Summary

A detailed study has been made of the precipitation phenomenon observed with high molecular weight DNA in the analytical ultracentrifuge. The dependence of the phenomenon upon molecular weight, density, viscosity, and rotor speed has been studied and an equation developed which fits the experimental data closely. The phenomenon can be described by a pseudo-phase-transition line relating ω^2 to the reciprocal of the monomer concentration. The slope of this line appears to be a function of $M^{3/2}$ and $(1 - \bar{v}\rho)$ as well as ω^2, but independent of the viscosity of the solution.

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Evidence for Ribonucleic Acid Molecules Restricted to the Cell Nucleus*

Ruth W. Shearer and Brian J. McCarthy

ABSTRACT: The molecules of ribonucleic acid (RNA) present in the mouse L-cell nucleus and cytoplasm were compared by competition for complementary sites on mouse deoxyribonucleic acid (DNA). All sequences of RNA in the cytoplasm were also represented in the nucleus. However, cytoplasmic ribonucleic acid (cRNA) was unable to compete against a fraction of the nuclear ribonucleic acid (nRNA) molecules, indicating that these are not present in the cytoplasm. Saturation of the homologous DNA showed that nRNA is complementary to about five times as much of the genome as is cRNA, further confirming the existence of RNA molecules present only in the nucleus. Turnover of the unique sequences was examined by measurement of the accumulation of label in the unique fraction and its rate of breakdown after the labeled precursor was removed from the medium. A maximum was reached after 100 min of label, indicating a short lifetime for the RNA molecules which are limited to the nucleus.

The label (80% of it) was lost after 150 min in unlabeled medium, and no label remained in the unique nuclear fraction after 20 hr. Competition of cRNA against nRNA was repeated using rabbit kidney cells, to test the possibility that the above results are peculiar to heteroploid cells or to cells in culture. Unique nRNA was again found. It was concluded that although cytoplasmic messenger ribonucleic acid (mRNA) originates in the nucleus, approximately 80% of the base sequences being transcribed into RNA in L cells are in unstable molecules which are retained in the nucleus.

The nucleus of mammalian cells has been shown to be the site of origin of at least the bulk of cRNA[1] (Prescott, 1964; Girard *et al.*, 1965). However, there is evidence that the nucleus is also the site of synthesis of RNA with a specifically nuclear function. Kinetic studies have indicated a high rate of turnover of RNA within the nucleus (Harris, 1963). An RNA of unusual base composition has been found complexed to histones

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[1] Abbreviations used: SDS, sodium dodecyl sulfate; SSC, standard saline citrate (0.15 M NaCl–0.015 M sodium citrate); cRNA and nRNA, cytoplasmic and nuclear ribonucleic acids, respectively.

in nuclei of peas (Huang and Bonner, 1965) and of rat liver (Benjamin *et al.*, 1966). Sedimentation of RNA from HeLa cell nuclei in a sucrose gradient shows rapidly labeled polydisperse RNA present in an amount far greater than would be expected for RNA that is to become cytoplasmic messenger (Penman, 1966).

Hybridization of RNA with homologous denatured DNA allows a comparison of populations of RNA molecules in terms of common complementary sites in the DNA. The method of immobilization of denatured DNA on nitrocellulose filters (Gillespie and Spiegelman, 1965) provides a simple, sensitive, and accurate way to make this comparison. The purpose of this study was to determine whether there exists a population of RNA molecules in the cell nucleus which is not present in the cytoplasm, and to further clarify the nuclear origin of cRNA.

Materials and Methods

Growth and Labeling of Cells. Mouse L cells were grown as monolayer cultures in Eagle's minimum essential medium (Eagle, 1959) supplemented with 10% calf serum. They were harvested on the day they became confluent.

Cells from the kidney cortex of a 4-week-old San Juan rabbit were dissociated by treatment with trypsin–EDTA (0.025:0.01) and cultured in the same way but with fetal calf serum. Rapidly growing fibroblast-shaped cells were harvested after 21–41 days in culture.

Cells were pulse labeled by addition of [^{3}H]uridine (New England Nuclear Corp.) (3 μc/ml) to the medium. Cells were randomly labeled by addition of [^{32}P]-orthophosphate (2 μc/ml) in phosphate-free Eagle's medium for 2 days.

Preparation of Cell Fractions. Cells were removed from the glass surface with trypsin–EDTA, collected by centrifugation at 200*g* for 5 min at 0°, and suspended in distilled water. Tween 80 (polyoxethylene sorbitan monoleate) (Fisher and Harris, 1962) was added to a concentration of 0.1% (v/v) and the solution of ruptured cells was homogenized with a motor-driven Teflon pestle in a glass tube with clearance 0.004–0.006 in. After about ten strokes at high speed, nearly all nuclei were entirely free of cytoplasmic tags when viewed by phase microscopy.

The homogenate was layered over 2.2 M sucrose and spun at 200*g* for 5 min. Cytoplasm was removed by pipet and frozen at −12°. The nuclear film was washed by slowly dripping 0.5 M sucrose down the side of the tube to lift the remaining cytoplasm so that it could be pipetted off without disturbing the film. The nuclei were resuspended in 0.25 M sucrose, layered over 2.2 M sucrose, and spun at 500*g* for 5 min. The film was again washed with 0.5 M sucrose and suspended in its 2.2 M sucrose cushion. Microscopic examination of this suspension shows undistorted nuclei in a clean field. Staining of the nuclei in the sucrose solution revealed normal nucleoli.

This procedure has been used successfully on different kinds of cultured cells and a variety of rabbit tissues. The trypsin is essential; in its absence, the cytoplasm peels off in large membranous pieces which will sediment with the nuclei and cause them to aggregate. Dead cells present in suspension cultures are resistant to rupture by this method and remain as whole cells among the clean nuclei, as shown by vital staining. The detergent method offers the advantage that it is faster than other published methods for isolation of nuclei for nucleic acid preparation, lessening the danger of nuclease damage. The time required is less than 30 min, with all steps done at 0°.

Extraction of RNA. The nuclear fraction was diluted with distilled water to facilitate phase separation after phenol extraction. To both cell fractions was added one-fourth volume of a solution of 1.0 M LiCl–0.01 M sodium acetate, pH 5.2, and 2.5% SDS.[1] After mixing, an equal volume of phenol (Mallinckrodt analytical reagent) saturated with a fivefold dilution of the above solution was added. This was heated to 60° to destroy RNase, mixed, cooled in ice, and shaken vigorously before centrifugation at 1000*g* to separate phases. The aqueous phase was reextracted with cold phenol and precipitated with two volumes of ethanol overnight at −12°. In very dilute nuclear fractions, 30 μg/ml of denatured bacterial DNA was added before ethanol, to promote quantitative precipitation of the RNA.

The precipitate was suspended in a buffer containing 0.01 M Tris (pH 7.8) and 0.01 M $MgCl_2$, reprecipitated, and resuspended. DNase (1 μg/ml) (Worthington Biochemical Corp.) was added and allowed to digest at 37° for 30 min. One-fourth volume of the LiCl–sodium acetate–SDS solution was added, followed by cold phenol extraction, two ethanol precipitations, and suspension in 2 × SSC.

The average yield of RNA from one 16-oz prescription bottle of monolayer culture was 200 μg from L cells and 100 μg from rabbit fibroblasts. The L-cell RNA (18%) and the rabbit RNA (40%) were present in nuclei.

Extraction of DNA. DNA for the present experiments was extracted from mouse embryos and rabbit liver. It has been shown that DNA from different mammalian tissues is identical, and that L-cell DNA is not distinguishable from mouse DNA by hybridization tests (McCarthy and Hoyer, 1964).

Tissue was minced with scissors and homogenized in 2 × SSC, and the DNA was extracted by a modification of the method of Marmur (1961). RNA was removed by incubation with boiled RNase, and further deproteinization done with hot phenol. DNA was harvested by spooling, denatured by boiling 5 min at 400 μg/ml in 0.01 × SSC, followed by chilling in ice, and stored over chloroform at 4°.

Annealing of RNA with DNA. Denatured DNA was immobilized on nitrocellulose filters as described by Gillespie and Spiegelman (1965) except that drying was done at 60° overnight. Retention of DNA by the filter was assayed by measuring the optical density of the filtrate and of the nucleotides removed by incubation of the filter with DNase (1 μg/ml) for 30 min.

Filters were cut into four equal segments, and each

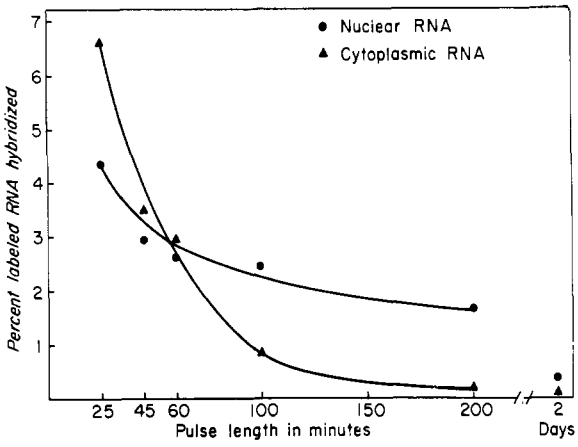

FIGURE 1: Hybridization of n- and cRNA. L-cell n- or cRNA labeled with [^{3}H]uridine for various periods was incubated with 12 μg of denatured mouse DNA immobilized on a nitrocellulose filter in 1 ml of 2 × SSC for 18 hr at 67°.

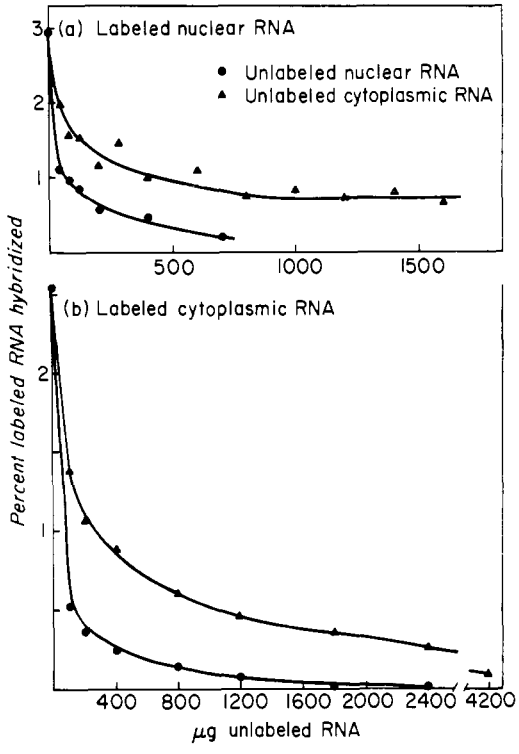

FIGURE 2: Competition between n- and cRNA for sites on homologous DNA. RNA (4 μg) from L cells labeled with [^{3}H]uridine for 45 min (5950 cpm/μg in upper curve, 1246 cpm/μg in lower curve) was incubated with mouse DNA (12 μg in upper curve, 8 μg in lower curve) in the presence of unlabeled RNA.

piece was immersed in labeled RNA in 1 ml of 2 × SSC in a small screw-capped glass vial which was incubated in a 67° water bath for 18–20 hr. After annealing, each filter was rinsed in 15 ml of 2 × SSC

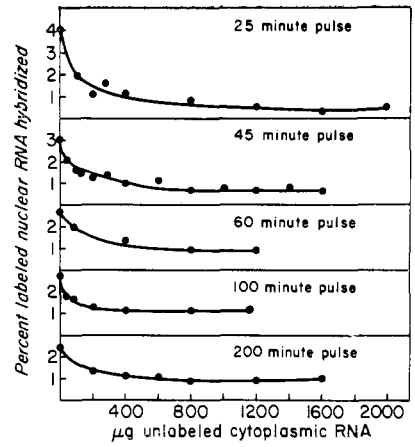

FIGURE 3: Competition of cRNA against nRNA after different pulse lengths. RNA (4 μg) from L cells pulse labeled with [^{3}H]uridine was incubated with 12 μg of DNA in the presence of unlabeled cRNA. Specific radioactivities of the various RNA preparations were in the range 5512–11,600 cpm/μg.

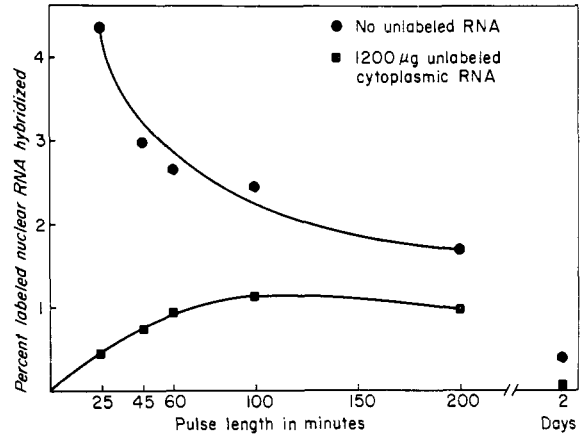

FIGURE 4: Competition plateau level as a function of pulse length. Data of Figure 3.

for 5 min (three times), dried, and assayed in a Packard Tri-Carb spectrometer.

Nonspecific binding of the RNA to the filter was determined by incubation of the labeled RNA with a filter containing an amount of bacterial DNA equal to the amount of homologous DNA to be used in the experiment. Values obtained were between 0.02 and 0.2% of input counts, and this background was subtracted from values obtained with homologous DNA. Bacterial DNA has been shown to have no long base sequences in common with mammalian DNA (Hoyer *et al.*, 1964).

Experimental Results

Effect of Pulse Length on Per Cent Hybridization

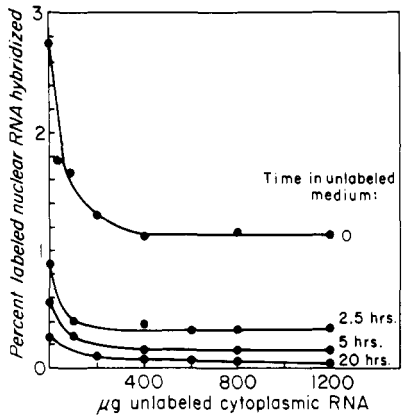

FIGURE 5: Competition of cRNA against nRNA after different times in unlabeled medium. Cells were labeled with [^{3}H]uridine for 100 min, then transferred to unlabeled medium. Labeled nRNA (4 μg) was incubated with 12 μg of DNA in the presence of unlabeled cRNA. Specific radioactivities of the various RNA preparations were in the range 11,600–1050 cpm/μg.

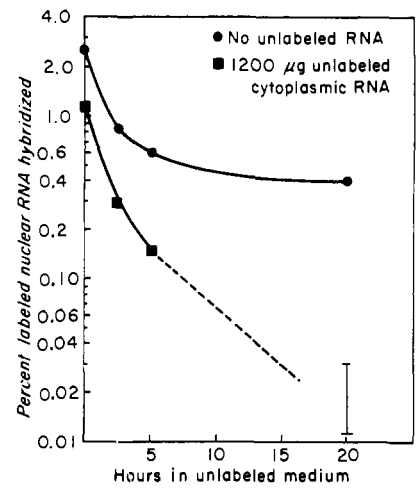

FIGURE 6: Competition plateau level as a function of time in unlabeled medium. Data of Figure 5.

without Competitor. The fraction of labeled RNA hybridized in the absence of competitor decreases with increasing pulse length in both the cytoplasmic and nuclear fractions (Figure 1). The higher per cent hybridization of the cRNA compared to the nRNA after a short pulse supports other evidence that there is a delay in movement of newly synthesized rRNA out of the nucleus, and during this time newly synthesized labeled mRNA is accumulating on old ribosomes in the cytoplasm (Penman *et al.*, 1963).

The greater hybridization of the nuclear RNA compared to the cRNA after longer periods of labeling reflects both the accumulation of label in the unique nuclear fraction and the accumulation of labeled ribosomes in the cytoplasm. DNA base sequences complementary to rRNA are saturated at very low RNA:DNA ratios, and at higher ratios the excess ribosomal RNA dilutes the other less plentiful molecules for which complementary sites are still available.

Existence of RNA Base Sequences Unique to the Nucleus. L cells were labeled with [^{3}H]uridine for 45 min and harvested, and n- and cRNA were prepared. Aliquots of each labeled RNA were incubated with filters containing mouse DNA in the presence of increasing amounts of unlabeled RNA from nuclei and cytoplasm of L cells.

cRNA was unable to compete against a fraction of the nRNA, as shown by the plateau at high ratios of competitor to labeled RNA (Figure 2a). The plateau level is a measure of the fraction of the hybridized RNA molecules whose reaction with the DNA is unaffected by the presence of large amounts of cRNA. This and other similar experiments detailed later support the view that there exists a population of RNA molecules in the mammalian cell nucleus which is not found in the cytoplasm.

The plateau level was unaffected by an increase in the amount of DNA on the filter from 12 to 24 μg, showing that the sites complementary to the unique nuclear RNA are not saturated at the RNA:DNA ratio used. The plateau level represented well-formed RNA:DNA hybrid as demonstrated by its resistance to RNase. Seventy per cent remained after treatment with 2 μg/ml of enzyme for 1 hr.

Presence of cRNA Base Sequences in nRNA. nRNA was able to compete against all sequences of cytoplasmic RNA which are complementary to nDNA (Figure 2b), and was in fact a more efficient competitor than the cRNA, as shown by the slope of the initial part of the curve. This may be attributed to a higher content of RNA hybridizable at high RNA:DNA ratios (non-rRNA) in the nucleus as compared to the cytoplasm.

A 1000-fold dilution of labeled by unlabeled cRNA is necessary to reduce the hybridization level by 96%. This results from the fact that only a very minute fraction of the unlabeled cRNA is identical with the mRNA which is preferentially labeled by the short pulse ($^1/_{32}$ of a cell generation).

Unstable Nature of RNA Restricted to the Nucleus. Competition of unlabeled cRNA against labeled nuclear RNA was repeated using different pulse lengths (Figure 3). Plateau levels were found to increase to a maximum at 100 min (Figure 4). The RNA restricted to the nucleus reached one-half of maximum labeling in only 30 min, implying that the RNA molecules which remain in the nucleus have a very short average lifetime. This does not preclude heterogeneity, and molecules with a lifetime many times the average could be present.

The decline in per cent of labeled RNA found in the unique nuclear fraction after the maximum indicates that RNA species common to nucleus and cytoplasm are still becoming labeled. This agrees with previous reports that 28S rRNA does not leave the nucleus immediately after its synthesis (Penman, 1966; Prescott,

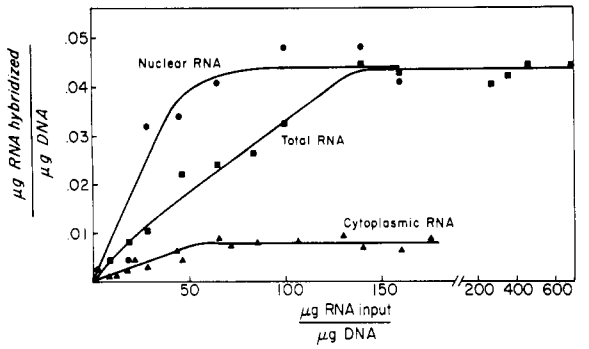

FIGURE 7: Saturation of complementary sites on DNA by randomly labeled total, n-, and cRNA. Various amounts of ^{32}P-labeled L-cell RNA (12,000 cpm/μg) were incubated with filters containing 12 μg of mouse DNA.

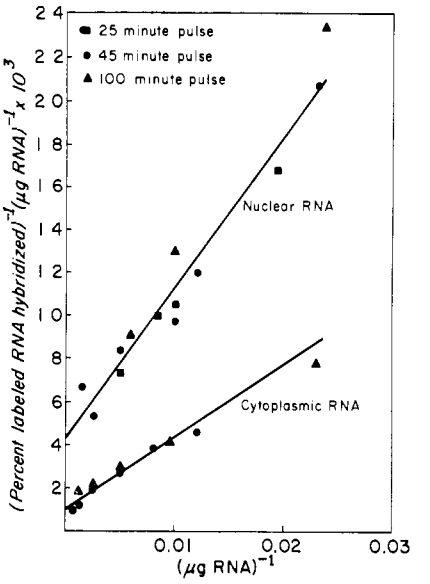

FIGURE 8: Saturation of complementary sites on DNA by pulse-labeled n- and cRNA. L-cell RNA (4 μg), labeled with [^{3}H]uridine for various periods, was incubated with 12 μg of DNA in the presence of identical unlabeled RNA. Specific radioactivities of the various RNA preparations were in the range 1246-11,600 cpm/μg.

1964). The plateau level after 2 days of labeling shows that the RNA molecules limited to the nucleus comprise an extremely small fraction of the total nuclear RNA.

Although these results suggest the possibility that the delay in maximum labeling of the bulk nuclear RNA could be due to a pool of rRNA in the nucleus, Penman's (1966) finding of no stable RNA in the nucleus after a 3-day chase makes this unlikely unless the pool is emptied into the cytoplasm when the nuclear membrane breaks down during mitosis.

Stability of the RNA molecules restricted to the nucleus was studied by measuring the decrease in 100-min pulse plateau level after varying periods of time in unlabeled medium (Figure 5). No plateau is found after 20 hr. The plot of these plateau levels (Figure 6) indicates a half-life of about 75 min for the unique nRNA. This is probably an overestimate because of the decrease in specific activity of the bulk nRNA with time, caused by the transfer of labeled RNA to the cytoplasm and the synthesis of unlabeled RNA molecules other than those specific to the nucleus. The curve also might be elevated by the preferential synthesis of new molecules from labeled breakdown products in a nuclear pool as suggested by Watts (1964).

Saturation of Complementary Sites on DNA by Total, n-, and cRNA. L cells were labeled with ^{32}P for 2 days before harvesting, at which time they appeared normal. They were divided into two fractions, one of which was used for extraction of total RNA and the other for n- and cRNA. Each RNA fraction was diluted with the like fraction from unlabeled L cells, and DNA filters were incubated with increasing amounts of each fraction (Figure 7). Amounts of RNA hybridized were calculated on the assumption that all of the mRNA is fully labeled, and that the generation time of the cells is 24 hr so that the stable RNA would be three-fourths labeled.

nRNA saturated over five times as much of the DNA as did cRNA, confirming the existence of RNA base sequences present only in the nucleus and showing that this minute fraction of the total RNA is coded by a very significant fraction of the genome. nRNA saturated the same fraction of the DNA as did total RNA, confirming that all cRNA base sequences complementary to nDNA are found also in the nucleus. The slow approach to saturation by the total RNA reflects

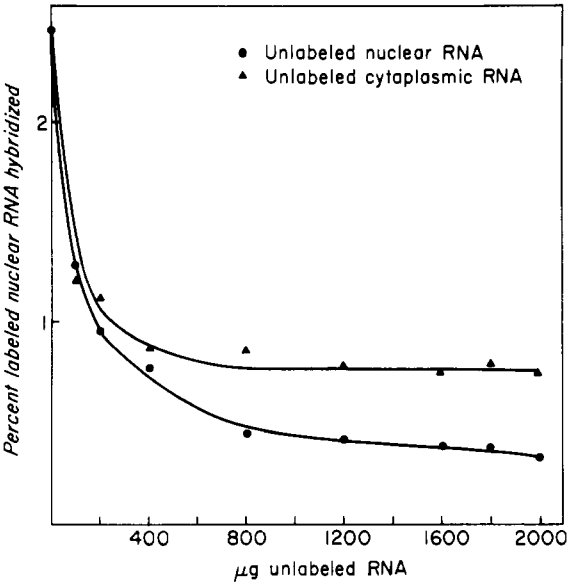

FIGURE 9: Competition against nRNA from diploid cells. nRNA (4 μg) from cultured rabbit kidney cells labeled with [^{3}H]uridine for 90 min (9800 cpm/μg) was incubated with 18 μg of denatured rabbit DNA in the presence of unlabeled RNA from rabbit kidneys.

the abundance of rRNA diluting the rarer molecules which are found only in the nucleus.

The ratio of nuclear to cRNA hybridized at saturation was also determined by a double-reciprocal plot (Figure 8) of the hybridization of n- and cRNA in the presence of identically unlabeled RNA. Data were pooled using nRNA labeled for 25, 45, and 100 min, and cRNA was labeled for 45 and 100 min. The intercepts indicate that nRNA saturates about 4.3 times as much of the genome as cRNA, further supporting the above conclusions.

RNA Restricted to the Nucleus in Diploid Cells in Vivo. Competition of cRNA against nRNA was repeated using nRNA from cells of rabbit kidney pulse labeled in culture and unlabeled RNA from the kidneys of 5-week-old rabbits. The plateau in Figure 9 confirms the existence of RNA base sequences in the nuclei of rabbit kidney cells which are not present in the cytoplasm. Thus the class of RNA molecules restricted to the nucleus exists in normal diploid cells *in vivo* as well as heteroploid cells in culture. Failure of the unlabeled nRNA to completely compete with the labeled nRNA may be due to the appearance in culture of new RNA molecules which are not synthesized *in vivo*.

Discussion

These results offer a possible resolution of two viewpoints on the relationship of n- to cRNA. Autoradiographic studies of Prescott and Bender (1962) using HeLa and cultured Chinese hamster cells show disappearance of label from nRNA and its appearance in cRNA following transfer of cells to unlabeled medium, implying that nRNA is the precursor of cRNA. Kinetic studies of Harris' group (Harris, 1963) using HeLa cells are interpreted to mean that the bulk of the rapidly labeled nRNA is broken down within the nucleus and cannot be the precursor of cRNA. The experiments described suggest that nRNA consists of two distinct classes: (1) molecules which are transported to the cytoplasm, and (2) molecules which are degraded in the nucleus without ever entering the cytoplasm. The first class is quantitatively much larger, but the second class represents a much larger fraction of the genome.

Although the precise location and function of the short-lived RNA molecules which remain in the nucleus are unknown, several possibilities are suggested and are being studied. It is reasonable that proteins which function only in the nucleus should be coded by RNA messages which remain in the nucleus. These might include the histones (Bonner, 1965) and the nucleic acid polymerases. Messages which code for the structures of the mitotic apparatus may be synthesized shortly before mitosis, be used in the nucleus, and subsequently disappear. This is being investigated by the comparison of nRNA of dividing and nondividing cells. However, it seems unlikely that as large a part of the active genome as that which codes for RNA unique to the nucleus should have its function limited to the mechanics of cell division.

During the isolation of nuclei, aggregated chromatin of metaphase cells sediments with the nuclei while the nuclear sap must mix with the cytoplasm. Since less than 2% of the L cells are in metaphase at the time of harvesting, leakage of small amounts of specifically nRNA into the great bulk of cytoplasm in this manner might not be detectable in these experiments. However, RNA has been shown to be attached to the chromosomes during metaphase in HeLa (human) cells (Feinendegen and Bond, 1963), and it is possible that the RNA which is restricted to the nucleus is either attached to the chromosomes or is not present at all during metaphase.

Although little RNA is synthesized during metaphase, this small amount may be specific to this stage of the cell cycle and have a significant function (Johnson and Holland, 1965). The presence of this RNA fraction as part of the unique nRNA may be revealed through studies of synchronized cultures.

The RNA which Huang and Bonner (1965) report associated with histones in pea seedling nuclei may represent a small portion of the unique nRNA. However, it is too small and too homogeneous to be the rapidly labeled polydisperse RNA which Penman (1966) found underlying the peaks of ribosomal precursor in his sucrose gradients of pulse-labeled RNA from clean nuclei of HeLa cells.

The existence of RNA molecules specific to the nucleus suggests a role as mediators of the regulation of gene transcription. It has been suggested (Frenster, 1965) that genes may be activated through the interaction of specific acidic proteins with the deoxynucleoprotein to displace histone repressors so as to allow transcription by RNA polymerase. In this system, repression would be the normal state and derepression a response to environmental conditions. The RNA which is restricted to the cell nucleus may code for these acidic nuclear proteins as well as histones. Although these functions are entirely speculative, the finding that the majority of the active genome codes for short-lived RNA molecules which are restricted to the nucleus opens up exciting possibilities for the study of the regulation of gene action in mammals.

Acknowledgment

We thank Dr. Stanley M. Gartler for valuable advice on cell culture and for the use of his laboratory to conduct these experiments.

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Isolation and Characterization of the *N*-Methyl Derivatives of 2-Aminoethylphosphonic Acid from the Sea Anemone, *Anthopleura xanthogrammica**

J. S. Kittredge, A. F. Isbell, and R. R. Hughes

ABSTRACT: The phosphonic acid analogs of choline phosphate (trimethyl-2-phosphonoethylammonium hydroxide inner salt) and 2-methylaminoethylphosphonic acid have been isolated in crystalline form from the ethanolic extracts of the sea anemone, *Anthopleura xanthogrammica*. The presence of a small amount of 2-dimethylaminoethylphosphonic acid was also detected.

The three *N*-methyl derivatives of 2-aminoethylphosphonic acid have been synthesized and the structures of the isolated phosphonic acids have been established by comparison with the synthetic compounds. The biological occurrence of the phosphonic acid analogs of ethanolamine phosphate, serine phosphate, and choline phosphate, the three major constituents of phospholipids, has now been established.

The two aminophosphonic acids previously isolated, ciliatine (2-aminoethylphosphonic acid) (Horiguchi and Kandatsu, 1959, 1960; Kittredge *et al.*, 1962) and phosphonoalanine (2-amino-3-phosphonopropionic acid) (Kittredge and Hughes, 1964), may be considered to be the phosphonic acid analogs of β-alanine and aspartic acid, or their structural similarity to the phosphate esters of ethanolamine and serine may be emphasized, *i.e.*, they are desoxyethanol aminophosphate and desoxyserine phosphate. Ciliatine occurs in phospholipids (Kittredge *et al.*, 1962; Rouser *et al.*, 1963; Hori *et al.*, 1964; de Koning, 1966). A survey of 19 species of *Coelenterata* for phosphonic acids (Kittredge, 1965) revealed a number of new phosphonic acids; among these were several ninhydrin-negative zwitterions. The possibility that one of the compounds detected might be desoxycholine phosphate[1] was examined.

Materials and Methods

Anthopleura xanthogrammica. Approximately 4.8 kg wet weight of this sea anemone was collected intertidally near Scripps Institution of Oceanography, La Jolla, Calif. They were drained briefly, freed of much of the adhering sand and shells, and diced into 6 l. of 95% ethanol.

Synthetic 2-Trimethylaminoethylphosphonic Acid. We are indebted to Dr. A. F. Rosenthal, Long Island Jewish Hospital, for a gift of this compound (Rosenthal and Geyer, 1958).

Ion-Exchange Resins. The resins employed for the

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[1] Trimethyl-2-phosphonoethylammonium hydroxide inner salt. For clarity we have considered the *N*-methyl derivatives as a series and have used the names: 2-aminoethylphosphonic acid, 2-methylaminoethylphosphonic acid, 2-dimethylaminoethylphosphonic acid, and 2-trimethylaminoethylphosphonic acid betaine.