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# Preferential expression of unique sequences adjacent to middle repetitive sequences in mouse cytoplasmic RNA

Atsushi Kuroiwa and Shunji Natori

Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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## ABSTRACT

Total single-copy DNA and single-copy DNA contiguous to middle repetitive sequences were isolated from mouse brain by successive hydroxylapatite column chromatographies. These DNAs, termed repeat-contiguous single-copy DNA, were found to constitute 48% of the total single-copy DNA. The saturation hybridization values of these two DNA probes to nuclear RNA and cytoplasmic RNA containing polyA of mouse brain and liver were measured. The saturation hybridization levels of total single-copy DNA to brain and liver nuclear RNA were 13.5% and 8.8%, respectively, and those of repeat-contiguous single-copy DNA to the same RNA samples were 13.3% and 8.5%, respectively.

On the contrary, the saturation hybridization levels of single-copy DNA to cytoplasmic RNA containing polyA of brain and liver were 3.8% and 2.0%, respectively, and those of repeat-contiguous single-copy DNA to the same RNA samples were 5.8% and 4.0%, respectively. Similar results were obtained with total cytoplasmic RNA.

These results indicate that about half the steady state nuclear RNA is transcribed from repeat-contiguous single-copy DNA, and that cytoplasmic RNA containing polyA is mainly derived from repeat-contiguous single-copy DNA.

## INTRODUCTION

It is well known that eukaryotic DNA contains single-copy sequences, middle repetitive sequences and highly repetitive sequences (1). A certain class of middle repetitive sequences was found to be interspersed between single-copy sequences (2-9). It is possible that these interspersed sequences of DNA are intimately related to regulation of eukaryotic gene expression, but their precise function and significance are largely unknown.

Nuclear RNA, which is thought to be a precursor of cytoplasmic messenger RNA, is many times larger than cytoplasmic

 

RNA and has 3- to 5-fold greater sequence complexity. It was found that at least 25% of nuclear RNA was a product of middle repetitive sequences and that this RNA was linked to the products of single-copy sequences; namely, a middle repetitive sequence and a single-copy sequence are transcribed simultaneously as an RNA molecule (10, 11). These large RNAs are processed and modified in the nucleus and some of them are transported to the cytoplasm as messenger RNA. Most of the middle repetitive sequences in nuclear RNA are digested during this process (12, 13). Therefore, cytoplasmic messenger RNA is mostly free from interspersed middle repetitive sequences.

These middle repetitive sequences were found to have organ specificity (14, 15). Thus, they may be related to the transcription or post-transcriptional expression of organ specific messenger RNA. In sea urchins, it was found that most of the polysomal RNA was derived from repeat-contiguous single-copy DNA (16). In the present work we examined whether cytoplasmic RNA containing polyA is transcribed from long stretches of single-copy DNA or from single-copy DNA adjacent to middle repetitive sequences by DNA: RNA hybridization under RNA excess conditions. We found that nuclear RNA was a mixture of sequences transcribed from repeat-contiguous single-copy DNA and long stretches of single-copy DNA, whereas cytoplasmic RNA containing polyA was mostly derived from repeat-contiguous single-copy DNA.

## EXPERIMENTAL PROCEDURES

Preparation of total single-copy DNA DNA was extracted from mouse brain nuclei as described by Natori et al. (17), but omitting the step of equilibrium density gradient centrifugation in CsCl solution. The DNA was sheared to 500 - 600 base pairs at 40,000 psi in a French Press (Ohtake Seisakusho). The sheared DNA was reassociated at Cot 200, under which conditions middle repetitive sequences should reanneal. It was then applied to a column of hydroxylapatite (Bio-Rad, DNA grade) at 60°C. The DNA was eluted successively with 0.03 M, 0.12 M and 0.4 M PB (PB=phosphate buffer, equimolar $Na_2HPO_4$ and $NaH_2PO_4$,

pH 6.8) containing 0.06% SDS. The single stranded DNA eluted with 0.12 M PB was reannealed at Cot 200. After three cycles of annealing at Cot 200 and hydroxylapatite chromatography, the single-stranded DNA was dialyzed against distilled water, precipitated with 67% ethanol in the presence of 0.1 M NaCl and stored at 4°C in distilled water. The recovery of total single-stranded DNA from the shared DNA was usually 50%.

Preparation of repeat-contiguous (RC-) single-copy DNA The DNA was sheared to 4 - 5 kilo-bases (kb) in a Polytron (Kinematica GNBH) at a power setting at 8. Its size was analyzed by alkaline sucrose density gradient centrifugation using the $^{3}$H-SV40/EcoRI fragment (5.2 kb) as an internal marker (18). Fragmentation differed in different batches of DNA, but samples of DNA from a single batch were consistently sheared to the same length when the conditions were kept constant. The sheared DNA was reassociated at Cot 200 and the DNA containing reannealed regions was collected by elution from a hydroxylapatite column with 0.4 M PB as described above. At this Cot value, 52% of the DNA was recovered as partially reannealed DNA. For removal of highly repetitive DNA, the resulting DNA fraction was reannealed at Cot 0.01 and rapidly passed through a column of hydroxylapatite equilibrated with 0.12 M PB. The DNA that failed to bind to hydroxylapatite was collected (99% recovery) and sheared in a sonifier (Branson B-12) using a micro-tip at a power setting 3, by 12 sonication treatments for 10-sec periods at intervals of 1 - 2 min. The average size of the sheared DNA was estimated to be 600 bases. The sheared DNA was reannealed at Cot 200 and single-stranded DNA was collected by column chromatography on hydroxylapatite. After three cycles of annealing at Cot 200 and hydroxylapatite chromatography, the final fraction of single-stranded DNA was dialysed against distilled water, precipitated with ethanol and stored as described above. Usually 24% of the shared DNA was recovered as this single-copy DNA which is thought to be adjacent to middle repetitive sequences.

Iodination of probe DNA in vitro Single-stranded DNA probes obtained as described above were iodinated *in vitro* by the method of Chikaraishi et al. (19) with a slight modifica-

tion. The specific activity of $^{125}$I-DNA was 5 - 6 x $10^6$ cpm/μg, and the average length of this DNA was estimated to be 600 nucleotides from its sedimentation profile on alkaline sucrose density gradient centrifugation.

Preparation of cytoplasmic RNA Frozen mouse brains were homogenized in 10 volumes of buffer (0.02 M Tris-HCl, pH 7.4, 0.14 M NaCl, 0.005 M $MgCl_2$, 0.25 M sucrose and 50 μg/ml of potassium polyvinyl sulfate) in a glass homogenizer by 7 strokes of a Teflon pestle. Freshly isolated mouse livers were homogenized in the same buffer supplemented with 0.25% of diethylpyrocarbonate. The homogenates were centrifuged at 1,000 x g for 10 min at 4°C. The resulting supernatants were mixed with SDS and EDTA at final concentrations of 0.5% and 5 mM, respectively. RNA was extracted by shaking the solutions with an equal volume of phenol/chloroform (1 : 1, v/v) and the aqueous phase was obtained by centrifugation. This process was repeated 3 - 4 times until no significant turbidity was detected between the aqueous phase and the phenol-chloroform phase, and then RNA was precipitated with 67% ethanol in the presence of 0.1 M NaCl. Total cytoplasmic RNA was obtained from this RNA fraction by the method of Grady et al. (20), which included treatments with DNase I, bentnite-treated α-amylase and pronase. RNA containing polyA-sequences was isolated from the crude RNA fraction described above by oligo-dT cellulose column chromatography, according to the method of Bantle et al. (21). About 60 - 70% of the polyA-RNA present in cytoplasmic RNA was recovered after two cycles of oligo-dT cellulose column chromatography. The average size of polyA-RNA was determined by sucrose density gradient centrifugation, assaying polyA sequences by hybridization with $^3$H-polyU according to the method of Bishop et al. (22).

Isolation of nuclear RNA Brain and liver nuclei were prepared by the method of Bantle et al. (21) and Brobel et al. (23), respectively. The purified nuclei were suspended in 0.05 M acetate buffer, pH 5.2, containing 0.15 M NaCl. After addition of SDS to a final concentration of 0.3%, RNA was extracted by the method of Edmond et al. (24). The nuclear RNA obtained was purified further by centrifugation in CsCl solution

according to the method of Chikaraishi et al. (19). The resulting RNA was treated with 50 µg/ml of DNase I for 30 min at 37°C and then DNase I was inactivated by shaking the preparation with an equal volume of water-saturated phenol. The aqueous phase was passed through a column of Sephadex G-50 and the material excluded was collected.

Assay of DNA : RNA hybridization using S1 nuclease The hybridization mixture containing $^{125}$I-labeled probe DNA and various RNAs was diluted to 500 µl with distilled water. Then a sample of 50 µl was removed and nucleic acid was precipitated from it by adding 1 ml of 5% TCA solution and 50 µg of yeast tRNA as a carrier. The radioactivity in the precipitate was counted to calculate the total input of TCA-precipitable radioactivity. The remaining 450 µl of the preparation was mixed with 550 µl of 0.06 M acetate buffer, pH 4.5, and final concentrations of ingredients were adjusted to 0.3 M NaCl, 0.001 M $ZnCl_2$ and 100 µg/ml of heat denatured salmon sperm DNA. Then 1600 units of S1 nuclease (Seikagaku Kogyo) was added and the reaction mixture was incubated for 60 min at 45°C. After incubation radioactive DNA was precipitated by adding 100 µl of 100% (w/v) TCA solution in the presence of 50 µg of carrier yeast tRNA. The radioactivity remaining in the TCA-precipitate after S1 nuclease treatment was calculated as a percentage of the total input TCA-precipitable radioactivity. For measurement of the background count, the hybridization mixture was heated for 5 min at 100°C, rapidly cooled in ice, and analyzed with S1 nuclease treatment as described above. The background count was subtracted from hybridization count as zero time S1 nuclease resistant count.

## RESULTS

Characterization of DNA probes Two DNA probes - total single-copy DNA and single-copy DNA from regions contiguous to middle repetitive sequences (RC-single copy DNA) - were isolated as described in the Experimental Procedures. The rationale of the procedures used for isolation of the latter probe is mostly based on the experiments of Davidson et al. (16).

First, DNA was sheared to 4 - 5 kb and annealed at Cot 200, at which middle repetitive sequences should anneal. The reannealed DNA was isolated and highly repetitive DNA and fold-back DNA were removed. The resulting DNA containing middle repetitive sequence was sonicated to give fragments of 600 bases. These DNA fragments were reannealed at Cot 200 and DNA that did not reanneal under these conditions was isolated and defined as RC-single copy DNA. It has been suggested that 5 kb DNA obtained in this way contains at least one interspersed middle repetitive sequence consisting of about 300 bases (25). Therefore, this probe should cover roughly 10 kb single-copy DNA adjacent to a middle repetitive sequence. The molecular size of RNA transcribed from 10 kb DNA was estimated to be 38S, which coincides with the size of rapidly labeled nuclear RNA. Thus, most of the RNA transcribed from single-copy DNA adjacent to middle repetitive sequences should be detected using this DNA as a probe.

It calculated that about 48% of the single-copy DNA was contiguous to repeated sequences in mouse from the following evidence. The recovery of RC-single copy DNA was 24% after four repeated hydroxylapatite column chromatographies followed by reassociation at Cot 200, whereas the recovery of total single-copy DNA was 50% after three repeated hydroxylapatite column chromatographics followed by reassociation at Cot 200. These recoveries were quite reproducible. Thus, the amount of RC-single copy DNA in total single-copy DNA was calculated to be 48%.

Both DNA probes were iodinated *in vitro*, yielding preparations with specific activities of $5 - 6 \times 10^6$ cpm/μg. The iodinated DNA had a broad size-distribution with a mean length of 600 bases, judging from the results of alkaline sucrose density gradient centrifugation (18).

The reassociation curves of these DNA probes obtained using S1 nuclease assay are shown in Fig. 1. As controls, total mouse DNA and T4 phage DNA were analyzed under the same experimental conditions. The reassociation curves of these two control DNAs were quite similar to those obtained by Britten et al. using hydroxylapatite (1). When reassociated with excess

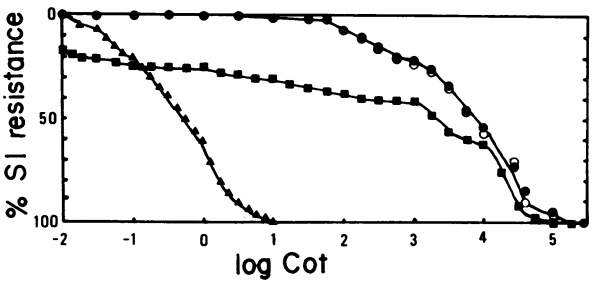


Fig. 1 Reassociation curves of DNA probes. Reassociation of $^{125}$I-DNA probes was measured with excess mouse total DNA that had been sheared to an average of 600 bp. DNA was reassociated in 0.12 M phosphate buffer at various Cot values and radioactivity of material that was resistant to nuclease S1 was measured. As controls, $^{14}$C-T4 DNA, and $^{3}$H-DNA prepared from mouse Friend leukemia cells were analyzed under the same conditions. The equivalent Cot is corrected to 0.18 M sodium concentration. (●—●), total single-copy DNA, (○—○), RC-single copy DNA, (▲—▲), T4 DNA, (■—■), mouse total DNA.

total mouse DNA having an average molecular length of 600 base pairs, 98 - 100% of the iodinated DNA reannealed. The association profiles of the two probes were almost completely identical giving $Cot_{1/2}$ values of $7.9 \times 10^3$ mol-sec$^{-1}$. It was estimated from the profiles that these DNAs were single copy DNAs and that their levels of contamination with repetitive sequences were both less than 1%. Thus, these probes seemed sufficiently pure for use in subsequent experiments as total single-copy DNA and single-copy DNA contiguous to middle repetitive sequences.

Hybridization of brain and liver nuclear RNA to DNA probes

Nuclear RNA was extracted from brain and liver nuclei and purified. The hybridizations of these nuclear RNAs to total single-copy DNA and RC-single copy DNA were measured in the presence of excess RNA by assaying the nuclease S1 resistant radioactivity of the iodinated probes. The results are shown in Fig. 2, where points are averages of duplicate measurements. With total single-copy DNA as a probe, saturation hybridization values of 13.5% for brain nuclear RNA and 8.8% for liver nuclear RNA were obtained. If transcription is asymmetric, these values are equivalent to 27.0% and 16.6% of the total single-copy sequences of brain and liver, respectively. These values

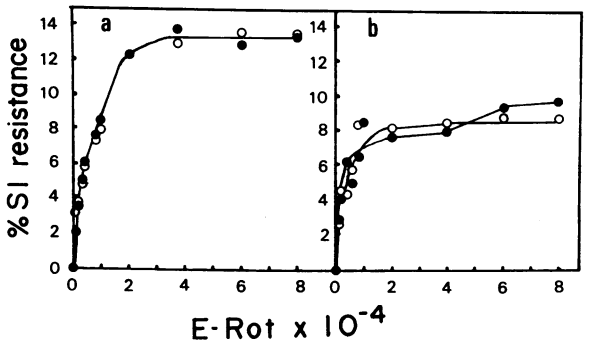


Fig. 2 Hybridization of DNA probes to nuclear RNA. Total single-copy DNA (●—●) and RC-single copy DNA (○—○) were hybridized with brain(a) and liver(b) nuclear RNA and analyzed by S1 nuclease assay as described under Experimental Procedures. Hybridization was done at RNA concentrations of 100 µg, 1mg and 10 mg/ml under $Na^+$ concentration of 0.18 M and/or 1.0 M, changing incubation time from 1 to 80 hrs at 68°C. The RNA equivalent Rot on the abcissa is corrected to that at 0.18 M sodium concentration, as described by Britten et al. (30).

obtained using total single-copy DNA as a probe were consistent with values for rat liver and mouse brain reported by Chikaraishi et al. (19) and Bantle et al. (21), respectively, obtained by DNA : RNA hybridization under RNA excess conditions.

When RC-single copy DNA was used as a probe, saturation hybridization values of 13.3% for brain nuclear RNA and 8.5% for liver nuclear RNA were obtained under the same conditions. Since RC-single copy DNA constitutes 48% of the total single-copy DNA, the amount of nuclear RNA derived from RC-single copy DNA in brain was calculated to be (13.3 x 0.48/13.5) x 100 = 47.3% of the product from total single-copy DNA. Therefore, the remaining 52.7% must be derived from long stretches of single-copy DNA. The values for liver were calculated similarly to be 46.3% for RC-single copy DNA and 53.7% for long stretches of single-copy DNA.

These results indicate that both single-copy DNA from regions contiguous to middle repetitive sequences and long stretches of single-copy DNA are expressed in the nuclei, and that nuclear RNA transcribed from single-copy DNA is a mixture of the products of RC-single copy DNA and long stretches of

single-copy DNA in a ratio of nearly 1 : 1.

Before evaluating these results it was necessary to show that DNA : DNA reassociation did not take place in the presence of excess RNA. Single-copy DNA probes were incubated with yeast tRNA under the conditions described above and then DNA : DNA reassociation was examined. As shown in Fig. 3, only 0.2 - 0.5% of the input radioactivity became resistant to digestion by S1 nuclease with increase in the Rot value, and no significant difference was detected between the two probes. Thus, it can safely be said that the amount of DNA : DNA reassociation occurring during the hybridization process was almost negligible.

Hybridization of brain and liver cytoplasmic RNA to DNA probes Cytoplasmic polyA-RNA was isolated using two cycles of oligo-dT cellulose column chromatography as described under Experimental Procedures. The recovery of polyA-RNA by this procedure was 60 - 70%. It was found that the cytoplasmic polyA-RNA in brain was 18S and that little degradation occurred during the purification. However, the size of cytoplasmic polyA-RNA in liver shifted from 18S to 14S during purification, indicating that significant degradation occurred during the procedure. Nevertheless, since the recovery of polyA sequences from liver RNA was almost identical with that from brain RNA, we concluded that this RNA could be used for subsequent experiments.

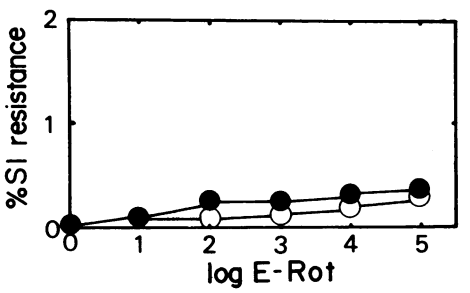


Fig. 3 Reassociation of $^{125}$I-DNA probes in the presence of excess yeast tRNA. Total single-copy DNA (●—●) and RC-single copy DNA (○—○) were reassociated in the presence of 10 mg/ml of yeast tRNA under the same conditions as for Fig. 2 and the amount of reassociation was measured by S1 nuclease assay. The equivalent Rot is corrected to that at 0.18 M sodium concentration.

Hybridization of these cytoplasmic polyA-RNAs to total single-copy DNA and RC-single copy DNA were assayed under RNA excess conditions. The results are shown in Fig. 4, where each point is the average of duplicate measurements. With total single-copy DNA as a probe, the saturation hybridization values were 3.8% for brain RNA and 2.0% for liver RNA. However, the saturation hybridization levels were much higher when RC-single copy DNA was used as a probe, being 5.8% for brain RNA and 4.0% for liver RNA.

As RC-single copy DNA constitutes 48% of the total single-copy DNA, it was calculated that (5.8 x 0.48/3.8) x 100 = 73.3% of the cytoplasmic polyA-RNA is transcribed from RC-single copy DNA in the brain. Similarly it was calculated that 96% of the cytoplasmic polyA RNA is transcribed from RC-single copy DNA in the liver.

To examine the possibility that a specific population of polyA-RNA might be obtained from oligo-dT cellulose, we repeated the experiments using total cytoplasmic RNA. The results were similar to those obtained with polyA-RNA, as shown in Fig. 5, the saturation hybridization values of total single-copy DNA for brain and liver RNA were 4.2% and 2.1%, respectively, and those of RC-single copy DNA for brain and liver RNA were 6.2% and 4.6%, respectively. These results indicate that total cyto-

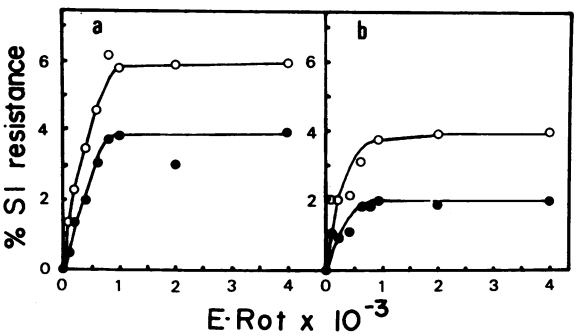


Fig. 4 Hybridization of DNA probes to cytoplasmic polyA-RNA. Total single-copy DNA (●—●) and RC-single copy DNA (○—○) were hybridized with brain(a) and liver(b) polyA-RNA and analyzed by S1 nuclease assay. The equivalent Rot is corrected to that at 0.18 M sodium concentration.

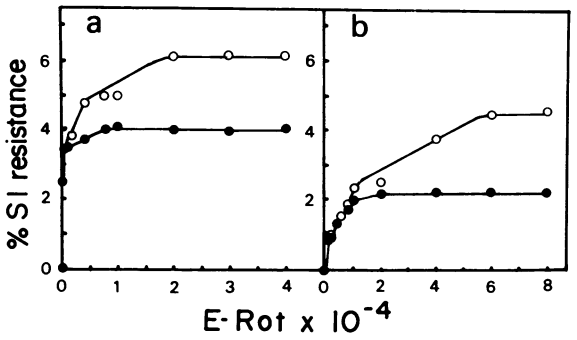


Fig. 5 Hybridization of DNA probes to total cytoplasmic RNA. Total single-copy DNA (●—●) and RC-single copy DNA (○—○) were hybridized with brain(a) and liver(b) total cytoplasmic RNA and analyzed by S1 nuclease assay. The equivalent Rot is corrected to that at 0.18 M sodium concentration.

plasmic RNA and polyA-RNA contain similar populations of RNA in terms of the sequences hybridized to single-copy DNA.

Thus, it is very likely that both RC-single copy DNA and long stretches of single-copy DNA are transcribed in nuclei, but that most of the RNA modified and transported to the cytoplasm is derived from RC-single copy DNA.

## DISCUSSION

In this work we measured the saturation hybridization values of single-copy DNA to nuclear and cytoplasmic RNA of mouse brain and liver. Davidson et al. distinguished two types of single-copy DNA: long stretches of single-copy DNA and single-copy DNA that is contiguous to middle repetitive sequences (25). It has been found that polysomal RNA, namely cytoplasmic messenger RNA of sea urchin embryo is mostly transcribed from RC-single copy DNA (16).

We showed here that in the liver and brain of adult mice, most cytoplasmic RNA containing polyA is derived from RC-single copy DNA. Results were similar with total cytoplasmic RNA and with cytoplasmic RNA containing polyA. The polyA-RNA used was probably messenger RNA, because 50 - 70% of the cytoplasmic messenger RNA contains polyA sequences (26-29).

To justify our conclusions, it was necessary to show that the materials and methods used were reliable. We isolated two DNA probes from mouse DNA, namely total single-copy DNA and RC-single copy DNA according to the procedure of Davidson et al. (16). The final preparations of these probes were sufficiently pure for use as single-copy DNA, because from their reassociation profiles we estimated that contamination with repetitive sequences, if any, was less than 1%.

Using these DNA probes we first measured the saturation hybridization values with nuclear RNA in the presence of excess RNA. From the values we drew the following conclusions.
(1) In liver and brain nuclei, RC-single copy DNA and long stretches of single-copy DNA are expressed equally.
(2) The nuclear RNA which hybridizes to total single-copy DNA is a mixture of the products of RC-single copy DNA and long stretches of single-copy DNA in a ratio of nearly 1 : 1.
These conclusions are consistent with those of others obtained using different animals under different conditions (10, 11). Thus, we conclude that our probes and methods are suitable for analysis of cytoplasmic RNA.

We isolated RNA containing polyA from brain and liver and purified the preparations by two cycles of oligo-dT cellulose column chromatography. During this process, little degradation of polyA-RNA from brain occurred, but degradation of RNA from liver was appreciable. We examined the isolation procedures to find conditions resulting in less degradation of polyA-RNA, and finally found that use of fresh liver and addition of 0.25% of diethylpyrocarbonate to the buffer prevented the degradation to some extent, although not completely.

We measured the saturation hybridization values of the DNA probes to cytoplasmic polyA-RNA. Results showed that 73.3% of the liver polyA-RNA and 96% of brain polyA-RNA were derived from single-copy DNA contiguous to middle repetitive sequences. Similar results were obtained using total cytoplasmic RNA. Since nuclear RNA was a mixture of products of long stretches of single-copy DNA and RC-single copy DNA, these results indicate that only the products of RC-single copy DNA are processed, modified and preferentially transported to the cytoplasm. The

products of long stretches of single-copy DNA may be selectively degraded in the nuclei.

Since it is known that RC-single copy DNAs are transcribed as single RNA molecules containing both middle repetitive sequences and single-copy regions, the repetitive sequences may have a function of acting as a signal for subsequent processing and modification. The results reported here are consistent with those of Davidson et al. obtained using sea urchin embryos. This is the first report that cytoplasmic polyA-RNA of mature mouse tissues is mainly transcribed from single-copy DNA adjacent to middle repetitive sequences.

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