‘RNA walk’ a novel approach to study RNA–RNA interactions between a small RNA and its target

Yaniv Lustig1, Chaim Wachtel1, Mark Safro2, Li Liu1 and Shulamit Michaeli1,*

1The Mina and Everard Goodman Faculty of Life Sciences, Bar Ilan University, Ramat-Gan 52900 and 2Department of Structural Biology, Faculty of Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel

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ABSTRACT

In this study we describe a novel method to investigate the RNA–RNA interactions between a small RNA and its target that we termed ‘RNA walk’. The method is based on UV-induced AMT cross-linking in vivo followed by affinity selection of the hybrid molecules and mapping the intermolecular adducts by RT–PCR or real-time PCR. Domains carrying the cross-linked adducts fail to efficiently amplify by PCR compared with non-cross-linked domains. This method was calibrated and used to study the interaction between a special tRNA-like molecule (sRNA-85) that is part of the trypanosome signal recognition particle (SRP) complex and the ribosome. Four contact sites between sRNA-85 and rRNA were identified by ‘RNA walk’ and were further fine-mapped by primer extension. Two of the contact sites are expected; one contact site mimics the interaction of the mammalian Alu domain of SRP with the ribosome and the other contact sites include a canonical tRNA interaction. The two other cross-linked sites could not be predicted. We propose that ‘RNA walk, is a generic method to map target RNA small RNAs interactions in vivo.

INTRODUCTION

Small non-coding RNAs (ncRNAs) have recently come to the center stage of eukaryotic biology, since it was realized that in most organisms a large amount of genetic information is dedicated to the synthesis of RNA molecules that do not code for proteins. ncRNAs exhibit a range of diverse functions, including ribosomal RNA maturation (small nucleolar RNAs or snoRNAs), splicing of pre-mRNA (small nuclear RNAs or snRNAs), DNA replication (telomerase RNA), protein translocation (7SL RNA) and gene silencing (microRNAs or miRNAs and small interfering RNAs or siRNAs). It has been suggested that ncRNAs are major determinants of the complexity of an organism, complementing the protein-based regulation (1,2). Another large group of regulatory RNAs, found only in recent years in eukaryotes, are the miRNAs and siRNAs; with a length of only 21 nucleotides; these RNAs constitute the smallest class of ncRNAs described to date. miRNAs mostly suppress translation via non-perfect pairing with the target 3’ UTR sequences of mRNAs (but can also elicit degradation of the target mRNA) and are central to a wide range of developmental and physiological processes in metazoa (3).

In the last couple of years, thousands of ncRNAs were described from bacteria to man based on tiling arrays, comparative genomics and more recently, deep sequencing (1,2,4). In some cases bioinformatic approaches suggest that these small RNAs interact with an RNA target to regulate their stability or translatability. In the case of microRNAs it was demonstrated that target mRNAs can either interact with the same microRNA in different domains or the 3’ UTR is bound by different microRNAs (3). Experimental methods are in demand to examine the validity of proposed small RNA–target interactions as well as to map on the target RNA the binding sites of the small RNA.

Trypanosomes are protozoan parasites whose gene expression is mediated by RNA-dependent processes. These organisms lack promoters for protein coding genes and regulate gene expression by post-transcriptional processes such as trans-splicing, RNA editing, mRNA stability and translation (5). Trypanosomes also possess a very unique signal recognition particle (SRP) that, similar to eukaryotes, functions in protein translocation across the ER (6–8). In eukaryotes, binding of the SRP leads to retardation of peptide elongation, known as elongation arrest (9–11). The SRP–ribosome-nascent chain complex (RNC) is then targeted to the ER membrane in a GTP-dependent manner (12). After docking to the membrane, the RNC is translocated to the translocon. Binding of the RNC to the translocon leads to insertion of the nascent chain into the channel; translation then
resumes and the protein is co-translationally translocated (13–15). In mammals the SRP is composed of a single RNA molecule, 7SL RNA and six proteins; the complex is divided into two distinct domains, the Alu domain on one side and the S domain at the opposite end. The Alu domain is responsible for the elongation arrest activity of the SRP (16,17). One of the intriguing questions in understanding the role of the SRP in protein translocation is elucidating how the SRP induces transient translational arrest upon its binding to the ribosome. A detailed cryo-electron microscopy (EM) study identified three interactions of the mammalian S domain with the ribosome, found in the vicinity of the peptide exit site. On the other hand, the Alu domain bridges, in a tight fit, the 6 Å space between the large and the small ribosomal subunits and directly contacts the ribosome (18). Although the Alu domain is essential for the elongation arrest function of the mammalian SRP, not all SRPs in nature carry an Alu domain; notably, the bacterial and archael SRPs lack this domain (19,20).

The trypanosome system may represent a unique solution for the arrest function. The trypanosome SRP is different from all other SRPs described so far in that it is composed of two RNA molecules, the 7SL RNA and a unique tRNA-like molecule, sRNA-76 in Trypanosoma brucei and sRNA-85 in Leptomonas collosoma (6,21). Structurally, the trypanosome 7SL RNA homolog resembles the mammalian 7SL RNA except for its Alu domain (22,23). The trypanosome SRP also lacks the Alu domain binding proteins (7). We therefore proposed that the tRNA-like molecule may functionally replace the missing Alu domain and mediate the arrest function of trypanosome SRP (6,7). sRNA-85 was shown to interact with the 7SL RNA by base pairing (6). The association of sRNA-85 and 7SL RNA was also demonstrated by co-afinity selection, and AMT cross-linking. sRNA-85 exists together with 7SL RNA by base pairing (6). The association of sRNA-85 and 7SL RNA was also demonstrated by co-afinity selection, and AMT cross-linking. sRNA-85 exists together with 7SL RNA by base pairing (6). Since there is currently no functional in vitro protein translation/translocation system in trypanosomes, we could not study the interaction of sRNA-85 with the ribosome during the translocation cycle in vitro. We therefore developed a novel approach termed ‘RNA walk’ to follow the interaction of this unique molecule with the ribosome in vivo. ‘RNA walk’ is a generic method that can be applied to study small RNA–target interactions in vivo and in vitro. The approach utilizes AMT-induced UV cross-linking, which links two RNA molecules that are located in close proximity. The cross-linked species are mapped by ‘walking’ on the RNA using reverse transcription polymerase chain reaction (RT–PCR) and primers spanning the region of interest, identifying domains that cannot be copied efficiently due to the presence of cross-linked adducts. Using this method, we suggest that sRNA-85 contacts the ribosome in at least four sites. The four cross-linked sites were fine mapped by primer extension and oligonucleotide-mediated RNase H cleavage. Of special interest is the one site within helix 95, the sarcin ricin loop (SRL) domain, which was shown to bind the mammalian SRP Alu domain. Another site reveals a tRNA-like interaction with the ribosomal P site, the third contact site is located within the peptidyl-transferase center (PTC) and the fourth site is within helix 63 of the large subunit. We propose that in trypanosomes, sRNA-85 may provide a novel solution for the SRP arrest function in the absence of a functional Alu domain. This study demonstrates how the ‘RNA walk’ method made it possible to unravel a complex interaction between a small RNA and its target that could not otherwise be solved.

MATERIALS AND METHODS

UV cross-linking of living cells treated with AMT

Cross-linking was performed essentially as described in Liu et al., 2003 (6). Briefly, L. collosoma cells were harvested at 5 × 10⁶ cells ml⁻¹ and washed twice with PBS. Cells (5 × 10⁵) were concentrated and incubated on ice. 4′-Aminomethyl-trioxalsalan hydrochloride (AMT) (Sigma) was added to the cells at a concentration of 0.2 mg ml⁻¹. Cells treated with AMT were kept on ice and irradiated using a UV lamp at 365 nm with an intensity of 10 milliWatts cm⁻² for 60 min. Next, the cells were washed once with PBS and deproteinized by digestion with protease K (Roche) (200 µg ml⁻¹ in 1% SDS for 60 min). RNA was prepared using TRIzol reagent (Sigma). For reversal of the cross-linking, the RNA was irradiated at 254 nm UV in the same intensity described above, for 10 min.

Affinity purification of sRNA-85 using 2′-O-methyl biotinylated oligonucleotide

Five hundred micrograms of total RNA in hybridization buffer (20 mM HEPES pH 8, 5 mM MgCl₂, 300 mM KCl, 0.01% NP40, 1 mM DTT) was heated for 2 min at 80°C and put on ice. Oligonucleotide 85-R-1 (wild-type) or oligonucleotide s85-mutant bio (mutant) (specified in S1) (8 µg) was added and the solution was incubated overnight at room temperature. Next, 50 µl of blocked Neutravidin Sepharose beads (Pierce) were added and the reaction was incubated for 2 h at 4°C. Blocking of the beads was in 1 ml blocking buffer (700 µl DEPC, 200 µl WB100 [20 mM HEPES pH 8, 10 mM MgCl₂, 100 mM KCl, 0.01% NP40, 1 mM DTT], 50 µl BSA (10 mg ml⁻¹), 10 µl Glycogen (20 mg ml⁻¹), 40 µl tRNA (11 mg ml⁻¹)). After binding of the RNA to the oligonucleotide-bound beads, the beads were washed five times in WB400 buffer (20 mM HEPES pH 8, 10 mM MgCl₂, 400 mM KCl, 0.01% NP40, 1 mM DTT). RNA was extracted from the beads using TRIzol reagent (Sigma) and analyzed by primer extension (24).

Primer extension and sequencing

Primer extension was performed as described previously (24). Total RNA, or affinity-selected RNA obtained from cells treated with AMT and UV cross-linked and untreated cells, were subjected to primer extension reaction with [γ⁻³²P]-ATP-labeled oligonucleotides. The reaction was analyzed on 6% polyacrylamide denaturing gel next to DNA sequencing reactions performed using the...
same primer. DNA sequencing was performed on plasmids containing the *Trypanosoma brucei* rRNA genes. These regions are 100% conserved between *T. brucei* and *L. major*. The secondary structure of *L. major* rRNA that is more closely related to *L. collosoma* was used. No changes in the DNA sequences presented next to the primer extension were observed between *L. major* and *T. brucei* and only minor changes were found outside these domains.

**RT–PCR**

The RNA was treated with ‘DNA-free’ reagent (Ambion) according to the manufacturer’s protocol for 30 min to remove DNA contamination. Reverse transcription was performed on RNA (1/10 of the affinity-selected RNA) by random priming (Reverse Transcription System, Promega). The samples were heated for 5 min at 70°C, followed by chilling on ice for 5 min. Next, 1 U of AMV-reverse transcriptase (Promega) was added, together with 1 U RNase inhibitor (Promega) and the elongation reaction was performed according to the manufacturer’s instructions at 25°C for 10 min and then at 50°C for 60 min (Promega kit). The cDNA was used for PCR amplification using primers as specified in S1. The cDNA was diluted 1:10, 1:100 and 1:1000 for each primer set, to determine the dilution that produces PCR in the exponential phase. For most of the analysis we found that 1:500 dilution of cDNA was in the exponential phase and resulted in detectable amounts of the PCR products. As a negative control PCR was performed using primers for snoRNA (sno-2), an RNA not expected to interact with sRNA-85 (25). PCR was performed on 1 μl of cDNA (diluted 1:500), 1 μM primers and ReadyMix™ Taq PCR Reaction Mix with MgCl₂ (Sigma). The PCR conditions were as follows: 95°C for 2 min followed by 25 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s.

**Real-time PCR**

Real-time PCR was performed in a two-step reaction. First cDNA was prepared from either 2 μg of total RNA or 1/10 of the affinity-selected RNA (from 500 μg total RNA as starting material) using random primer and the RevertAid™ First Strand cDNA synthesis kit (Fermentas) according to the manufacturer’s instructions. Next, real-time PCR was performed using 1 μl of cDNA described above, diluted 1:100,000, 1 μM primers and Absolute Blue QPCR SYBR™ Green ROX mix (Thermo Scientific). The PCR conditions were as follows: 95°C for 2 min followed by 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. A calibration curve was established using a PCR product produced with the same primers examined by the RT–PCR. The fragment was purified by QIAquick PCR purification kit (Qiagen) and its concentration was determined by NanoDrop ND-1000 spectrophotometer (Thermo). Calibration curves were prepared for each of the domains examined.

**Plasmid construction and transformation**

The sRNA-85 mutant was generated by PCR mutagenesis using a primer carrying the mutation and oligonucleotides situated upstream and downstream of the gene. The different oligonucleotides used for the PCR amplification are listed in S1. The mutated sRNA-85 gene was subcloned into the BamHI site of the pX-neo expression vector. The mutation was confirmed by DNA sequencing. Stable cell lines carrying the mutated genes were established as described previously (26).

**Splint labeling**

Ten to twenty micrograms of RNA was mixed with 150 pmol of oligonucleotide (see S1 for primer sequence) and heated for 2 min at 85°C in 50 mM Tris–HCl (pH 7.8), 10 mM MgCl₂ and 1 mM DTT. The annealing reaction was quenched on ice for 30 min. 50 μCi of [α-32P]-dCTP (3000 Ci/mmol) and 5 units of T7 DNA polymerase (Sequenase v. 2.0, USB) were added, and the reaction was incubated for 1 h at 37°C. RNA was separated on a 6% polyacrylamide–7M urea gel.

**RNase H cleavage**

RNase H cleavage of rRNA was performed by annealing 200 pmol of oligonucleotides as indicated in figures (see S1 for specific primer sequences) with 20 μg of total RNA at 65°C for 10 min in 75 mM KCl, 50 mM Tris–HCl (pH 8.3), 3 mM MgCl₂ and 10 mM DTT. The samples were then placed on ice and two units of RNase H (NEB) were added. The samples were further incubated at 37°C for 60 min, phenol/chloroform extracted and ethanol precipitated. Samples were then labeled by splint labeling (see above) and separated on a 6% polyacrylamide–7 M urea gel.

**Extract preparation and fractionation on sucrose gradients and northern analysis**

*Leptomonas collosoma* growth and extract preparation were as previously described (24). Cells were disrupted using nitrogen cavitation, and RNPs were extracted at 0.1 M KCl. The low-salt extracts were layered on continuous 10–30% sucrose gradients containing 100 mM KCl. Gradients were centrifuged at 4°C for either 3 or 22 h at 35 000 rpm using a Beckman SW41 rotor. RNA prepared from the sucrose gradient fractions was subjected to northern analysis.

**RESULTS**

**In vivo cross-linking with AMT and affinity selection of sRNA-85**

In the absence of an *in vitro* translation–translocation system in trypanosomes, it is not possible to elucidate the function of sRNA-85 *in vitro*. We therefore sought to elucidate the function of this molecule by mapping its interaction with the ribosome *in vivo*. We propose that sRNA-85 may functionally replace the missing Alu domain of the trypanosome SRP.

To investigate this complex system, we made use of the methodology of AMT treatment followed by UV cross-linking. AMT compounds are unique in their ability to freeze helical regions of nucleic acids. First, the planar
AMT molecule intercalates within the double-helical region; upon irradiation at 365 nm, covalent adducts form with pyrimidine bases (27). The covalent linkage can be reversed by irradiation at 254 nm. This methodology was widely used to study RNA and DNA structures (28, 29). This method was also used in vivo in trypanosomes to identify interactions between the spliced leader RNA (SL RNA) and other small RNAs (30). AMT intercalates within stems formed either inter or intramolecularly. To identify interactions on the target due to interactions with small RNA and to avoid mapping intramolecular interactions that are more abundant on the target, we had to devise a protocol to enrich the cross-linked adducts that result from intermolecular cross-linking. To achieve this goal, we affinity purified sRNA-85 together with its covalently attached rRNA target and compared the adducts on the rRNA enriched with sRNA-85 to those on total RNA obtained from the same sample. Only adducts that were enriched by the affinity selection were considered to be adducts emerging from the interaction between sRNA-85 and rRNA. To calibrate the cross-linking in our experimental set-up, we repeated the experiment using the optimal set-up, we repeated the experiment using the conditions previously reported for T. brucei (30), and followed the SL RNA cross-linked species by northern hybridization (data not shown). The same conditions were then tested on L. collosoma cells; we first performed a time course of irradiation at 10 milliWatts cm⁻² from 15 to 60 min, and found that 60 min of irradiation was optimal for capturing the cross-linked SL RNA species (data not shown). We then confirmed that the cross-linking procedure did not affect our ability to affinity select sRNA-85. Total RNA was subjected to affinity selection with 2'-O-methyl biotinylated oligonucleotide complementary to the 3'-end of sRNA-85. After elution, the RNA was subjected to primer extension to detect the presence of sRNA-85 and tRNA Arg, which was used to control for the specificity of the selection. As can be seen in Figure 1A, and based on the amount of RNA examined (see details in the figure legend), 20% of sRNA-85 was selected using this procedure. No tRNA Arg was selected, confirming the specificity of the selection (Figure 1A; lanes 3 and 4). Results (Figure 1B) show little difference in the yield of affinity selection using cross-linked RNA compared with RNA extracted from untreated cells, suggesting that the cross-linking did not interfere with the ability to affinity select sRNA-85 from the irradiated cells.

We next quantified the procedure of affinity selection with respect to the enrichment of sRNA-85 and the levels of rRNA and snoRNA (negative control). To quantify, sRNA-85 primer extension was used, and the level of sRNA-85 was calculated based on a calibration curve generated from picomoles of labeled primers. The results indicate that 500 μg of total RNA contains ~0.2 pmol of sRNA-85. After affinity selection we selected ~0.04 pmol of this RNA. The same amount of total RNA contains ~30 pmol of rRNA based on the percentage of rRNA in total RNA and that the amount of mRNA (present at 0.1%) in 10 μg of total RNA is 0.6 fmol. Because only 0.1% of RNA was selected (Figure 1C) only 0.03 pmol of rRNA was recovered. Although this is a rough estimation the calculations suggest the enrichment of sRNA-85 and the relative low level of rRNA in the sample.

Since the presence of rRNA contamination can interfere with mapping the intermolecular adducts, we examined the contamination of rRNA in the affinity-selected sRNA-85 preparation. The results (Figure 1C) suggest that if we perform two rounds of affinity selection we obtain preparations which are highly enriched for sRNA-85 (compare lanes 1–3) and carry less of the contaminating rRNA (compare lanes 4–6). However, after a single round of affinity selection we lost ~90% of the rRNA and after two rounds of selection rRNA contamination was hardly detected (~0.1% of the initial amount). The specificity of the selection and contamination was also examined by the level of sno-2 (25). snoRNAs involved in methylation bind to rRNA during
rRNA maturation and thus should not exist on rRNA extracted from cytoplasmic ribosomes. Indeed, snoRNA-2 can easily be detected by RT–PCR in total RNA but cannot be detected in RNA prepared from cells treated with AMT and UV and after affinity selection with sRNA-85 oligonucleotide (Figure 1D; compare lanes 1–3).

‘RNA walk’ identifies four potential contact sites between sRNA-85 and rRNA

To map the potential contact sites between sRNA-85 and rRNA we developed a novel method that we termed ‘RNA walk’, and is illustrated in Figure 2A. The method utilizes the in vivo cross-linking described above and is based on the fact that upon cross-linking the adducts formed between the target and small RNA will not allow the reverse transcriptase to copy the cross-linked region. In the next step, the entire target can be scanned for the presence of such adducts by simply amplifying small portions of the cDNA by PCR. Such an experiment is illustrated in Figure 2B. Briefly, total RNA from irradiated cells (500 μg) and from control untreated cells were subjected to sRNA-85 affinity selection, and cDNA was produced from the affinity-selected RNA using random primers. We performed a calibration curve to examine at which dilution the amplification is exponential as described in ‘Materials and Methods’ section. Next, PCR analysis was performed on the cDNA (diluted...
(1:500), using primers that divide the rRNA target into 12 ~500 nucleotide domains (depicted in Figure 2B). The results show that 4 out of the 12 domains were not amplified by PCR, suggesting that these contain contact sites between sRNA-85 and rRNA. As can be seen, interactions between sRNA-85 and the rRNA possibly exist in domains 2, 9, 10 and 12. The results in Figure 2B show enrichment of rRNA that was calculated by examining the level of the RT–PCR product produced on affinity-selected sRNA-85 with and without UV irradiation. Comparison of the level of the PCR products covering the entire rRNA suggests its higher levels in irradiated cells. For instance 250, 900 and 480% increase in domains 3, 6, 7, respectively was observed (Figure 2B), demonstrating the enrichment of the rRNA target due to its cross-linking with sRNA-85. Next, we compared the amplification of the four cross-linked domains to sRNA-85 (D2, 9, 10, 12) to two non-cross-linked domains (D3, D11). This time, the affinity-selected RNA was spiked with synthetic luciferase mRNA. cDNA was prepared, diluted (1:500) and PCR was performed with the domain-specific primers. The results demonstrate that with an internal luciferase control, we could observe major differences between the production of PCR in cross-linked domains versus non-cross-linked domains (Figure 2C).

To verify that the interactions discovered by the ‘RNA walk’ method are indeed AMT cross-linking adducts, the cross-linking was reversed in vitro, by irradiating at 254 nm which reverses the cross-linking (31). The RNA was then subjected to reverse transcription using random primers, and the cDNA was amplified by PCR. The results (Figure 2D) demonstrate that after reversing the cross-linking, the rRNA from the treated cells can be amplified by RT–PCR, suggesting that the four unamplified sites represent genuine AMT cross-linked sites on the rRNA target. Several additional controls were performed and the ‘stop’ was examined in cells UV cross-linked in the absence of AMT. Under these conditions, no stop could be observed (Figure 2E; lane 3). The stop generated by cross-linking in domain 12 can be detected if instead of using cDNA prepared with random primers, we prepared cDNA with a specific primer situated in this domain (Figure 2F). However, the use of cDNA prepared with random primer is more useful because the same cDNA preparation can be used to examine cross-linking at different sites.

Next, we performed real-time PCR on cDNA prepared from cells that were UV cross-linked or from non-cross-linked cells. The RNA was subjected to affinity selection and the ability to amplify the rRNA was determined based on the amplification of the four cross-linked domains and two non-cross-linked domains (domains 3 and 12*). The results presented in Figure 3 demonstrate that after affinity selection with sRNA-85 of RNA from the irradiated cells it was possible to amplify domains that are not involved in cross-linking with sRNA-85 (domains 3 and 12*). For such domains tens of femtomoles of PCR product were generated. However, in the same conditions only a few femtomoles of PCR product were produced for domains cross-linked to sRNA-85. The results demonstrate that rRNA–sRNA-85 hybrids are enriched only in samples derived from irradiated cells. cDNA prepared from non-irradiated cells, yielded only 1–2 fmol of PCR product.

### sRNA-85 mimics the mammalian SRP RNA Alu domain in its interaction with the ribosome

In the next step, we sought to analyze the cross-linked adducts on the rRNA target in greater detail. The positions of the cross-linked adducts were narrowed down by RT–PCR to domains of 100 nucleotides (data not shown). The cross-linked adduct in domain 9 was revealed to be in helix 95 of rRNA (SRL). The homolog of helix 95 in trypanosome RNA is situated in srRNA-2, which is processed from the LSU to produce an RNA molecule of 181 nucleotides. This srRNA-2 fragment maintains its interaction within the rRNA by base pairing (32, 33). To fine map the cross-linked site between sRNA-85 and the SRL of rRNA, we used a primer extension assay. Reverse transcriptase, as mentioned above, will not pass the cross-linked adducts and the enzyme will fall off the template one nucleotide before the cross-linking site, creating a stop in the reaction (30). This stop will not be seen in the RNA samples extracted from the untreated cells. To distinguish between intermolecular and intramolecular cross-linking, samples of both total RNA and affinity-selected sRNA-85, were analyzed side by side. The rationale, as stated above, is that intermolecular interactions would only be seen in the affinity-selected RNA, whereas intramolecular interactions will be detected in both samples. In order to ensure that the cross-linking adducts we identify are indeed caused by AMT cross-linking, the affinity-selected sRNA-85 extracted from irradiated cells and from untreated cells was irradiated at 254 nm which reverses the cross-linking (31). All the samples were subjected to primer extension, and the products were separated on a 6% polyacrylamide denaturing gel alongside a DNA-sequencing ladder of the corresponding rDNA region of *L. major*. The results (presented in Figure 4A; lanes 1–6) showed a cross-linking adduct at U68 of srRNA-2 (helix 95, the SRL domain; see Figure 4B). Note that there are multiple stops by primer extension on total RNA, but these were reduced in the affinity-selected RNA. These stops may stem from secondary structure, degradation products and even intramolecular cross-links on the target. However, after affinity selection, these stops are reduced and the specific cross-link at position U68 is highly enriched. In all organisms (from bacteria to man) helix 95 interacts with elongation factors EF1 and EF2 (34). Interestingly, the mammalian Alu domain interacts with this domain. These data therefore suggest that sRNA-85, like the mammalian Alu domain, interacts with the SRL. The mammalian Alu domain also interacts with helix 43. However, no evidence for cross-linking between sRNA-85 and trypanosome helix 43 was obtained (data not shown). Thus, either sRNA-85 does not interact with this domain in trypanosomes or the AMT cross-linking technique is unable to demonstrate such an interaction. Note, pyrimidines (mostly U) have
to be present in the interaction domain to be captured by the AMT cross-linking technique (27).

**sRNA-85 interacts with the ribosome like a tRNA molecule**

The position of the second adduct on rRNA (domain 2) was narrowed down to helix 31 of the small subunit (SSU) rRNA, and the fine mapping by primer extension is presented in Figure 5A. This cross-linking adduct at position U1539 is a *bona-fide* intermolecular interaction, since it is enriched by affinity selection (Figure 5A; compare lanes 2 and 4), and can be photoreversed upon irradiation at 254 nm (Figure 5B; lanes 2 and 4). Interestingly, tRNA nucleotide at position 34 was shown to interact with helix 31 (35,36). To examine which position of sRNA-85 might interact with helix 31, the cross-linked adducts on sRNA-85 were mapped by primer extension. The corresponding position to U34 on sRNA-85 is U36, due to the insertion of nucleotides in the anti-codon stem of this unusual tRNA (6). Indeed, mapping of cross-linked sites on sRNA-85 indicate a major stop at position U36 (Figure 5D), suggesting that sRNA-85 most probably interacts with SSU U1539, possibly via its U36 position. Note, a second stop can be observed at U34, however this may be a structural stop since it is present in the absence of UV (Figure 5D; lane 1). While the fact that the stop observed on rRNA was reversed upon irradiation at 254 nm (Figure 5B; lane 4), it is still possible that the stop observed was not due to the cross-linking but rather resulted from a post-transcriptional modification on the RNA present in this domain. To exclude this possibility, reverse transcription was performed under non-optimal reaction conditions, which has been used to map 2'-O-methyls, and induces stops of the reverse transcriptase at low dNTP concentrations (37). The results (Figure 5F) demonstrate the presence of a modification at position 1550, since the stop one nucleotide before the modified nucleotide was increased upon decreasing the dNTP concentration (compare lanes 1–3; Gm1550). The UV cross-linking at position U1539, however, decreased (lanes 1–3; U1539) suggesting that this nucleotide is not modified and the stop stems from cross-linking and not modification on this nucleotide. Primer extension stops due to modification or structural stops that appear in the primer extension mapping of the cross-link sites seem not to interfere with the ‘RNA walk’ method since only distinct stops of RT were observed with this method.

To further demonstrate the authenticity of the cross-linking between U36 and helix 31, we sought to examine the cross-linked pattern of sRNA-85 lacking position U36. We generated a mutant of sRNA-85 in which the

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**Figure 3.** Quantitative real-time PCR analysis of the ‘RNA Walk’ method. Real-time PCR was performed as described in ‘Materials and Methods’ section using cDNA (diluted 1:100,000). Concentration curves were prepared for each domain studied. The curves were used to determine the amount of PCR product amplified from each domain. PCR was performed on DNA prepared from RNA extracted from non-irradiated cells (−UV, gray bars) and from irradiated cells (+ UV, black bars). The different rRNA domains examined are indicated and depicted schematically underneath the graph.
The anti-codon loop was deleted. The mutation was created by PCR (see ‘Materials and Methods’ section) and is depicted in Figure 6A. A cell line expressing the mutation from the pX expression vector was generated and the expression of the mutant was examined by northern analysis. The results (Figure 6B) demonstrate efficient expression of the mutant compared with the wild-type RNA. Next, the association of wild-type and mutant sRNA-85 with the ribosome was examined. Extracts were prepared from the transgenic cell line expressing the mutant sRNA-85 and fractionated on a sucrose gradient. The gradient fractions were examined by northern analysis for the distribution of particles carrying the sRNA-85 wild-type and mutant molecules. The results (Figure 6C) demonstrate that the mutated sRNA-85, like the wild-type molecule, is present mainly in small RNPs (shorter exposure) but also on ribosomes (longer exposure). The presence of mutant sRNA-85 on the ribosomes suggests that sRNA-85 can associate with the ribosomes even if it cannot interact with h31 of the SSU. We next examined the interaction of the mutant sRNA-85 with rRNA by ‘RNA walk’. Cells expressing mutant sRNA-85 were subjected to UV cross-linking in the presence of AMT. RNA isolated from the cross-linked cells was affinity selected with an anti-sense biotinylated oligonucleotide that can specifically select only the mutant, but not the wild type, since the primer used for selection is complementary to positions 24–49, which includes the new junction created by the deletion of nucleotides 36–40. Primer extension on total RNA and affinity-selected RNA showed that selection with the primer was indeed specific for the mutant sRNA-85 (Figure 6D, compare lane 1–2). The affinity-selected RNA was then subjected to analysis using the ‘RNA walk’ method. The results (Figure 6E) demonstrate that the stop, previously observed with wild-type sRNA-85 in the h31 domain that interacts with the anti-codon of sRNA-85, was eliminated. However, all the other stops were present. This data strongly suggests that the ‘RNA walk’ analysis is a specific and reliable method for mapping the interaction of sRNA-85 with rRNA. The data also suggest that sRNA-85 lacking the interaction with h31 can still stably interact with the ribosome (see below).

Fine mapping of the interactions with the large subunit of rRNA

As described above for the cross-links in domains 2 and 9 (Figure 2B), we narrowed down the interaction in domain 10 to 100 nt and then mapped the exact cross-linking positions by primer extension. The interaction of sRNA-85 in domain 10 is located at U1369 of helix 92 of the LSUβ (our unpublished results) which is part of the PTC. The PTC is where peptide bond formation takes place (38,39). Such an interaction may interfere with or retard peptide bond formation.
We next narrowed down the interaction domain within domain 12 and mapped the interaction precisely. The interaction found in domain 12 (Figure 7) is located at U349 of helix 63 of the LSU. This helix is not evolutionarily conserved and its primary sequence has no known function. Moreover, this helix is one of five helices in the ribosome which exhibit variable lengths over evolution; in eukaryotes it is longer than in prokaryotes (40). Interestingly, among these five non-conserved helices, helix 63 is the only one that cannot tolerate size changes, suggesting it has an important role in ribosome function (41).

Figure 5. The sRNA-85 interaction with helix 31 of SSU is a canonical tRNA interaction in the P site. (A) Cells (10^10) were treated with AMT and irradiated with UV light at 365 nm with an intensity of 10 milliWatts cm^-2 for 60 min. RNA was prepared from irradiated cells and from control untreated cells and subjected to affinity selection using a 2'-O-methyl biotinylated oligonucleotide of sRNA-85 as described in ‘Materials and Methods’ section. Total RNA (2 μg; lanes 1 and 2) or 10% of the affinity-selected RNA (lanes 3 and 4) from untreated and irradiated cells was subjected to primer extension with the oligonucleotide indicated schematically in (C). The products were separated on a 6% polyacrylamide denaturing gel alongside a DNA-sequencing ladder (lanes 5–8) of the corresponding SSU rDNA. The sequence in the vicinity of the cross-linked site is indicated. The left arrow indicates the stop of the reverse transcriptase and the right arrow indicates the position of the cross-linked adduct on the rRNA. (B) As in (A), but the cross-linking was reversed by UV irradiation at 245 nm (10 milliWatts cm^-2) for 10 min. Affinity-selected RNA (10% of total; lanes 1 and 2) and the same amount of RNA that was subjected to photoreversal cross-linking (lanes 3 and 4) was analyzed by primer extension as described in (A). The treatments are indicated and the arrow marks the cross-linked adducts on the rRNA. (C) A portion of the secondary structure of the L. major SSU rRNA is shown. The cross-linked site of SSU helix 31 is indicated by an arrow and the location of the oligonucleotide primer used for primer extension by a line. (D) As in (A), but samples were subjected to primer extension using oligonucleotide 18490 complementary to positions 39–53 of sRNA-85 (lanes 1 and 2). RNA sequencing of sRNA-85 is shown (lanes 3–6). The left arrow indicates the stop of the reverse transcriptase and the right arrow indicates the position of the cross-linked adduct on sRNA-85. (E) Secondary structure of sRNA-85. Arrows indicate the two cross-linked sites on the RNA. (F) RNA isolated from irradiated cells was subjected to affinity selection and primer extension was performed as in (A) except that a gradient of dNTP concentration (0.05, 0.5 and 5 mM dNTPs; lanes 1–3, respectively) was used. The products were separated on a 6% polyacrylamide denaturing gel. The arrows on the right indicate the cross-link site identified in (A) as well as the modified Gm1550, the stops are one nucleotide before the cross-linked or modified site.

Oligonucleotide/RNase H cleavage demonstrates genuine intermolecular cross-linking between sRNA-85 and its interaction domains on rRNA

To verify that the cross-linked species mapped by ‘RNA walk’ and primer extension are indeed bona fide intermolecular cross-linked species between sRNA-85 and rRNA, the hybrid molecules were confirmed by RNase H mapping. To map the interaction between these molecules sRNA-85 and its associated rRNA were tagged by the splint labeling technique. In this method, a primer complementary to sRNA-85 carrying a run of 9 G
nucleotides at the 3’-end of the oligonucleotide was used as a template to incorporate [α-32P]-dCTP at the 3’-end of the sRNA-85 using the enzyme T7 DNA polymerase. In such an experiment we anticipate to identify cross-linking species between sRNA-85 and the different rRNA species (srRNA-2, LSU and SSU). To be able to detect such cross-linking in a denaturing gel that separates molecules in the size range of ten to hundreds of nucleotides, it is necessary to ‘release’ the rRNA domain that is cross-linked to sRNA-85. Indeed, we can observe that after cross-linking and labeling the cross-linked species, large amount of the label was trapped at the top of the gel and was unable to enter it. To release the cross-linked adducts, the cross-linked species were digested with RNase H and oligonucleotides flanking the predicted cross-linked sites. Next, these fragments were labeled by splint labeling of sRNA-85. This labeling should detect free sRNA-85 present in the sample and the rRNA fragments linked to this molecule. In addition and as previously demonstrated (6), the sample should also contain the 7SL RNA-sRNA-85 cross-links. The results of such experiments are presented in Figure 8. A fragment common to lanes 2–7 (marked with an arrow) represent two co-migrating fragments, 7SL RNA-sRNA-85 and
srRNA-2-sRNA-85 cross-linked species. Cleavage with either srRNA-2 (lane 4) or 7SL RNA (lane 7) oligonucleotides reduced the intensity of this fragment and produced new fragments (number 2 and 5) for srRNA-2 and 7SL RNA, respectively, suggesting that this fragment is composed of these two cross-linked species. Furthermore, the cleavage with rRNA-specific oligonucleotide flanking the cross-linked species released different size fragments; fragments number 1, 3 and 4. The size of these fragments was larger than the distance between the oligonucleotides used for digestion (marked in Figure 8B) because of the cross-linking to sRNA-85, which causes aberrant mobility of the cross-linked hybrid molecule in denaturing gels. To further verify that indeed these fragments were cleaved from their expected positions in the rRNA, the cleaved-labeled fragments were eluted from the gel and were further subjected to cleavage with an oligonucleotide situated between the 3′-end of the fragment (oligonucleotide K) and the cross-linked nucleotide (lane 2) was used, but not when an oligonucleotide situated downstream of the 3′ end of the fragment (oligonucleotide K) was used (lane 3). These data clearly demonstrate that the cross-linked sites represent an intermolecular linkage between sRNA-85 and the different sites on the rRNA.

To investigate if the four contact sites of sRNA-85 may represent interactions that can take place simultaneously, or whether these interactions may require the movement of this molecule within the ribosome, we sought to obtain structural information that will enable us to locate the interactions within the tertiary structure of the ribosome. Since there is no available crystal structure of eukaryotic ribosomes but only cryo-EM data of the yeast ribosome (35), we based our mapping on the crystal structure of the *Thermus thermophilus* 70S ribosome (PDB files 2J00 and 2J01) (42). To spatially locate the various cross-linked sites within the ribosome, we matched the rRNA sequence of *L. collosoma* (L.c) rRNA and their corresponding sites in *T. thermophilus*. Our analysis indicates that there is only one possible way to position a tRNA into the ribosome that agrees well with three of the contact sites. To construct our model, we used a tRNA^Phe^ which is 76 nucleotides long to simulate the interaction. Note that sRNA-85 is longer (85 nucleotides) and hence the proposed distances based on tRNA^Phe^ might be slightly different. Modeling tRNA^Phe^ suggests distances of ~11 Å with the Alu-like domain (helix 95), ~12.5 Å with the PTC (helix 92) and ~11 Å with the SSU of the rRNA (helix 31) (data not shown). Note, that the universal tRNA interaction with the SSU in helix 31 mapped in Figure 5 helped us to determine the orientation of the molecule within the ribosome. The interaction with A1745 (helix 63) seems
to be too far from the three other sites forming the triangle (helices 31, 92, 95) to allow its concomitant interaction. Thus, it is possible that this interaction reflects another conformation of sRNA-85 within the ribosome. The model further demonstrates that sRNA-85 occupies the interspace between the two ribosomal subunits and suggests that sRNA-85 may move during the translocation cycle.

DISCUSSION

In this study, we present a novel approach to identify RNA–RNA interactions between a small RNA and a complex target, the entire rRNA. Our approach is based on in vivo UV cross-linking in the presence of AMT. Enrichment of the target via affinity selection of the small RNA enables the use of RT–PCR to map interactions even with a target as large as the entire rRNA. This novel approach enabled us to map the interactions between the tRNA-like RNA of the trypanosome SRP, and the ribosome. The mapping identified four cross-linked sites that were verified by primer extension and oligonucleotide/RNase H cleavage. Three of the cross-linked sites are located in domains of known functions. Most intriguing is the finding that sRNA-85 interacts with helix 95, the SRL, an interaction analogous to the mammalian Alu domain; this suggests molecular mimicry between sRNA-85 and the mammalian Alu domain. Interestingly, a contact site was also found with the PTC. These contact sites with the SRL and the PTC may suggest that this novel tRNA can transiently block translation. The strength of the ‘RNA walk’ method is that it is performed in vivo and therefore can capture interactions which are transient and often weak. The methodology is simple, quantitative and affords quick scanning of a large target for the binding sites of small RNAs. The method was used in this study to elucidate a complex interaction in trypanosomes, but it should be applicable to any biological system from bacteria to man.
sRNA-85 interactions with the ribosome: possible implications on attenuating protein synthesis

The trypanosome 7SL RNA lacks the Alu domain L1.2 loop as well as the Alu domain-binding proteins, SRP9 and 14 (7,22). This raises the question of how such an SRP, lacking a functional Alu domain, can mediate a translation pause. sRNA-85 may represent an elegant solution for the arrest function by interacting with two functional domains of the ribosome, which affect translation, the binding site of the elongation factors (SRL) and the PTC. The SRL binds to elongation factors EF1 and EF2, which are required for efficient and accurate translation (43,44). Astonishingly, the sRNA-85 interaction with h95 (SRL), is identical to one of the contacts of the mammalian Alu domain with the ribosome (18). sRNA-85 may therefore represent a molecular mimic of the mammalian Alu domain and mediate the translational arrest function of trypanosome SRP. However, the actual arrest may involve a trypanosome-specific mechanism that may elicit an even stronger effect on the translating ribosome. Indeed, sRNA-85 was shown to interact with the PTC, where peptide bond formation is carried out. The interaction of sRNA-85 with the PTC seems to be unique. The binding sites of tRNA located in the A site or P site and the PTC site are well documented. C74 and C75 of the P-site tRNA base pair to G2252 and G2251, respectively of the 23S rRNA (45). The CCA end of the A-site tRNA is fixed by pairing of C75 with G2553 of the 23S A-loop (46,47). The contact site of sRNA-85 with the PTC is different from these interactions and is located at h92 (position 2561). Recent, comprehensive genetic studies of functional domains within the PTC showed that position 2573 (adjacent to the cross-linked site- position 2561) plays a role in guiding the rotating tRNA from the A to the P site. In addition, h92 interacts with h71 (domain IV) of rRNA (48,49) and a mutation in C2556 (also close to the sRNA-85 cross-linked site) induces steric hindrance, which disrupts the interaction between h92 and h71, also affecting the function of the ribosome (50). Thus, the pause in translation that the trypanosome SRP may elicit on the translating ribosome might be even stronger than that induced by the mammalian SRP.

The interaction of sRNA-85 with helix 31 in the SSU is a conventional tRNA interaction which places the tRNA anti-codon stem loop in the small subunit and allows the CCA end of the molecule to interact with the large subunit. Note, however that three out of the four contact sites mapped in this study are non-canonical tRNA interactions. In addition, 14 canonical tRNA interactions with the ribosome could not be detected between sRNA-85 and rRNA (data not shown). Our inability to detect these cross-linked sites may stem from the limitations of the method, as discussed above. However, these results may also reflect the inability of sRNA-85 to interact with conventional sites due to its deviations from the canonical structure; sRNA-85 is uncharged, and has a longer anti-codon stem with several bulges (6). These deviations may have caused the loss of the conventional interactions and helped this molecule to acquire special interactions necessary to fulfill its function in translation arrest. Interestingly, deletion of the anti-codon of sRNA-85 eliminated its ability to interact with h31, yet sRNA-85 can still interact with the ribosome via the other binding sites (Figure 6).

Our previous studies suggest that sRNA-85 interacts with the 7SL RNA by base pairing between positions 26 and 42 of the 7SL RNA (truncated Alu domain) and the sRNA-85 molecule (positions 15–30) (6). Can sRNA-85 interact simultaneously with 7SL RNA and the ribosome? The interactions of sRNA-85 with the LSU are predicted to occur at the 3'-end of the tRNA. The four interactions of sRNA-85 with the rRNA, however, are not mutually exclusive to the interactions of 7SL RNA with sRNA-85, since the latter interactions were mapped to the 5' end of the molecule. We therefore propose that sRNA-85 potentially interacts with the 7SL RNA throughout the translocation cycle.

Using the cross-linking positions of sRNA-85 and rRNA, we made attempts to position a known tRNA crystal to the crystal of eubacterial crystal structure (PDB file 2J01). Although the model is speculative, it suggests that while the interactions of sRNA-85 with the SSU, the PTC, and the SRL domains can take place simultaneously, the interaction of sRNA-85 with h63 is too far away from the triangle interactions. If indeed h63 is located far from the triangle interaction, the interaction with this domain may require other conformational changes of this molecule within the ribosome. sRNA-85 might have at least two conformations within the ribosome, the functional binding to the PTC and SRL (the triangle interaction) and another conformation which predominates either before or after the triangle functional interaction.

Pros and cons of the ‘RNA walk’ approach

‘RNA walk’ can be viewed as a generic method to map the interaction sites between a small RNA and its target, even with a target as large as the entire rRNA. The method is reliable only after enrichment of the cross-linked species by affinity selection with the small RNA as a ‘hook’. The affinity selection is a very critical step for the ‘RNA walk’ method. Only the selection of hybrid molecules enable the enrichment of specific rRNA molecules that were bound by sRNA-85, avoiding RT–PCR stops emerging from intermolecular cross-linking.

Statistically, at the level of irradiation used in this study each molecule will be cross-linked only once. However, since there are potentially four sites of cross-linking, the affinity-selected RNA should contain molecules that were not cross-linked at the tested cross-linked domain. Indeed, we were unable to efficiently amplify rRNA using primers spanning each of the cross-linked sites.

One of the drawbacks of the ‘RNA walk’ method is the efficiency of capturing the molecules that can teach us about the RNA–RNA interactions. The efficiency of selection can vary considerably for different small RNAs (in this study only 20% of sRNA-85 was selected). In some cases, it will not be possible to use the small RNA as the hook for affinity selection, like in the case of the miRNA
that is only 21-nt long, and part might be involved in cross-linking to the target. In such cases, the affinity selection should be mediated via the target.

The low abundance of the hybrid molecules could make it difficult to detect RNA–RNA interactions by the ‘RNA walk’. The interaction examined in this study is between rRNA that is found at 200 000 copies per cell and sRNA-85 that is ~100-fold less abundant. If on the other hand, mRNAs are considered as the target, the ability to capture hybrid molecules may be reduced by up to ~1000-fold. However, it should be possible to detect the interaction between mRNA and a small RNA that interacts with it using larger quantities of cDNA. The authenticity of ‘RNA walk’ has been demonstrated by showing that the adducts revealed by this method were confirmed by both primer extension and RNase H cleavage mapping. However, there might be additional contact sites that the ‘RNA walk’ failed to identify because the AMT cross-linking is limited to U which are in close proximity within the interaction domain, although Cs were also shown to be cross-linked (27).

In summary, this study describes the ‘RNA walk’, a generic method to identify small RNA target interactions based on in vivo cross-linking with AMT, affinity selection of the target and mapping the adducts by a variety of techniques. This method led to identification of novel interaction sites that could not be guessed by modeling or predicted bioinformatically. The method was used to probe the interaction between a unique and novel tRNA-like molecule in trypanosomes, sRNA-85 and the entire rRNA. The study led to discovery of molecular mimicry between this novel molecule and the Alu domain of the mammalian SRP. This study therefore suggests that several different solutions might exist in nature to fulfill the arrest function of the SRP; such possible mechanisms should be particularly investigated in bacteria and in lower eukaryotes whose SRP lacks the Alu domain. ‘RNA walk’ is a simple, quick and quantitative method that can be used to examine RNA–RNA interactions between a small RNA and its target being rRNA or less abundant mRNA. Each pair of interactions will need a special calibration to find the optimal target for affinity selecting the hybrid small RNA with its target.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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