B2 RNA and 7SK RNA, RNA polymerase III transcripts, have a cap-like structure at their 5′ end

G.P.Shumyatsky, S.V.Tillib and D.A.Kramerov*
Institute of Molecular Biology, USSR Academy of Sciences, Moscow, B-334, Vavilov str. 32, USSR

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ABSTRACT

We found that hydrolysates of poly(A)$^+$ RNA from Ehrlich ascites carcinoma cells which were transcribed by RNA polymerase III contained an unusual component designated as X. It was part of B2 RNA representing a transcript of B2 retroposon, typical of rodents. The component X possesses a cap-like structure, *x*ppp5′G, where *x* has a non-nucleotide structure. About half of all B2 RNAs contained this group at the 5′ end. Previously, Epstein *et al.* (1) detected a similar structure at the 5′ end of small nuclear U6 RNA. Later, Singh and Reddy (2) showed methyl to be the blocking group in the component x of U6 RNA. Besides B2 RNA, we found 5′ ends containing methyl groups in 7SK RNA.

INTRODUCTION

It is generally accepted that all poly(A)$^+$ RNA of eukaryotic cells is transcribed by RNA polymerase II and represents mRNA. However, we found that a significant fraction (up to 20%) of all newly synthesized polyadenylated RNA from mouse tumor cells were transcribed by RNA polymerase III (3). The major part of this RNA was represented by short transcripts from mouse retroposons B1 and B2 (4–6). They did not relate to mRNA. The biological role of B1 and B2 RNAs is still unknown, but possibly, they are not involved in functioning of the normal cells. The most abundant transcript is B2 RNA, which contains posttranscriptionally added poly(A)-segment at the 3′ end (3, 7, 8, 9). B2 RNA is the only example of polyadenylated RNA transcribed by RNA polymerase III. As shown in the present paper, B2 RNA also possesses other unusual features.

It is well known that RNA polymerase II transcripts contain caps at their 5′ ends (10). 7-Methylguanosine blocks gamma-phosphate of the 5′ nucleotide triphosphate in mRNAs, while 2,2,7-trimethylguanosine does so in small nuclear RNAs U1, U2, U3, U4, U5, U7, and U8. Typically, RNA polymerase III transcripts are not capped at their 5′ ends, but instead contain free triphosphate groups (11).

In this work, we found that many B2 RNA molecules possessed a cap, though very different from usual ones. Epstein *et al.* (1) found similar caps in U6 small nuclear RNA. A similar cap could also be detected in non-polyadenylated B2 RNA and 7SK RNA. No other studied RNA polymerase III transcripts contained cap structures.

MATERIALS AND METHODS

Cell labeling

Ehrlich ascites carcinoma cells were incubated and labeled as previously described (3).

RNA isolation and analysis

Total cellular RNA was isolated according to (12) with modifications (3). Poly(A)$^+$ RNA was selected using chromatography on poly(U)-sepharose (13) with modifications as described (3). Two cycles of chromatography were usually done.

Sucrose gradient centrifugation experiments were performed according to (14) with modifications (3).

For isolation of B1 and B2 RNAs, corresponding plasmid DNAs (ca. 100 μg) from Mm 31 (5) and Mm 14 (6) clones were fixed on nitrocellulose filters and hybridized with labeled cellular RNA for 20 hours at 42°C in 250 μl of solution containing 50% formamide, 50 mM Hepes-NaoH (pH 7.0), 0.5 M NaCl, 4 mM EDTA and 1% SDS. Unbound RNA was collected. The filters were washed in 15 mM NaCl, 1.5 mM sodium citrate and 0.1% SDS and then RNA was eluted by incubation in water for 2 min at 100°C.

Electrophoresis was carried out in 5% polyacrylamide 8M urea gels followed by autoradiography.

RNA was treated with nuclease P1 (Sigma) according to (15). Digest was spotted on the polyethylenimine (PEI) cellulose thin layer plate (Merck, Schleicher & Schull) and dried. The products of nuclease P1 digestion were resolved by two-dimensional ascending chromatography as described (15) with modifications in the developing time at first dimension while using PEI-plates from different batches and suppliers. The first dimension was developed in 0.2 M LiCl–5 mM EDTA. The plate was dried and then washed with anhydrous methanol to remove LiCl. The second dimension was developed for 1 min (from the time the solvent front reached the sample) in 0.2 M LiCl–5 mM EDTA, for 5 min in 1.0 M LiCl–5 mM EDTA and, finally, in 2.0 M LiCl–5 mM EDTA until the solvent front reached the top of the plate. After autoradiography of the plates the products of interest were scraped off and eluted from the cellulose with 3.0 M triethylammonium carbonate (pH 9.7) according to (15, 16).

Digestions with alkaline phosphatase and nucleotide pyrophosphatase (both from Boehringer-Mannheim) were performed as recommended by the supplier.

* To whom correspondence should be addressed

Thin-layer chromatography on cellulose plates (Merck, Schleicher & Schull) was done according to Silberklang *et al* (17). The first-dimension solvent was isobutyric acid/water/NH_4OH, 66:33:1 (vol/vol), and the second-dimension solvent was 0.1 M sodium phosphate buffer, pH 6.8/ammonium sulfate/1-propanol, 100:60:2 (vol/wt/vol).

B2 plasmid DNA was transcribed in S100 extract prepared as described in (18) from Ehrlich ascites carcinoma cells.

The monomethyl ^{32}P-phosphoric ester (CH_3-O-P) was prepared by incubating 1uCi of ^{32}P-orthophosphate in 10 μl of 10mM Tris-HCl (pH 8) with 1 ml of methanol at 65°C for 16 hr (2).

RESULTS

Finding component X in RNA polymerase III poly(A) transcripts

The component X was detected in our experiments demonstrating the existence of long poly(A)$^+$ RNAs transcribed by RNA polymerase III (polIIIpoly(A)$^+$ RNA). It is known, that polIII primary transcripts contain triphosphorylated 5′ ends, while polII transcripts 5′ ends are capped (10). Therefore, if alpha-amanitin resistant polyadenylated RNAs also possess 5′-triphosphorylated nucleoside, they are obviously transcribed by RNA polymerase III.

Ehrlich ascites carcinoma cells were incubated for 4.5 hrs either with or without alpha-amanitin (50 μg/ml) and then ^{32}P-orthophosphate was added and the incubation was prolonged for 2 hrs. Poly(A)$^+$ RNA was isolated by two cycles of chromatography and ultracentrifuged through sucrose gradient in DMSO. Three fractions were collected: light (L), RNA size was less than 500 n.; intermediate (M), 500−1800 n.; and heavy (H), RNA size was higher than 1800 n. Each fraction was digested with nuclease P1 and underwent two-dimensional chromatography on PEI-cellulose plates followed by autoradiography. Figure 1 shows, that 5′ end pppG and pppA were present in all RNA fractions, but their content in fraction L is significantly higher than in the other two fractions. For example, the difference between L and M fractions was 3.5-fold. Treatment of cells with alpha-amanitin did not decrease the pppG and pppA content in poly(A)$^+$ RNA.

Additionally, caps were not detected in the RNA from alpha-amanitin treated cells, though they were readily seen in the H-fraction from untreated cells. They were also detectable in the M-fraction from untreated cells after longer exposure. These observations showed that RNA polymerase II was completely inhibited in alpha-amanitin treated cells.

A high amount of low-molecular-weight polIIIpoly(A)$^+$ RNA was synthesized in Ehrlich ascites carcinoma cells in agreement with our previous results (3). Probably, high-molecular-weight (>18S) polIIIpoly(A)$^+$ RNA was also transcribed in these cells, but its abundance was rather low (3).

In the present experiments, we detected three new spots of unknown nature, in addition to the above mentioned components, designated as X, Y and Z. Analysis of the Y and Z spots, which included several enzyme treatments and chromatographic separations showed that they represent 5′-phosphate-cytidine-2′,3′-cyclophosphate and 5′-phosphate-uridine-2′,3′-cyclophosphate, respectively. We suggest, that these components are formed as a result of specific RNases' action on mRNAs at the first stages of their intracellular degradation. It is known, that nucleoside-2′,3′-cyclophosphate is formed at the 3′ end upon RNase action. Later, it is converted into nucleoside-3′-phosphate (19).

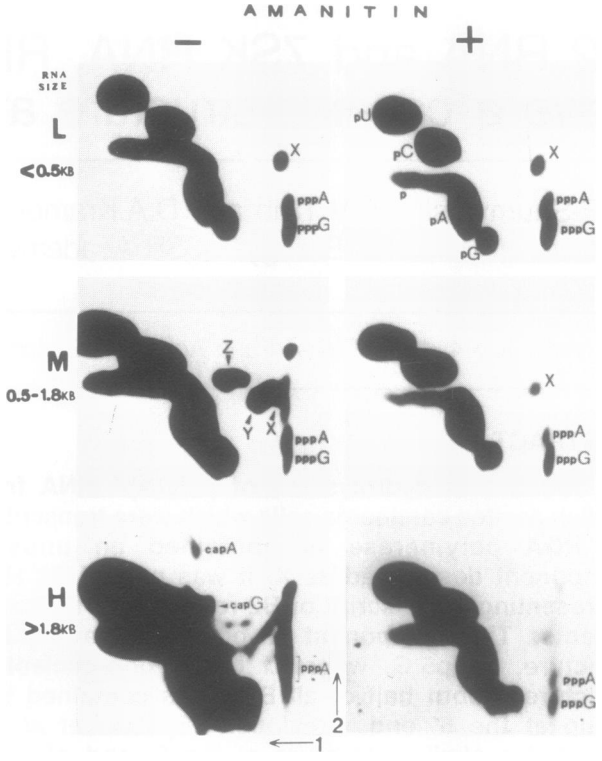

Fig. 1. Detection of 5′-terminal triphosphorylated nucleotides and identification of the X structure in polIIIpoly(A)$^+$ RNA of ^{32}P-orthophosphate labeled Ehrlich ascites carcinoma cells treated (+) or not (−) with alpha-amanitin. Poly(A)$^+$ RNA was fractionated into three fractions (L, M, and H) using sucrose gradient centrifugation . RNA from each fraction was treated with nuclease P1 followed by chromatography on PEI-cellulose plates. In case of L-fraction only half of the material was digested by nuclease P1. The markers used were ATP and 7mGpppA, their positions were detected by UV-absorption (254 nm). After chromatography the plates were autoradiographed. The exposure time for H/alpha-amanitin(+) fraction was two times longer than for five other fractions. The spots were identified according to their positions on the chromatograms (15) and using markers. pppG, pppA and capA (7mGpppAm) were additionally determined using enzymatic treatments and chromatographies. The spot, designated as capG, was not proved completely to correspond to the cap 7mGpppGm. The smear on the chromatography of the digest of the H-fraction observed at the area of pppA and pppG position is due to the chromatography being overloaded with radioactive material.

Distribution of the spot X material correlated with that of pppG and pppA (Fig. 1). It is predominantly located in L fraction and its amount was not changed upon administration of alpha-amanitin to the cells. Thus, X spot obviously originated from polIIIpoly(A)$^+$ RNA.

The X component is a part of B2 RNA

The characteristic feature of the X component is its resistance to alkaline phosphatase treatment. As shown in Fig. 2 (A and B), alkaline phosphatase treatment of the polIIIpoly(A)$^+$ RNA predigested with nuclease P1 did not remove the X spot while pppG and pppA disappeared.

The component X could readily be detected in B2 RNA isolated by hybridization of polIIIpoly(A)$^+$ RNA with immobilized B2 DNA (Fig. 2, C and D).The ratio of the X radioactivity to that of pppG was higher in B2 RNA as compared to original polIIIpoly(A)$^+$ RNA.

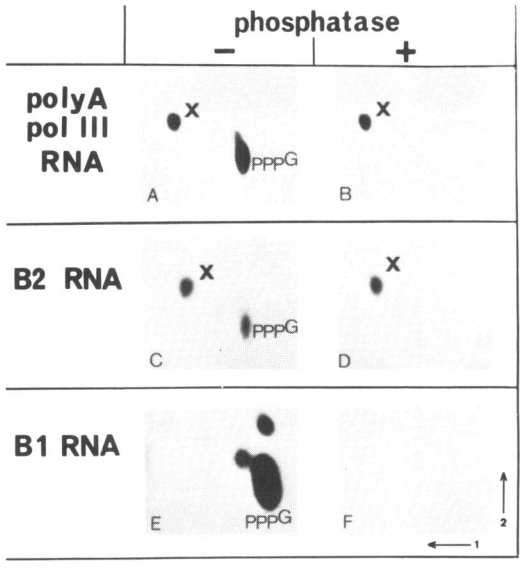

Fig. 2. Chromatography on PEI-cellulose plates of nuclease P1 digests of ^{32}P-labeled low-molecular-weight polIIIpoly(A)$^+$ RNA (A, B), B2 RNA (C, D) and B1 RNA (E, F; analysis of B1 RNA was done in a separate experiment). In each case, half of the material digested with nuclease P1 was additionally treated with alkaline phosphatase (B, D, F). The first dimension of chromatography was performed with a paper wick (15) and much longer than chromatography presented on Fig. 1. Only a part of each chromatogram is shown. Pieces of the chromatograms carrying nucleoside monophosphate spots were removed. This was done with the following figures too.

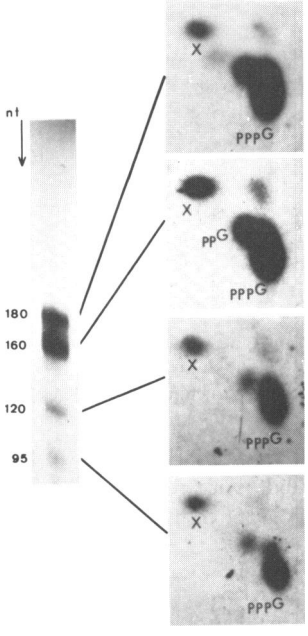

Fig. 3. PEI-cellulose chromatography of nuclease P1 digests of four poly(A)$^-$ B2 RNAs. Autoradiograph of the polyacrylamide gel which was used for separation of B2 RNA types is shown left. Numbers are sizes of RNAs in nucleotides.

A similar experiment was performed with B1 RNA isolated by hybridization of polIIIpoly(A)$^+$ RNA with B1 DNA. No radioactivity was detected in the X spot (Fig. 2, E, F).

The X component was observed in non-polyadenylated B2 RNAs (Fig. 3), which have been recently described (3). However. it will be noted, that the X component content in these

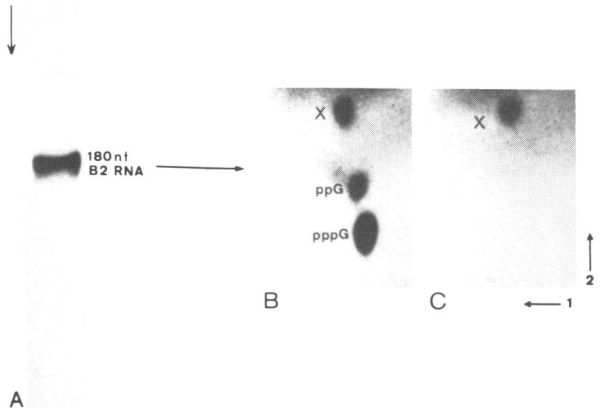

Fig. 4. (A). Polyacrylamide gel electrophoresis of RNA synthesized from DNA template (Mm 14) by S100 extract (18) of Ehrlich ascites carcinoma cells in presence of alpha-^{32}P-GTP. (B and C). PEI-cellulose chromatography of nuclease P1 digests of the RNA isolated from the gel. (C). The digest was treated (C) or not (B) with alkaline phosphatase.

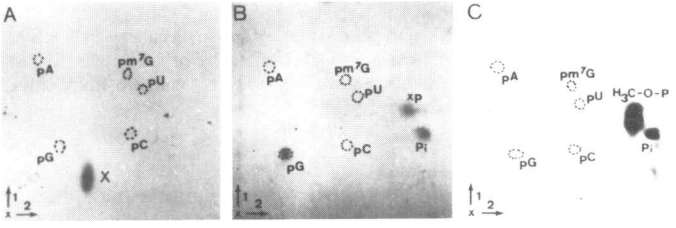

Fig. 5. Chromatography on cellulose plates of the native (A), and treated with nucleotide pyrophosphatase (B) material X. The material X was isolated by PEI-cellulose chromatography of nuclease P1 digest of ^{32}-labeled B2 RNA. (C). Chromatography on cellulose plate of monomethyl phosphate (CH_3-O-P) synthesized by reaction of ^{32}P-orthophosphate with methanol (2). (Unreacted orthophosphate is also visible on the autoradiograph). Positions of nucleotide 5′-phosphates markers are shown by dotted line.

RNAs was lower in comparison with that in poly(A)$^+$ B2 RNA. We also observed the X component in RNA transcribed from B2 DNA clone in a cell-free transcription system from Ehrlich ascites carcinoma cells (Fig. 4). Thus, all obtained data confirmed the existence of the X component in B2 RNA.

Structural studies on the X component

It is known that caps of mRNAs and small nuclear RNAs are resistant to alkaline phosphatase (10, 20). Therefore, X component resistance to this enzyme allowed us to suggest a cap-like structure of X, i.e. nucleoside triphosphate with blocked gamma-phosphate. To check this suggestion, we digested the X component with nucleotide pyrophosphatase, which is known to cleave mRNA caps (7mGpppN) with formation of p7mG, p (orthophosphate) and pN_1 (10).

As shown in Fig. 5 (A, B), thin layer chromatography on cellulose of the X component treated with nucleotide pyrophosphatase revealed three new spots. One corresponded to 5′-pG, another, to orthophosphate (Pi). However, the third spot did not coincide with any usual or modified nucleotide. It was located close to orthophosphate spot, but far from the region of nucleotide migration, suggesting that it might contain a non-nucleotide component. Thus, nucleotide pyrophosphatase treatment of the X component leads to formation of pG, p and

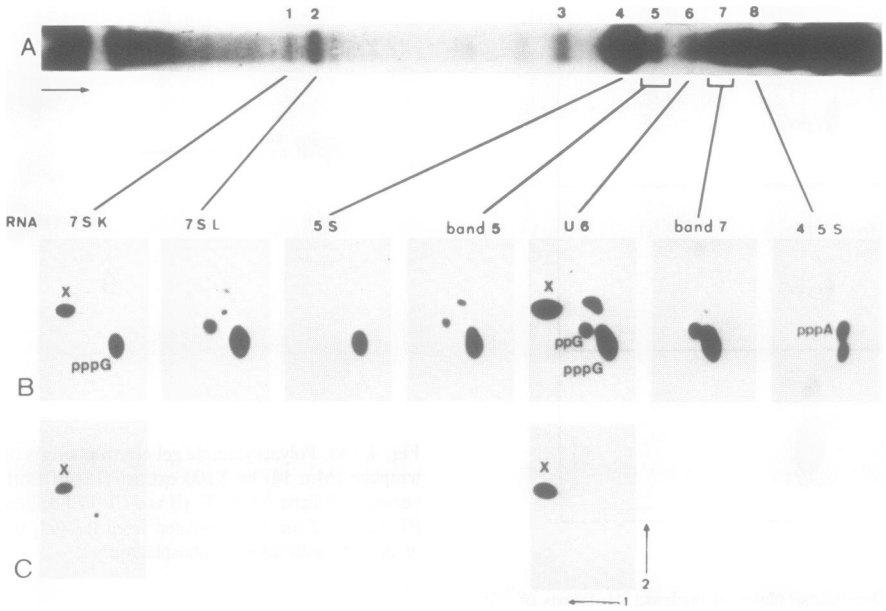

Fig. 6. Polyacrylamide gel electrophoresis (A) of low-molecular-weight RNAs of Ehrlich ascites carcinoma cells labeled by ^{32}P-orthophosphate in presence of alpha-amanitin (50 ug/ml) and PEI-cellulose chromatography of nuclease P1 digests of this RNAs (B). (C), is the same as (B), but digests was treated additionally with alkaline phosphatase (the data are shown for 7SK RNA and U6 RNA only). Some RNAs were not identified (bands 3, 5 and 7; RNA isolated from band 3 did not contain X, data not shown).

*x*p. Thus, X seems to be pppG where gamma-phosphate is blocked by non-nucleoside group *x*. The general scheme of the X component structure is *x*pppG. It is most probable that such structure is located at the 5′ end of B2 RNAs. About half of B2 RNA molecules possess this blocked triphosphate group at the 5′ end.

The X component in other RNAs transcribed by RNA polymerase III

Next question was whether some other RNA polymerase III transcripts contain similar components. Epstein *et al.* (1) described similar structure at the 5′ end of U6 RNA which was later found to be transcribed by RNA polymerase III (21, 22). They also found that the blocking group did not absorb UV-light (260 nm), i.e. it did not contain heterocyclic bases. However, until this time its structure remained unknown.

Recently, the same authors (2) found that gamma-phosphate on the 5′ end of U6 RNA was blocked by a methyl group. We isolated X component by a different chromatographic method, than Epstein *et al.* The mobility of B2 RNA and U6 RNA caps was compared using our system. Some other RNAs transcribed by RNA polymerase III were also tested for the presence of X component. The chromatograms of nuclease P1 digests of low-molecular-weight polIIIpoly(A)$^-$ RNAs are shown in Fig. 6. The X component was detected in U6 RNA and 7SK RNA. (Identification of 7SK RNA was proved by fingerprinting, data not shown). Other small RNAs contained pppG and pppA only. In some cases ppG and pppA were also detected.

Finally, methylphosphate (CH_3-O-P) was synthesized according to the method described in (2). The synthetic CH_3-O-P and *x*p isolated from B2, U6 and 7SK RNAs migrated to the same place on two-dimensional cellulose chromatograms (Fig. 5, data of U6 and 7SK RNAs not shown). Thus, all three RNAs contain the same structure, CH_3pppG. These chromatographic data exactly coincide with results of Singh and Reddy (2) on cellulose chromatography of CH_3-O-P (synthetic and isolated from U6 RNA).

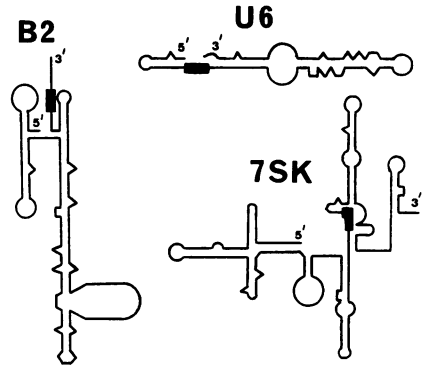

Fig. 7. Predicted secondary structures of B2 RNA (28), U6 RNA (1) and 7SK RNA (22). The (A/G)UAUA(C/A) sequence is shown as closed boxes.

DISCUSSION

We found in this paper that in contrast to most RNAs transcribed by RNA polymerase III, B2 RNA and 7SK RNA of mouse cells contained a cap-like component, *x*pppG, at their 5′ end. The blocking group had a non-nucleoside nature; the found component was shown to be identical to the cap detected by Epstein *et al.* (1) in U6 RNA.

Originally, U6 RNA was considered to be a RNA polymerase II transcript, and therefore, the presence of a blocked 5′ end was not surprising (10, 20). However, U6 RNA was later found to be a RNA polymerase III transcript (21, 22) where the capped 5′ end had not yet been detected. Thus, U6 RNA became the

first while B2 RNA and 7SK RNA were two other exceptions to the above mentioned rule.

Interestingly, both polyadenylated and non-polyadenylated B2 RNAs may contain 5'-cap structure. The caps were found both among larger (180 nt) and among smaller (160, 120, 95 nt) poly(A)$^-$ B2 RNAs. Finding the cap in other minor RNA polymerase III transcripts cannot be excluded either. Previously, 7SK RNA was shown to carry free pppG at the 5' end (24). According to our data, about half of the 7SK RNA molecules possessed 5' pppG while the other half was capped. Possibly, the difference between capped and non-capped structures was missed in the analysis used by these authors. Another possibility was that we used different type of cells and labeled them for a shorter period of time.

Recently, Singh and Reddy (2) showed that the blocking group in U6 RNA was a methyl group. Similarly, we found that B2 RNA and 7SK RNA have this structure at their 5' ends.

The mechanism and the role of the gamma-phosphate methylation in these RNAs remains open. Possibly, the methyl group protects the molecule from exonucleases, increasing the life time of corresponding RNAs. However, our preliminary data indicated that the life time was the same for capped and non-capped B2 RNAs. We compared the ratios of the X radioactivity to that of pppG on PEI-cellulose plates after nuclease P1 digestion of B2 RNA molecules isolated both from cells labeled in the usual way and those additionally treated for 1 hr with actinomycin D, which is known to terminate RNA transcription. The intensities of the X and pppG radioactivities reflect the amount of capped and non-capped B2 RNA molecules, respectively. The ratios were the same in these two cases (0,93 and 0,96 for untreated and treated cells, respectively). We concluded that the presence of methyl group had no influence on the stability of B2 RNA molecules.

The U6 RNA was found to participate in the mRNA splicing reactions (25), while the 7SK RNA function is still unclear. Although, these RNAs are non-homologous, they have the following common feature. Usually, RNA polymerase III promoter is localized inside the gene sequence. In contrast, U6 RNA and 7SK RNA genes do not have an internal promoter. It is located within 5' flanking sequence (26, 27). Possibly, this feature is connected with the capping ability. However, the capped B2 RNA is transcribed using an internal promoter (5).

It was found, recently, that the first 25 nucleotides of U6 RNA were necessary for its capping (23). This area contains a hairpin-like structure 19 nucleotides long followed by the AUAUAC sequence. Damage to the hairpin structure and dinucleotide substitutions in the AUAUAC sequence inhibited U6 RNA capping in a cell-free transcription system. Interestingly, B2 RNA and 7SK RNA possess hexanucleotides differing from AUAUAC by one nucleotide substitution (AUAUAA in B2 RNA and GUAUAC in 7SK RNA). However, they are located at a very long distance from the 5' end (154 and 189 nucleotides, respectively).

According to the existing second structure models of B2 RNA (28) and 7SK RNA (22), the 5' ends of these RNAs form hairpin-like structures, similar to U6 RNA. In addition, the first nucleotide in these RNAs is predicted to be adjacent to (A/G)UAUA(C/A) hexanucleotide which is not involved in formation of secondary structures (Fig. 7). Possibly, these common features of U6, B2 and 7SK RNAs determine this capping. However, additional structural studies are necessary to formulate the major rules determining capping of these RNA polymerase III transcripts.

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