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Small Noncoding 4.5SH and 4.5SI RNAs and Their Binding to Proteins

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Abstract—The functions of small noncoding RNAs 4.5SH and 4.5SI found in murine-like rodents are unclear. These RNAs synthesized by RNA polymerase III are widely expressed in rodent organs and tissues. Using crosslinking assays, it was shown that approximately half of all 4.5SI and 4.5SH RNA molecules were bound to proteins provisionally called X and Y, respectively. An immunoprecipitation experiment showed that both these RNAs were associated with the La protein, which did not crosslink to them. The termini of 4.5SI RNA form a long duplex stem, which makes the molecule more stable than 4.5SH RNA. Modification of the 5'-end sequence destructing the stem of 4.5SI RNA altered its protein-binding properties; after the 3'-end sequence was changed to the complementary, both the stem structure and the RNA binding to protein X were restored. Presumably, this protein plays a role in increasing the half-life of 4.5SI RNA.

Keywords: 4.5S RNA, small noncoding RNA, RNA-binding proteins, La protein, rodents

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INTRODUCTION

In eukaryotic cells, noncoding RNAs (ncRNAs) are involved in a number of different processes: initiation of DNA replication (Y RNA), regulation of transcription (7SK RNA), splicing (U1, U2, U4, U5, U6, U11, and U12 RNA), translation (tRNAs, rRNAs, and 7SL RNA), and mRNA degradation (small interfering RNAs and long ncRNAs) [1, 2]. However, very little is known about the role of two small ncRNA species detected in mouse and rat cells as long ago as in the 1970s [3, 4]. One of them (4.5SH RNA) supposedly prevents the export of certain mRNA species from the nucleus into the cytoplasm [5]; the function of the other one (4.5SI RNA) currently remains unknown. The 4.5SH and 4.5SI RNA molecules are similar in length (94 and 98 b, respectively), but their sequences are different. These ncRNAs synthesized by RNA polymerase III are found in all organs and are located predominantly in the nucleus; the number of molecules per cell may reach several millions [6]. 4.5SI RNA was found in rodents of three families: Muridae (mice, rats, and gerbils), Cricetidae (hamsters and voles), and Spalacidae (blind mole-rats, bamboo rats, and zokors) [7]. 4.5SH RNA is present in the same families, as well as in Dipodidae (jerboas and jumping mice) [8]. Apparently, the genes that encode these ncRNAs originate from mobile genetic elements termed SINEs [9]. The nucleotide sequences of 4.5SH RNA and SINE B1 are very similar, implying that they are evolutionary related [10, 11]. At the same time, the 5' end of 4.5SI RNA resembles the corresponding fragment of SINE B2, which suggests their common origin [12]. The organization of these two ncRNA genes is very different. A rodent genome contains 600–800 genes of 4.5SH RNA, each of them making part of long (4–5 kb) tandem repeats [8]. At the same time, mouse and rat genomes apparently contain only three 4.5SI RNA genes located at a considerable distance from each other (40 kb) [13]. Another important difference between these RNAs is that 4.5SI RNA is fairly stable, whereas 4.5SH RNA disintegrates rapidly with a half-life of 20 min [14]. The abundance of 4.5SH RNA in a cell significantly increases in response to heat shock, due to both enhanced transcription and slower disintegration of the molecule [15].

4.5SI RNA exhibits higher stability than 4.5SH RNA, probably because its terminal fragments are complementary to each other and form a double-helix stem [16]. Experiments with other short-living RNAs also showed that sequence modifications that resulted in the formation of a terminal double-helix stem significantly increased the lifetime of these RNAs [14].

Most RNA species present in a cell are associated with proteins. However, mature tRNA molecules do not form stable protein complexes. Currently, little is known about the association of 4.5SH and 4.5SI RNAs with proteins. It was reported that these RNAs bind with the La protein [17, 18], in agreement with the notion that La associates with the UUU trinucleotide present at the 3' end of all primary transcripts synthesized by RNA polymerase III (UUU results from the transcription of the terminator sequence)

[19]. In the present work, we show that most 4.5SH and 4.5SI RNA molecules are associated with proteins that can be crosslinked to RNA as a result of UV irradiation of cells or nuclear extracts. The La protein is also associated with 4.5SH and 4.5SI RNAs, but does not crosslink to them.

EXPERIMENTAL

Cells. For Krebs ascites carcinoma (KAC) cells, outbred mice were injected intraperitoneally with 0.7 mL ascites fluid; after seven to ten days, ascites fluid was collected and cells were isolated by centrifugation.

For transfection experiments, HeLa cells were cultured in DMEM supplemented with 10% fetal calf serum and 40 μg/mL gentamicin to 70–90% confluence in petri dishes (d = 100 mm). A transfection mixture was prepared as follows: 15 mg of plasmid was mixed with 1.5 mL DMEM without serum or an antibiotic and with 30 μL of TurboFect reagent (Thermo Fisher Scientific, United States) and incubated for 20 min at room temperature as recommended by the manufacturer. The mixture was added to the cells, and they were incubated overnight at 37°C.

Nucleoplasm isolation from KAC and transfected HeLa cells. KAC fluid (1 mL) was diluted with phosphate-buffered saline (PBS), centrifuged for 5 min at 1000 rpm, and the cells were suspended in 10 mL of cold HTM-C buffer (0.1 M NaCl, 50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, and 0.25 M sucrose) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was diluted with 10 mL cold HTM-C containing 1% NP-40, thoroughly mixed, incubated on ice for 5 min, and centrifuged (3500 rpm, 10 min, 4°C). The pellet of nuclei was resuspended in 4 mL of the buffer containing 50 mM NaCl, 2.5 mM $MgCl_2$, and 20 mM Tris-HCl pH 7.4. Transfected HeLa cells were harvested from the petri dishes with Versene solution, and the nuclei were isolated using the same technique, except that the volumes of solutions used at each stage were 10 times smaller. The nuclear suspensions were ultrasonicated on ice using a Sonopuls Bandelin disintegrator (Germany): for KAC cells, a 73-mm probe with 2-min exposure was used; for HeLa cells, a 72-mm probe with six series of 10-s exposure at 20-s intervals. Next, the suspensions were centrifuged at 15000 rpm (10 min, 4°C); the supernatant was collected and further centrifuged in an SW50 rotor for 2 h at 30000 rpm and 4°C. The resulting supernatant (nucleoplasm fraction) was collected, divided into 30-μL aliquots, and stored in liquid nitrogen until the start of the experiment.

RNA–protein crosslinking. Specimens of KAC or HeLa cell nucleoplasm containing nuclear RNP particles were UV-treated [20] on ice in open one-way Eppendorf tubes. The distance from UV light source to the specimen was 4 cm; irradiation time was 8 min, unless specified otherwise.

Alternatively, intact KAC cells were irradiated in 35-mm petri dishes (10^7 cells in 1 mL PBS, 4-cm distance towards the lamp, 8 min) with subsequent nucleoplasm isolation.

Immunoprecipitation of RNA–La complexes, RNA isolation. Aliquots of protein G-sepharose suspension (20 μL, Amersham, Great Britain) were washed three times with 100 μL IP buffer (0.025 M Tris-HCl pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol), with 1.5-min centrifugation at 13400 rpm, 4°C. After resuspending the pellet in 30 μL IP-buffer, 2 μg antibodies to La/SSB (sc-80656, Santa Cruz Biotechnol., United States) or control mIgG-2a antibodies were added, and the particles were incubated with stirring for 30 min at 4°C. Antibody-carrying particles were washed twice with IP buffer as described above.

Nucleoplasm isolated from KAC cells (30 μL) was mixed with 1.5 μL of a protease inhibitor cocktail (Sigma), 20 units RNase inhibitor, and 240 μL IP buffer. The mixture was transferred into a tube with sepharose particles and incubated in a rotating shaker for 2 h at 4°C. After immunoprecipitation, the tubes were centrifuged for 5 min at 13400 rpm and the supernatant was collected in clean tubes. The pellet was washed with 200 μL PBS for 5 min and centrifuged for 1.5 min (the procedure was repeated three times). The pellet was resuspended in 200 μL of elution buffer (50 mM Tris-HCl pH 8.0, 20 mM DTT, 1% SDS), incubated for 5 min at 95°C, and centrifuged again. The eluate (further referred to as "precipitate") was collected in clean tubes.

RNA isolation from precipitate and supernatant specimens after immunoprecipitation was performed by adding phenol–chloroform (1 : 1, pH 8.0), mixing for 10 min, incubating on ice for 15 min, and centrifuging for 15 min at 10000 rpm. RNA was isolated from the supernatant fraction with isopropanol and analyzed by denaturing PAGE in an urea-containing gel (see below).

In some experiments, UV-irradiation and SDS–PAGE were performed prior to nucleoplasm immunoprecipitation (see below).

Northern blotting of RNA and RNP. RNA isolated from the precipitate and supernatant specimens was separated by PAGE in a 6% gel containing 8 M urea.

RNP particles were separated by PAGE in a 10% gel containing 0.1% SDS conventionally used for protein analysis [20]. In our work, the method was modified as follows: the separating gel comprised two layers with acrylamide : bisacrylamide ratios of 19 : 1 in the lower layer and 37 : 1 in the upper layer. The lower layer was twice narrower than the upper and served for retardation of RNA not linked to proteins.

Following electrophoresis, RNA and RNP were transferred onto a Hybond-XL nylon membrane for 2–2.5 h at 3 V using a semidry electroblotting system. After transfer, RNA was fixed on the membrane by UV irradiation (365 nm, 3.6 J).

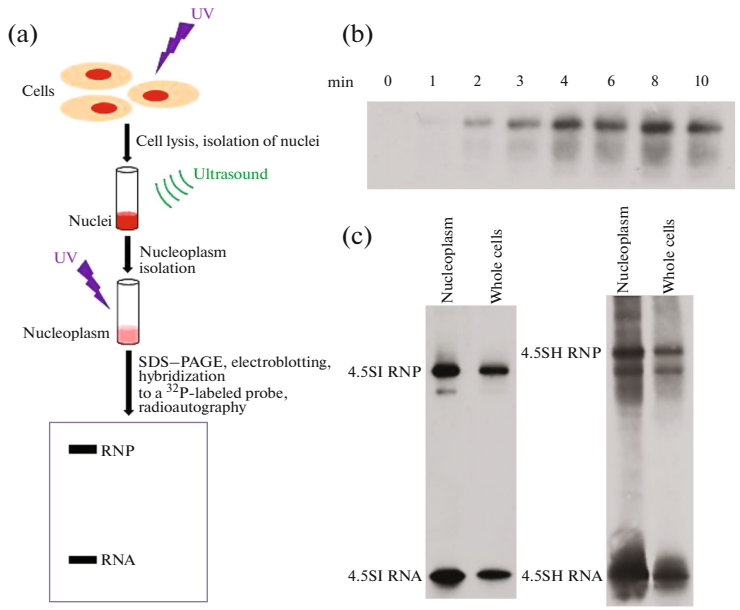

Fig. 1. 4.5SI and 4.5SH RNA crosslinking to proteins. (a) Design of the experiment. (b) Determining the optimal duration of nucleoplasm exposure to UV radiation. Complexes of 4.5SI RNA with proteins were detected by Northern blot hybridization. In this experiment, free RNA was not monitored. (c) Detection of RNA and RNPs after UV-irradiation of nucleoplasm or whole KAC cells by Northern blot hybridization with ^{32}P-labeled probes complementary to 4.5SI RNA (left panel) or 4.5SH RNA (right panel). 4.5SI RNP, 4.5SI RNA complex with protein X; 4.5SH RNP, 4.5SH RNA complex with protein Y.

4.5SH RNA/RNP and 4.5SI RNA/RNP were detected by hybridization with a ^{32}P-labeled probe synthesized by PCR [7, 8]. The membrane was incubated overnight with the probe in a hybridization buffer (50% formamide, 5× Denhardt's solution, 4× SSC, 1% SDS, and 0.1 mg/mL salmon sperm DNA) at 42°C. Finally, after washing the membrane with 0.1× SSC and 0.1% SDS for 1 h at 42°C, an X-ray film was exposed to it and the membrane was scanned using a Cyclone phosphorimager.

RESULTS

Proteins associated with 4.5SH and 4.5SI RNA were detected using the UV irradiation of the nucleoplasm fraction of KAC cells (Fig. 1a). It is known that exposure to UV rays usually causes RNA crosslinking to the proteins directly interacting with them. The irradiated nucleoplasm was separated using SDS–PAGE, after which RNPs and free RNA were transferred onto a membrane. Specific RNPs and RNAs were detected by Northern hybridization. As shown in Fig. 1b, an increase in the duration (dose) of UV-irradiation first enhanced the intensity of the 4.5SI RNA–protein band, but after 4 min the yield of crosslinked complexes reached a plateau. In further experiments, 8-min-long UV-irradiation was used to ensure efficient RNA–protein crosslinking. Approximately half of the 4.5SI RNA molecules were crosslinked to a protein and migrated significantly slower than free RNA during electrophoresis (Fig. 1c). For 4.5SH RNA, the portion of such molecules was slightly lower. The fact that a considerable fraction of RNA was not crosslinked to proteins probably indicates that not all 4.5SI and 4.5SH RNA molecules make a part of RNPs. For both 4.5SH and 4.5SI RNAs, two bands could be observed in the RNP migration zone, the upper band being clearly a dominant one (further referred to as 4.5SH RNP and 4.5SI RNP, respectively). The mobility of 4.5SH RNP was slightly lower than that of 4.5SI RNP, which may mean that these RNAs interact with two different proteins (Fig. 1c). Proteins associated with 4.5SI and 4.5SH RNAs were provisionally termed X and Y. To verify that 4.5SH and 4.5SI RNA association with these proteins exists in vivo, and does not represent a nucleoplasm isolation artifact, we also irradiated intact KAC cells, and their nucleoplasm was isolated and analyzed by electrophoresis with subsequent Northern hybridization. This experiment revealed the same 4.5SH and 4.5SI RNP bands (Fig. 1c), which proved that the corresponding RNPs exist within living cells.

It is known that transcripts synthesized by RNA polymerase III are associated with the La protein, which interacts with the 3-terminal UUU sequence of these RNAs [19]. To determine whether one of the polypeptides X or Y was in fact a La protein, we performed immunoprecipitation experiments, incubating the nucleoplasm fraction with sepharose particles carrying antiLa antibodies. Next, RNA was isolated from the sepharose-bound material (precipitate) and from the supernatant and analyzed by Northern hybridization. It was found that approximately 40% 4.5SI RNA and 25% 4.5SH RNA were associated with the La protein (Fig. 2).

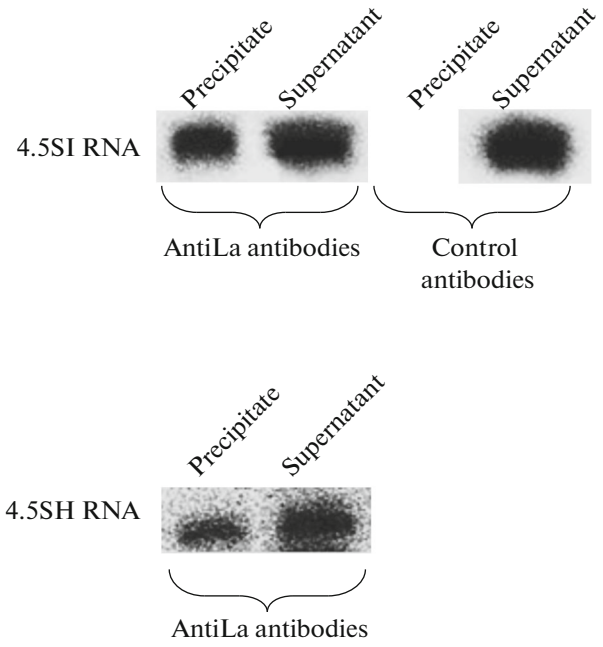

Fig. 2. Immunoprecipitation of RNPs from the nucleoplasm of KAC cells with antibodies to protein La. Northern blot analysis of 4.5SI RNA (upper panel) and 4.5SH RNA (lower panel) isolated from the precipitate (antibody-bound material) and the supernatant (material remaining in the solution).

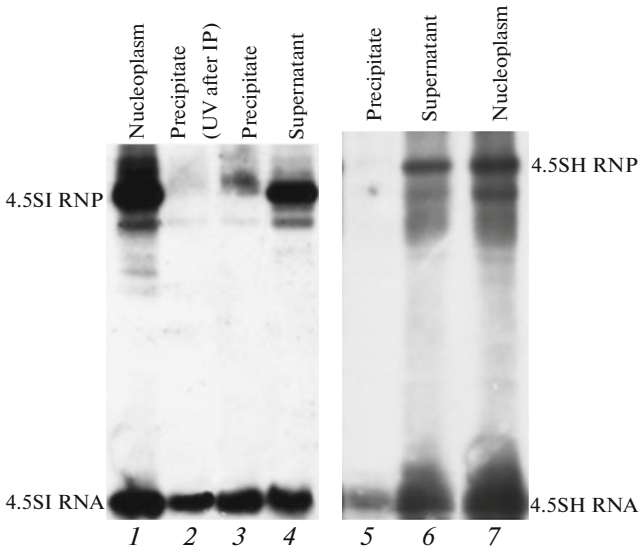

Fig. 3. Immunoprecipitation of UV-crosslinked RNA–protein complexes with antibodies to the La protein. RNP and RNA detection by Northern hybridization with probes to 4.5SI RNA (left) and 4.5SH RNA (right): (*1*) and (*7*) UV-irradiated nucleoplasm of KAC cells; (*2*) precipitate that was UV-irradiated after immunoprecipitation with antibodies to the La protein; (*3–6*) precipitate and supernatant from the immunoprecipitation of UV-crosslinked RNPs using antibodies to the La protein ; IP, immunoprecipitation.

Next, we performed immunoprecipitation of UV-irradiated nucleoplasm fraction with antiLa antibodies. The precipitate and supernatant fractions were subjected to SDS–PAGE and subsequent Northern hybridization (Fig. 3). As a result, the bands of 4.5SI and 4.5SH RNPs were detected in the supernatant but not in the precipitate. This suggests that neither X nor Y polypeptide crosslinked to the RNA molecules concerned was a La protein. Moreover, it can be concluded that those 4.5SI and 4.5SH RNA molecules that interact with the La protein are not bound to proteins X or Y, respectively.

In a further experiment, we performed the immunoprecipitation of 4.5SI RNA and subsequent UV irradiation of RNP bound to the sepharose particles. Thus, it could be determined whether RNA–La protein crosslinking interfered with La interaction with antibodies. However, in this setting, the precipitate did not contain RNPs either (Fig. 3, *2*). Therefore, in our experiments, the La protein was associated with 4.5SI RNA, but could not be crosslinked to it.

In our previous study, it was shown that 4.5SI RNA is more long-living than 4.5SH RNA thanks to a double-helix structure formed by its complementary terminal regions [16]. In this context, we undertook to find out how the presence of the terminal stem structure affected the binding of X and Y proteins using the following constructs of modified 4.5SH and 4.5SI RNA genes (Fig. 4a). Construct A encoded a chimeric 4.5SH RNA sequence where the terminal fragments were substituted with the corresponding fragments of 4.5SI RNA. In construct B, the 3'-end of the 4.5SH RNA gene was substituted with a sequence complementary to the 5'-end. Construct C was a 4.5SI RNA gene where the sequence of the 5'-end was made non-complementary to the 3'-end; as a result, this RNA lacked the terminal double-helix stem. Finally, construct D was derived from construct C by replacing the 3'-end sequence with the one complementary to the 5'-end; thus, in the corresponding RNA transcript, the stem structure was restored. These constructs, as well as the plasmids carrying unmodified genes of 4.5SH and 4.5SI RNA, were used to transfect HeLa cells. Since human cells do not synthesize 4.5SH or 4.5SI RNA, RNAs transcribed from the plasmid constructs could be easily detected. Nucleoplasm isolated from the transfected cells was exposed to UV radiation and separated by electrophoresis in SDS–PAGE. Northern blot hybridization showed that protein-crosslinked constructs A, B, and D and 4.5SI RNA molecules had similar mobility (Fig. 4b), indicating that all of them were bound to the same protein (X). Importantly, in spite of the difference in their nucleotide sequences, all these RNA molecules featured a 16-bp double-helix structure formed by the complementary ends of the molecule (Fig. 4a); apparently, it is this structure that determines RNA interaction with

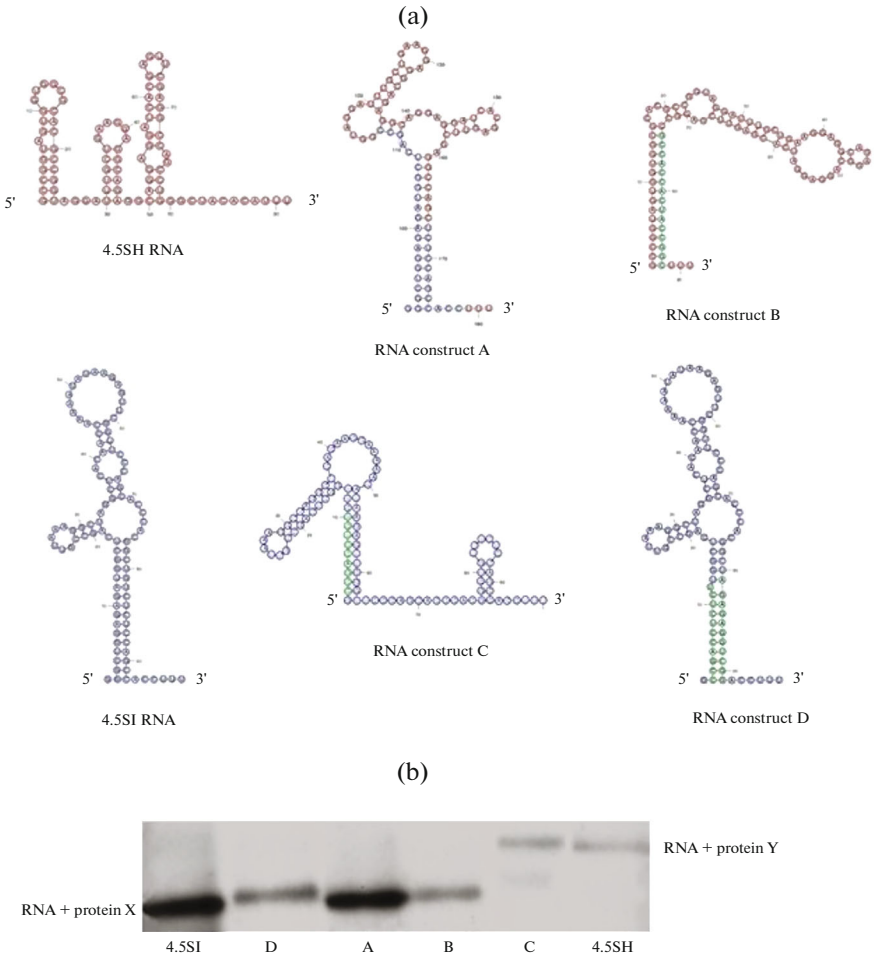

Fig. 4. RNA–protein UV-crosslinking in the nucleoplasm isolated from HeLa cells transfected with genes encoding 4.5SH RNA, 4.5SI RNA, and constructs A–D. (a) Predicted secondary structures of 4.5SH RNA, 4.5SI RNA, and RNAs transcribed from constructs A–D. Sequences derived from the 4.5SH RNA and the 4.5SI RNA gene are shown in red and in blue, respectively; artificial substitutions are shown in green. (b) RNP detection by Northern blot hybridization with ^{32}P-labeled probes to 4.5SH RNA (4.5SH RNA, constructs A and B) or 4.5SI RNA (4.5SI RNA, constructs C and D).

protein X. Construct C and 4.5SH RNA formed protein complexes with lower mobility, possibly because they were associated with a different protein (Y). Interestingly, RNAs transcribed from these constructs do not possess complementary terminal regions. Thus, our results suggest that the presence or absence of the terminal double-helix stem may determine RNA association with one of the detected proteins.

DISCUSSION

Apparently, most RNA species form stable complexes with proteins, which is important for their functioning [21–23]. However, cytoplasmic tRNAs are free and form only short-living complexes with aminoacyl-tRNA synthetases and ribosomes [24]. This work was undertaken to find out whether small noncoding 4.5SI and 4.5SH RNAs were associated with proteins in vivo. It was shown that nearly half of these molecules was associated with proteins and could be crosslinked to them by UV irradiation of nuclear extracts or intact cells. The crosslinking implies direct interaction between RNA and proteins. Northern blot analysis revealed a single dominant band of an RNA–protein complex for either RNA species studied. Proteins that form complexes with 4.5SI and 4.5SH RNA were provisionally denoted X and Y.

It is known that primary transcripts synthesized by RNA polymerase III bind to the La protein via the UUU triplet at their 3'-end [19, 25]. Available data suggest that both 4.5SI and 4.5SH RNA are also associated with this protein [17, 18]. Immunoprecipitation experiments with antiLa antibodies showed that 40% of 4.5SI RNA and 25% of 4.5SH RNA are associated with this protein. However, RNPs precipitated with antiLa antibodies did not contain proteins X or Y crosslinked to RNA. This fact indicates that some 4.5SI and 4.5SH RNA molecules present in a cell are associated with La proteins, while others are bound to protein X or Y, respectively. The inability of La to cross-

link to 4.5SI or 4.5SH RNA was probably due to the small length of the protein-binding region in these molecules.

Previously, we showed that 4.5SI RNA comprises a 16-bp terminal stem structure, which is not in 4.5SH RNA [16]. To find out whether this structure affects RNA binding to X and Y proteins, we performed UV-crosslinking experiments on 4.5SI and 4.5SH RNA molecules with terminal sequences modified in such a way that the 16-bp stem was either destructed or restored. It was found that all RNAs that carried the terminal double-helix stem could bind to protein X, similarly to 4.5SI RNA, whereas those that lacked the stem did not bind it, similarly to 4.5SH RNA. It is known that RNA molecules that possess complementary stem-forming terminal fragments are long-lived, while those that lack it are short-lived [16]. These data suggest that X protein binds to the terminal double-helix stem of RNA and may play an important role in its stabilization.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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