NUCLEAR AND CYTOPLASMIC RIBONUCLEIC ACIDS OF CALF THYMUS

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The findings that isotopic precursors incorporate in much higher rate into nuclear ribonucleic acid (nRNA) than into any fraction of cytoplasmic RNA (cRNA)[1-5] may be interpreted by assuming nRNA as the precursor of cRNA[1,2]. The problem is of considerable biological interest in view of the possible role of nRNA as the mediator in the nuclear control of cytoplasmic protein synthesis. BARNUM *et al.*[6,7], however, are of the opinion, on the basis of their tracer experiments, that nRNA does not satisfy the theoretical requirements of the precursor of cRNA. Another objection to this hypothesis is derived from the known differences in average nucleotide composition between nRNA and cRNA[8-10]. This appears to preclude the possibility of a simple diffusion of nRNA into the cytoplasm. However, neither of these objections seems to be conclusive, because heterogeneity of RNA might obscure the situation. It is also possible that a secondary synthesis and/or turn-over of cytoplasmic RNA takes place rather independently from the nucleus. These possibilities may complicate the interpretation of the tracer experiments. At any rate, the transfer of nRNA into the cytoplasm seems to occur in the case of *Amoeba* as is evidenced by the autoradiographic experiments of GOLDSTEIN AND PLAUT[11], and of PRESCOTT[12], although their experiments do not exclude the possibility of decomposition or alteration of nRNA prior to or during the transfer.

One way of approaching the problem may be to find out, firstly whether any kind of ribonucleic acid exists that is common to the nucleus and cytoplasm, and secondly whether this RNA in the nucleus, if it exists, satisfies the requirements of a precursor of cRNA in the tracer experiments. The work described below has been conducted on calf thymus tissue. The first step has been to show that there are at least two kinds of nRNA, one of which reveals the same nucleotide composition as the cytoplasmic RNA. In the next step, both RNAs have been shown to occur as ribonucleoproteins, which can be purified by streptomycin. The final point we have dealt with is whether *all* cytoplasmic RNAs are derived from the nucleus, as tested by the incorporation of ^{32}P into nRNAs and cRNAs.

A preliminary account of this work has been published[13].

EXPERIMENTAL

Calf thymus

In all the experiments described in this paper, Holstein male calf of two to seven days old was used throughout. Thymus tissue was taken from the animal about 3 min after death. The tissue was immediately chopped fine with scissors and placed in 9 volumes of ice-cold 0.275 *M* sucrose–0.0036 *M* $CaCl_2$ solution that had been kept in a jar surrounded with crushed ice. This was then transported to the laboratory within 30 min. The details of the procedure for ^{32}P experiments will be described separately.

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Preparation of sucrose nuclei and nuclear protein fractions

Sucrose nuclei were prepared according to the method of ALLFREY *et al.*[14]. For the preparation of nuclear protein fractions, sucrose nuclei were homogenized in a glass homogenizer with 5 volumes of 0.1 *M* potassium phosphate buffer at pH 7.1. The homogenate was then centrifuged at 20,000 × *g* for 20 min in an International Refrigerated Centrifuge. A slightly opalescent supernatant was obtained ("pH 7.1 fraction"). The gelatinous pellet formed was dispersed with a homogenizer in a large volume of the same phosphate buffer, and centrifuged as above. The supernatant was discarded. The pellet ("residue fraction") was suspended in 30 volumes of 1 *M* NaCl solution, homogenized in a blendor at top speed for 30 sec, and stirred with a high-speed mechanical stirrer for 2 h. The viscous solution was centrifuged at 30,000 × *g* for an hour in a Spinco L ultracentrifuge. The colored pellet was homogenized in a blendor with 10 volumes of 1 *M* NaCl solution, and centrifuged as above ("1 *M* fraction").

Preparation of cytoplasmic fractions

The cytoplasmic fraction was prepared from the tissue homogenate in sucrose–$CaCl_2$ medium (see above) by centrifuging at 2,000 × *g* for 8 min to remove the nuclei. The microsomal fraction was spun down at 90,000 × *g* for 90 min in a Spinco L ultracentrifuge from the cytoplasmic fraction, from which the mitochondrial fraction had previously been removed by 20 min centrifugation at 7,500 × *g*.

Preparation of mononucleotide mixture of RNA for nucleotide composition analyses

Each fraction was successively treated with a large volume of cold 2% perchloric acid (PCA) (twice), 95% ethanol (three times), ether (twice), and finally dried in air. The dry powder so obtained was treated with 0.5 *N* KOH (in the proportion of 100 mg dry powder per 1 ml KOH) at 37° for 20 h, permitting quantitative conversion of RNA into a mixture of mononucleotides. This was then acidified with 60% PCA in the cold to precipitate proteins and DNA, if present. The sediment was washed with a small volume of cold 2% PCA, and the supernatant was combined. The combined supernatant containing all RNA mononucleotides was then neutralized with 6 *N* KOH (pH 7–8). After the removal of precipitated $KClO_4$ by centrifugation in the cold, each nucleotide in the solution was separated by ion-exchange chromatography.

Chromatographic determination of RNA nucleotide compositions

The "formic acid system" of HURLBERT *et al.* [15] was used throughout. The eluting system was adapted for the complete separation of a mixture of 5 to 7 mg of RNA mononucleotides by using a 50 ml mixing flask and a 0.6 × 20 cm Dowex-1 column (Dowex-1, formate form, cross linkage 2%, 200–400 mesh). Adsorbed nucleotides were eluted with a continuously increasing concentration of eluent, that is, first with 30 ml of 1 *N* formic acid, followed by 200 ml of 4 *N* formic acid. Samples of the effluent containing 3 ml were collected automatically every 15 min. The extinction at 260 mμ and 280 mμ was measured for each tube. After the readings, the contents of the tubes in each peak were quantitatively transferred to a volumetric flask, and the extinctions were read again. For the calculation of each nucleotide content, the following millimolar extinction coefficients were used: adenylic acid, 14.2 at 260 mμ; guanylic acid, 11.8 at 260 mμ; cytidylic acid, 13.0 at 280 mμ; uridylic acid, 9.9 at 260 mμ.

Purification of ribonucleoproteins from microsomes and nuclear "pH 7.1 fraction"

The extract of microsomes and the nuclear "pH 7.1 fraction" were used as the sources of ribonucleoproteins. For the preparation of the microsomal extract, a microsomal pellet was homogenized with 5 volumes of 0.1 *M* potassium phosphate buffer at pH 7.1. The content was then centrifuged at 20,000 × *g* for 20 min in an International Refrigerated Centrifuge. The procedure for preparing nuclear "pH 7.1 fraction" has already been described. To the microsomal extract or to the "pH 7.1 fraction", a one tenth volume of 0.2 *M* streptomycin sulfate solution in 0.1 *M* potassium phosphate buffer was added and the contents mixed. After 2 hours' standing in the ice bath, the contents, now whitish and turbid, were centrifuged at 15,000 × *g* for 15 min. The precipitate was washed 4 to 5 times with 0.02 *M* streptomycin in 0.1 *M* phosphate buffer at pH 7.1, with a homogenization each time. The final precipitate was then dispersed in 3 to 10 ml of 0.33 *M* potassium phosphate buffer at pH 7.1, and dialyzed for 24 h against the same buffer, followed by dialysis against 0.1 *M* potassium phosphate buffer at the same pH.

Electrophoresis

A Tiselius electrophoresis apparatus was used throughout. All electrophoretic analyses were performed in 0.1 *M* potassium phosphate buffer ($\mu = 0.24$) at pH 7.1 in a field of 7.3 V/cm.

Chemical determinations

RNA and DNA were estimated by the method of SCHNEIDER[16] (orcinol test for RNA, diphenylamine test for DNA). Phosphorus was determined by ALLEN's method[17].

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^{32}P-incorporation experiment

15 mC of inorganic ^{32}P (in 10 ml 0.14 *M* NaCl buffered with bicarbonate) were injected intravenously into a 3 days' old calf weighing about 50 kg. After 3 h, the animal was sacrificed, and the thymus tissue was quickly taken out. The nuclear and cytoplasmic fractions listed in Table IV were obtained by the procedure described before. For the counting of radioactivity, RNA was isolated from each fraction following the duponol method of KAY *et al.*[18], modified by AMANO *et al.*[19] for the purpose of tracer study. Mononucleotides were separated after alkaline hydrolysis of each RNA sample on a Dowex-1-formate column, using the same method as was adopted for the nucleotide composition analyses.

RESULTS AND DISCUSSION

Nucleotide composition of cytoplasmic and nuclear RNAs

It has been mentioned that the average nucleotide composition of nRNA is different from that of cRNA when compared in bulk[8–10]; this is often taken as an objection to the idea of a simple diffusion of nRNA into the cytoplasm. The question arises whether any fraction of nRNA of calf thymus has an average nucleotide composition comparable to that of the cRNA of the same tissue. Evidence has been presented showing that there are at least two different RNAs in calf-thymus nuclei isolated in sucrose–$CaCl_2$ medium with respect to the extractability[14,20] and the metabolic activity[21,22]. A protein fraction containing RNA can be readily extracted with neutral phosphate buffer, leaving unextractable RNA in the residue. In the experiments to be described below, the nuclei were isolated in sucrose–$CaCl_2$ medium, and the protein fractions containing RNA were prepared as the sources of nucleotide analyses. The first fraction was obtained by extracting sucrose nuclei with neutral phosphate buffer (pH 7.1 fraction). The gelatinous pellet was once more washed with the same buffer. It was shown that this washing removed only one tenth of the material, as compared with the first extract. The quantity of the pH 7.1 fraction is equal to 7–13 % of the nucleus (dry weight). The fraction contains about 12 % of RNA by weight. It was also estimated that about one third of the nuclear RNA is associated with this fraction. The pellet after phosphate extraction was sometimes used *per se* as the source for RNA nucleotide analyses; but in other cases it was dissolved in 1 *M* NaCl solution, followed by high speed centrifugation. The high-speed pellet is termed "1 *M* fraction", and used for the nucleotide analyses. The quantity of this fraction is equal to 7 to 10 % of the nucleus, and it contains 20 % RNA by weight. Cytoplasmic fraction and microsomes were also analysed for the nucleotide composition of RNA. It should be mentioned that the microsomes here obtained would be only part of this fraction. In the medium employed, a considerable proportion of the microsomes may be centrifuged down together with mitochondria. Actual chemical analyses of each fraction revealed that "microsomes" obtained by our fractionation procedure contained only 65 % of the cytoplasmic RNA.

RNA mononucleotides obtained after alkaline hydrolysis of each fraction were quantitatively separated on a Dowex-1 column. Fig. 1 and Table I show a sharp separation of four RNA mononucleotides and demonstrate the good reproducibility of this ion-exchange technique. 98 to 100 % of the ultraviolet-adsorbing material at 260 mμ applied on the column were usually recovered on the chromatogram. It may be noteworthy that, in our experiments, Dowex-1 x 10 or once-used Dowex-1 x 2 (both of formate form) give a relatively broad peak of each mononucleotide and do not seem to be suitable for precise quantitative purposes. The analytical data on the

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nucleotide composition of RNAs from different sources of calf-thymus tissue are given in Table II. It is at first sight clear that the nucleotide composition of nRNA of both residue fraction and 1 *M* fraction are characterized by a higher uridylic and a lower guanylic acid content as compared with that of cytoplasmic RNA. The results are in harmony with the data on the nuclear and cytoplasmic RNAs reported from other laboratories[8-10]. The point of interest here, however, lies in the fact that the RNA of the pH 7.1 fraction reveals practically the same nucleotide composition as that of

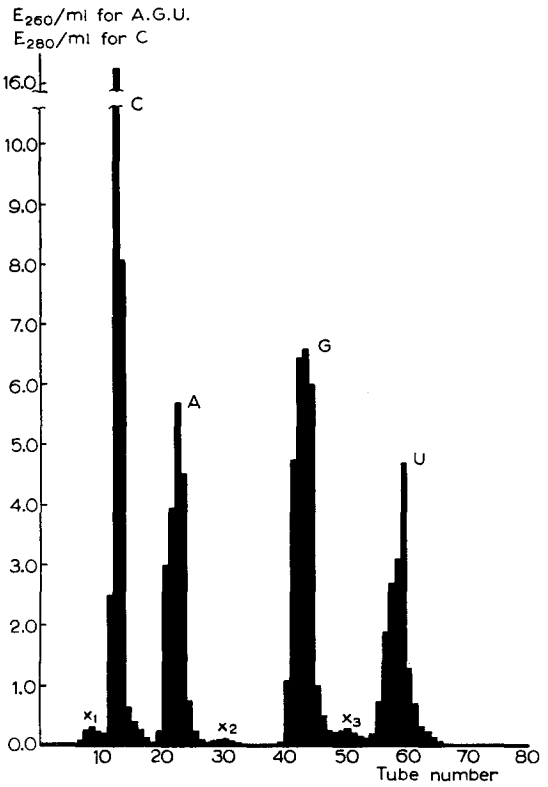

TABLE I

REPRODUCIBILITY OF THE CHROMATOGRAPHIC SEPARATION OF INDIVIDUAL RNA MONONUCLEOTIDES

MOLAR RATIO (ADENYLIC ACID = 10.0)

	Guanylic	*Cytidylic*	*Uridylic*
1st assay*	17.9	14.1	10.2
2nd assay*	17.9	14.2	10.1

* Samples from a single alkaline hydrolysate of the pH 7.1 fraction.

Fig. 1. Chromatographic separation of individual mononucleotides of RNA of calf thymus microsomes. C = Cytidylic acid; A = Adenylic acid; G = Guanylic acid; U = Uridylic acid; X_1, X_2, X_3 = unknown ultraviolet adsorbing substances.

TABLE II

NUCLEOTIDE COMPOSITION OF RNAs FROM DIFFERENT NUCLEAR AND CYTOPLASMIC FRACTIONS OF CALF-THYMUS TISSUE

MOLAR RATIO (ADENYLIC ACID = 10.0)

Fraction	*No. of determinations*	*Guanylic*	*Cytidylic*	*Uridylic*	$\frac{Purine}{Pyrimidine}$
Whole thymus tissue	1	17.3	13.5	11.4	1.11
Sucrose nuclei	1	15.7	13.5	12.5	0.99
Residue fraction (nucleus)	3	15.1	12.5	12.9	0.92
1 *M* fraction (nucleus)	2	15.1	13.4	12.5	0.97
pH 7.1 fraction (nucleus)	4	18.0	14.1	10.1	1.16
Cytoplasmic fraction	3	17.9	14.4	10.3	1.13
Microsomes	1	17.6	14.6	10.1	1.12

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cytoplasm and microsomes. This is a clear indication that in the nucleus of calf thymus RNA is not homogeneous, and that at least two structurally different RNAs exist. One of these is similar to the cytoplasmic RNA with respect to the nucleotide composition.

Purification of ribonucleoprotein from nucleus and cytoplasm

It is a general belief that most RNAs in the cells are associated with proteins to form ribonucleoprotein complex. It is therefore reasonable to assume that RNA occurs in the nucleus as ribonucleoprotein, like the ribonucleoprotein particles of microsomes. Comparison of the nuclear with cytoplasmic ribonucleoproteins might help us to understand the relationship of nRNA to cRNA. Microsomal ribonucleoprotein has been isolated by the use of streptomycin from rabbit appendix tissue in our laboratory[23]. The same procedure has been applied in isolating calf-thymus microsomal ribonucleoprotein particles, as well as the ribonucleoprotein in the pH 7.1 fraction of nuclear origin. Purification of ribonucleoprotein in 1 *M* fraction has so far been unsuccessful, because it does not go into solution when subjected to the mild treatment we have tried. In Fig. 2 is shown the electrophoretic pattern of a purified ribonucleoprotein from the phosphate extract of sucrose nuclei and that of microsomes. Both ribonucleoproteins revealed a considerable electrophoretic homogeneity.

TABLE III

ELECTROPHORETIC MOBILITY AND RNA CONTENT OF RIBONUCLEOPROTEINS FROM NUCLEAR "pH 7.1 FRACTION" AND MICROSOMES

Source of ribonucleoprotein	*Electrophoretic mobility* at pH 7.1, μ = 0.24*		*% RNA***
	Ascending	*Descending*	
pH 7.1 fraction (nucleus)	—11.5	—10.7	54.7
Microsomes	—11.9	—10.9	56.8

* $cm^2/sec/V \cdot 10^{-5}$.
** Percent RNA in RNA plus protein.

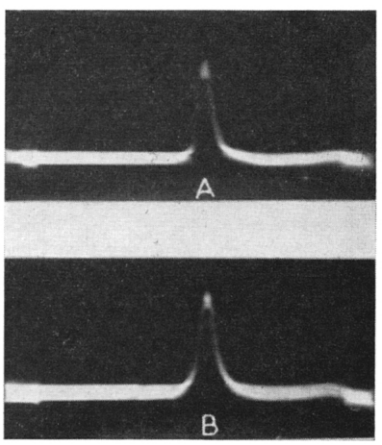

Fig. 2. Electrophoretic patterns of purified ribonucleoproteins from calf thymus microsomes (A) and from "pH 7.1 fraction" of calf-thymus sucrose nuclei. Descending patterns in 0.24 μ potassium phosphate buffer at pH 7.1 in a field of 7.3 V/cm.

The electrophoretic mobilities and RNA content of these two ribonucleoproteins have been compared, and the results are included in Table IV. It is seen that these are not distinguishable from each other, at least as far as the properties examined here are concerned.

The observations, together with the data on nucleotide compositions might be taken to mean that RNA in the pH 7.1 fraction passes into the cytoplasm in the form of ribonucleoprotein.

^{32}P incorporation into nuclear and cytoplasmic RNAs of calf thymus in vivo

It is one of the requirements for the precursor–product relationship that incorporation of isotope into the precursor RNA must occur at a higher rate than into the product RNA, if the early phase of the reaction is examined. In our case, however,

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TABLE IV

INCORPORATION OF ^{32}P INTO RNAs OF DIFFERENT FRACTIONS OF CALF THYMUS *in vivo* 3 h AFTER INTRAVENOUS INJECTION OF 15 mC ^{32}P

Fraction	*Specific activity (counts/min/mg RNA-P)*					
	Mixed nucleotides	*Mixed nucleotides (calculated)**	*Adenylic*	*Guanylic*	*Cytidylic*	*Uridylic*
pH 7.1 fraction (nucleus)	1360	1370	990	2760	685	960
1 *M* fraction (nucleus)	3330	3500	3800	3260	3910	3100
Cytoplasmic fraction	850	880	866	905	710	1140
Microsomes	880	—	—	—	—	—

* Calculated from the specific activity of mononucleotides.

such a relation would not be expected when cRNA exchanges phosphate independently from the nucleus, even if the RNA of the pH 7.1 fraction is the sole precursor of cRNA. The rule might also not be applicable when part of the cRNA is synthesized in the cytoplasm, even if the RNA of the pH 7.1 fraction is transferred from the nucleus to the cytoplasm. At any rate, it is ideal in such experiments to follow the time course of the incorporation of isotope into the two RNAs in question. The present situation in our laboratory, however, does not allow such a large scale experiment on calf thymus *in vivo* to be undertaken.

About 15 mC of ^{32}P were injected into a calf. After 3 h, the animal was killed, and the thymus was removed. The fractions listed in Table IV were prepared by the method described under EXPERIMENTAL. It is clearly seen from the table that the RNA in the 1 *M* fraction is the most active site of ^{32}P incorporation of all the fractions examined. It is also obvious that the RNA in the pH 7.1 fraction gained ^{32}P almost twice as actively as the RNA of cytoplasmic origin. However, examination of the data on the incorporation into individual mononucleotides indicates that the situation is not at all simple. Specific activities of adenylic, cytidylic, and uridylic acids are not very much different between the RNA of the pH 7.1 fraction and that of the cytoplasmic fraction. The higher specific activities of the mixed nucleotides in the pH 7.1 fraction over cytoplasmic is not due simply to the higher activity of all four nucleotides, but to the unusually high specific activity of the guanylic acid. This is of some interest, because, a relative inertness of guanylic acid phosphorus (after alkaline hydrolysis of RNA) has often been pointed out[1,21,25]. Several possible explanations may be given for the relation between the RNA in the pH 7.1 fraction and cytoplasmic RNA. An explanation will be that they are synthesized independently of each other at a different rate despite their chemical similarities. Two other possibilities mentioned at the beginning of this section should not be overlooked, however. Experiments recently conducted in our laboratory (to be reported in detail later) indicated that ^{32}P incorporation into microsomal RNA in mitotic tissues (rabbit appendix or thymus) takes place rather independently of the RNA of the pH 7.1 fraction from the quantitative aspect, but roughly in parallel with the synthesis of DNA. However, in non-mitotic tissue (adult rat liver) the RNA of the pH 7.1 fraction does satisfy a requirement of the precursor of microsomal RNA in the tracer experiments. The results strongly suggest that, upon mitosis, microsomal RNA is synthesized largely in the cytoplasm, and that the transfer of the RNA of the pH 7.1 fraction into the cytoplasm, even if it occurs, is covered by mitotic synthesis of cRNA and cannot be detected by the tracer experiments.

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SUMMARY

1. It has been shown that there are at least two kinds of nuclear ribonucleic acid in calf thymus tissue, one of which is associated with a fraction soluble in neutral phosphate buffer and has the same nucleotide composition as that of cytoplasmic RNA. On the other hand the RNA associated with the nuclear residue has a different nucleotide composition.

2. Electrophoretically homogeneous ribonucleoprotein samples were isolated from a nuclear fraction soluble in neutral phosphate buffer and from microsomal fractions. Both showed the same electrophoretic mobilities and RNA content.

3. The experiment of ^{32}P incorporation into nuclear RNA and cytoplasmic RNA indicated that not all the RNA in the cytoplasm can be derived from the nuclear RNA soluble in phosphate buffer, assuming that no exchange reaction of cytoplasmic RNA phosphorus taked place.

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