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MOLECULAR CELL BIOLOGY

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4.5SI and 4.5SH RNAs: Expression in Various Rodent Organs and Abundance and Distribution in the Cell

K. A. Tatosyan, A. P. Koval, I. K. Gogolevskaya, and D. A. Kramerov*

Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, 119991 Russia

**e-mail: kramerov@eimb.ru*

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Abstract—Studying the structure, functions, and cell physiology of small RNAs remains important. The 4.5SI and 4.5SH small RNAs, which were among the first to be discovered and sequenced, share several features, i.e., they are both approximately 100 nt in size, are synthesized by RNA polymerase III, and are found only in rodents of several related families. Genes coding for these RNAs are evolutionarily related to short interspersed elements (SINEs). However, the two RNAs differ in nucleotide sequence, half-life in the cell, and the organization of their genes in the genome. Although the 4.5SI and 4.5SH RNAs have been identified more than three decades ago, several aspects of their metabolism in the cell are still poorly understood. The 4.5SI and 4.5SH RNA levels were measured in various organs of three rodent species (mouse, rat, and hamster). Both of the RNAs were found to occur at high levels, which were much the same in different organs in the case of the 4.5SI RNA and varied among organs in the case of the 4.5SH RNA. Both 4.5SI and 4.5SH RNAs demonstrated a predominantly nuclear localization with a detectable presence in the cytoplasm. The copy number per cell for the RNAs was estimated at $0.4–2.4 \times 10^6$. A quantitative study for the 4.5SI and 4.5SH RNAs was performed for the first time and resolved a number of contradictions in data from other studies.

Keywords: 4.5SH RNA, 4.5SI RNA, small noncoding RNAs, SINE, rodents

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INTRODUCTION

Studies of small cell RNAs started with the discovery of tRNAs and the 5S rRNA. Their roles in mRNA translation were established quite soon afterwards. In the 1970s, a number of new small RNAs (70–300 nt) were identified in eukaryotic cells. It has become clear with time that small RNAs play important roles in pre-mRNA splicing (U1, U2, U4, U5, U6, U11, and U12 RNAs), pre-rRNA processing (U3 and U14 RNAs), pre-rRNA modification (C/D-box and H/ACA-box RNAs), protein secretion (7SL RNA), transcriptional regulation (7SK RNA), and DNA replication initiation (Y RNA) [1–5]. A discovery of numerous microRNAs of approximately 22 nt has opened a special area of research, wherein microRNAs have been shown to regulate gene expression via mRNA silencing [6].

The 4.5SI and 4.5SH RNAs were among the first to be identified and sequenced [7, 8]. These RNAs have been detected in mouse and rat, but not human, cells [9, 10]. More recently, the 4.5SI RNA has been shown to occur in cells of only three related rodent families: Muridae (mice, rats, and gerbils), Cricetidae (hamsters and voles), and Spalacidae (mole rats, bamboo rats, and zokors) [11, 12]. The 4.5SH RNA is found in the same rodent taxa and additionally in jerboas, jumping mice, and birch mice (Dipodidae), that is, in all members of the clade Myodonta [13].

The two RNAs are approximately 100 nt in length each, but have only a low nucleotide sequence similarity. Both of the RNAs are synthesized by RNA polymerase III, and this circumstance determines the presence of short similar sequences: an internal promoter, which consists of A and B boxes (11 nt each), and a 3'-terminal region of three or four U residues, which result from transcription of a terminator (TTTTTT) [14, 15].

The 4.5SI and 4.5SH RNA genes are differently organized in the genome. In mice, the 4.5SI RNA is synthesized from three genes, which occur in chromosome 6 and are 40 kb away from each other [12]. Far more (700–800) 4.5SH RNA genes have been found in all rodent genomes examined, each occurring in a tandem repeat of 4–5 kb [13, 16]. It is of interest that the two RNAs are evolutionarily related to short interspersed elements (SINEs). The 4.5SI RNA genes probably originate from the SINE B2 [17]; and the 4.5SH RNA genes, from a copy of the ancient SINE pB1 [18, 19]. SINEs are nonautonomous mobile genetic elements typical of the majority of multicellular eukaryotes [20]. The SINE length varies from 100 to 500 bp, and a SINE copy number in the genome can

reach one million. The relationship between SINEs and the 4.5SI and 4.5SH RNA genes is not unique; evolutionary relationships with SINEs are known for other small noncoding RNA genes as well [21–23].

The functions of the 4.5SI and 4.5SH RNAs remain unclear. However, a recent study has shown that the 4.5SH RNA is capable of interacting with antisense SINE B1 sequences contained in mRNAs and thereby render these transcripts in the nucleus [24]. The 4.5SH RNA level in the cell has been found to increase in response to heat shock, and slower 4.5SH degradation contributes substantially to the increase [25].

In spite of the long history of research focusing on the 4.5SI and 4.5SH RNAs, several important issues are still poorly understood, and the available data are discrepant. For the first time, we systematically assayed the relative contents of the 4.5SI and 4.5SH RNAs in various mouse, rat, and hamster organs and studied the 4.5SI and 4.5SH RNA distributions between the nucleus and cytoplasm. The copy number in the cell was estimated at several millions for both of the RNAs.

EXPERIMENTAL

Biological material. Tissue and organ samples were examined in the house mouse *Mus musculus* strain C57BL/6 (four mice), gray rat *Rattus norvegicus* strain Wistar (three rats), and golden hamster *Mesocricetus auratus* (three hamsters). Cells of the Rat1 [26], L929 (ATCC CCL-1), NIH/3T3 (ATCC CRL-1658), and 4T1 (ATCC CRL-2539) lines were cultured in DMEM supplemented with 10% fetal bovine serum. To obtain Krebs II ascites carcinoma (KAC) cells [27], 0.7 mL of ascites fluid were injected intraperitoneally to random-bred mice, ascites fluids were taken 7–10 days after, and cells were collected by centrifugation (700 *g*, 5 min).

Cell fractionation. Cells (4–24 million) were suspended in 1 mL of buffer HTM-C (0.1 M NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.25 M sucrose) containing 1 mM PMSF; the suspension was diluted with 1 mL of buffer HTM-C containing 1% NP-40, incubated in ice for 5 min, and centrifuged (1900 *g*, 10 min). The nuclear fraction (pellet) was separated from the cytoplasmic fraction (supernatant).

RNA isolation. Total and nuclear RNAs were isolated from cultured cells and tissue and organ samples with guanidine thiocyanate [28]. To isolate RNA from the cytoplasmic fraction, we used deproteinization with a mixture (1 : 1) of phenol (pH 8.0) and chloroform.

Northern blot hybridization. RNA samples were resolved by PAGE in 6% gel containing 8 M urea. After PAGE, RNA was transferred onto a Hybond XL nylon membrane via semi-dry electroblotting (3–5 V, 2–2.5 h) and immobilized by exposing the membrane to UV light (365 nm). Probes were labeled with [α-^{32}P]dATP in PCR. Hybridization was carried out as in [29]. To visualize the signal, the membrane was exposed with a screen, which was then scanned using a Cyclone Storage Phosphor System phosphorimager. In addition, the membrane was exposed with an X-ray film in a cassette with an intensifying screen at –70°C for 1–48 h.

Estimation of the RNA copy number per cell. Prior to isolating RNA, cells were counted in a Goryaev chamber. An RNA aliquot corresponding to 4×10^5 cells was applied on an 8% polyacrylamide gel. Deoxyoligonucleotides corresponding to fragments (50 nt) of the RNAs under study were applied in various amounts on the same gel. The following deoxyoligonucleotides were used:

4.5SI AAAATATAAGAGTTCGGTTCCCAGCACCCACGGCTGTCTCTCCAGCCACC;

4.5SH TGGCGCACGCCGGTAGGATTTGCTGAAGGAGGCAGAGGCAGGAGGATCAC;

U1 CTGCGATTTCCCCAAATGCGGGAAACTCGACTGCATAATTTGTGGTAGTG.

RNA and DNA were transferred onto a Hybond XL membrane and hybridized with the following labeled deoxyoligonucleotides:

4.5SI GGTGGCTGGAGAGACAGCCGTGGGTGCTGGGAACCGAACT;

4.5SH GTGATCCTCCTGCCTCTGCCTCCTTCAGCAAATCCTACCG;

U1 CCACAAATTATGCAGTCGAGTTTCCCGCATTTGGGGAAAT.

Probes complementary to both the RNAs under study and the deoxyoligonucleotide immobilized on the membrane were 5'-endlabeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Hybridization was carried out as described previously [29]. The intensities of the radioactive signals from the 4.5SI, 4.5SH, and U1 RNAs and the respective deoxyoligonucleotides were determined with a phosphorimager. Data obtained for the deoxyoligonucleotides were used to construct calibration plots (deoxyoligonucleotide band radioactivity as a function of the deoxyoligonucleotide amount applied on gel). The copy number of the RNA in question was calculated as

$$N = \frac{R \times N_A}{K \times M \times 10^{12}},$$

were N is the copy number of the RNA in given cells; R is the radioactivity (conventional units) of the RNA band for given cells; N_A is Avogadro's number (6×10^{23}), M is the cell count corresponding to the RNA amount applied on gel ($M = 4 \times 10^5$); $K = (Y_2 - Y_1)/(X_2 - X_1)$, where Y_1 and Y_2 are the radioactive hybridization signal intensities (conventional units), and X_1 and X_2 the deoxyoligonucleotide amounts (pmol) corresponding to two different points of the calibration plot.

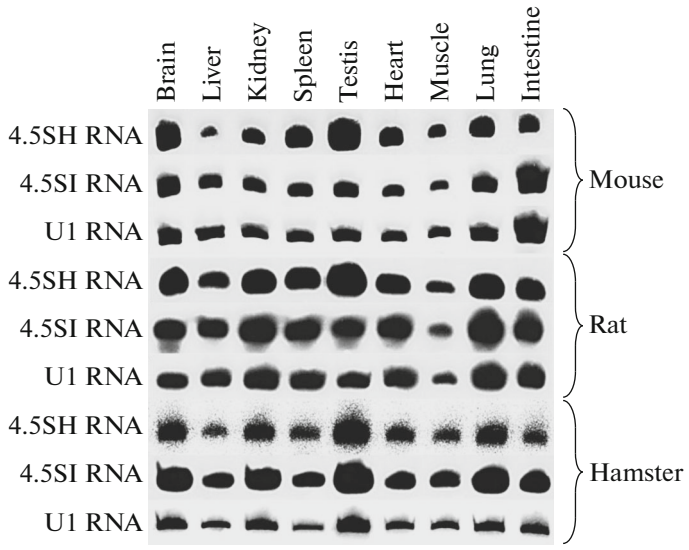

Fig. 1. Detection of the 4.5SH, 4.5SI, and U1 RNAs in total RNA samples isolated from nine organs in the house mouse *M. musculus* strain C57BL/6, gray rat *R. norvegicus* strain Wistar, and golden hamster *Mes. auratus*. Northern blot hybridization data obtained for one animal are shown for each species.

The RNA and deoxyoligonucleotide losses during the transfer from polyacrylamide gel onto a membrane were estimated using a labeled marker. For the marker, the difference in losses was no more than 15% between short (50 nt) and long (100 nt) fragments.

RESULTS

Relative Abundances of the 4.5SI and 4.5SH RNAs in Various Rodent Organs

Estimating the contents of various RNAs and proteins in organs and tissues can be of importance for understanding the roles that the molecules in question play in the body. For this purpose, total cell RNA was isolated from nine organs (brain, liver, kidney, spleen, heart, muscle, lung, testis, and small intestine) in the house mouse *M. musculus*, gray rat *R. norvegicus*, and golden hamster *Mes. auratus*. RNA was resolved by denaturing PAGE, transferred onto a membrane, and hybridized with a ^{32}P-labeled probe specific to the 4.5SI or 4.5SH RNA. As a control, the U1 RNA (a widespread nuclear RNA involved in splicing) was detected by hybridization with a specific probe. The membrane was exposed with an X-ray film or scanned with a phosphorimager. Autoradiography results are shown in Fig. 1. As is seen, the 4.5SI and 4.5SH RNAs were easily detectable in all samples, suggesting their high-level expression in various tissues and organs. Signal intensities obtained with a phosphorimager were analyzed quantitatively (Fig. 2). Hybridization signals from the 4.5SI and 4.5SH RNAs were normalized to the U1 RNA signal. The muscle levels of the 4.5SI and 4.5SH RNAs were taken as unity for convenience. The 4.5SI RNA content varied among organs insignificantly, no more than twofold (the liver and spleen in hamster). A greater variation was observed for the 4.5SH RNA levels; i.e., an almost fivefold difference was seen between the small intestine and testis in mouse. The evaluation of contents in different organs for the two RNAs showed that their expression profiles are similar in different rodents (Fig. 2).

Intracellular Localization of the 4.5SH and 4.5SI RNAs

To estimate how the RNAs are distributed between the nucleus and the cytoplasm, nuclear and cytoplasmic fractions were isolated from cells of several lines and used to isolate RNA. The RNA samples were tested by Northern blot hybridization, and the hybridization signal was measured with a phosphorimager (Fig. 3).

The 4.5SH and 4.5SI RNAs were found to accumulate in the nucleus to 75–79 and 66–77.5% of their respective contents in the four cell lines (Table 1). The finding suggests a predominantly nuclear localization for the two RNAs.

Estimation of the 4.5SH and 4.5SI Copy Numbers per Cell

The amount in the cell is important to know when studying metabolism and functions for a particular RNA. We developed a simple method to estimate the RNA copy number per cell. Total RNA isolated from a known number of cells was resolved by PAGE in one gel with serial dilutions of a deoxyoligonucleotide corresponding to a fragment (50 nt) of the RNA of interest. RNA and DNA were transferred onto a membrane and hybridized with a 5'-endlabeled deoxyoligonucleotide (40 nt) complementary to both the target RNA

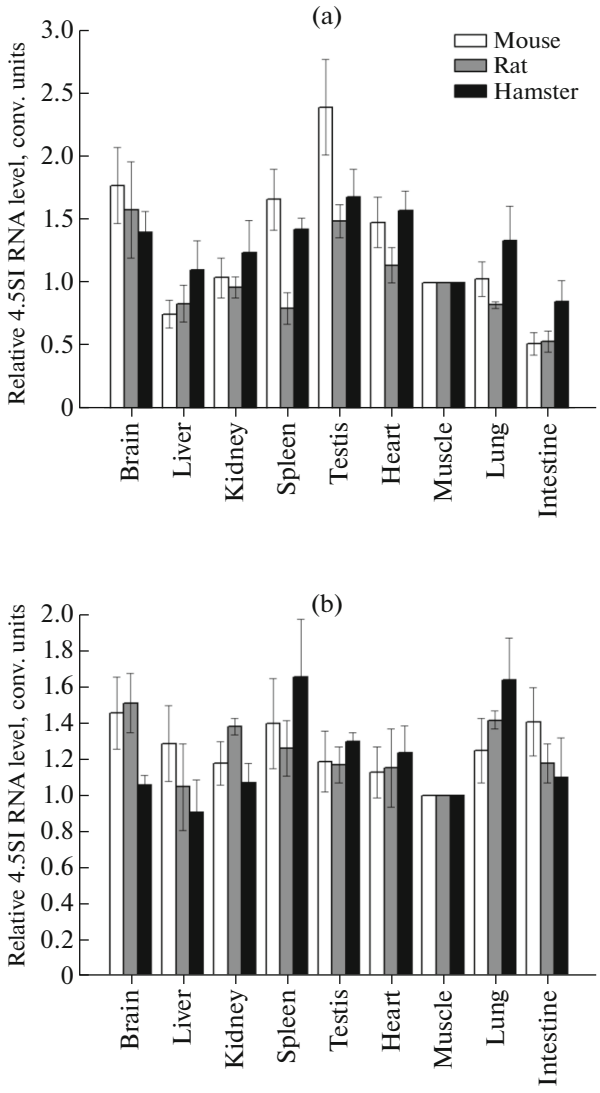

Fig. 2. Relative levels of the (a) 4.5SH and (b) 4.5SI RNAs in nine organs of mice (four individuals), rats (three individuals), and hamsters (three individuals). Hybridization signals from the 4.5SI and 4.5SH RNAs were normalized to the U1 RNA signal. The level in muscles (conv. units) was taken as unity for each RNA. Whiskers show the standard deviation.

and the deoxyoligonucleotide immobilized on the membrane. The intensity of the radioactive signal, which is proportional to the RNA and deoxyoligonucleotide amounts applied on gel, was measured by scanning the membrane with a phosphorimager (Fig. 4). The results were used to construct a calibration plot of signal intensity as a function of deoxyoligonucleotide amount (Fig. 5). A special equation was used to estimate the copy number per cell for the RNA under study (see Experimental).

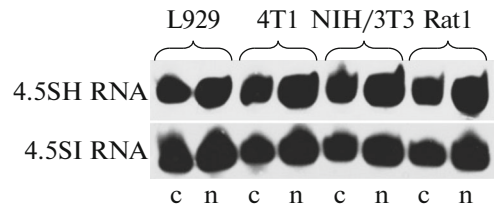

Fig. 3. Northern blot hybridization of the 4.5SH and 4.5SI RNAs in the nuclear (n) and cytoplasmic (c) RNA samples isolated from mouse (L929, 4T1, and NIH/3T3) and rat (Rat1) cell lines.

The method was tested with the U1 RNA, which occurs at several millions of copies per cell [9]. Similar estimates were obtained with the above method (Table 2). Then the copy numbers of the 4.5SH and 4.5SI RNAs were estimated for the four rodent cell lines. The copy number was estimated at 0.4–2.1 million for the 4.5SH RNA and 0.9–2.4 million for the 4.5SI RNA, varying among the cell lines. Thus, the two RNAs are

Table 1. Distribution of the 4.5SH and 4.5SI RNAs between the nucleus and the cytoplasm in four rodent cell lines

Cell line	4.5SH RNA, %	4.5SI RNA, %		
	nucleus	cytoplasm	nucleus	cytoplasm
L929	75.1	24.9	77.5	22.5
4T1	77.6	22.4	70.5	29.5
NIH/3T3	76.0	24.0	66.4	33.6
Rat1	74.9	25.1	66.8	33.2

Table 2. Copy number per cell estimated for the 4.5SH, 4.5SI, and U1 RNAs

Cell line	Copy number per cell		
	4.5SH RNA	4.5SI RNA	U1 RNA
AKK	0.4×10^6	0.9×10^6	3.0×10^6
4T1	1.2×10^6	2.0×10^6	13.6×10^6
L929	0.8×10^6	1.8×10^6	4.9×10^6
NIH/3T3	2.1×10^6	2.4×10^6	7.9×10^6

abundant in the cell, and their copy numbers are no more than one order of magnitude (3- to 11-fold) lower than the copy number of the U1 RNA.

DISCUSSION

We demonstrated in this work that the 4.5SI and 4.5SH RNAs occur in all organs examined in the house mouse, gray rat (family Muridae), and golden hamster (family Cricetidae). The 4.5SI RNA levels in organs were highly similar in different animals. The 4.5SH RNA is especially abundant in the testis and brain in all three species and occurs at somewhat lower levels in the kidney, spleen, heart, muscle, and lung. The lowest levels (2–5 times lower than in the testis) of the 4.5SH RNA were observed in the small intestine and liver.

Recently, Ishida et al. [24] have similarly detected the 4.5SH RNA in all of the eight mouse organs examined. Although quantification has not been performed, the data reported indicate that the 4.5SH RNA level is extremely high in the embryonic brain. Bachvarova [30] has earlier found that the 4.5SH RNA occurs in minor amounts in mature mouse oocytes.

A quantitative analysis of the RNA contents received special attention in our study. Tissue samples were obtained from several animals; the hybridization signal was quantified with a phosphorimager; and the results obtained for the 4.5SI and 4.5SH RNAs were normalized to the U1 RNA, which is a widespread RNA with a predominantly nuclear localization and occurs in spliceosomes. This normalization makes it possible to allow for differences in nuclear-to-cytoplasmic volume ratio among different tissues.

Discrepant data are available on the distribution of the 4.5SH RNA in the cell. A predominantly cytoplasmic localization has been reported for the 4.5SH RNA in one study [16], while the 4.5SH RNA has been detected almost exclusively in the nucleus in another study [24] (Table 3).

We think that the data reported in 1986 [16] are less reliable because an indirect method has been employed in the analysis and only one cell line has been examined. Our results, which were obtained with four cell lines, demonstrate that the 4.5SH RNA is present in both the nucleus and cytoplasm at a ratio varying from 3 : 1 to 4 : 1 (Table 1).

Table 3. Comparison of the methods and results of three studies that have addressed the distribution of the 4.5SH and 4.5SI RNAs between the nucleus and the cytoplasm in rodent cells

Study	Cell line	Method	Distribution in the cell
Schoeniger and Jelinek, 1986	MEL (mouse erythroleukemia)	Nuclear and cytoplasmic RNAs were labeled and hybridized with an excess of immobilized 4.5SH gene DNA; measurements were performed by scintillation counting	Nucleus, 30% Cytoplasm, 70%
Ishida et al., 2015	Neuro2A (moue neuroblastoma)	Northern blot hybridization of RNAs isolated from cytoplasmic and nuclear fractions, visual evaluation	Nucleus (approximately 100%)
This work	• 4T1 (mouse breast cancer) • L929 (mouse immortalized fibroblasts from connective tissue) • 3T3/NIH and Rat1 (mouse and rat immortalized embryonic fibroblasts, respectively)	Northern blot hybridization of RNAs isolated from cytoplasmic and nuclear fractions, measurements with a phosphorimager	Nucleus, 75–78% Cytoplasm, 22–25%

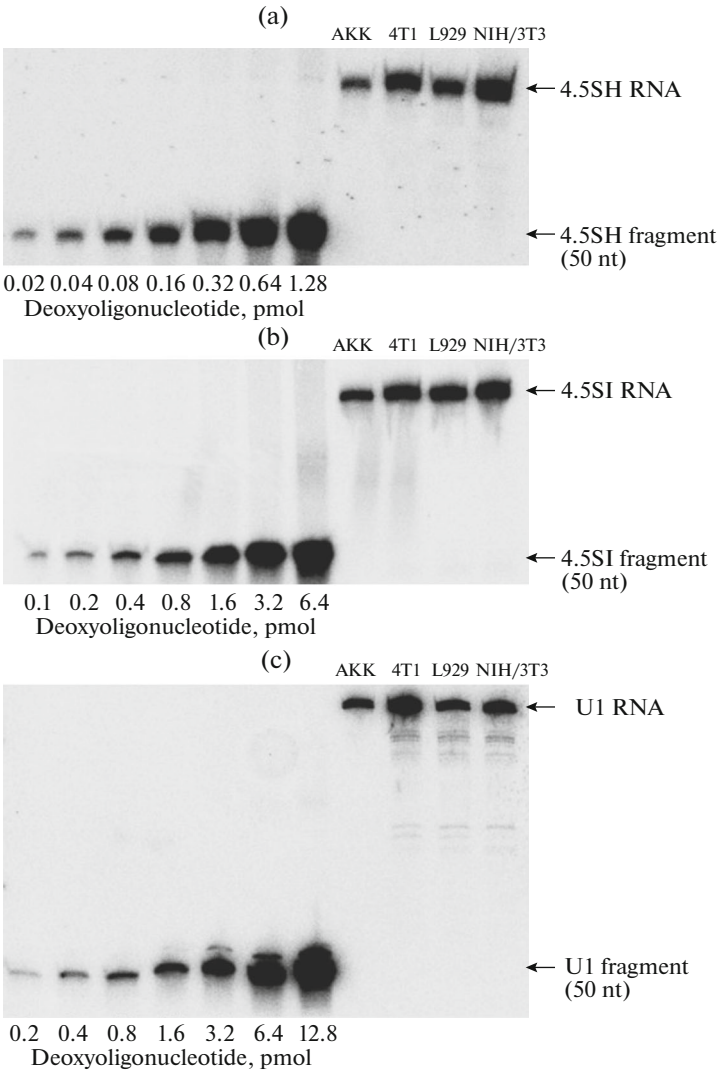

Fig. 4. Northern blot hybridization of the (a) 4.5SH, (b) 4.5SI, and (c) U1 RNAs in total RNA samples from cells of four cell lines (4×10^5 cells for each line) and deoxyoligonucleotides corresponding to 50-nt fragments of the respective RNAs. The deoxyoligonucleotides were applied on gel in the amounts specified at the bottom.

The discrepancy in evaluating the intracellular localization of the 4.5SH RNA might be explained by the differences in cell lines examined, technics used to separate the nuclear and cytoplasmic cell fractions, and methods used to estimate the 4.5SH RNA levels (Table 3).

A predominantly nuclear localization of the 4.5SI RNA was observed in our experiments; i.e., 66.4–77.5% of this RNA was detected in the nucleus (Table 1).

Shoeniger and Jelinek [16] have estimated the 4.5SH RNA copy number at 1.3×10^4 copies per cell. We checked the estimate with a more accurate straightforward method in four cell lines. Surprisingly, our estimates of the 4.5SH RNA copy number were two orders of magnitude higher than the estimate reported in [16] (Table 2). Factors responsible for the difference are unclear. The difference might be due to differences in study protocol or specifics of the MEL cell line, which has been examined in [16], but the latter seems less likely.

Estimates of the 4.5SI RNA copy number have not been reported in the available literature. It has only been mentioned that the 4.5SI RNA occurs at approximately one million copies in the cell nucleus [31]. Our results are similar in order of magnitude to this estimate (Table 2).

Thus, the 4.5SI and 4.5SH RNAs should be classed with abundant noncoding RNAs, being comparable in copy number with the U1 RNA, which is one of the most abundant small noncoding RNAs in the cell apart from the 5S rRNA and tRNAs. The result is of importance for further studying the 4.5SI and 4.5SH RNAs.

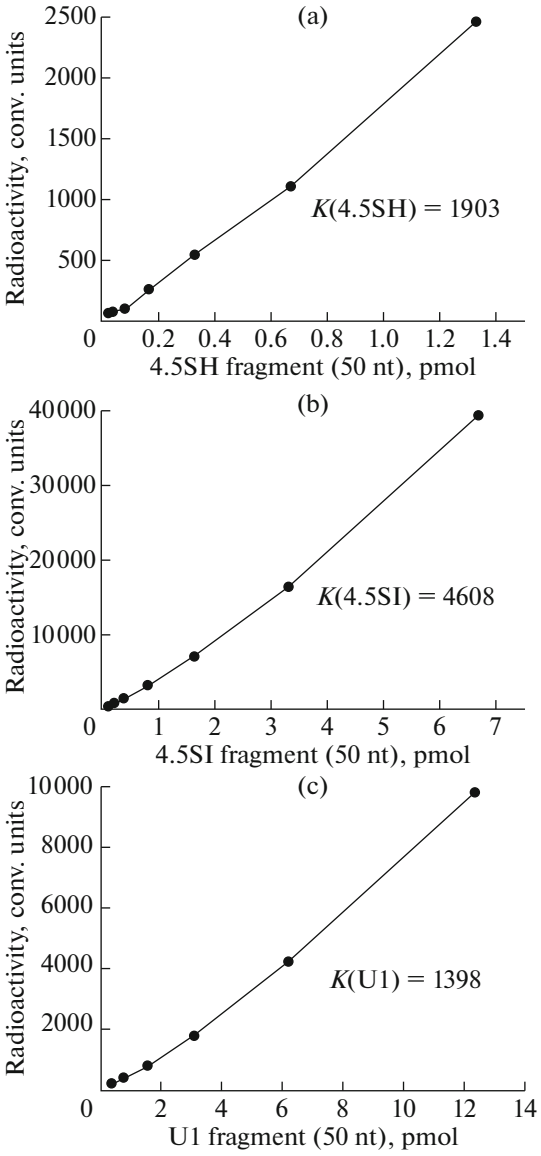

Fig. 5. Calibration plots of the radioactive signal intensity from electrophoretic bands of deoxyoligonucleotides corresponding to fragments of the (a) 4.5SH, (b) 4.5SI, and (c) U1 RNAs as a function of deoxyoligonucleotide amount. The parameter *K* was obtained from the calibration plots (see Experimental).

While the possible function has recently been studied for the 4.5SH RNA (which presumably regulates the mRNA export from the nucleus by interacting with the antisense SINE B1 sequences) [24], there is no data on the role that the 4.5SI RNA may play in the cell. However, it should be noted that the two RNAs are restricted to only few rodent families (although these account for 65% of species of the order) and, therefore, are most likely involved in molecular mechanisms that are additional to the standard regulatory system characteristic of mammalian cells.

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