INTRANUCLEAR ACCUMULATION OF RNA RESEMBLING THE SMALLER RIBOSOMAL RNA COMPONENT

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SUMMARY
A study was made of the nuclear RNA in HeLa cells with particular reference to the rapidly labelled fractions. It was found that if cells were incubated at a high density, that is, under 'step-down' conditions, there was a rapid accumulation of RNA in the nucleus. The fraction of the nuclear RNA which includes rapidly labelled RNA and which binds tightly to columns of methylated albumin on kieselguhr increased in amount and reached levels which permitted enough of the material to be isolated for direct measurement of its base composition. This was found to be very similar to that of 16s ribosomal RNA.

When cells growing logarithmically were treated with low concentrations of actinomycin D and then incubated in the presence of [3H]uridine it was found that an RNA fraction which bound tightly to methylated albumin on kieselguhr again accumulated in the nucleus. This fraction resembled that which accumulated under 'step-down' conditions. It contained over 85% of the total radioactivity in the nuclear RNA and again had a base composition very similar to 16s ribosomal RNA. Since nucleolar RNA synthesis was inhibited by the concentrations of actinomycin D used, it appeared that an RNA closely resembling 16s ribosomal RNA was synthesized outside the nucleolus.

Sedimentation patterns on sucrose density gradients and thermal denaturation profiles lent support to the view that the RNA which binds tightly to columns of methylated albumin on kieselguhr probably represents 'nascent' 16s ribosomal RNA.

INTRODUCTION
Certain rapidly labelled fractions of RNA (rl-RNA) isolated from animal cells have been found to bind very strongly to columns of methylated albumin on kieselguhr (MAK) (Ellem & Sheridan, 1964; Ellem, 1966; Bramwell & Harris, 1967; Lingrel, 1967; Lichtenstein, Piker & Shapot, 1967; Roberts & D'Ari, 1968). These fractions can be removed only by drastic means. Other workers have found labelled fractions which are eluted after the main ribosomal RNA peaks (r-RNA), but still within the normal salt gradient (Yoshikawa, Fukada & Kawade, 1964; Kimura, Tomando & Sibatini, 1965; Kubinski & Koch, 1966). In plants, also, short periods of labelling result in the bulk of the radioactivity eluting after the main ribosomal RNA peaks (Chroboczek & Cherry, 1965; Inglc, Key & Holm, 1965; Jachymczyk & Cherry, 1968).

These rapidly labelled fractions are thought to contain precursors of r-RNA and

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messenger RNA (m-RNA). Base compositions determined by $[^{32}P]$-labelling have shown that the fractions become progressively more 'DNA-like' the more difficult they are to remove from the column (Yoshikawa et al. 1964; Ellem & Sheridan, 1964; Ingle et al. 1965; Ellem, 1966; Kubinski & Koch, 1966; Lichtenstein et al. 1967; Roberts & D’Ari, 1968). In addition, the RNA isolated from plants and fractionated in this manner has been shown to have a high template activity in cell-free amino acid incorporating systems, compared with the bulk of the RNA (Jachymczyk & Cherry, 1968).

RNA possessing the properties of rapid labelling, 'DNA-like' base composition and high template activity (suggested criteria for identifying m-RNA) has been isolated using other methods, which usually involve hot phenol extractions over a temperature range of 45-95 °C (Georgiev & Mantieva, 1962; Brawerman, 1963; Brawerman, Gold & Eisenstadt, 1963; Scherrer, Latham & Darnell, 1963; Krsmнович, Kanazir & Errera, 1965; Kimura et al. 1965; Dukes, Sekeris & Schmid, 1966; Hadjiolov, 1966; Soeiro, Birnboim & Darnell, 1966; Arion, Mantieva & Georgiev, 1967; Mizuno, Collin & Hof, 1967; Morrison & McCluer, 1967). The general experience seems to have been that those RNA fractions in which the three properties mentioned above are most pronounced are also the most difficult fractions to extract from animal cells. It is, therefore, very likely that such RNA fractions can be equated with those which bind strongly to MAK columns.

Under 'step-down' conditions, for example, amino-acid starvation, in Escherichia coli, there is a preferential increase in the amount of m-RNA, as judged by increased template activity and increased ability to hybridize with DNA (Hayashi & Spiegelman, 1961; Mandel & Borek, 1962; Willson & Gros, 1964; Bellamy, 1966; Friesen, 1968; Sarkar & Moldave, 1968; Venetianer, Berberich & Goldberger, 1968). Similarly, animal cells in a stationary phase of growth have been shown to accumulate a ‘DNA-like’ RNA fraction (Kubinski & Koch, 1966). Treatment of both bacteria and animal cells with low levels of actinomycin D leads to an accumulation of an RNA fraction with the properties ascribed to m-RNA and a cessation of r-RNA synthesis (Georgiev, Samarina, Lerman & Smirnov, 1963; Samarina, 1964; Harel et al. 1964; Perry, Srinivasan & Kelley, 1964; Trakatellis, Axelrod & Montjar, 1964; Kubinski & Koch, 1966; Samarina, Lukandidin, Molnar & Georgiev, 1968). The fact that an RNA fraction with the properties ascribed to m-RNA can be preferentially synthesized under certain conditions, coupled with the fact that the bulk of the rapidly-labelled RNA in HeLa cell nuclei binds to MAK columns very strongly, suggested a method by which the rl-RNA could be isolated and its base composition measured directly without recourse to the $[^{32}P]$-pulse procedure, which has been shown by several workers to be of doubtful value (Spencer, 1962; Harris et al. 1963; Roberts, 1965; Marbaix, Burny, Huez & Chantrenne, 1966; Hadjiolov, Venkov, Dolapchiev & Genchev, 1967).
Intracellular accumulation of RNA

MATERIALS AND METHODS

Cultivation of cells

HeLa cells were grown in suspension culture as described by Harris & Watts (1962).

Step-down conditions

The cells were spun out of the suspension medium at a low speed and resuspended in a
smaller volume of a modified Eagle's medium (Eagle et al. 1956). The effect of increasing the
cell density by a factor of 5 to 10 produced the necessary 'step-down' conditions. Cells
were then labelled and harvested as required.

Isolation of cell nuclei

The nuclei were isolated as described previously (Bramwell & Harris, 1967), using the

Extraction of nuclear RNA

HeLa cell nuclei obtained from 1 l. of suspension culture medium were washed twice with
buffered saline (0.15 M NaCl + 0.01 M tris-HCl, pH 7.4) and suspended in distilled water
(10 ml). One ml of 20 % (w/v) sodium dodecyl sulphate was added and the suspension stirred.
The nuclei lysed rapidly to form a viscous solution. An equal volume of aqueous phenol was
added and the mixture rotated at 5 °C for 30 min. After centrifugation, the supernatant was
precipitated with 2 volumes of cold ethanol containing a few drops of a 2 M NaCl solution.
The pellet was washed twice with ethanol and drained. Five ml of 10⁻⁴ M MgCl₂ solution were
added, together with 125 μg deoxyribonuclease. The pellet dissolved rapidly leaving an
opalescent solution. This was made 2 % with respect to sodium dodecyl sulphate and extracted
twice with aqueous phenol. The final supernatant was mixed with 2 volumes of ethanol, spun
down and the pellet extracted with 2 M NaCl at 0 °C to remove glycogen and DNA fragments.

Extraction of ribosomal RNA

This was done as described previously, by extraction of the ‘Tween’ supernatant with
aqueous phenol and 2 % (w/v) sodium dodecyl sulphate (Bramwell & Harris, 1967). For
comparative purposes, 16S RNA was also extracted from the small subunit of the ribosome.
The Tween supernatant was made 0.4 M with respect to NaCl and samples put on to linear
sucrose gradients (30-5 % w/v) containing 0.25 M NaCl + 0.01 M tris-HCl, pH 7.4. After
centrifugation for 15 h at 22,500 rev/min in the SW 25 rotor of a Spinco L-50 centrifuge, 15-
drop fractions were collected on a fraction collector. The 30S peak fractions were pooled, made
2 % (w/v) with respect to sodium dodecyl sulphate and shaken with an equal quantity of phenol
saturated with water. After centrifugation, the supernatant was mixed with cold ethanol to
precipitate the RNA. The pellet was washed twice with ethanol, drained and either used
immediately or stored at −18 °C.

Preparation of methylated albumin on kieselguhr

The MAK was prepared as described by Mandell & Hershey (1960), except that Hyflo
Super-Cel (Johns-Manville, New York, N.Y., U.S.A.) was used in place of crude kieselguhr.

Extraction of MAK-bound RNA

A single-layer MAK column was prepared and washed with 0.15 M NaCl + 0.01 M tris-HCl,
pH 7.4. A solution of nuclear RNA (n-RNA) in the same buffer was passed through the
column, which was further washed with the 0.15 M NaCl buffer. This procedure resulted in
complete adsorption of the RNA to the MAK. A large proportion of the n-RNA could be
eluted by washing the column exhaustively with 2 M NaCl + 0.01 M tris-HCl, pH 7.4. This fraction is designated 2 M NaCl-eluted n-RNA. The fraction of the RNA which remains tightly bound to the MAK after successive washings with 2 M NaCl is designated MAK-bound RNA. Conditions of the elution are rather critical: complete removal of an RNA sample, known to contain a minimum of MAK-bound RNA, could only be achieved at a temperature of 35-40 °C with a step-wise increase of the salt content from 0.15 M to 2 M NaCl.

For recovery of the MAK-bound RNA two methods were employed, depending on the purpose of the experiment. For base-composition determinations, the MAK containing the bound RNA was washed once with 0.15 M NaCl to reduce the salt concentration. The MAK pellet was then resuspended in 0.3 M KOH of sufficient volume to yield a smooth suspension. At this point it should be noted that the Hyflo Super-Cel has an actual volume which is only about 1/10 of its apparent volume, so that 2 M KOH was added to allow for the dilution effect of the liquid contained in the MAK pellet. After digestion for 18 h at 37 °C the suspension was centrifuged and the supernatant removed. The pellet was washed once with a small volume of water, which was pooled with the supernatant. Perchloric acid was added to a concentration of 10% and the solution left for 30 min in an ice-bath. Insoluble protein was removed by centrifugation and the solution brought to pH 6-7 with 2 M KOH. Before chromatography, the volume was reduced by evaporation.

For recovery of intact MAK-bound RNA, the MAK pellet was shaken with a mixture of 2% (w/v) sodium dodecyl sulphate solution and phenol saturated with water for 30 min. After centrifugation, the supernatant fraction was diluted with 2 volumes of cold ethanol and left overnight at -18 °C. The precipitate which formed was collected by centrifugation and dissolved in a small volume of 2% sodium dodecyl sulphate solution. Re-extraction with aqueous phenol was necessary since there was still some protein present. The clear supernatant was again treated with 2 volumes of ethanol and left overnight at -18 °C. The precipitate was washed twice with ethanol, drained and stored at -18 °C.

**Measurement of MAK-bound RNA and 2 M NaCl-eluted RNA**

The precipitate of n-RNA was dissolved in 2 ml of 0.15 M NaCl + 0.01 M tris-HCl, pH 7.4. Samples were taken for measurement of total radioactivity and optical density. To the remaining solution 1 ml of a suspension of MAK was added. This was repeated until the n-RNA had been completely adsorbed to the MAK as judged by the loss of radioactivity and optical density from the supernatant liquid. Any trace of unadsorbed material was later subtracted from the measurement of the total amount in the sample. The supernatant liquid was decanted off after centrifugation and 5 ml of 2 M NaCl solution added. The mixture was stirred for 5 min in a water-bath at 37 °C and again centrifuged. The supernatant now contained the n-RNA extractable with 2 M NaCl (2 M NaCl-eluted RNA) and the measurement of optical density and volume allow the amount of 2 M NaCl-eluted RNA to be calculated. (As noted earlier, the volume of the MAK must be taken into account, since the actual volume it occupies is only 10% of its apparent volume.) The amount of RNA still bound to the MAK (MAK-bound RNA) is, therefore, the difference between the total and the 2 M NaCl-eluted RNA.

**Base analysis**

This was performed according to the method of Katz & Comb (1963). Success in this method is dependent on complete removal of all protein contamination, which otherwise elutes with uridylic acid. Particular attention was, therefore, paid to the purity of each sample analysed, and only those with 220-260 nm ratios less than 1 were used.

**Sucrose gradients**

Linear sucrose gradients from 20 to 2% (w/v) were prepared in appropriate buffers. Samples of 0.2-0.3 ml of solution were applied to the top of the gradients. Centrifugation was carried out in the SW 39 or 50 rotors of a Spinco Model L-50 ultracentrifuge at 5 °C for an appropriate time. Fractions were collected on an LKB UltraRac fraction collector and were diluted for measurement of extinction at 260 nm.
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Measurement of radioactivity

The Nuclear Chicago liquid-scintillation system 725 (Nuclear Chicago Corp., Des Plaines, Ill., U.S.A.) was used. Samples 0.1–0.5 ml in volume were added to counting vials containing 15 ml of scintillation liquid (600 ml of ethanol + 1 l of toluene containing 0.1 g of 1,4-bis-(5-phenylloxazol-2-yl) benzene and 0.6 g of 2,5-diphenylloxazole).

Spectrophotometry

Measurements of the extinction of solutions were made in a Zeiss PMQII spectrophotometer. Heating profiles were made with a water-heated cell holder, the temperature being measured by means of a thermistor probe fitted into the blank cell (Grant Instrument Co., Cambridge, England).

Reagents

EDTA (reagent grade), phenol (analytical reagent grade) and sucrose (analytical reagent grade) were obtained from British Drug Houses Ltd., Poole, Dorset. The phenol was redistilled before use. Sodium dodecyl sulphate was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks, and TRIZMA buffer from Sigma Chemical Co. Ltd., London, S.W.6. Bovine plasma albumin was obtained from the Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex. Deoxyribonuclease I was an electrophoretically purified enzyme from the Worthington Biochemical Co., Freehold, N.J., U.S.A. This was further purified by extraction with 200 μg of bentonite (B.D.H.) which was added to a solution of 5 mg of the enzyme in 5 ml of distilled water. The purified enzyme contained no ribonuclease activity.

[5-3H]Cytidine, specific activity 250 Ci/mM, [5-3H]uridine, specific activity 265 Ci/mM and [8-14C]adenine, specific activity 277 mCi/mM were obtained from the Radiochemical Centre, Amersham, Bucks.

Autoradiography

 Autoradiographs were made of cells labelled in suspension culture. A small sample of cells was taken from the culture vessel, washed twice with saline and flattened on to glass slides in a cytocentrifuge (Shandon-Elliot, S.C.A.-0020, Shandon Scientific Co. Ltd., London, N.W.10). After fixation in methanol for 10 min the slides were washed with distilled water and extracted with 5 % trichloroacetic acid at 5 °C for 45 min. Stripping film (Kodak AR 10) was applied in the usual way and the slides were exposed for several days.

Observations

Effect of 'step-down' on the amount of nuclear RNA

HeLa cells growing in suspension culture (1.5 l. at a cell density of 1 × 10⁶/ml) were centrifuged at low speed and resuspended in 200 or 500 ml of the modified Eagle's medium containing 2 μCi/ml of [5-3H]uridine. Samples (40 or 100 ml) were taken at the times indicated. n-RNA was extracted and the amount measured from the extinction at 260 nm. Figure 1 shows the effect of transferring the cells to the poorer environment ('step-down'). The amount of n-RNA has increased 3–4 fold in 80 min at the higher cell density (7.5 × 10⁶ cells/ml) and has more than doubled at the lower density (3 × 10⁶ cells/ml). Incubation at the higher density thus results in a greater increase in n-RNA content.

This situation was analysed further by making use of the property of MAK to bind certain fractions of RNA very tightly. Figure 2 shows the result of such an analysis. The n-RNA has been separated into MAK-bound RNA and 2 M NaCl-
eluted RNA and the amount of RNA present in each fraction determined. Both fractions increase in amount with time and then decrease. The amount of MAK-bound RNA increases most: at zero time, the MAK-bound RNA accounts for less than 1% of the total r-RNA, but as incubation proceeds this proportion increases almost linearly to reach over 50% (Fig. 3). This must surely reflect a profound change in

Fig. 1. Accumulation of nuclear RNA under ‘step-down’ conditions. The cells were grown in suspension medium to a density of $1 \times 10^6$ cells/ml, centrifuged at low speed and resuspended in modified Eagle’s medium at a density of $3 \times 10^6$ cells/ml (●), or $7.5 \times 10^6$ cells/ml (○).

Fig. 2. Accumulation of nuclear RNA fractions under ‘step-down’ conditions. The cells were grown in suspension medium to a density of $1 \times 10^6$ cells/ml, centrifuged at low speed and resuspended at a density of $7.5 \times 10^6$ cells/ml in modified Eagle’s medium. ○, 2 M NaCl-eluted RNA; ●, MAK-bound RNA.

Fig. 3. Accumulation of MAK-bound RNA under ‘step-down’ conditions. The cells were grown in suspension medium to a density of $1 \times 10^6$ cells/ml, centrifuged at low speed and resuspended at a density of $7.5 \times 10^6$ cells/ml in modified Eagle’s medium.
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the metabolism of the cell and suggests that a block has occurred which results in the accumulation of MAK-bound RNA.

It was previously found that, after a short period of radioactive labelling under conditions which were essentially 'step-down', the bulk of the labelled RNA in the HeLa cell nucleus was bound to MAK very tightly (Ellem & Sheridan, 1964; Ellem, 1966; Bramwell & Harris, 1967). Figure 4 shows the amount of radioactivity incorporated into the MAK-bound and 2 M NaCl-eluted RNA fractions in the present experiment. There is again an increase in both fractions followed by a decrease, which is more marked in the case of the MAK-bound RNA. Figure 5 shows the increase in specific activity of the RNA fractions over the course of the experiment. The MAK-bound RNA has the higher specific activity, but, in both fractions, the specific activity continues to rise even after the total amount of n-RNA has begun to decrease. This suggests that there is an increased rate of degradation of n-RNA, rather than a reduction in the rate of its synthesis after 80 min.

Fig. 4. Incorporation of radioactivity into nuclear RNA fractions under 'step-down' conditions. The cells were grown in suspension medium to a density of 1 x 10^6 cells/ml, centrifuged at low speed and resuspended at a density of 7.5 x 10^6 cells/ml in modified Eagle's medium containing 2 μCi/ml of [5-3H]uridine. ●, MAK-bound RNA; ○, 2 M NaCl-eluted RNA.

Fig. 5. Specific activity of nuclear RNA fractions accumulated under 'step-down' conditions. The cells were grown in suspension medium to a density of 1 x 10^6 cells/ml, centrifuged at low speed and resuspended at a density of 7.5 x 10^6 cells/ml in modified Eagle's medium containing 2 μCi/ml of [5-3H]uridine. ●, MAK-bound RNA; ○, 2 M NaCl-eluted RNA.

MAK-bound RNA in cells growing logarithmically

The zero time measurements of the amount of MAK-bound RNA in cells in 'step-down' indicated that this fraction contained less than 1% of the total optical density, but the amount of radioactivity incorporated was not enough to permit meaningful measurement. Clarification of this point was achieved by the following experiment.
To 1 l. of HeLa cells growing logarithmically in Earle's medium 20 μCi of [8-14C]-adenine were added. Samples (200 ml) were taken at the times indicated in Fig. 6. The n-RNA was extracted as before and separated into the 2 m NaCl-eluted and MAK-bound fractions. The results show that over the period of the experiment about 80% of the radioactivity was eluted with 2 m NaCl whereas only about 20% was bound to the MAK. This occurred even at 30 min which is, in terms of the generation time of the cell, almost equivalent to a pulse label. By comparison, more than 60% of the radioactivity was bound to MAK in 30 min in the 'step-down' situation. Under conditions previously employed (Bramwell & Harris, 1967), in which cells were labelled at a much higher density than in the present experiments (2 x 10⁷ as compared with 7.5 x 10⁶ cells/ml), over 90% of the radioactivity was MAK-bound.

These results strongly suggest that the MAK-bound RNA represents an initial transitory stage in RNA maturation, and that any interference with the cell results in a slowing down of the maturation process and an accumulation of the primary products of the RNA synthetic system. Since RNA cannot be extracted from cells without their being centrifuged and washed, it is possible that the 20% of the radioactive n-RNA which is found to be MAK-bound in growing cells may accumulate during the 'step-down' produced by the procedures necessary for extraction of the RNA. The amount of MAK-bound RNA in the growing cell may be much less. This view is supported by the fact that the proportion of the radioactive RNA which is MAK-bound remains constant over the whole period of the experiment. On kinetic grounds, the proportion of radioactivity in the precursor material should decrease relative to the product with
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time. On the other hand, it might be expected that the physical conformation of m-RNA molecules would not be influenced to any great extent by environment, as it is supposed that these molecules do not undergo the same secondary modifications and shape changes as do the r-RNA precursors. If the MAK-bound RNA in cells in logarithmic growth is m-RNA, it represents less then 1% of the total n-RNA and less than 0.2% of the total cell RNA.

Effect of actinomycin D on MAK-bound RNA

To 1 l. of HeLa cells growing logarithmically in Earle's medium, actinomycin D was added to a concentration of 0.05 μg/ml and, after 30 min, 20 μCi of [8-14C]adenine. This interval allows the actinomycin D to penetrate the cell (Perry, 1963). Samples were taken at 30-min intervals thereafter, n-RNA was extracted and fractionated into 2 M NaCl-eluted RNA and MAK-bound RNA as before. Figure 7 shows the result of such an experiment in terms of the amount of radioactivity incorporated into each fraction. It is apparent that there is a much greater proportion of radioactivity incorporated into the MAK-bound RNA than into the 2 M NaCl-eluted RNA, and the situation is very similar to that in the ‘step-down’ experiment shown in Fig. 4, except that there is no decrease in incorporation after the initial increase. The result is, thus, the reverse of that obtained in the absence of actinomycin D (Fig. 6).

In terms of optical density there is a very slight overall increase in RNA content in the nucleus, but nothing comparable to that obtained in ‘step-down’ conditions. The greatest increase is in the MAK-bound RNA, as before, which rises from less
than 1% to about 20% of the total n-RNA (Fig. 8). At this low level of actinomycin D, there is some incorporation of radioactivity into cytoplasmic ribosomal RNA (Fig. 9). The sedimentation pattern of the cytoplasmic RNA is rather polydisperse, but the most prominent peak is in the 16s component with a shoulder at 28s. Compared with the control (Fig. 10) the pattern of labelling is clearly aberrant and the level much reduced (9% of the total radioactivity is incorporated into high-molecular-weight RNA, compared with 60% in the control). This incorporation into ribosomal RNA is abolished at a concentration of actinomycin D of 0.1 µg/ml (Fig. 10).

The residual incorporation of radioactivity into the RNA in the nucleus in the presence of low levels of actinomycin D has been shown to be largely 'extranucleolar' and to have a 'polydisperse' pattern of sedimentation in a sucrose gradient (Perry, 1963). On these grounds it has been suggested that continued synthesis of m-RNA takes place in the presence of actinomycin D (Perry et al. 1964). In the present experiments the RNA which accumulated in the presence of actinomycin D was also found to have a 'polydisperse' sedimentation in conventional sucrose gradients (Fig. 11).

The accumulation of this RNA in the nucleus again permitted a direct measurement of its base composition. The measurements shown below indicate that a 'DNA-like' RNA does not accumulate in the presence of actinomycin D.
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The base composition of MAK-bound RNA

In 'step-down' conditions and in the presence of actinomycin D the amount of MAK-bound RNA increases sufficiently to permit direct measurement of its base composition. The results of the determinations are set out in Table 1. It can be seen

![Graph](image)

Table 1. The base composition of MAK-bound RNA

<table>
<thead>
<tr>
<th>MAK-bound RNA accumulated under 'step-down' conditions (min)</th>
<th>MAK-bound RNA accumulated in the presence of 0.1 μg/ml of actinomycin D</th>
<th>16s RNA isolated from the small subunit of the ribosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>18.6  21.1  18.0  19.9  20.9</td>
<td>23.6  24.1</td>
</tr>
<tr>
<td>UMP</td>
<td>24.7  24.0  25.8  29.8  29.0</td>
<td>29.8  29.0</td>
</tr>
<tr>
<td>GMP</td>
<td>30.9  31.1  29.8  26.7  26.0</td>
<td>26.7  26.0</td>
</tr>
<tr>
<td>CMP</td>
<td>25.8  23.8  26.4  3       3</td>
<td>3       3</td>
</tr>
<tr>
<td>No. of determinations</td>
<td></td>
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</tr>
</tbody>
</table>

that the MAK-bound RNA which accumulates under the two conditions has the same base composition, within the limits of experimental error. Comparison with the cytoplasmic components isolated from the ribosomal subunits reveal a striking similarity between the MAK-bound RNA and the 16s r-RNA. Two possibilities exist to
account for this: (1) MAK-bound RNA is a mixture of many species of high molecular weight RNA and its overall base composition is coincidentally similar to one of these species; (2) MAK-bound RNA consists of immature 16s RNA.

In considering the first alternative, if it is assumed that both 28s and 16s RNA are synthesized at similar rates, the amount of m-RNA or 'DNA-like' RNA needed to produce the required shift to the observed 16s r-RNA-like base composition would be about equal to the combined amount of 28 + 16s RNA synthesized. This is conceivable, but one would also have to assume that, in the presence of actinomycin D, all three species were synthesized in the 'extranucleolar' region of the nucleus, since nucleolar incorporation of radioactivity is greatly reduced in this situation (Perry, 1963; Table 2).

The second possibility, that MAK-bound RNA is, in fact, immature 16s RNA, seems the more likely for the following reasons. (1) The base composition of MAK-bound RNA is very similar to that of 16s ribosomal RNA over a range of times in the 'step-down' environment, and in the presence of actinomycin D. (2) MAK-bound
RNA and rl-RNA from HeLa cells can be sedimented in sucrose gradients as a single peak with a sedimentation coefficient of about 16s (Fig. 13B, Bramwell & Harris, 1967). (3) End-group analysis of rl-RNA from L cells indicates that its mean chain length is very similar to that of 16s ribosomal RNA (Tamaoki & Lane, 1967). (4) Comparative chain length analysis of rl-RNA and 16s ribosomal RNA from HeLa cells, by means of a specific exonuclease, has also shown them to be equal (Riley, 1969). That MAK-bound RNA can be indentified with rl-RNA is indicated by the similarity in sedimentation behaviour (Figs. 12A, 13A) of the two materials, and by the fact that rl-RNA under severe ‘step-down’ conditions binds very tightly to MAK.

The physical state of MAK-bound RNA

The physical state of MAK-bound RNA may be studied in several ways. From a consideration of the properties of polyuridylic acid and denatured DNA, which have little secondary structure and which bind tightly to MAK columns (Hershey, Goldberg, Burgi & Ingraham, 1963; Asano, 1965), it would be expected that MAK-bound RNA would have a high molecular weight and a low degree of secondary structure. The first point is established by its insolubility in 2 M NaCl and its high sedimentation rate in sucrose gradients. Fig. 12A shows the sedimentation pattern of MAK-bound RNA extracted from cells incubated under ‘step-down’ conditions for 60 min. The predominant feature is the peak of RNA with a sedimentation coefficient greater than the 28s RNA in the control gradient (Fig. 12B). This is followed by more polydisperse material. Mature 28s RNA, which normally is found in the nuclear RNA as a whole, does not bind to the MAK. The pattern of optical density in Fig. 12A resembles that of the radioactivity incorporated into MAK-bound RNA after pulse labelling of a sample of HeLa cells under ‘step-down’ conditions (Fig. 13A). In this case, the period in ‘step-down’ was short and there is therefore very little RNA present in terms of optical density. When the MAK-bound RNA was dissolved in 5 mM EDTA and centrifuged on a sucrose gradient containing 5 mM EDTA only, the pattern seen in Fig. 13B was obtained. A symmetrical peak of radioactivity is now seen which sediments at slightly less than 16s. The initial sedimentation pattern is not, therefore, due to the presence of larger amounts of some other kind of RNA which sediments rapidly and with which the radioactivity might co-sediment. The conversion of rapidly sedimenting ‘polydisperse’ RNA to a single component sedimenting in the 16s position has been previously described for rl-RNA (Bramwell & Harris, 1967). As pointed out by these authors, this effect is reversible and cannot therefore be due to the action of contaminant ribonucleases as suggested by Fujisawa & Muramatsu (1968).

Another way of showing differences in secondary structure between groups of polynucleotides is to compare the heating profiles obtained by measuring the variation of optical density produced by changes in temperature. Figure 14 shows such a profile obtained from the sample of MAK-bound RNA shown in Fig. 12A. The upper heating curve indicates a lower degree of hypochromic change than is normal for RNA (19.0% compared with 27.0%). However, on cooling, a lower absorbance than the initial value was obtained, suggesting an increase in hypochromicity. As
shown in Fig. 14, a second curve was obtained on reheating, which coincided with the first only at the highest temperatures. One explanation of this might be that the effect was due to contamination by a protein which became denatured on heating and precipitated out, thus lowering the overall absorbance. However, there was no visible evidence of protein precipitation, and the maximum difference between the two curves occurs below 50 °C, which is not normally a temperature at which proteins denature. Alternatively, the effect may be due to an annealing process which occurs over the range 20–50 °C. Thus, prior to extraction from the cells, the MAK-bound RNA may exist in an extended form and, after extraction, intermolecular and intramolecular hydrogen bonding may occur in a random fashion. This would prevent the formation of more specific and stable regions which are known to exist after the extensive modification of shape which takes place when an extended polynucleotide chain is converted into a ribosomal subunit.

Comparison of the heating profiles of MAK-bound RNA and 16s r-RNA (Fig. 15) shows that MAK-bound RNA has a lower T_m and a more gradual transition than 16s r-RNA. The central portion of the curve which shows a relatively sharp rise in 16s r-RNA (45–65 °C) is absent in the MAK-bound RNA, suggesting that the latter possesses fewer regions containing long stretches of hydrogen-bonded base pairs. Methylation is thought to play a part in stabilizing regions of RNA, and it is interesting to note that Tamaoki & Lane (1967) have shown that the degree of methy-
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Intranuclear accumulation of RNA from L cells is only 25% of that in 16 or 28s r-RNA. But all three species of RNA contain a common methylated sequence (Tamaoki & Lane, 1968). It has also been found that ‘extranucleolar’ RNA from rat liver cells (Muramatsu & Fujisawa, 1968) and from HeLa cells (Zimmerman & Holler, 1967) has a much lower degree of methylation than nucleolar RNA.

**Autoradiography**

In order to determine the location of the RNA which accumulates in the nucleus in ‘step-down’ and in the presence of actinomycin D, the pattern of labelling in the HeLa cell nucleus was examined autoradiographically. Samples of cells in various growth conditions were taken after labelling with [5-3H]cytidine for 15 min and autoradiographs prepared. Table 2 shows the proportion of grains over the nucleolus compared with those over the rest of the nucleus. In cells growing logarithmically it is apparent that the bulk of the rl-RNA is found in the nucleolus. However, after incubation in ‘step-down’ conditions for 75 min, followed by a 15-min pulse label, the bulk of the counts are in the ‘extranucleolar’ region. In cells which have not yet achieved logarithmic growth, the results are intermediate between the two. Prolonged incubation of HeLa cells in the presence of actinomycin D and [5-3H]cytidine results exclusively in ‘extranucleolar’ labelling.

The proportions of grains found outside the nucleolus under these three different situations closely parallels the proportions of radioactively labelled RNA which binds to MAK tightly after similar treatments. There appears, therefore, to be a direct corre-
lation between 'extranucleolar' RNA and MAK-bound RNA, indicating that both in 'step-down' and in the presence of actinomycin D, MAK-bound RNA accumulates outside the nucleolus.

Table 2. Distribution of labelled RNA within the nucleus, under different conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean no. of grains over nucleolus</th>
<th>Mean no. of grains over the 'extranucleolar' region</th>
<th>'extranucleolar' region (%)</th>
<th>MAK-bound RNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Log-growth'</td>
<td>7·5 ± 0·64</td>
<td>3·6 ± 0·6</td>
<td>35·6</td>
<td>19·5</td>
</tr>
<tr>
<td>15 min label</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>'Step-down'</td>
<td>3·5 ± 0·4</td>
<td>25·7 ± 1·76</td>
<td>88·0</td>
<td>90·0</td>
</tr>
<tr>
<td>15 min label</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actinomycin D 0·1 µg/ml for 3 h</td>
<td>0·25 ± 0·10</td>
<td>21·3 ± 1·06</td>
<td>98·8</td>
<td>82·7</td>
</tr>
</tbody>
</table>

DISCUSSION

The main conclusions to be drawn from this paper are: (1) there is a rapid accumulation of MAK-bound RNA in the nucleus of HeLa cells when they are transferred to a 'step-down' situation and when they are treated with low concentrations of actinomycin D; (2) the MAK-bound RNA has a base composition very similar to 16s ribosomal RNA; (3) this MAK-bound RNA is synthesized and accumulates outside the nucleolus. There are two possible interpretations of these conclusions: (a) that MAK-bound RNA is the precursor of 16s ribosomal RNA, and that it is made outside the nucleolus; (b) that MAK-bound RNA represents a mixture of precursor RNA molecules and that the overall base composition is coincidentally similar to 16s ribosomal RNA.

The evidence against the first view and in favour of the second is mainly derived from measurements of the apparent base composition of rl-RNA bound to MAK columns, made by means of the $^{[32P]}$-pulse method (Yoshikawa et al. 1964; Ellem & Sheridan, 1964; Ingle et al. 1965; Ellem, 1966; Kubinski & Koch, 1966; Lichtenstein et al. 1967; Roberts & D'Ari, 1968), and from similar measurements of the rl-RNA isolated from whole cells (Brawerman, 1963; Hadjivassiliou & Brawerman, 1965; Houssais & Attardi, 1966; Watson & Ralph, 1967). The results obtained by the use of this method indicate that rl-RNA has a base composition quite unlike the bulk of the RNA in the cell, but somewhat similar to that of the DNA. However, the $^{[32P]}$-labelling procedure has been shown to be of doubtful value for short pulses (Spencer, 1962; Harris et al. 1963; Roberts, 1965; Marbaix et al. 1966; Hadjiolov et al. 1967). Even so, in certain cases, the base composition of the rl-RNA determined in this way appears to be very similar to 16s r-RNA (Hadjiolov, Venkov & Dolapchiev, 1965; Venkov & Hadjoilov, 1967; Mizuno et al. 1967; Beiderbeck & Richter, 1968). It has also been observed that after longer periods of labelling with $^{[32P]}$ the MAK-bound RNA in HeLa cells has an apparent base composition very similar to 16s r-RNA (Ellem, 1966).
Intranuclear accumulation of RNA

That the rl-RNA can be equated with a fraction of the MAK-bound RNA is clear from the fact that, under 'step-down' conditions, the bulk of the rl-RNA binds tightly to columns of MAK. But the whole of the MAK-bound RNA behaves very similarly to rl-RNA on sucrose density-gradient centrifugation, showing a characteristically polydisperse sedimentation pattern, which is resolved to a single peak at about 16s on gradients containing 5mM EDTA (Bramwell & Harris, 1967). This fact, together with the evidence that rl-RNA from animal cells has a mean chain length equal to 16s r-RNA (Tamaoki & Lane, 1967; Riley, 1969) argue in favour of the view, suggested by its base composition, that MAK-bound RNA is a precursor to 16s r-RNA.

The fact that RNA with a base composition similar to 16s r-RNA may be synthesized outside the nucleolus, is clearly contrary to a current view that both 16 and 28s r-RNA are synthesized co-ordinately in the nucleolus (Ritossa & Spiegelman, 1965; Birnstiel, Wallace, Sirlin & Fischberg, 1966; Brown, 1966). These experiments are, however, based on the technique of RNA-DNA hybridization the validity of which has been recently questioned in the case of animal cells (Melli & Bishop, 1969). These authors have shown that only a very small proportion (5%) of the total DNA in the cell nucleus will readily form hybrids with RNA, even when the RNA used for hybridization is known to be complementary in sequence to at least 50% of the DNA. The small fraction of the DNA which does hybridize with RNA appears to contain a high proportion of repeating sequences, as judged by the speed of its renaturation after melting. It is this property which apparently confers on this DNA the ability to form hybrids with all types of high molecular weight RNA including 'DNA-like' RNA. Although ribosomal RNA cistrons may well be present in this small fraction of DNA, the possibility that they occur elsewhere on the genome cannot now be excluded. Indeed, it has been shown that in Chinese hamster cells RNA can be synthesized on a large number of different chromosomes at sites which are clearly identifiable as micronucleoli (Phillips & Phillips, 1969). Another objection to co-ordinate synthesis of 16 and 28s RNA at the one site is the fact that 16s r-RNA always labels before 28s r-RNA. Moreover, the differential effects of certain compounds on the synthesis of the two ribosomal RNA components point to these being synthesized separately (Ennis, 1966; Wagner & Roizman, 1968; Mayo, Andrean & De Kloet, 1968).

The fact that the initial precursor of 16s RNA and not 28s RNA binds to MAK so strongly may reflect a fundamental difference in the rate at which the secondary and tertiary structures are imposed on the two species of molecule. On the other hand, the degree to which MAK binds RNA molecules depends on molecular weight, secondary structure and base composition. Base composition is, of course, closely related to the secondary structure. A higher G+C/A+U ratio would confer a higher degree of secondary structure on the molecule and consequently ensure earlier elution from MAK. Since the proportion of A+U is much higher in the 16s RNA than in 28s RNA it will have a less stable secondary structure and will therefore be eluted later, but one would expect this to be cancelled out to some extent by the difference in molecular weight. In cells growing logarithmically there is very little MAK-bound RNA, but in an unfavourable situation, it accumulates. The present experiments suggest that this accumulation represents 16s RNA which has not
matured. If 16s RNA is synthesized outside the nucleolus it may, therefore, have to pass into the nucleolus to be matured. The 16s RNA which accumulates outside the nucleolus in ‘step-down’ and in the presence of actinomycin D may thus fail to be matured. The 28s RNA, on the other hand, appears to be synthesized inside the nucleolus and might, therefore, mature more rapidly.

There is at present no decisive evidence against the view that the rapidly labelled, ‘polydisperse’, MAK-bound RNA is nascent 16s r-RNA, and it appears that this RNA is synthesized in the ‘extranucleolar’ regions of the nucleus.

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REFERENCES


Intranuclear accumulation of RNA


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