV71 5'-UTR is considered to be a nanostructure with interconnected domains, then a requirement that cognate partners induce a conformational change in the structures would impart a level of regulation to ensure the correct biological signals are activated [56]. While the exact set of ITAFS and IRES elements involved in ribosome assembly remains unsettled, this work has shown that hnRNP A1 induces a change in the structure of SLII²²³¹ to simulate EV71 translation. Thus, the stage is set to more quantitatively characterize protein-IRES interactions that contribute to EV71 replication.

Materials and Methods

RNA Preparation

SLII²²³¹ and SLII^{CCC} were prepared by *in vitro* transcription using recombinant T7 RNA polymerase that was overexpressed and purified from BL21 (DE3) cells. Synthetic DNA templates, corresponding to the EV71 2231 isolate, were purchased from Integrated DNA Technologies (Coralville, IA). Transcription reactions were carried out using standard procedures [57] and consisted of 10–15 mL reaction volumes containing unlabeled ribonucleotide triphosphates (rNTPs; Acros Organics), (¹³C/¹⁵N)-labeled rNTPS (Cambridge Isotope Laboratories), or ²H-labeled rNTPs (Cambridge Isotope Laboratories) wherein positions 3'-5' and H5 are deuterated. Following synthesis, samples were purified to homogeneity by 12% denaturing PAGE electrophoresis, excised from the gel, electroeluted, and desalted via exhaustive washing of the samples with RNase free water using a Millipore Amicon Ultra-4 centrifugal filter device. Purified RNA was dried under vacuum and stored at -20 °C until further use. Solution properties of both RNAs were examined as described previously [20]. All NMR samples were resuspended in 100% D₂O or 10% D₂O/90% H₂O. Samples were annealed by heating at 95 °C for 2 min and flash cooled on ice. Samples were subsequently concentrated to desired NMR levels using a Millipore Amicon Ultra-4 centrifugal filter followed by addition of buffer salts [5mM K₂HPO₄, pH 6.5]. Concentrations of samples were determined using each RNAs theoretical molar extinction coefficient, NMR samples ranged from 0.1 to 0.6 mM at 200 uL.

RNA for all titration experiments was annealed as described above. Post annealing all RNAs were washed into their respective buffers (see below) using a Millipore Amicon Ultra-4 centrifugal filter device. Independent of the method of preparation, the predominant (>95%) species of all RNAs examined migrated as a hairpin on native PAGE; however, a small population (<5%) of duplex or dimer was observed at NMR concentrations.

NMR Data Acquisition, Processing and Analysis

NMR spectra were recorded on Bruker Avance (800 and 900 MHz) high field spectrometers equipped with cryogenically cooled HCN triple resonance probes and a z-axis pulsed field gradient accessory. All NMR data was processed with NMRPipe/NMRDraw[58] and analyzed using NMRView J[59]. Assignments of non-exchangeable protons were carried out following well-established procedures[60]. ^1H - ^1H NOESY ($\tau_m = 250\text{ms}$) and ^1H - ^1H TOCSY ($\tau_m = 75\text{ms}$) spectra recorded in 100% D_2O at 303 K on SLII²²³¹ and SLII^{CCC} samples prepared with either unlabeled or selectively-deuterated rNTPs; rRTP $^2\text{H}(3',4',5'5'')$ and rYTP $^2\text{H}(5,3',4',5'5'')$. Assignments were further verified through acquisition of ^1H - ^{13}C heteronuclear multiple quantum coherence (HMQC) spectra of SLII²²³¹ constructs selectively-labeled with $^{13}\text{C}(\text{rATP})$ and $^{13}\text{C}(\text{rGTP})$. All reported chemical shift assignments correspond to the numbering system of the EV71 2231 isolate.

Residual Dipolar Couplings (RDCs) were measured using ^1H - ^{13}C Transverse Relaxation Optimized Spectroscopy Heteronuclear Single Quantum Coherence (TROSY-HSQC) experiments[61] for both isotropic and anisotropic ^{13}C -selectively labeled SLII²²³¹ and SLII^{CCC} samples. Partial alignment was achieved via the addition of Pf1 filamentous bacteriophage (ASLA) to a concentration of 10 mg/mL; phage concentration was verified via ^2H splitting at 900 MHz. RDC values were determined by taking the difference in $^1\text{J}_{\text{CH}}$ couplings under anisotropic and isotropic conditions.

EV71 SLII²²³¹ and SLII^{CCC} structure calculations

Distance restraints utilized in structure calculations for SLII²²³¹ and SLII^{CCC} constructs were derived from ^1H - ^1H NOESY ($\tau_m = 250\text{ms}$) spectra. Restraint boundaries were obtained qualitatively by the intensity of the NOE and grouped into strong (1.8 – 3.0 Å); medium (2.5 – 4.5 Å); weak (3.5 – 6.0 Å) bins. Sugar pucker restraints were obtained from analysis of H1'-H2' ^1H - ^1H TOCSY ($\tau_m = 75\text{ms}$) peak intensities. Residues displaying strong TOCSY peaks were set to C2'-endo conformation in SLII²²³¹ (A136, C151, C152) and in SLII^{CCC} (C138, C151, C152). Glycosidic restraints were obtained from ^1H - ^{13}C HMQC spectra collected on selectively labeled ^{13}C rNTPs constructs. Glycosidic angle restraints were set to the *anti* conformation ($180 \pm 90^\circ$) for all residues in SLII²²³¹ except A136 and G137. A136 and G137 in SLII²²³¹ were set to the *syn* ($0 \pm 90^\circ$) conformation based on observed downfield chemical shift and/or peak intensity, respectively. All residues in the SLII^{CCC} mutant were set to the *anti* conformation. Standard A-form backbone dihedral angle restraints ($\pm 20^\circ$) were applied to all residues in both RNAs. Hydrogen bond and planarity restraints (Xplor-NIH only, 20 kcal/mol Å) were applied to all WC and GU base pairs displaying NOE cross peak patterns in the in H_2O and D_2O ^1H - ^1H NOESY spectra.

Structure calculations for both SLII²²³¹ and SLII^{CCC} constructs were performed in four steps and incorporated both NMR and SAXS data. The four general steps were performed as follows: generation of a structure pool using Xplor-NIH followed by filtering with experimental Small Angle X-ray Scattering (SAXS) data; extended simulation of selected structures in Amber followed by re-filtering with SAXS data; 1 ns of simulation with inclusion of RDCs; and a final refinement stage that included 100 ps of simulation at 0K

with RDCs and the *ab initio* SAXS molecular envelope. The four-stage protocol resulted in good agreement between final structures and both NMR and SAXS data and is described in detail below.

An initial pool of 1024 structures was generated using Xplor-NIH 2.34[62] as previously described[63]. During this stage, hydrogen-bond, NOE, dihedral and planarity restraints were used. Converged structures were fit to experimental SAXS data using Crysol[64] in batch mode, allowing for constant subtraction and a maximum q value of $0.23 \text{ \AA}^{-1}$. The resulting χ^2 value from SAXS fitting was plotted along with the total energy for each structure, and the 10 structures that best satisfied both values (lowest energy and χ^2) were used for further simulation. The selected structures were then simulated for 20 ns each in Amber 12[31] using the ff99bsc0 χ_{OL3} force field[65–67]. In both minimization and production, simulations were performed in implicit solvent using the generalized Born model[68] ($igb = 1$) and a salt concentration of 10 mM. Structures were prepared for Amber using tLEaP and minimized in sander using 2000 steps of steepest descent followed by 2000 steps of conjugate gradient. A $24 \text{ \AA}$ cutoff for non-bonded interactions was used along with a $10 \text{ \AA}$ cutoff for calculation of the Born radii. After minimization, simulations were performed using GPU-accelerated pmemd[69] on NVIDIA Tesla K40 GPUs. During this stage, only hydrogen-bonding and NOE ($20 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$), sugar pucker restraints ($300 \text{ kcal mol}^{-1} \text{ rad}^{-1}$) generated using Pseudorotation Phase Angle, and chirality restraints[70] were used. For the 20 ns simulation (10,000,000 steps of 2 fs each), a non-bonded cutoff of $999.9 \text{ \AA}$ was used alongside a $10 \text{ \AA}$ cutoff for calculation of the Born radii. SHAKE was used to constrain bonds involving hydrogen, and Langevin dynamics with a collision frequency of 2.0 ps^{-1} was used to control temperature. The RNA was heated from 0 to 300 K over 100 ps and simulated for the remainder of the 20 ns simulation at 300K while writing the trajectory every 100 ps.

After 20 ns of simulation, structures were written from the trajectory files using cpptraj[71], providing a structure for every 100 ps of simulation (200 per starting structure). Only structures simulated for longer than 5 ns were considered, resulting in a pool with a total of 1500 structures. These structures were filtered by SAXS using Crysol in batch mode, allowing for constant subtraction and a maximum q value of $0.22 \text{ \AA}^{-1}$. The structures that had the lowest χ^2 value were chosen for the next stage of simulation.

The selected structures were simulated for 1 ns in Amber tools 15 using sander and the ff99bsc0 χ_{OL3} force field. At this stage, both minimization and production used implicit solvent ($igb = 1$), a salt concentration of 10 mM, a non-bonded cutoff of $24 \text{ \AA}$, and a $10 \text{ \AA}$ cutoff for calculation of the Born Radii. Residual Dipolar Couplings (RDCs) were incorporated into the production simulation with a weak-weighting coefficient of 0.05 as single-value restraints[70] alongside the hydrogen bond, NOE, chirality, and sugar pucker restraints. Structures were prepared in tLEaP and minimized over 4000 steps (2000 steps of steepest descent followed by 2000 steps of conjugate gradient). After minimization, the experimentally determined RDC values were fit to the structures by freezing the coordinates of the structure while allowing optimization of the alignment tensor. The tensor components were then simultaneously optimized alongside the atomic coordinates during the production simulation. The structures were subjected multiple rounds of 900 ps of simulation at 300 K

followed by cooling from 300 K to 0 K over 100 ps using a 2 fs timestep (500,000 steps) and Langevin dynamics with a collision frequency of 2.0 ps^{-1} was used to control temperature.

At the final stage of simulation, both SAXS and NMR data were incorporated for final refinement in Amber Tools 15. NMR restraints (RDC, NOE, hydrogen bond, sugar pucker and chirality) and simulation parameters were identical to those used for the production run of the previous step, with the exception that the temperature was held at 0K for 100 ps. Inclusion of the SAXS data was accomplished by utilizing the SAXS *ab initio* model (creation of which is described in SAXS materials and methods section) and the emap function[72], incorporated in a manner as previously described[63]. First, Supcomb[73] was used to align the SAXS molecular envelope to each structure while allowing the search for enantiomers. The aligned bead model was then read into the simulation and used as a shape constraint using the emap function, using a $5 \text{ \AA}$ map around the SAXS envelope (to minimize loss of information from the already low-resolution model), and incorporated with a fcons of 0.005. Once completed, the 10 lowest energy structures for each construct were visualized in PyMol[74] and checked using MolProbity[75]. Final χ^2 values were calculated using Crysol in batch mode with a maximum q value of $0.22 \text{ \AA}^{-1}$ and allowing for constant subtraction.

Preparation and Calorimetric Titrations of UP1

The UP1 construct used in these studies was prepared and purified as previously described [20, 63]. Calorimetric titrations for C_p were performed on a VP-ITC calorimeter (Malvern) at each temperature examined into 10 mM K_2HPO_4 , 40 mM KCl, and 0.5 mM sodium ethylene-diaminetetraacetic acid (EDTA), pH 6.5. (His)₆-UP1 at 40 μM was titrated into ~1.4mL of 1.0–1.5 μM RNA over a series of 42 injections set at 6 μL . To minimize the accumulation of experimental error associated with batch-to-batch variation, titrations were performed in duplicate at each temperature. Prior to non-linear least squares fitting in Origin v7.0, the raw data was corrected for dilution as previously described [20]. All data was fit to a two independent sets of sites binding isotherm.

NMR Titrations of UP1-SLII²²³¹

TROSY HSQC titrations were performed via titration of unlabeled UP1 into $^{13}\text{C(A)}$ -labeled SLII²²³¹. Spectra were collected at the following protein:RNA molar ratios: 0.25:1.0, 0.50:1.0, and 0.75:1.0. All titrations were carried out in 5mM K_2HPO_4 , pH 6.5 buffer at 303 K.

Steady State Fluorescence Titrations of UP1-SLII²²³¹

All fluorescence measurements were performed on a Varian Cary Eclipse Fluorescence Spectrophotometer using selectively 2-aminopurine labeled SLII²²³¹ (Dharmacon) and unlabeled UP1 in a 1.75 mL sample cell. Emission spectra were collected from 320–540 nm with an excitation wavelength of 310 nm at the following protein:RNA molar ratios: 0.25:1.0, 0.50:1.0, and 0.75:1.0. All titrations were carried out using 2 μM RNA in 5mM K_2HPO_4 , pH 6.5 at room temperature.

SEC-SAXS Collection and Processing

RNA and protein samples were prepared as described above. All Size Exclusion Chromatography – Small Angle X-ray Scattering (SEC-SAXS) experiments were collected at BioCAT (beamline 18-ID; Advanced Photon Source) in 5mM MES, 10 mM KCl and 2mM TCEP at pH 6.5. The SEC-SAXS setup was the same as previously described[29]. 200 μ L of each sample at a concentration between 4–10 mg/mL was loaded on the SEC column and scattering data were collected by one-second exposures collected every two seconds during the chromatography run. Points coinciding with a single peak (in both scattering intensity and on the UV chromatogram) were considered sample plus buffer while baseline before the peak was taken as buffer only.

Primus[76] from the ATSAS suite of programs[77] was used to analyze the scattering data. Guinier fitting was used to check for aggregation or repulsion and measure the Radius of Gyration (R_g) from Guinier fitting ($R_g \times q < 1.3$). GNOM[78] was used to fit the scattering curve and generate the $p(r)$ plot for molecular reconstruction. DAMMIF[79] in fast mode was used to generate 20 individual models that fit the scattering data (χ^2 between 1 and 1.1 for the RNA constructs and between 1.3 and 1.4 for the complex). The models for each sample were averaged using DAMAVER[80] and the most populated model was generated using damfilt. The overall NSD values for the RNA constructs and protein-RNA complex were ~0.51 and ~0.54, respectively.

Structural modeling of the UP1-SLII²²³¹ complex

Using the lowest energy SLII²²³¹ structure and the UP1-(5'-AGU-3') crystal structure (PDB code 4YOE), the UP1-SLII²²³¹ complex was modeled using HADDOCK 2.2[27, 28] on the WeNMR Grid[27] followed by MD simulation in Amber 12[31]. In preparation for docking, the 5'-AGU-3' trinucleotide, along with any solvent molecules, were removed from the UP1-bound crystal structure.

Restraints for the complex were obtained by chemical shift perturbations (CSPs) in ¹H-¹³C TROSY-HSQC titrations as described above. Residues A136 and G137 were considered active residues on the RNA along with residues F17, F57, F59, R92, Q12, K15, and H101 on the protein. Passive residues were automatically detected for both the protein and RNA, along with regions for semi-flexible refinement of the protein. 1000 rigid body complexes were calculated, and the 200 most favorable structures underwent semi-flexible and water refinement. The structures were clustered with a 7.5 Å RMSD and a minimum cluster size of 4, resulting in 10 total clusters.

Structures were selected via HADDOCK-SAXS scoring[81], which utilizes both HADDOCK scores along with χ^2 values from Crysol. Three structures representative of the three best scoring HADDOCK-SAXS clusters were selected for further simulation in Amber. These structures were also the three best scoring structures overall in terms of χ^2 values. The three models were subjected to 20 ns of simulation in Amber 12 using the ff12SB force field for protein and the ff99bsc0 χ_{OL3} force field for RNA in explicit solvent. Using tLEaP, all three models were neutralized using potassium ions (K⁺), followed by solvation in a TIP3P octahedron with no less than 8 Å between all atoms of the molecule

and the edge of the water octahedron. This gave rise to three systems that contained 41 K⁺ ions and, due to differences in size depending on the docking pose, between 18,124 and 25,690 water molecules.

Water was first minimized in sander using 500 steps of steepest descent followed by 500 steps of conjugate gradient. During this stage, the coordinates of the protein-RNA complex were held fixed with a constant of 500 kcal mol⁻¹ Å⁻² and a 10 Å cutoff was used for non-bonded interactions. This was followed by minimization of the unrestrained systems, using 2000 steps of steepest descent, followed by 2000 steps of conjugate gradient and a non-bonded cutoff of 10 Å. The three systems were then equilibrated by heating from 0 to 300 K over 60 ps, followed by 40 ps of simulation at 300 K for a total of 100 ps (50,000 steps of 2 fs each) using GPU-accelerated pmemd[69]. During this stage, the coordinates of the protein-RNA complex were restrained with a weak constant of 10 kcal mol⁻¹ Å⁻². SHAKE was used to constrain bonds involving hydrogen, Langevin dynamics with a collision frequency of 2 ps⁻¹ was used for temperature control, and constant volume periodic boundaries were used.

Finally, the systems were simulated at 300 K for 20 ns (10,000,000 steps of 2 fs each) using GPU-accelerated pmemd. During this stage, all NOE, hydrogen bond, sugar pucker, and chirality restraints used in the SLII²²³¹ RNA structure calculations were incorporated into the simulation except for those in the internal loop. Standard chirality restraints and trans-omega constraints were also included for the protein. Stacking restraints derived from the crystal structure that agree with the NMR CSPs were incorporated into the simulation at 20 kcal mol⁻¹ Å⁻¹. These were comprised of two restraints per stacking interaction (F17 and H101 with A136 and F59 and R92 with G137) and one between A136 and the backbone oxygen of R88. Again, SHAKE was used to constrain bonds involving hydrogen, the temperature was controlled using Langevin dynamics with a collision frequency of 2 ps⁻¹, and a non-bonded cutoff of 10 Å. A constant pressure periodic boundary was also used with an average pressure of 1 atm, a 2 ps pressure relaxation time, and isotropic position scaling. The trajectory file was written every 10 ps.

After simulation, water molecules and K⁺ ions were stripped and a structure was written for every 10 ps using the trajectory file and cpptraj[71], giving rise to 2,000 structures per system. The structures were fit to the experimental SAXS data using Crysol[64] in batch mode, with a maximum q value of 0.25 Å⁻¹ and allowing for constant subtraction. The structures were filtered by the resulting χ^2 values. The lowest-scoring structures were ordered according to overall energy, resulting in the final three representative models. The models were visualized and compared using PyMol[74].

Femtosecond Transient Absorption Spectroscopy

Samples of 2AP-SLII²²³¹ were prepared in 5mM K₂HPO₄, pH 6.5 buffer with an absorbance of 0.04 at 311 nm in a 2 mm quartz cuvette. Transient absorption was examined at the following molar ratios (protein:RNA): 0 to 1, 0.2 to 1 and 0.50 to 1. The femtosecond transient absorption instrumentation has been described in detail previously.[82, 83] Briefly, the fundamental source is based on a Ti:Sapphire regenerative amplifier (Libra-HE, Coherent, Inc.: 4W, 1 kHz) producing 800 nm pulses with a pulse width of ~100 fs.

Approximately 95% of the fundamental beam is directed through an optical parametric amplifier (TOPAS, Quantronix/Light Conversion) to produce the 311 nm excitation pulses. Wavelengths other than 311 nm are removed from the excitation beam using an all-reflective wavelength filter and a Glann-Taylor polarizer. The remaining 5% of the fundamental beam is attenuated and focused into a continuously-translated 2 mm CaF₂ crystal to produce broadband (i.e., ~320 to 710 nm) white-light continuum probe pulses. The excitation beam is attenuated to 1.7 μ J and directed through a polarization randomizer before being focused into the sample at a 3:1 diameter ratio with the probe beam. The instrument response function is 200 ± 50 fs based on the signal of neat methanol[84] and data is collected at time intervals up to 3 ns by mechanically delaying the time at which the probe pulses reach the sample via a delay stage. Transient absorption data was collected using a Helios spectrometer (Ultrafast Systems, LLC) and a home-made LabView program (National Instruments, Inc.).

All broadband transient data was corrected for group velocity dispersion of the white light continuum using a homemade LabView program, as previously described.[82] Kinetic traces were taken from the 550 to 610 nm probe region where the S₁-state of the 2AP nucleoside has been demonstrated to have a maximum transient absorption.[37, 39, 85] Global fitting analysis of these traces was performed in Igor 6.32A (WaveMetrics, Inc.) using a three lifetime sequential kinetic model.[39, 86] In each case, the final lifetime was beyond the time window of the experimental setup and was thus held to a value of 10 ns based on the reported decay of the S₁ state of the 2AP nucleoside free in solution.[39] The traces reported in Figure 7 are an average of the traces collected between 550 and 610 nm under the respective protein concentration. Aside from the picosecond decay dynamics, each lifetime and amplitude from a global analysis of the data sets were found to be equal within the experimental uncertainty. Therefore, a final global fit was performed using the average traces in Figure 7 allowing only the second lifetime to be independent among the data sets. The values reported in Table S2 are the result from this global analysis across all data sets. Supporting Figure S7 demonstrates the necessity of allowing the second lifetime to be independent in this analysis. A mechanistic model that attempts to interpret how UP1 affects the excited-state population dynamics of 2AP-SLII²²³¹ is provided in supporting information.

EV71 functional Studies

Cells—HeLa (human cervical carcinoma) and Vero (African green monkey kidney) cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) (Mediatech). RD (human embryonal rhabdomyosarcoma) cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS.

Plasmid construction and in vitro transcription—The bicistronic reporter plasmid pRHF-EV71-5'UTR which contains the EV71 IRES between Renilla and Firefly luciferase open reading frames was described previously[47]. Plasmid pRHF-EV71-5'UTR-AS contains the IRES in the antisense orientation between the two Luc open reading frames[47]. In plasmid pRHF-EV71-5'UTR-Del, the bulge nucleotides 134–138 within the EV71 IRES were deleted (prepared by GenScript). Plasmids pRHF-EV71-5'UTR, pRHF-EV71-5'UTR-

Del, and pRHF-EV71-5'UTR-AS were linearized by *Drd I* digestion and used as templates for the synthesis of RLuc-EV71-5'UTR-FLuc RNA, RLuc-EV71-5'UTR-Del-FLuc RNA, and RLuc-EV71-5'UTR-AS-FLuc RNA using the MAXIscrip kit (Life Technologies).

Determination of EV71 IRES activity—HeLa cells were seeded in 12-well plates in antibiotic-free MEM and transfected with the respective Luc reporter RNAs as described[20]. IRES activity was determined two days after transfection by measuring Renilla luciferase (RLuc) and Firefly luciferase (FLuc) activities with a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Viral growth and plaque assay—Full-length viral RNAs containing the wild type IRES or the SLII bulge loop deletion were synthesized by *in vitro* transcription using infectious cDNAs as templates and transfected into Rd cells. Supernatants were collected 2 days after transfection. Virus titers were determined by plaque formation on Vero cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research Highlights

- The SLII IRES domain adopts a compact structure in solution wherein the high-affinity hnRNP A1 binding site is sequestered in a bulge loop
- The overall structure of SLII is determined by the sequence of its bulge loop since mutation leads to a global structural rearrangement
- A large negative heat capacity change is a thermodynamic signature of specific hnRNP A1-SLII recruitment
- Binding of hnRNP A1 induces a change in the conformation of the SLII bulge structure

Figure 1.

NMR evidence that EV71 SLII²²³¹ folds into a stable and independent structural domain. (A) Schematic of the consensus secondary structure of the EV71 5'-UTR determined using phylogenetic comparisons. Phylogenetic consensus logos show the level of conservation of the bulge and hairpin sequences. (B) NMR-determined secondary structure of SLII²²³¹ derived from EV71 2231 strain[20]. (C) ¹H-¹H NOESY (800 MHz and $\tau_m = 250$ ms) spectrum of EV71 SLII²²³¹ collected in 5 mM K₂HPO₄, pH 6.5 at 303 K. Assignments of the intranucleotide NOE (H8/H6/H2-H1') interactions are indicated for every other nucleotide. Numbers are colored according to secondary structural elements shown in B. Note that G* corresponds to the non-native guanosine added to increase transcription yield. Solid lines denote sequential walk pathway and dashed lines denote H2 spin systems. The spectrum was collected on a ²H-selectively labeled SLII²²³¹ construct wherein rRTPs are deuterated at positions 3'-5'' and rYTPs at positions 5 and 3'-5''.

Figure 2.

NMR and SAXS refined solution structure of SLII²²³¹. (A) Ensemble of 10 lowest energy AMBER structures superimposed using WC and GU base pairs only. The structures are rotated by 90 degrees to reveal continuous stacking throughout and docked into the SAXS molecular reconstruction. Color-coding of secondary structural elements are the same as in Figure 1B. (B) Correlation plot between measured and back-calculated RDCs for the lowest energy AMBER SLII²²³¹ structure. (C) Crysol back-calculated scattering curve (red) of lowest energy SLII²²³¹ structure fit to experimental SAXS data (black circles). SAXS data were collected on a SLII²²³¹ sample at 5 mg/ml in 5mM MES, 10 mM KCl and 2mM TCEP buffer at pH 6.5.

Figure 3.

NMR and SAXS refined solution structure of SLII^{CCC}. A) Ensemble of 10 lowest energy AMBER structures superimposed using WC and GU base pairs only. The structures are rotated by 90 degrees to reveal continuous stacking throughout and docked into the SAXS molecular reconstruction. Color-coding of secondary structural elements are the same as in Figure 1B. (B) Correlation plot between measured and back-calculated RDCs for the lowest energy AMBER SLII^{CCC} structure. (C) Crysol back-calculated scattering curve (red) of lowest energy SLII²²³¹ structure fit to experimental SAXS data (black circles). SAXS data were collected on a SLII^{CCC} sample at 9.5 mg/mL in 5mM MES, 10 mM KCl and 2mM TCEP buffer at pH 6.5.

Figure 4.

Mutating the SLII²²³¹ AUAGC bulge loop to ACCCC induces a reorientation of the upper helix. (A) Zoomed view of the AUAGC bulge loop environment shows continuous base stacking wherein A136 and G137 adopt *syn* conformations to cause a sharp redirection of the phosphodiester backbone. (B) Zoomed view of the ACCCC bulge loop shows less stacking interactions and a more extended backbone topology. (C) Superimposition of the lower helices of native SLII²²³¹ (surface representation) onto the ACCCC mutant (cartoon representation) illustrates how the respective bulge loop structures determine the orientation of the upper helices.

Figure 5.

Specific interactions of hnRNP A1 with SLII²²³¹ are required for EV71 translation and replication. (A) Calorimetric titrations of UP1 into SLII²²³¹ reveal a specific and biphasic interaction that is stable over a wide temperature range. Titrations were performed in 10 mM K₂HPO₄, 40 mM KCl, and 0.5 mM sodium ethylene-diaminetetraacetic acid (EDTA), pH 6.5. Red lines represent non-linear regression fits to a two-independent set of sites binding isotherm. (B) Plot of the thermodynamic parameters (ΔG , ΔH and $-T\Delta S$) versus temperature for the titrations of UP1 into SLII²²³¹. The temperature dependence of ΔH for the bulge loop interaction shows a large negative heat capacity change ($C_{p,1} = -1044 \pm 21$ cal mol⁻¹ K⁻¹) indicative of site-specific binding. Titrations at each temperature were performed in duplicate. Thermodynamic parameters for the second lower affinity site are not reported. (C) Effect of SLII²²³¹ bulge loop deletion on EV71 IRES activity. Top, the diagram depicts the bicistronic Luc reporter plasmid used to synthesize RNA for transfections. A control plasmid contained the IRES in the antisense (AS) orientation. HeLa cells were transfected with RLuc-EV71-5'UTR-FLuc RNAs containing the wild-type IRES (SLII²²³¹), the IRES harboring the SLII bulge loop deletion (Del), or the IRES in the antisense (AS) orientation (as a control for IRES activity). Luciferase activities were measured two days later. Mean values and standard deviations from three independent experiments are shown in the bar graph. ***, $P < 0.001$.

Figure 6.

Structural model of the hnRNP A1-SLII²²³¹ complex. (A) TROSY ^1H - ^{13}C HSQC titration of A(^{13}C)-selectively labeled SLII²²³¹ construct with the UP1 domain of hnRNP A1. The spectrum is zoomed on the C8–H8 correlations wherein black peaks represent free SLII²²³¹ and red peaks correspond to SLII²²³¹ in the presence of a sub-stoichiometric amount of UP1. The spectra were collected at 900 MHz at 303 K. (B) Change in fluorescent intensity of 2AP-labeled SLII²²³¹ wherein A136 was replaced with 2AP. The plot shows an increase in the 2AP fluorescence intensity as a function of increasing UP1 concentration (0.25 molar increments). The increase in 2AP fluorescence intensity indicates that the bulge undergoes a conformational change wherein A136 becomes more solvent exposed. (C) SAXS scattering curve of a UP1-SLII²²³¹ complex (~5 mg/ml). (D) Representative HADDOCK-SAXS models of the UP1-SLII²²³¹ complex depicted as three different poses wherein SLII²²³¹ is differentially colored red, green and blue, respectively.

Figure 7.

Decay of transient absorption at a probe wavelength of ca. 580 nm (i.e., the average of the traces collected between 550 and 610 nm probe wavelengths) for 2AP-SLII²²³¹ in buffer following 311 nm excitation without (top) and with the addition of protein at 1:0.2 (middle) and 1:0.5 (bottom), 2AP-SLII²²³¹:UP1 ratios.

Table 1Thermodynamic parameters of UP1-SLII²²³¹ interactions

Temperature (K)	G° (kcal mol ⁻¹)	H° (kcal mol ⁻¹)	-T S° (kcal mol ⁻¹)
283	-13.1±0.3	-36.0±1.1	22.9±0.8
288	-13.2±0.5	-37.1±0.1	23.8±0.4
293	-13.0±0.3	-47.4±3.7	34.5±03.9
298	-13.2±0.3	-50.0±2.9	36.8±3.2

Calorimetric titrations were performed in 10 mM K₂HPO₄, 40 mM KCl, and 0.5 mM sodium ethylene-diaminetetraacetic acid (EDTA), pH 6.5 using a VP-ITC. Reported thermodynamic parameters correspond to the average +/- standard deviation of two replicates.