lands of the Galapagos comes from the observation (Fig. 2) that prediction error is greater for the more isolated islands. That correlation between area and isolation ($r_{13} = -0.28$) is slight hints that the distance effect may contribute to floral species variation independently of factors associated with reduced insular area.

That better predictions of $Y$ should result from multiple rather than single regression analysis is not surprising. Of interest, however, is that linear, rather than curvilinear, multiple regression analysis gives more accurate predictions. In fact, multiple curvilinear analysis for these data gives predicted values less accurate than those determined by single curvilinear analysis with area only (Fig. 1). This raises the question of why area alone can be used to predict variation (in model 2) with an accuracy approaching that obtained by use of several factors (in model 1). Another question is why area by model 2 gives better predictions than it does by model 1? The answers may relate to the obvious: that the *number* of factors determining richness of insular floras or faunas increases progressively with increase in insular area. Thus use of logarithms and the model of $y = bx^z$, rather than of the actual numbers and $y = bx$, may give a prediction "curving in the right direction" for progressive, overlapping accumulation of elements of ecologic diversity associated with increased area. It is now clear that groups whose insular variation in species numbers have previously been studied by the Arrhenius approach (model 2) need to be examined by multiple regression analysis, utilizing linear, curvilinear, or mixed linear-nonlinear models.

Our study deals with insular variations in number of plant species for a cluster of small islands remote in the eastern Pacific, and two interrelated sets of problems are undoubtedly intermingled by the analysis: (i) insular production of endemic species versus insular increase of nonendemic species and (ii) whether insular number of species and number of individuals regulate, in part, each other. Preston's discussion (*4*) is the most recent one to approach the topic, and we will soon discuss it elsewhere (*10*). The present report, however, suggests that area itself exerts little control on insular species abundance in strong centers of endemic differentiation (*1*), with isolation and ecologic diversity being more important regulators. Area may be more important as a regulator in regions of larger land mass (large islands, continents) where barriers to dispersal are reduced and the degree of isolation is decreased. In this context the possibility arises that on small islands another aspect of the colonization barrier is the regulation of species numbers by numbers of individuals maintained or permitted by reduced ecologic diversity and competition, influenced in turn by vagaries of interisland dispersal (*11*).

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7. We are indebted to Beverley Lee for programming assistance and computer feeding at the Harvard Computing Center. All regression coefficients were evaluated by Student's $t$-distribution, and may be considered insignificant unless accompanied by asterisk* (significant: $.05 \geq P > .01$) or asterisks** (highly significant: $P < .01$).
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10. T. H. Hamilton, R. H. Barth, Jr., I. Rubinoff, in preparation. While the finding that small islands deviate from prediction more so than larger ones is not obvious and is not anticipated by our presuppositions resulting from a comparable study of the Darwin finches (*1*), we note Ernst Mayr's exact prediction of our finding in conversations about the biogeography of the Australopapuan biota prior to computer analysis of the present data.
11. Study was supported in part by a National Science Foundation research grant. We gratefully acknowledge the assistance and interest of W. F. Blair, E. Mayr, R. H. MacArthur, F. A. Pitelka, R. K. Selander, W. J. Smith, O. Solbrig, E. E. Williams, and E. O. Wilson.

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## Nucleolus: A Center of RNA Methylation

Abstract. *The ubiquitously distributed complex of enzymes, the RNA methylases, the apparent function of which is the alteration of the structure of transfer RNA at the macromolecular level by the introduction of methyl groups into the component bases, are concentrated in the nucleolus, an organelle previously implicated in RNA synthesis.*

Transfer RNA (tRNA) is structurally characterized by the presence of methylated bases and of pseudo-uridine. The synthesis of the methylated bases of tRNA is achieved by methylation of preformed RNA by an enzyme system, RNA methylase (*1*).

Purification of the RNA methylase revealed that methylation is performed by a complex of enzymes with highly restricted substrate specificities (*2*). Moreover, the enzymes are species specific as well (*3*).

Purified preparations of pea nuclei have been shown to synthesize in vitro an RNA with the attributes of transfer RNA (*4*). We have evidence now that the enzyme system which effects the methylation to tRNA is localized within the nucleolus.

Nuclei were prepared from 36-hour-old pea seedlings (*5*), purified by centrifugation through an empirically established sucrose gradient (2.0 to 0.6$M$ sucrose; 0.0005$M$ $MgCl_2$) in a Spinco rotor No. 25 at 8000 rev/min for 15 minutes. The cell-free nuclei were disintegrated by rapid stirring in saturated sucrose and the subnuclear fractions were recovered by differential centrifugation (*6*). The fractions were dialyzed for 2 to 3 hours against 0.01$M$ tris ($p$H 7.8) and 0.005$M$ mercaptoethanol at 0°C and were homogenized with 6 strokes in a glass teflon homogenizer, and incubated (Tables 1–3). As a control, samples containing identical incubation mixtures were kept at 0°C for the duration of the incubation and were then washed in the same way as incubated samples. The reaction was stopped by the addition of an equal volume of ice-cold 20 percent trichloroacetic acid (TCA). The precipitates were redissolved in 2 ml of 0.2$M$ tris ($p$H 10) and were incubated at 30°C for 15 min. The precipitation was repeated with 4 ml of 15 percent ice-cold TCA, and the precipitate was washed twice with 10

Table 1. Intranuclear distribution of RNA methylase activity (per cell nucleus). Each nucleus-free subnuclear fraction was incubated with the standard mixture individually in the presence of 0.75 mg *E. coli* (methyl-deficient) tRNA for 15 minutes at 30°C.

| Subnuclear fraction | Fraction of rho (*9*) | Protein per nucleus ($\mu\mu$g) | Methyl-$C^{14}$ per $10^9$ nuclei ($\mu\mu$mole) |
|---|---|---|---|
| Nucleoli | 2-4 | 20.4 | 625 |
| DNA-rich nucleoplasm * | **6, 7** | 19.8 | 99 |
| RNA-rich nucleoplasm † | 8 | 5.2 | 0 |

* Chromatin. † Ribosomal fraction.

percent TCA, extracted once with 66 percent ethanol and dissolved in 0.4 ml of water to which a drop of $NH_4OH$ had been added. The samples were plated out and counted in a Nuclear-Chicago micromill window gas-flow counter or in a Packard scintillation counter.

For the physical characterization of the methylated RNA the product of the incubation was purified as follows. To the incubation mixture sodium lauryl sulfate (SLS) at *p*H 7.4 was added to make a 0.05-percent concentration and the solution was homogenized with an equal volume of phenol (88 percent) at 0°C. To the aqueous phase 2 volumes of ethanol were added in the presence of 2 percent sodium acetate (*p*H 5.0). The precipitate was dissolved in 0.05*M* acetate-tris buffer at *p*H 6.5 and was dialyzed for 4 hours against the same medium at 0°C. The deproteinization was repeated a second time (*7*). The RNA was centrifuged in a sucrose gradient (*8*) and in CsCl (*9*).

Table 2. Occurrence of RNA methylase and RNA methylase substrate in nuclei and subnuclear fractions. Incubation mixture was standard except that in the incubations indicated 1.5 mg *E. coli* (methyl-deficient) tRNA and 2 mg *E. coli* methylase preparation were added. Two milligrams of nuclear fractions were incubated at 30°C for 30 min.

| Additions | Methyl-$C^{14}$ incorporated ($\mu\mu$mole) |
|---|---|
| *Nuclei* | |
| None | 8 |
| *E. coli* methylase | 15 |
| *E. coli* (methyl deficient) tRNA | 37 |
| *Nucleoli* | |
| None | 20 |
| *E. coli* methylase | 31 |
| *E. coli* (methyl deficient) tRNA | 63 |
| *DNA-rich nucleoplasm* | |
| None | 0 |
| *E. coli* methylase | 0 |
| *E. coli* (methyl deficient) tRNA | 13 |

The RNA methylases that methylate tRNA in the macromolecular state in vitro were first found in *Escherichia coli* (*1*). To show the presence of a similar enzyme in peas 1 mg of cell-free extract was incubated with or without methyl-deficient RNA and the methyl donor, *S*-adenosylmethionine-methyl-$C^{14}$. Physical fractionation of this homogenate showed that the nucleus is the principal site of the RNA methylase activity. For example, in measurements of methyl incorporation into endogenous RNA substrate the nucleus would yield a specific activity four times that of the total cellular homogenate. A similar ratio of activities was observed when an exogenous substrate, methyl deficient tRNA from *E. coli*, was added. The incubation mixtures in this and subsequent experiments contained 0.01*M* tris, .01*M* $MgCl_2$, 0.005*M* mercaptoethanol, 1 $\mu$c *S*-adenosylmethionine-methyl-$C^{14}$ (30 $\mu$c/$\mu$mole), and 1 mg of protein fraction, all in a final volume of 2 ml at *p*H 7.8. This was incubated at 30°C for 20 min.

When nuclei were fractionated and the nucleus-free subnuclear fractions were incubated in vitro in the presence of tRNA from methionine-starved *E. coli* (methyl-deficient tRNA) (*10*), virtually all of the methylase activity was associated with the nucleolar fractions (Table 1). Thus the DNA-rich nucleoplasm (chromatin) has only slight activity and the RNA-rich nucleoplasm (nuclear ribosomal fraction) is completely inactive. Almost all the activity of intact nuclei can be accounted for in the nucleolar preparation from the same number of nuclei. In the nucleolar fractions the rate of incorporation of methyl-$C^{14}$ is maximal for 15 to 20 min at 30°C and then levels off. When ribonuclease (50$\mu$g/ml) was included in the incubation mixture the incorporation was reduced by at least 90 percent, though a small fraction of the incorporation remained resistant to ribonuclease.

Furthermore, RNA is implicated as the recipient of the labeling in vitro since the product does not dissolve in phenol and is rendered unprecipitable by TCA after exposure to 0.3*N* KOH at 37°C for 24 hours. Finally, the identity of the methylated RNA was unequivocally established by the isolation of radioactive methylribonucleotides from the hydrolyzed product (Table 3).

Table 3. Incorporation of methyl groups into bases of RNA incubated with nuclear enzyme preparation and *S*-adenosylmethionine labeled with methyl-$C^{14}$. The standard incubation mixture was used. The RNA was extracted with phenol, hydrolyzed in alkali, treated with prostatic phosphatase (*16*) and chromatographed as nucleosides. A combined method of electrophoresis followed by chromatography in a different direction devised by Ingram and Pierce (*17*) was also used.

| Nuclei | $C^{14}H_3$ incorporated in $\mu\mu$mole per mg RNA | | |
|---|---|---|---|
| | Thymine | 1-Methylguanine | 6-Methyladenine |
| Plus *E. coli* methyl-deficient tRNA | 7 | 14 | 5 |
| Plus *E. coli* normal tRNA | 1 | 5 | 9 |
| Plus pea RNA insoluble in MNaCl | 2 | 3 | 1 |

In early experiments it became obvious that methyl-$C^{14}$ incorporation is not completely dependent on the presence of methyl deficient tRNA; apparently some endogenous RNA in the nuclear extracts can serve as a substrate for the RNA methylase in the extract (Table 2). A similar observation was made independently by Comb (*11*) on the presence of an RNA fraction available for methylation in the nuclei of a water mold. Apparently the synthesis of the primary nucleotide chain of tRNA and its methylation are sequential processes with a sufficient time lag to yield unmethylated products in disrupted nuclei. The localization of the unmethylated tRNA within the nucleolus (Table 2) is in accord with the views of Sirlin *et al.* (*12*) and of Swift (*13*) that the nucleolus is the site of tRNA synthesis.

The addition of an RNA-free RNA methylase from a heterologous source, that is, from *E. coli*, enhanced even further the incorporation of methyl groups by the endogenous RNA. Such species specificity of RNA methylases has been independently observed in other laboratories as well (*3*).

To determine whether methylation is restricted to the fraction of RNA capable of amino acid acceptance, nucleolar homogenates were simultaneously exposed to $C^{14}$ adenosylmethionine and $H^3$ leucine. The RNA was isolated and fractionated by sucrose gradient centrifugation (Fig. 1). The incorporation of methyl $C^{14}$ was confined to fractions of RNA smaller than 15*S* and there is good coincidence of total ultraviolet absorption and methyl-$C^{14}$ content in

this region. However, the $H^3$-leucine RNA is considerably displaced towards slower sedimenting fractions. Thus only about half of the methyl-$C^{14}$-content is recovered in the known functional tRNA fraction as identified by its $H^3$-leucine acceptance and by its sedimentation constant ($S_{20,w} = 4.5$). To exclude the possibility that the leucyl-RNA might not be representative of RNA in general, another double-labeling experiment was carried out on the cytoplasmic fraction (20,000*g* supernatant) with a complete amino acid mixture labeled with $C^{14}$ and leucine-$H^3$. In this case (Fig. 2) both $C^{14}$- and $H^3$-activity coincide with the ultraviolet absorption pattern. Leucyl RNA therefore is a characteristic marker for the tRNA in our sedimentation studies.

Although part of the methyl-$C^{14}$ incorporation is into tRNA, a significant amount of the incorporated radioactivity sediments faster than 4.5*S*. Thus analytical centrifugation of a sample, which contained $C^{14}$ activity but which was nearly free of $H^3$-leucyl RNA (fraction 14 of Fig. 1) yielded a sedimentation constant of $S_{20,w} = 10.3$ We conclude that RNA species other than tRNA are present in pea nucleoli and these RNA's also serve as substrates for the RNA methylase.

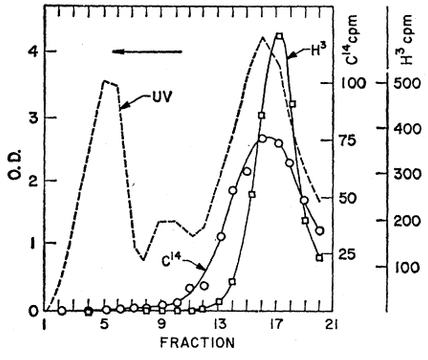


Fig. 1. Sucrose density gradient centrifugation of nucleolar RNA labeled in vitro. Circles represent the $C^{14}$-methylation of RNA and the squares the formation of $H^3$-leucyl-RNA. Nucleoli (25 mg of protein) were incubated with 0.01*M* tris, 0.005*M* mercaptoethanol, 0.01*M* $MgCl_2$, 20 $\mu$c *S*-adenosylmethionine-methyl-$C^{14}$ (30 $\mu$c/$\mu$mole), 1 mc DL-leucine-$H^3$ (5400 $\mu$c/$\mu$mole), 0.001*M* adenosine triphosphate, 0.001*M* cytidine triphosphate, 0.01*M* phosphocreatine, 2 mg creatine kinase, final volume 20 ml, *p*H 7.4, incubation at 28°C for 10 minutes. RNA placed on a linear sucrose gradient (20–25 percent sucrose, containing 0.01*M* tris *p*H 6.8) was centrifuged in a Spinco rotor No. 25 at 23,000 rev/min for 14 hours in the cold. One-ml fractions were collected, the RNA was precipitated with acetate-alcohol, and washed twice with 1 ml of 10 percent TCA, and twice with ethanol. The RNA was dissolved in 0.6 ml water, and portions were used for the determination of ultraviolet absorption (260 m$\mu$) and radioactivity in a Packard Scintillation counter.

It had been previously shown that isolated nuclei carry out the polymerization of ribonucleotides to yield tRNA (*4*). It is consistent therefore that the nucleoli also contain an enzyme capable of methylating tRNA. The methylase activity is nearly exclusively confined to the nucleolus in agreement with the reports of Sirlin *et al.* (*14*) who used autoradiographic methods for the intracellular localization of the incorporated methyl-$C^{14}$.

Fractionation of the RNA of the nucleolus indicates a large pool of tRNA, which was identified by its ability to incorporate amino acid and by analytical ultracentrifugation. About half of the incorporated methyl-$C^{14}$ is recovered in the tRNA species, the remainder sediments more rapidly. Nonlabeled 28*S* and 17*S* RNA are also found.

The nature of the faster moving $C^{14}$-methylated RNA (8 to 14*S*) is obscure. It cannot be derived from the 28*S* and 17*S* (ribosomal) RNA since these particles show no methyl acceptor activity in vitro. It might be derived, however, from other high-molecular and more labile RNA through degradation, since it does not appear in sedimenation patterns of RNA preparations extracted immediately from fresh nucleoli. The elucidation of the function of this larger methyl-containing moiety—whether it is a precursor RNA or endowed with some function of its own—awaits further study.

The relative amounts of specific methylated bases synthesized on different RNA substrates by the enzymes from pea nuclei is unexpected. Thus almost twice as much N-6-methyl adenine is produced in tRNA derived from normal *E. coli* as in methyl-deficient tRNA. Such unexpected results have been observed earlier in the interaction of tRNA's and RNA methylase from a heterologous source. For example, RNA methylase of liver introduces as many methyl groups into normal tRNA from *E. coli* as into methyl-deficient tRNA. The probable explanation for these anomalous findings must reside in the unusual nature of the interaction between the methylating enzyme system and its substrate tRNA: they are apparently both endowed with a directive specificity. Thus tRNA, while fully sat-

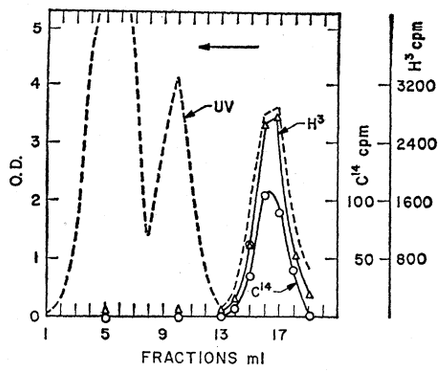


Fig. 2. Sucrose gradient centrifugation of cytoplasmic RNA labeled in vitro. The triangles represent $H^3$-leucyl-RNA, the circles, $C^{14}$-aminoacyl-RNA. Cytoplasm (20 mg of protein) from a 20,000g supernatant was incubated as described in Fig. 1, but 50 $\mu$c of uniformly labeled algal protein hydrolysate (obtained from Isotope Specialties) and 1 mc DL-leucine-$H^3$ were used in a final volume of 20 ml. Fractionation on a sucrose gradient and analysis of the 1-ml fractions as in Fig. 1.

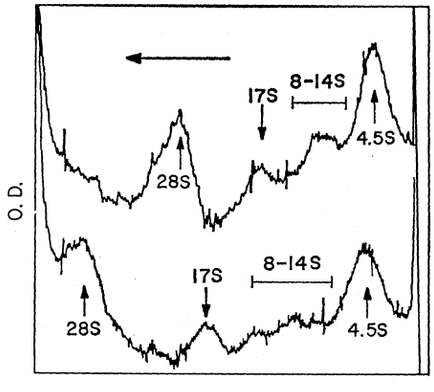


Fig. 3. Band centrifugation of nucleolar RNA. Tracings of ultraviolet patterns obtained by centrifugation at 56,100 rev/min (8°C) of 0.015 ml of RNA (120 $\mu$g/ml) overlayered on 0.72 ml of 2*M* CsCl (*13*). The time lapse between the two pictures is 8 min. The sedimentation constants for the RNA peaks were determined in a parallel centrifugation, by the standard boundary centrifugation techniques; the sedimentation values thus determined were applied to the ultraviolet tracings.

urated with respect to its homologous enzyme system, will offer new sites for methylation to a heterologous RNA methylase. Thus a normally methylated tRNA, because of the structural configuration conferred on it by its own complement of methyl groups, may possibly expose more specific sites for methylation to a heterologous enzyme than would a tRNA whose structure is different because of the deficiency of prior methylation. The interaction of purified specific methylating enzymes with homogeneous specific tRNA's should prove to be a rewarding model for the study of the enzymatic alteration of macromolecular structure (*15*).

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## Ultraviolet Absorption in Epidermoid Cancer Cells

Abstract. *The "excessive functional activity" of some cancer cells first found by Caspersson has been observed in fixed, stained smears of cervical epidermoid carcinomas from four patients. Preliminary results suggest that there may be a characteristic difference between the absorption profiles of some epidermoid cancer cells and other cells found in cytological smears. It is our belief that with an appropriate electronic scanning system such cells can be detected by measurements of their absorptions at two different wavelengths. However, the effect on the absorptions of cells with abnormalities other than cancer, and whether every epidermoid carcinoma will contain such cells, must yet be determined.*

The techniques developed by Papanicolaou (*1*) for preparing cytological smears of body surfaces or fluids are efficient for demonstrating the presence of cancer cells and are now widely used as a means of diagnosing certain cancers in an early and curable stage. It is known that use of the Papanicolaou techniques in a mass screening of the population may lead to early diagnosis of cancer of the uterine cervix with resulting reduction of morbidity and mortality (*2*). The wider application of the techniques is limited, however, by the requirement for highly trained technicians to engage in the time-consuming and tedious search for abnormal cells in each preparation. This limitation could be circumvented if a device were available for screening out a major portion of the clearly negative cases so that the cytotechnologist need only be concerned with a small fraction of the total cases.

Attempts have been made to automate the screening process based on measuring the size and visible light absorption of the nuclei of specially stained and isolated cells (*3*) and the fluorescence of cells stained with a fluorescent dyestuff (*4*). These studies have not yet resulted in a usable device. Furthermore, they require stains other than the standard Papanicolaou stain, which may be disadvantageous because they make unavailable the wealth of experience already accumulated with this stain. Thus, suspicious or problem cases must also be processed by the Papanicolaou technique for review and final evaluation. Also, many of these other preparations are not permanent and are awkward to handle on a community-wide basis.

This report is based on the work of Caspersson (*5*) and Mellors (*6*). From their work and that of others, it is now established that (i) both RNA and DNA have an absorption maximum near 2600 Å and certain proteins have an absorption maximum at 2800 Å; (ii) the average amount of DNA in the nucleus of a cancer cell, as well as the volume of the nucleus, is greater than these parameters in a normal cell; and (iii) the increased rate of protein synthesis in the cancer cell results in a greater concentration of cytoplasmic RNA than in a normal cell.

Our work with both stained and unstained material has shown that (i) absorptions with high optical contrast due to the nucleic acids and proteins of the cells were obtained even after alcohol-ether fixation and Papanicolaou staining of smears; (ii) the variability due to staining was avoided by using ultraviolet wavelengths; and (iii) particles of foreign matter, which may absorb visible light and thus become confused with cancer nuclei, did not show the specific absorptions of nucleic acids and proteins. All these facts constituted reasons for investigating the morphology of cells in Papanicolaou-stained smears at ultraviolet wavelengths. Cancer cells, as well as the various types of normal and abnormal benign cells present in a smear, were identified by well-established criteria with visible light. These same cells were then studied with ultraviolet light and relationships were established between their ultraviolet and visible light morphology. The absorption profiles of cells obtained at different wavelengths were compared to determine whether ultraviolet wavelengths offered any advantages that could be adapted to automated techniques.

Cervical and vaginal smears were fixed and stained according to the Papanicolaou technique (*1*). Quartz slides and cover slips were used. The obser-