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The sequences homologous to major interspersed repeats B1 and B2 of mouse genome are present in mRNA and small cytoplasmic poly(A)+RNA

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ABSTRACT

Heavy nuclear RNA contains high amounts of transcripts from repetitive sequences B1 and B2. Cytoplasmic poly(A)$^+$RNA and, particularly, polysomal poly(A)$^+$RNA (mRNA) also include these sequences but in smaller amounts. The abundant 2 kb mRNA of mouse liver are found to have a B2 sequence. These sequences are also found in a new class of low-molecular-weight heterogeneous (200-400 nucleotide long) poly(A)$^+$RNAs. These RNAs are located mostly in cytoplasm rather than in polysomes. The amount of small B2$^+$ RNAs is noticeable larger than that of small B1$^+$RNAs. Tumour cells seem to contain more small B2 RNA than normal cells. The hybridization tests show that extended homology exists between the B1 sequence and 4.5S small nuclear RNA, which is predicted from comparison of their base sequences. Also, we have found homology between B1 and small cytoplasmic 7S RNA. Hybridization of B2 to sn or sc RNAs has not been observed, although the sequencing reveals partial homology between B2 and 4.5S sn RNAI /1/.

INTRODUCTION

Short interspersed repetitive sequences are known for over ten years. However, their intensive investigation has become possible only recently owing to the newly-developed technique for DNA cloning and sequencing. Mammalian cells are found to contain a few dominating types of short interspersed repetitive sequences which are scattered throughout the genome. Two such ubiquitous repetitive sequences represented by ca. $5x10^4$ copies per genome each, are discovered in mice /2/. They are designated as B1 and B2 sequences as they have first been selected on the basis of hybridization to double-stranded RNA of the B type isolated from mouse hnRNA. DsRNA-B is 100-200 bp long and capable to fold back after hnRNA melting /3/. Over 50% of dsRNA-B is transcribed from the B1 and B2 sequences /2/.

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A similar ubiquitous repeat, the so-called Alu sequence, is isolated from human cells /4/. The mouse B1 nucleotide sequences /5/ and human Alu sequences /4, 6/ reveal certain homology suggesting the Alu sequence originates from duplication of B1. The B2 sequences are quite different /1/. These short interspersed repeats are quite reported to be homologous to certain functionally significant sequences of mammalian genome, i.e. (i) intron-exon and exon-intron junctions, (ii) replication origins of papova viruses, (iii) RNA polymerase III promoters, and (iv) several small RNAs /1, 5, 6, 7, 8/. Furthermore, some copies of interspersed repeats are flanked with short 10-20 bp direct repeats resembling in this respect transposable elements of genome /1, 6/.

The biological role of these sequences remains unclear. However, their homologies allow to speculate about their possible involvement in DNA rearrangements, replication, transcription or RNA processing.

This work is another attempt to clarify the role of B1 and B2 sequences by studying their transcription and further alteration in the cell. It has been found by us earlier that dsRNA consisting mostly of B1 and B2 sequences efficiently hybridizes not only to nuclear RNA but also to cytoplasmic poly(A)$^+$RNA /3, 9/. This is also confirmed by other authors /10, 11/. In this paper, we describe the properties of RNAs from different cellular compartments hybridized to B1 and B2 sequences. The hybridization patterns are found to be dependent on the RNA fraction and on the tissue from which the RNA was isolated. It is of interest that small cytoplasmic poly(A)$^+$RNA containing interspersed repeats were found, too.

METHODS

Clones. For hybridization, we used the DNAs of clones Mm31 and Mm35 containing the B1 sequence and those of clones Mm14 and Mm61 with the B2 sequence /2/. Also, the DNA of the Mm31c clone whose B1 fragment extends from the 1st to the 72nd nucleotide was used in some experiments. The isolation of plasmids and mouse DNA insertions was described earlier /2/. DNA was labeled with [^{32}P] by nick translation /18/.

Isolation of RNA fractions. Ehrlich ascites carcinoma cells were suspended in 0.25 M sucrose with 200 µg/ml heparine prepared in TSN buffer (0.05 M tris-HCl, pH 8.0, 0.05 M NaCl, 0.005 M $MgCl_2$). To the suspension NP40 was added to a final concentration of 0.5%. After a 5 min incubation and 5 min centrifugation at 4,000 rpm, the nuclear pellet was collected, suspended in 0.1 M sodium acetate, pH 5.0, and the total nuclear RNA was isolated according to Scherrer and Darnell /13/.

The total cytoplasmic RNA was prepared by the chloroform-phenol (1:1), pH 8, treatment of the supernatant after addition to the latter of EDTA to 0.005 M and sodium dodecyl sulfate to 0.5%.

Mouse liver and MOPC 104E cells were homogenized in 0.25 M sucrose in TSM buffer using the Potter homogenizer. The cytoplasmic supernatant was centrifuged at 12,000 g and then at 25,000 rmp in a SW-27 rotor (2°C, 17 h) through 1 M and 2 M sucrose layers /14/. The sediment (polysomes) and the supernatant were deproteinized by adding EDTA to 0.005 M and sodium dodecyl sulfate to 1% and by shaking with chloroform-phenol (1:1), pH 8.

All of the RNA preparations were purified by two precipitations with 3 M sodium acetate. No B1 or B2 hybridizing RNAs were lost in the procedure.

Poly(A)$^+$RNAs were isolated by chromatography on poly(U) Sepharose. RNA dissolved in NETS (0.1 M NaCl, 2 mM EDTA, 20 mM tris-HCl, pH 7.6, 0.2% SDS) was passed through a poly(U) Sepharose column, washed with NETS, and eluted with 0.2% SDS at 52° C. For stepwise elution, the column was washed with solutions at increasing concentrations of deionized formamide in NETS as described by Dubroff & Nemer /15/.

RNA electrophoresis and hybridization. Electrophoresis was performed in a 1.5% agarose gel in 7 M urea in citrate buffer (pH 3.5). /16/. DBM filters were prepared according to Alwine et al. /17, 18/. The RNA was transferred to an RNA paper in 1 M sodium acetate, pH 4.2, for 18 h. The filters were then placed in a medium containing 50% formamide, 6xSSC, 0.2% sodium dodecyl sulfate, 2x Denhardt solution, 0.005 M EDTA, 1 mg/ml

salmon sperm DNA, 0.01 M phosphate buffer, 1% glycine, and incubated for 24 h at 37°C. The hybridization with [^{32}P]-DNA was performed in the same medium (but without glycine) at 37°C for 48 h. The filters were then washed 5 times with 2xSSC, 0.2% SDS at room temperature (5 min), and twice with 0.1 SSC, 0.1% SDS at 42°C (30 min). Thereafter radioautography was performed.

RESULTS

In the first series of experiments, different preparations of cellular RNA were electrophoresed in agarose gel under denaturing conditions, and transferred to DBM-filters. These Northern blots were hybridized to [^{32}P]-DNA of the clones containing either B1 or B2 sequences.

Fig. 1 shows the radioautographs. One can see that both poly(A)$^+$ and poly(A)$^-$RNA from the nuclei of Ehrlich carcinoma cells efficiently hybridized to B1 and B2 sequences (slots 1, 3). The major part of RNA molecules containing B1 and B2 (designated as B1$^+$RNA and B2$^+$RNA) was characterized by a high molecular weight (>18S). Many of them reached 7 kb or more.

Cytoplasmic poly(A)$^-$RNA did not hybridize to B1 and B2 sequences at all. However, that was not the case with cytoplasmic poly(A)$^+$RNA from carcinoma cells. Although the content of chains hybridizing to B1 and B2 sequences was much lower than in nuclear RNA, yet a significant hybridization took place (Fig. 1, slot 4). There were detected two major regions of hybridization. They are (i) molecules of the mRNA size, i.e. 0.7-3.0 kb long, and (ii) short RNAs only 200-400 nucleotides long. For B1, the hybridization to a rather high-molecular-weight material predominated (Fig. 1a, slot 4). However, B2 sequences hybridized mostly to a fraction of small poly(A)$^+$RNAs (Fig. 1b, slot 4). Even in nuclear RNA, small B2$^+$RNAs could be observed (slot 3), their content being low.

Similar experiments were conducted with RNA fractions prepared from mouse liver cells. Again, nuclear RNA had a high amount of B1 and B2 sequences but, comparing to carcinoma cells, their distribution was shifted to the zone of low-molecular-weight chains (Fig. 1, slot 7). For liver cytoplasm, polysome-

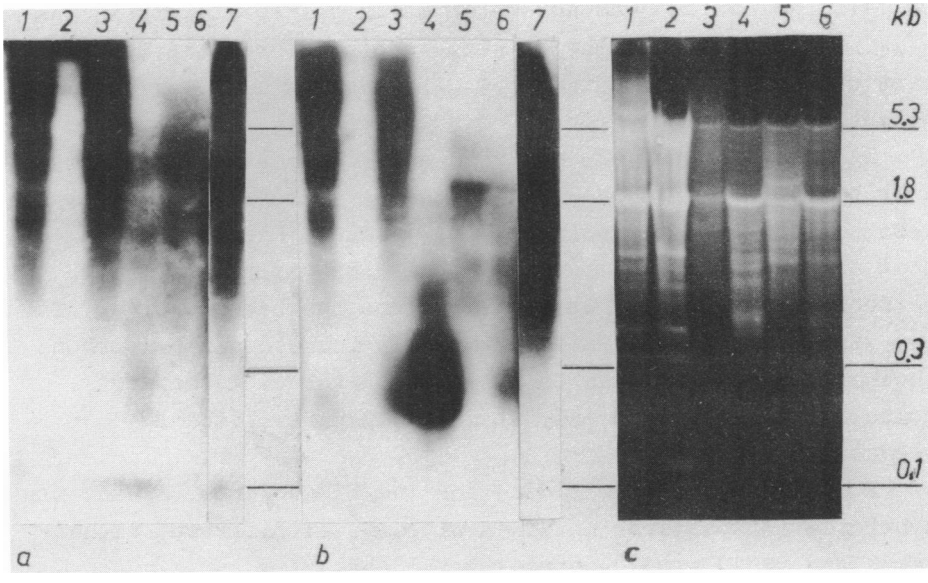

Fig. 1. Northern blot hybridization of mouse cell RNA to B1 and B2-containing clone [^{32}P]-DNA (clones Mm31 and Mm14, respectively).
(a) Hybridization pattern of B1$^+$RNAs;
(b) Hybridization pattern of B2$^+$RNAs;
(c) Gel stained with ethidium bromide.

1 - nuclear poly(A)$^-$ and 3 - poly(A)$^+$RNAs of Ehrlich carcinoma cells; 2 - cytoplasmic poly(A)$^-$, and 4 - poly(A)$^+$RNAs of Ehrlich carcinoma cells; 5 - polysome, and 6 - free cytoplasmic poly(A)$^+$RNA from liver cells; 7 - nuclear liver poly(A)$^+$RNA.

The arrows show the positions of 28S RNA, 18S RNA, 7S RNA, and 4.5S RNA.

bound and free poly(A)$^+$RNAs were analyzed separately. The content of B1-hybridizing sequences in both fractions was much lower than in nuclear RNA. Small B1$^+$RNAs were completely absent. All hybridization with B1 sequences was revealed in the fractions whose size was typical of mature mRNA.

On the other hand, B2 hybridizing sequences were characteristic of a more heterogeneous distribution. The bulk of material was found in the mRNA size fraction. However, significant hybridization was also revealed with the 200-400 nucleotide size fraction of free liver poly(A)$^+$RNA (Fig. 1b, slot 6). The amount of this small B2$^+$RNA was lower than in Ehrlich carcno-

ma cells. Small RNA was not detected in polysomal poly(A)$^+$RNA (Fig. 1b, slot 5). It may be believed therefore that the major part of small cytoplasmic poly(A)$^+$B2$^+$RNAs is not bound to polysomes.

In polysomal poly(A)$^+$RNA, one can find a strong discrete 2 kb band binding to the B2 sequence. A weaker 2 kb band can also be recognized in polysome-free poly(A)$^+$RNA. A high signal does not depend on the part of cloned DNA different from the B2 sequence as exactly the same picture has been observed with two different clones (Mm14 and Mm61) which contain no common sequences but B2. It can be believed that one of the major mouse liver mRNAs contains, at least, a part of the B2 sequence.

A similar control was done for the B1 sequence. Again, the hybridization patterns obtained with DNA of different clones (Mm31 and Mm61) were assentially the same.

Another control was aimed at checking the possibility of RNA degradation as accounting for the presence of small RNAs containing B2. To this end, we hybridized Northern filters also with other probes. These included the DNA of clone Mm22 which had the so-called A1 sequence /19/ corresponding to a long transcribed repeat of the mouse genome. It can be seen in Fig. 2a that A1-DNA hybridized only with the high-molecular-weight bands of cytoplasmic poly(A)$^+$RNAs from Ehrlich carcinoma cells. (One could observe two major bands and the background formed by several weakly resoluted bands.) The result indicates that there was no significant degradation of the RNA sample taken for analysis. Another evidence against the origin of small B2$^+$RNA via degradation of heavier molecules follows from the experiment with poly(A)$^+$RNA from the cytoplasm of plasmocytoma (MOPC 104E) (Fig. 2b). In this case, the hybridization of the B2 sequence to mRNA-size molecules and to the 200-400 nucleotide fraction was well resolved. The absence of B2$^+$ molecules having an intermediate size questions the validity of the degradation hypothesis for the origin of small B2$^+$RNA. The content of small B2$^+$RNA in the cytoplasm of MOPC 104E cells was much higher than that in liver cells (see Fig. 2b; at this exposition, small B2$^+$RNA was not visualized in liver cells).

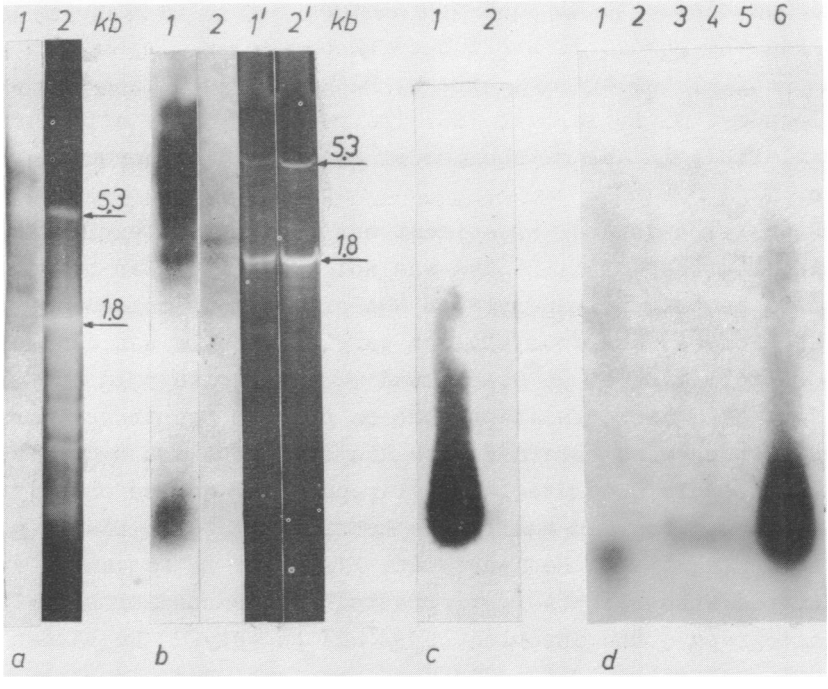

Fig. 2. Northern blot analysis:

(a) 1 - hybridization of the clone Mm22 DNA (the long repeat of mouse genome) to cytoplasmic poly(A)$^+$ Ehrlich carcinoma cell RNA (the same preparation which was used for hybridization with the B2-probe); 2 - gel stained with ethidium bromide;

(b) hybridization of the B2 DNA probe (clone Mm14) to cytoplasmic poly(A)$^+$RNA from MOPC 104E cells (1) and liver (2); gel stained with ethidium bromide (1' and 2');

(c) hybridization of the B2 probe (the DNA of Mm14) to cytoplasmic poly(A)$^+$ Ehrlich carcinoma cell RNA which was denatured and rechromatographed on poly(U)-Sepharose; RNA bound (1) and unbound (2) in the course of rechromatography;

(d) the same as (c), but rebound RNA was eluted with formamide solutions: 1 - unbound RNA; 2, 3, 4, 5 - RNA eluated with 10, 15, 20, and 25% formamide, respectively; 6 - RNA eluated at 52°C.

On the other hand, the cells of Ehrlich carcinoma and of plasmocytoma are similar. It seems likely that the high level of small poly(A)$^+$B$^+$RNA is typical for tumour cells. The same may be thought of B1$^+$RNA.

Comparison of the data presented in Fig. 1b and 2b shows that the quantities and properties of B2$^+$RNA of the mRNA size

vary from tissue to tissue, its content being the highest in MOPC and the lowest in Ehrlich carcinoma cells. In Ehrlich carcinoma cells, the average size of $B2^+$RNA is less than 18S while in plasmocytoma it is over 18S. The results may be attributed to variations in the metabolism of $B2^+$RNAs in different cell types.

Finally, a control experiment was designed to exclude the possibility that small $B2^+$RNA was not polyadenylated but just bound to another polyadenylated RNA of a higher molecular weight. Poly(A)$^+$RNA from Ehrlich carcinoma cells was denatured in 100% formamide at 75°C, diluted 100-fold with cold buffer (NETS), and again chromatographed on poly(U) Sepharose. Northern filters were obtained with RNA fractions and hybridized to B2 DNA. Only the material that repeatedly sorbed on poly(U) Sepharose did contain small RNAs hybridizing to B2 DNA (Fig.2c).

According to the sequence data /1/, some B2 sequences are terminated with oligo$(A)_{12-14}$ tracts. In our conditions of chromatography, the sorption of $B2^+$RNA to poly(U) Sepharose through such oligo(A) sequences was not excluded. To check such a possibility, cytoplasmic poly(A)$^+$RNA from Ehrlich carcinoma cells was denatured, bound to poly(U) Sepharose, and eluted using a discontinuous formamide gradient in NETS at 20°C. The RNA which remained bound after the increase of the formamide concentration to 25% was eluted at 52°C. The presence of $B2^+$RNA in each fraction was determined by the Northern blot hybridization to a B2 DNA probe. Fig. 2d shows that only a minor fraction of $B2^+$RNA was eluted with the formamide NETS solution. According to Dubroff and Nemer /15/, 25% formamide elutes the RNA containing oligo(A) tracts up to 25-30 nucleotides long. Thus, the major fraction of small $B2^+$RNA contains poly(A) tracts longer than 25 nucleotides which is possibly of the post-transcriptional origin.

We investigated then whether the hybridization could reveal homology between B1 and B2 sequences and non-polyadenylated discrete small nuclear and cytoplasmic RNAs. Such homology between 4.5S snRNA and the B1 sequence was established by comparing the sequences. Non-polyadenylated RNA was isolated from nuclei and cytoplasm, electrophoresed, transferred to DBM

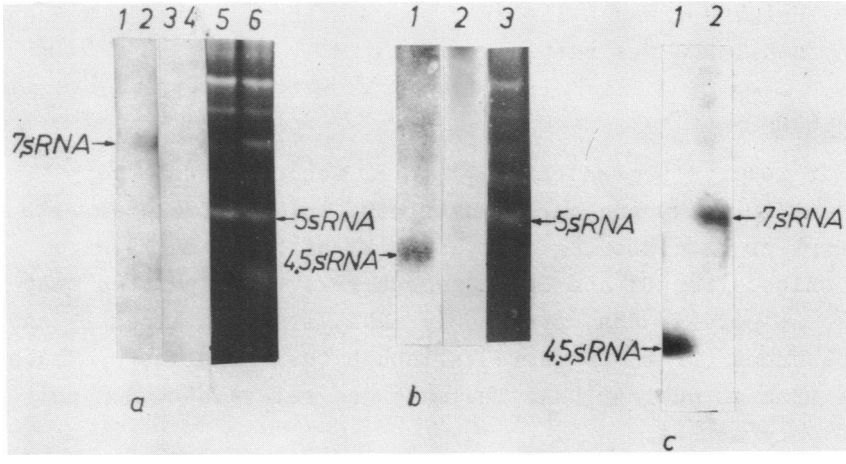

Fig. 3. Northern blot hybridization of the B1 and B2 probes to poly(A)⁻ cytoplasmic and nuclear RNA (shown is only the zone of small RNAs).
(a) polysomal (1, 3, 5) and free cytoplasmic (2, 4, 6) liver RNA was hybridized to the B1 probe DNA of Mm31 (1, 2) and the B2 probe DNA of Mm14 (3, 4); 5 and 6 - gel stained with ethidium bromide;
(b) nuclear RNA of Ehrlich carcinoma cells was hybridized to the B1 (1) and B2 (2) probes; gel stained with ethidium bromide (3);
(c) nuclear (1) and cytoplasmic (2) RNAs of Ehrlich carcinoma cells were hybridized to the DNA of clone Mm31c which contained only the 5'-half of B1.

filters, and hybridized to B1 and B2 DNAs.

Fig. 3 shows that the B1 sequence has a homology with 4.5S small nuclear RNA and 7S small cytoplasmic RNA (non-polysomal). No hybridization of the B1 sequence to small polysomal RNA could be detected. The hybridization between 7S RNA and the human Alu sequence is described by Weiner /20/.

We also hybridized filters containing small RNAs to a fragment of clone Mm31 which included only half of the B1 sequence (from the 1st to the 72nd nucleotide). This fragment hybridized to both 4.5S RNA (as was expected from the structure) and 7S RNA. Thus, 7S RNA (whose structure is not yet known) possesses a homology to the same part of the B1 sequence as 4.5S snRNA does.

Similar experiments with the B2 probe revealed no hybridi-

zation (Fig. 3) suggesting that the homology of the B2 sequence to any small RNA was rather low.

DISCUSSION

Our results proved that B1 and B2 sequences were very abundant in heavy nuclear transcripts, which correlated with the earlier data showing that about 2% of total hnRNA were transcribed from B1 and B2 sequences /2/. We found then that polysomal poly(A)$^+$RNA, presumably mRNA, also contained B1 and B2 sequences (or their parts) although their content was much lower than in nuclear RNA. The same was relevant to non-polysomal poly(A)$^+$RNA.

Using the similar method, Elder et al. /21/ show a high content of the Alu sequence (human analog of B1) in nuclear RNA and much lower content in total cytoplasmic poly(A)$^+$RNA. Calabretta et al. /22/ demonstrate the existence of Alu sequences in polysomal poly(A)$^+$RNA by means of electron microscopy of hybrids.

The above-listed data confirm our earlier observations on the presence of sequences hybridizing to nuclear dsRNA (that mostly consists of B1 and B2 sequences) in the free and polysome-bound poly(A)$^+$RNA of cytoplasm of different cells /3, 23/.

It can be suggested that many genes contain B1 (Alu) and B2 sequences located within either introns or 5'- and 3'-nontranslated sequences (Fig. 4). All of them are transcribed and can be detected in primary transcripts. Then the B1 and B2 transcripts inside the introns are eliminated in the course of splicing while those located in the nontranslated parts of exons survive and can be recovered in mature mRNA.

In the first-cloned eukaryotic genes such as globin, immunoglobin and ovalbumin ones, no repetitive sequences have been detected (see review 24). They can be found only in spacer regions. However, as follows from the above-mentioned results as well as from our earlier data it is not common. B1 and B2 sequences are present in some genes and, consequently, in some mRNAs. In particular, abundant 2 kb mRNA from mouse liver polysomes contains the B2 sequence. Some recent data demonstrate

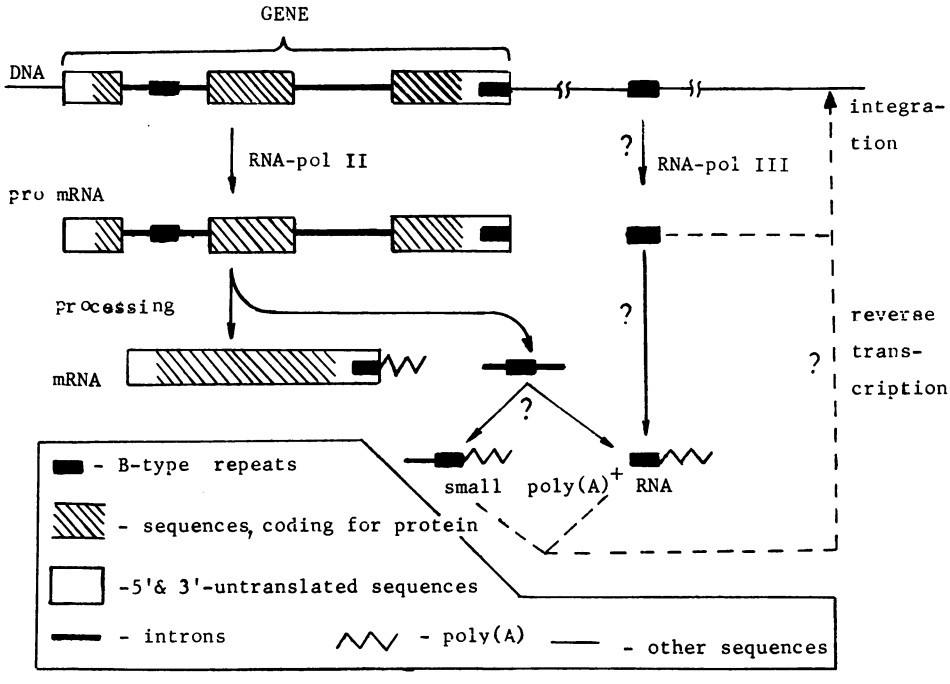

Fig. 4. A scheme showing possible metabolic pathways of RNA containing B-type repeated sequences (B^+RNA). It illustrates two ways via which small B^+RNA could be formed: in the processing of pre-mRNA and/or directly, by RNA polymerase III transcription. The scheme shows a hypothetic gene containing two B-type repeats which are located in the intron and 3'-untranslated region. The dotted line designates the presumptive process of B-type repeat amplification by reverse transcription of B^+RNA /30/. The question marks denote steps whose existence require additional proofs.

the presence of repetitive sequences within the introns of chicken conalbumin and Xenopus vitellogenin genes/25, 26/. The 3'-nontranslated part of mRNA transcribed from the chicken X-gene also induces a repeat /27/. One of the introns of the rat growth hormone gene possesses a repetitive sequence identical to B2 /28/. In D. melanogaster, a short repetitive sequence designated as suffix is found at the 3'-ends of many different mRNAs /29/.

Thus, it is clear that many genes include repetitive sequences, sometimes as a part of 5'- or 3'-exons. However, it is completely obscure whether repetitive sequences in genes

play a specific role at some level of gene expression.

Besides hnRNA and mRNA, ubiquitous repeat B1 hybridizes to small RNA of the nucleus (4.5S) and the cytoplasm (7S). According to the sequencing data, B2 contains regions of homology to 4.5S RNA I /1/, although it has not been visualized by the hybridization test. This can be explained by the fact that the average homology of B2 to 4.5S RNA I is lower (50%) than that of B1 to sn 4.5S RNA (68%).

Successful hybridization of B1 to 4.5S RNA may depend on the presence of two regions having 19 and 14 nucleotides in length with a 100% homology. Possibly, using milder conditions of hybridization, one will be able to detect the binding of B2 to 4.5S RNA I and to some other small RNAs.

Evidently, these small RNAs are not transcribed from B1 or B2 sequences. First, their sizes are different. Second, the homology is limited by a part of the sequence. Third, various B1 sequences differ by 8% point substitutions while all small RNAs of the given class are identical. However, it is possible that the genes for certain small RNAs have an ancestor in common, with ubiquitous repeats.

The homology between small RNAs and B1 and B2 sequences suggests that the corresponding RNAs are involved in the processing of hnRNA interacting with repetitive sequences.

In addition, in the present work, we discovered a novel type of small RNA, viz. small poly(A)$^+$RNAs with sequences homologous to B1 and B2 present both in the nucleus and in polysome-free cytoplasm. Those containing the B2 sequence are much more abundant.

These RNAs are heterogeneous in size (200-400 nucleotides long), but their heterogeneity can either completely or partly depend on the heterogeneity of the poly(A) tail. Their content in the cytoplasm is higher than in the nucleus.

It is noteworthy that the content of small poly(A)$^+$B2$^+$RNAs depends on the tissue under examination. For instance, in Ehrlich carcinoma and MOPC cells it is high but in mouse liver cells it is much lower. Apparently, the high content of these RNAs is related either to a high proliferation rate of carcinoma and plasmocytoma or to their transformed state.

Elder et al. /21/ studied the Northern blot hybridization of Alu-sequences to nuclear and cytoplasmic poly(A)$^+$RNAs of human cells. The hybridization is found to occur with the mRNA-size fraction and low-molecular-weight RNA. However, the high content of intermediate-size Alu-containing RNAs did not allow the authors to detect a special discrete fraction of small Alu$^+$ poly(A)$^+$RNA.

The origin of small B1$^+$ and B2$^+$RNAs is not clear. It can be the primary transcripts of B1 and B2 sequences which are synthesized with the aid of RNA polymerase III. Alternatively, they can be transcribed by RNA polymerase II as parts of long transcripts whence they are excised later in the course of processing (Fig. 4). After polyadenylation, they can appear in the cytoplasm. The process may somehow be involved in the regulation of processing.

In the present study, we demonstrated the existence of a rather long ($>$ 25 residues) poly(A) in small B2$^+$RNA. Another proof is our preliminary data obtained with the in vivo labeled small B2$^+$RNA. The direct measurement of the size of poly(A) isolated from the latter gave the figures up to 100-120 indicating the post-transcriptional formation of these tracts. Closely to the end of the B2 element, an AATAAA sequence was detected /1/ which is known to serve as a signal for polyadenylation.

It seems interesting that small B2$^+$RNA is accumulated in the cytoplasm at a much higher concentration than B1$^+$RNA. It is possible therefore that the enzyme systems destroying these sequences can discriminate between the two types of repeats. To understand the origin of small B1$^+$ and B2$^+$ RNAs, one has to clone and sequence them. This work is in progress now.

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ABBREVIATIONS

kb, kilobases; nt, nucleotides; DBM, diazobenzyloxymethyl; snRNA and scRNA, small nuclear and small cytoplasmic RNA;

dsRNA, double-stranded RNA; EDTA, ethylenediamine tetraacetic acid.

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