Volume 13 Number 18 1985 Nucleic Acids Research

Nucleotide sequence of small polyadenylated B2 RNA

D.A.Kramerov, S.V.Tillib, A.P. Ryskov and G.P.Georgiev

Institute of Molecular Biology, USSR Academy of Sciences, Vavilov str. 32, 117984 Moscow B-334, USSR

Received 7 August 1985; Accepted 5 September 1985

ABSTRACT

Small poly(A)-containing RNA molecules which hybridize to the ubiquitous short repetitive sequence B2 and which are transcribed by RNA polymerase III have been identified in the cytoplasm of mouse cells. Here, we describe the structure of this small B2 RNA. A cDNA library was prepared from low-molecular-weight cytoplasmic poly(A)$^+$RNA isolated from Ehrlich carcinoma cells and the clones which hybridized to B2 sequence were selected. The clones were sequenced and shown to contain B2 sequences followed by a poly(A) tract. The sequences of the cloned B2 RNAs different from each other by 3-10%, being similar in this respect to genomic B2 copies. Thus, B2 RNA is transcribed from many different B2 sequences in the genome. The 5'-ends of B2 RNA at least in most molecules coincide with the beginning of B2 genomic sequence. The poly(A) segments located at the 3'-end of small B2 RNA are the same size as in mRNA molecules, suggesting posttranscriptional formation. In some clones, additional sequences were detected between the 3'-end of B2 sequence and the poly(A) stretch. They seem to result from a lesion in the RNA polymerase III terminator in the corresponding B2 sequences. The possible significance of B2 sequences and small B2 RNA is discussed.

INTRODUCTION

B1 and B2 sequences represent two major classes of short repeats dispersed throughout the mouse genome /1/. They consist of 130 and 190 nucleotides, respectively /2, 3/, and each family is present in about fifty thousands copies per haploid genome. Repetitive sequences very similar to B1 and B2 have also been detected in Chinese hamster and rat genomes /4-6/. B1 sequences are also very homologous to Alu sequences, the major repeat in the human genome /7/.

All of these sequences have features in common. (i) They are usually flanked by short direct repeats typical of mobile genetic elements. (ii) Different copies within a family differ

in 5–10% of their bases. (iii) They contain an A-rich region at one end. (iv) They all contain sequences homologous to the split RNA polymerase III promoter /8, 9/. (v) The repeats also possess certain homology to cellular low-molecular-weight RNAs. The homologies between B1 (and Alu) sequences and 4.5S RNA and 7S RNA are most prominent. Homology between the B2 sequence and 4.5S.I RNA has been described /3/.

B1/Alu and B2 sequences occur in different parts of the genome including protein encoding genes. They are usually located within introns but are sometimes also in 3'-end non-translated regions. As a result, these repeats are very numerous in the nuclear pre-mRNA but rare in the mature mRNA /6, 10-14/.

Recently, we have described an additional fraction of RNA which efficiently hybridizes to B1 and B2 sequences, low-molecular-weight cytoplasmic poly(A)$^+$RNAs /11/. They were designated as B1 RNA and B2 RNA. The small B1 and B2 RNAs are heterogeneous in size, varying from 200 to 400 nucleotides. The amount of small B1 RNA is very low and we therefore have concentrated our efforts on the study of B2 RNA which is readily detectable in many tissues. It was found that B2 RNA is not formed in the course of pre-mRNA splicing (processing) but is transcribed independently by RNA polymerase III /15/. In the cell, B2 RNA is associated with proteins as a constituent of light RNP particles as well as of free informosomes /15/. B2 RNA is present in different mouse tissues and is especially abundant in several tumors /16/. Similar RNA has also been detected in rat brain cells /17, 18/ and mouse embryonal carcinoma cells /19/.

Although B2 RNA hybridizes with cloned copies of the B2 repeat, no direct evidence proving the identity of their nucleotide sequences has previously been reported. Therefore, it has not yet been shown that this RNA is transcribed from genomic B2 elements. Also, the extent of heterogeneity between different B2 RNAs has not yet been reported. Clarification of this point is important for understanding whether B2 RNA is transcribed from many different B2 repeats or from one or only a few special copies.

To answer these questions, we have cloned cDNA from B2 RNA and have sequenced some of these. The comparison of ten cDNA

sequences with known genomic B2 sequences clearly demonstrated that small B2 RNA is transcribed from many different genomic B2 elements. B2 RNA polyadenylation occurs posttranscriptionally.

MATERIALS AND METHODS

RNA isolation. Approximately 10^9 Ehrlich ascite carcinoma cells were lysed in the presence of Nonidet P-40 and the cytoplasmic extract was isolated as described earlier /15/. The extract was centrifugated in a 7-25% sucrose gradient at 20000 rpm for 25 h in SW27 rotor. A fraction of light ribonucleoprotein particles (7S-25S) was collected and deproteinized with a chloroform/phenol (pH 8.0) mixture three times. Subsequently, poly(A)$^+$RNA was isolated by oligo(dT)-cellulose column chromatography.

In some experiments, low-molecular-weight poly(A)$^+$RNA was used. Total cytoplasmic RNA of Ehrlich carcinoma cells was fractionated on a poly(U)-Sepharose column. Then poly(A)$^+$RNA was denatured in 90% dimethyl sulfoxide at 55° and centrifugated in a 5-20% sucrose gradient at 20000 rpm for 19 h in the SW27 rotor /15/. The low-molecular-weight RNA (lighter than 12S) was collected.

cDNA synthesis, cloning and sequencing. cDNA was synthesized with AMV reverse transcriptase using 10 µg poly(A)$^+$ RNA from 7-25S RNP particles as template and oligo(dT)$_{12-18}$ as primer /20/. The second strand was synthesized with the Klenow fragment of DNA polymerase I. Oligo(dC) was synthesized at the 3'-ends using terminal nucleotidyl transferase. The DNA of plasmid pUR222 /21/ was restricted by HindII and oligo(dG) was synthesized at the 3'-ends. E.coli cells (K 802) were transfected with DNA obtained by annealing vector DNA and cDNA. The B2-containing clones were selected by colony hybridization with a nick-translated insertion of clone Mm14 /1, 3/. Plasmid DNA used for sequencing was linearized by BamHI, 5'-labeled with polynucleotide kinase and γ-^{32}P ATP treated by PstI endonuclease and electrophoresed in 5% polyacrylamide gel prior to isolation. The sequencing was performed according to Maxam

and Gilbert /22/ using immobilization of DNA of DEAE cellulose /23/.

5'-end mapping in small B2 RNA. The procedure was performed as described by Norton et al. /24/. The SduI/HinfI fragment of the clone pcB2.20 was used as primer. The plasmid was first treated with BamHI and PstI, and the cDNA insertion isolated by sucrose gradient ultracentrifugation. Then the insertion was restricted by HinfI endonuclease, 5'-labeled with polynucleotide kinase and /γ-^{32}P/ATP and further restricted by SduI endonuclease. A labeled SduI/HinfI fragment was isolated by polyacrylamide gel electrophoresis. The fragment (20 ng) was denatured and annealed with poly(A)$^+$RNA (7-20 μg) in 80% formamide, 0.4 M NaCl, 50 mM HEPES (pH 7.0), 1 mM EDTA at 48°C. After three precipitations with ethanol, cDNA was synthesized in the presence of non-labeled nucleoside triphosphates (0.5 mM). Nucleic acids were deproteinized by chloroform, precipitated with ethanol and electrophoresed in 6% polyacrylamide gel, as usually done for DNA sequencing.

Measurement of poly(A) length. Low-molecular-weight (>12S) poly(A)$^+$RNA was 3'-labeled with the aid of RNA lygase and 5'-^{32}P-pCp /25/. The labeled RNA was hybridized to Mm14 DNA immobilized on a nitrocellulose filter in 50% formamide, 4xSSC, 0.04% ficoll, 0.04% polyvinylpyrrolidone, 0.2% SDS, and 200 μg/ml E.coli DNA at 42°C for 2 h. B2 RNA was eluted by incubation of the filter in 0.2% SDS at 95°C for 3 min, ethanol precipitated three times with tRNA carrier (25 μg) and treated by RNAase A (5 μg/ml) and RNAase T1 (100 units/ml) at 37°C for 1h in 0.2 ml solution containing 0.1 M NaCl, 10 mM Tris-HCl (pH 7.5), 2 mM EDTA. After deproteinization, poly(A) was purified by poly(U)-Sepharose chromatography and elestrophoresed in 10% polyacrylamide sequencing gel.

RESULTS

Cloning and sequencing of B2 RNA

To analyze the structure and heterogeneity of B2 RNA, we have cloned cDNA homologous to B2 RNA. Poly(A)$^+$RNA was isolated from light cytoplasmic ribonucleoprotein (RNP) particles (lighter than 25S) of Ehrlich carcinoma cells. These RNP par-

Fig. 1. Nucleotide sequences of ten cloned cDNAs derived from B2 RNA. The second line of each row is consensus cDNA sequence. Below, only differences between the individual cDNAs and the consensus sequence are indicated with letters.

(·) - Nucleotides of the individual cDNAs which are identical to nucleotides of the consensus sequence. ( ) - Nucleotides that are not represented in individual cDNAs.

(▲) - The additional tetranucleotide GACT in clone pcB2.9. The additional sequence in clone pcB2.20 is shown at the top of the figure. The upper line of each row is consensus genomic sequence B2. It is constructed from four genomic copies of the B2 element /3, 26/ and a B2 copy located within the gene encoded liver $mRNA_X$ /14/. The asterisks mark differences between genomic and cDNA consensus B2 sequences.

ticles are enriched in small B2 RNA /15/. The cDNA was synthesized by reverse transcriptase using oligo(dT) as primer. After synthesis of the second DNA strand and oligo(dC) tailing at the 3'-end with terminal transferase, the DNA was inserted into the plasmid pUR222 which had been restricted by HindII and tailed with oligo(dG). The clones hybridizing with B2 DNA (clone Mm14) were selected by colony hybridization. The DNA of ten clones (designated as pcB2 with a corresponding number) was isolated and the nucleotide sequences of the cloned DNA determined. The sequencing was always started at the BamHI site of the plasmid.

In Fig. 1, the nucleotide sequences of the cloned cDNAs and the cDNA consensus sequence is compared to that of the genomic B2 element. The cloned cDNAs varied in length because the 5'-ends of B2 sequence were lost in the course of cloning. All cloned sequences are highly homologous to the consensus genomic B2 repeat. They do not contain any significant insertions or deletions except for one GACT tetranucleotide insertion in the clone pcB2.9. On the other hand, different cloned sequences are not identical in contrast to what was found for other small RNAs. A sequence divergence ranging from 3 to 10% is revealed by comparing any pair of sequences. The same differences also exist among genomic copies of B2 element. The consensus sequence of the cDNA and that of the genomic B2 element differ in only three nucleotides (marked by asterisks in Fig. 1). One can conclude that small B2 RNA is transcribed from different copies of the B2 family.

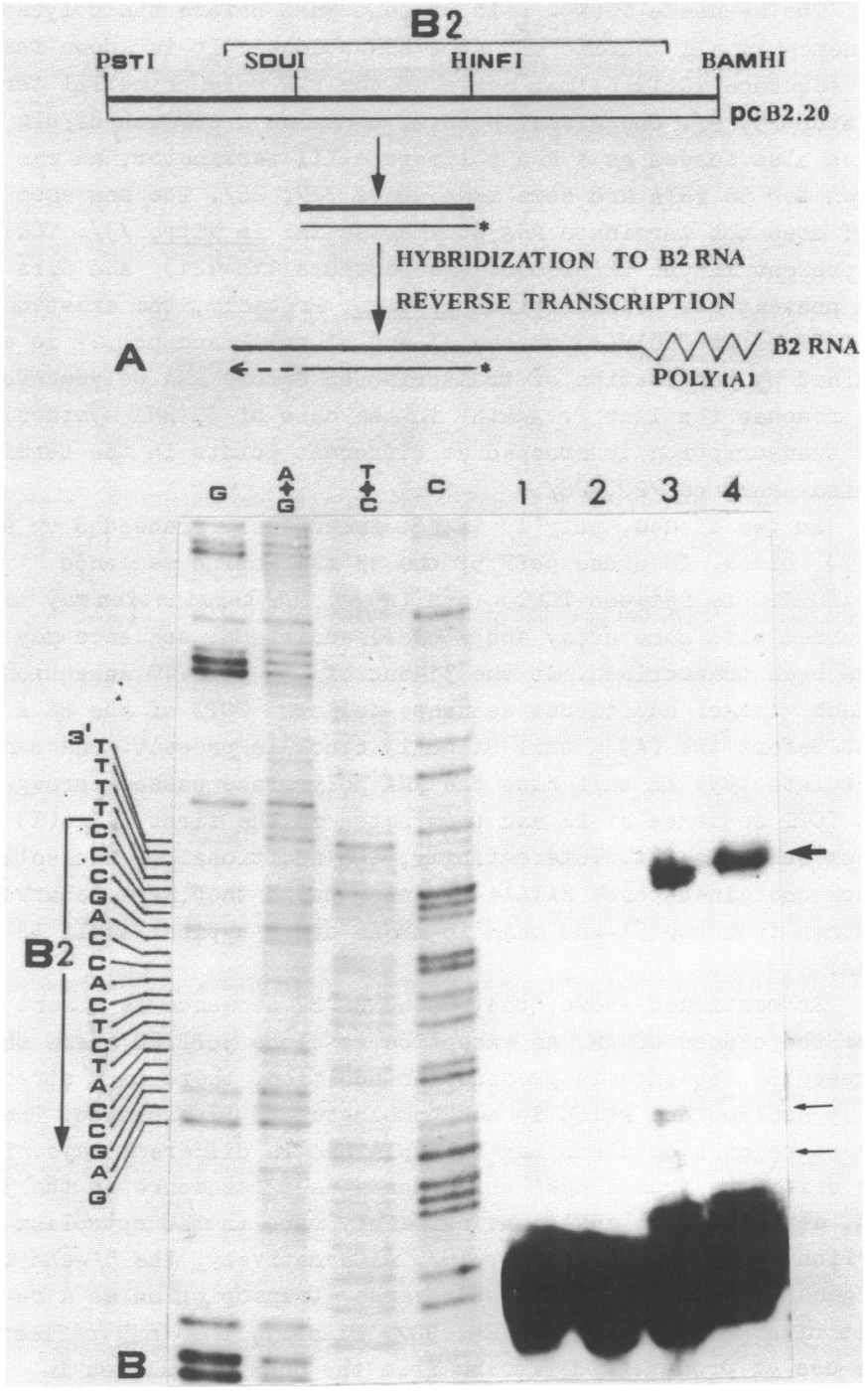
Most of the cDNA clones contained at least two thirds of the B2 sequence and this region was analyzed in detail. There are several conserved sequences in this region. One is located between nucleotides 57 and 97 and includes the second part of the split RNA polymerase III promoter. A second conserved region extends from nucleotides 127 to 152.

All clones contain a poly(dA) tail. The size varies from 10 to 52 nucleotides, the average length is 33 nucleotides. This is significantly longer than found in genomic B2 elements where the length ranges from 7 to 14 nucleotides. This suggests posttranscriptional addition of poly(A) to the transcript (see also below).

The sequence $TC(T)_{2-5}$ is present just before the poly(A) sequence in all genomic and most cDNA clones. It is known that the sequence $TC(T)_{3-5}$ may serve as the RNA polymerase III terminator /5, 9/. One clone, pcB2.9, contains a stretch of dTs which also serves as a RNA polymerase III terminator, as was shown for 5S rRNA and some tRNA genes /27, 28/. The sequence TCTT does not terminate RNA polymerase III in vitro /3/. TCTT is present in the B2 element (nucleotides 118-121) and does not prevent its transcription in vivo. Probably, the existence of TCTT before poly(A) at the 3'-end of pcB.2 and pcB.17 is explained by termination of transcription before RNA polymerase III reaches the last T. Again in the case of 5S RNA synthesis, the transcription is stopped at different points in the termination sequence /27, 28/.

In two clones, poly(A) is not immediately preceeded by a $TC(T)_3$ block. In clone pcB2.5, the 13 nucleotide sequence $(A)_7TCTATC$ is between $TC(T)_3$ and $(A)_{12}$. The termination may have occurred with some delay and a small additional sequence may have been transcribed. At the 3'-end of the pcB2.10 insertion, a much longer additional sequence follows TCTT of the B2 element. Before the $(A)_{22}$ tail oligo(T) block is present. One can speculate that in this case the RNA polymerase passes through the TCTT sequence of B2 and terminates in the first oligo(T) block which is met. Interestingly, the additional 49 nucleotide block contained three AATAAA signals though they were located further from the 3'-end than in mRNAs or in typical small B2 RNAs.

As mentioned above, the 5'-end of B2 sequence is absent from the cloned cDNAs. An exception is clone pcB2.20 where the compete B2 sequence is present. In addition, there is a stretch of 35 nucleotides which is not homologous to B2 sequence. The structure of such clone may be explained in different ways. It may originate from a mRNA which contains B2 sequence at the 3'-end, although such mRNAs are extremely rare in the cytoplasm of Ehrlich carcinoma cells /11, 15/. Alternatively, the 5'-end may be generated in the course of reverse transcription as a result of a template switch /29, 30/. Finally, this may reflect the use of promoters differing from the ones postulated in


B2
PSTI
SDUI
HINFI
BAMHI
pcB2.20
HYBRIDIZATION TO B2 RNA
REVERSE TRANSCRIPTION
A
B2 RNA
POLY(A)
G
A+G
T+C
C
1
2
3
4
3'
TTTTCCCGACCACTCTACCGAG
B2
B

small B2 RNA transcription. To exclude the third possibility, one has to position the 5'-end of B2 RNA more precisely.

Mapping of the 5'end in small B2 RNA

To map the 5'-end of B2 RNA, we have used the following approach. The HinfI/SduI fragment, 84 nucleotides long, was isolated from clone pcB2.20 (see Fig. 2A). Its 5'-HinfI end was ^{32}P-labeled with polynucleotide kinase. Subsequently, low-molecular-weight poly(A)$^+$RNA isolated from Ehrlich carcinoma cells was reverse transcribed using the above-mentioned fragment as primer. To identify the positions of nucleotides, the synthesized DNA was electrophoresed in a sequencing gel in parallel with the 175 nucleotide HinfI/PstI fragment treated according to Maxam and Gilbert /22/. The major product of reverse transcription is a fragment whose end exactly coincides with the beginning of the B2 sequence (Fig. 2B). No longer cDNA chains were observed on autoradiograph even after overexposure. The minor shorter bands were detected. Most likely they are formed as a result of premature termination of reverse transcription at regions containing primary or secondary structure obstacles which retards enzyme propagation. A conclusion can be made that at least a major part, if not all, of B2 RNAs are initiated *in vivo* exactly at the beginning of B2 sequence. This observation lends additional support to the idea that RNA polymerase III is responsible for B2 RNA synthesis.

Fig. 2. Mapping of the 5'-end of B2 RNA by primer extension. A. Scheme of the experiment. B2 RNA and the RNA-like strand of cDNA are shown with thick lines, the complementary strand of cDNA - with a thin line. (*) - The /^{32}P/-labeled 5'-end of DNA. The SduI site - GAGCAC (28-33). The HinfI site - GACTC (11-115). B. The radioautograph of gel after electrophoresis. 1 - The SduI/HinfI fragment of clone pcB2.20 DNA (primer). 2 - Primer incubated with reverse transcriptase in the absence of RNA template. 3 and 4 - Primer extension against template of cytoplasmic low-molecular-weight (3) or total (4) poly(A)$^+$RNAs. A thick arrow indicates position of the major primer extension product; thin arrows - minor products. Four left-handed lanes - the /^{32}P/-labeled HinfI/PstI fragment of pcB2.20 DNA treated according to Maxam and Gilbert /22/. The nucleotide sequence of the beginning of B2 element is shown. This sequence is complementary to B2 RNA or the cDNA strand represented in Fig. 1.

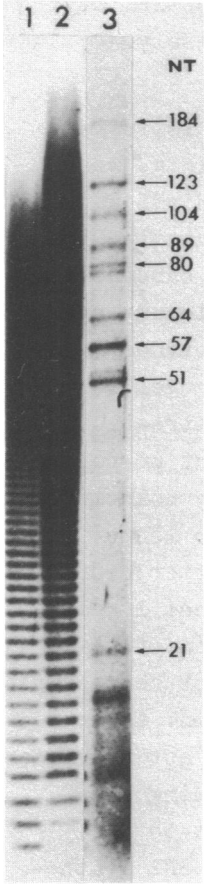


Fig. 3. Measuring the size of 3'-end poly(A) segments in B2 RNA by electrophoresis in polyacrylamide gel under denaturing conditions. 1 - poly(A) isolated from heavy (>12S) cytoplasmic mRNA. 2 - poly(A) isolated from B2 RNA molecules, 3 - marker, pBR322 DNA digested with BspI and labeled with ^{32}P. NT - nucleotides.

Measurement of the length of poly(A)-tail in B2 RNA

The length of poly(A)-tails in B2 RNAs should be longer than is found in DNA clones since the oligo(dT) primer does not necessarily bind at the 3'-end. To measure its real length, we labeled low-molecular-weight cytoplasmic poly(A)$^+$RNA with ^{32}P-pCp, purified B2 RNA by hybridization with B2 DNA, treated by a mixture of RNAase A and RNAase T1, and electrophoresed the material in polyacrylamide gel. In parallel, the poly(A) isolated from heavy cytoplasmic mRNA was similarly analyzed (Fig. 3). The poly(A) stretches of B2 RNA are very heterogeneous in size and vary from 10 to 120 nucleotides in length. The maximum of distribution is at the 80 nucleotide. A similar picture was obtained with poly(A) segments obtained from mRNA.

The similarity of these pictures as well as the long length of most of poly(A) stretches proves that the poly(A)-tailing B2 RNA is formed posttranscriptionally.

DISCUSSION

The structure and heterogeneity of small B2 RNA

The structure of small polyadenylated B2 RNA was determined in the present work. First, most of the B2 RNAs are comprised of full length B2 sequences with a poly(A)-tail at the 3'-end added posttranscriptionally. The beginning and the end of B2 RNA exactly coincide with the beginning and the end of B2 sequence. Such a structure was predicted earlier on the basis of indirect data /11, 15/. It is consistent with the hypothesis that RNA polymerase III is responsible to B2 RNA synthesis as was suggested by previous α-amanitin inhibition experiments /15/. It is most likely that the sequences ATGGCTC AGTGG(12-23) and GAGTTCAAATCC(57-68) of the B2 repeat are used as the RNA polymerase III promoter, and $TC(T)_{3-5}$ block as the RNA polymerase III terminator.

The unusual property of B2 RNA distinguishing it from other known RNA polymerase III transcripts is its polyadenylation. Our data indicate that this is a posttranscriptional process. The size of poly(A) in B2 RNA may be as long as 100-120 nucleotides while in genomic copies of B2 repeats the oligo(A) length does not exceed 7-15 nucleotides. The occurrence of RNA polymerase III transcription with polyadenylation seems to depend on the close association of the RNA polymerase III termination signal $TC(T)_{3-5}$ with several AATAAA sequences in the 3'-end of B2 repeat. B2 RNA contains AAUAAA, but not CAPyUG which was also postulated to be involved in mRNA polyadenylation /31/. One may speculate that this difference is related to the way the substrate for polyadenylation is formed; RNA polymerase III termination forms the 3'-end in B2 RNA, while in mRNA, RNA is cut at the polyadenylation site.

The second important conclusion is that B2 RNA is heterogeneous and therefore most likely transcribed from many different B2 sequences dispersed throughout the genome. Thus, there are no dominating sequences which can be considered as genes

while the others as pseudogenes. Many different B2 sequences are transcriptionally active.

In addition to sequence heterogeneity, we found that the size heterogeneity does not depend solely on the poly(A) length. Size heterogeneity results from the extension of transcription beyond the usual 3'-terminus due to the loss of one T from the $TC(T)_3$ sequence. Sequence TCTT does not terminate transcription and RNA polymerase moves until it meets another oligo(T) block. In one case, an additional 49 nucleotides have been read. Haynes and Jelinek /4/ have shown the formation of extended RNA polymerase III transcripts in vitro when cloned hamster B2-like sequences were used as templates. We have shown a similar phenomenon occurs in vivo. We suggest the loss of RNA polymerase III terminators from B2 repeats or other repeated sequences explains the long poly(A)$^+$RNA synthesis, which is rather resistent to treatment of cells with α-amanitin /15/.

On the possible function of B2 RNA

The significance of B2 RNA remains obscure. One possibility is that it is a transcription product of selfish B2 DNA which serves as a substrate for reverse transcriptase on the way to the retroposition of B2. Recently, Ullu and Tschudi /32/ have obtained some data indicating that Alu/B1 sequences represent processed 7SL RNA pseudogenes. However, for the B2 sequence such a possibility is very problematic since there is little homology to 4.5S.I RNA /3/. The existence of conserved regions in B2 RNA (besides the RNA polymerase III promoters) cannot be easily explained by the hypothesis of B2 selfishness. We have shown that about a quarter of B2 RNA molecules is bound to heavy cytoplasmic RNP. They seem to be associated with the mRNA of the RNP complex /15/. By analogy with other small RNA, this suggests the involvement of B2 RNA in the regulation of mRNA transport or translation /33, 34/. Another interesting possibility is the participation of B2 sequences and B2 RNA in regulation of transcription. Carlson and Ross /35/ and Bak and Jørgensen /36/ described sequences homologous to RNA polymerase III located before certain protein-coding genes. They suggested that RNA polymerase III-dependent transcription may in

some way modulate the transcription of the gene by RNA polymerase II.

Sutcliffe et al. /17, 18/ found in rat nerve cells a set of mRNA molecules which share the same 82 nucleotide sequence at the 3'-end, which they designated ID-sequence. The latter possess 65% homology with the 5'-part of B2 sequence (homology reaches 78% in a 35 bp stretch of these repeats). Also, small RNA hybridizing to the ID-sequence DNA probe was detected in nerve cells. In general, ID-sequences are more numerous in hnRNA and mRNA molecules isolated from brain than from liver or kidney. The authors suggest that RNA polymerase III-dependent synthesis of small ID-RNA may be the first step in activating ID-containing genes in a tissue-specific manner. This hypothesis could be extended to a B2 RNA. However, we found no correlation between the synthesis of small B2 RNA and B2-containing hnRNA or mRNA in different tissues. Also, in the case of ID-sequences, nothing is known about the structure of small ID RNA. Therefore the hypothesis that short dispersed repeats are involved in transcription regulation requires further experimentation.

One cannot exclude the participation of B2 sequences and small B2 RNA in processes such as DNA replication or recombination. Finally, it should be emphasized that the "selfish DNA" concept and the hypothesis that B2 sequence has function are not mutually exclusive. It is possible that some copies of B2 play certain functional roles while others represent "junk DNA", or pseudogenes.

ACKNOWLEDGEMENTS

We thank Dr. A.Janulaitis (Institute of Applied Enzymology, Vilnjus) for a kind gift of endonuclease SduI and Dr. V.Kavsan (Institute of Molecular Biology and Genetics, Kiev) for reverse transcriptase.

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